THE INFLUENCE OF WHEY PEPTIDES AND FENRETINIDE ON INFLAMMATION AND APOPTOSIS IN IMMORTALIZED WILD TYPE AND MUTANT ΔF508 CFTR HUMAN TRACHEAL EPITHELIAL CELLS

Regina Maria Vilela

School of Dietetics and Human Nutrition
McGill University, Montreal,
Quebec, Canada
January, 2006

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Your file Votre référence
ISBN: 978-0-494-25279-6
Our file Notre référence
ISBN: 978-0-494-25279-6

TABLE OF CONTENTS

ABSTRACT

Studies were conducted using cultured immortalized wild type (non-CF) and mutant (CF) ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) tracheal epithelial cells on the anti-inflammatory impact of agents that may alter ceramide and glutathione (GSH) metabolism. The CF cells demonstrated abnormally high levels of GSH and glutathione disulfide (GSSG), which could diminish intracellular production of ceramide, a key modulator of inflammation and apoptosis. Hence, additional cell culture studies were carried out with a known inducer of *in situ* ceramide synthesis, *N*-4(4-hydroxyphenyl) retinamide (fenretinide) on interleukin (IL)-8 release, intracellular ceramide content, and cellular proliferation in both the basal state and following the inflammatory stimuli of tumor necrosis factor (TNF) -α. Fenretinide treatment was associated with a dose-dependent increase in the cellular content of ceramide in both CF and non CF cells. Also, an inhibition of IL-8 release in the inflammatory condition of TNF-α treatment was observed following fenretinide treatment in the CF cells. As hyperbaric treatment of whey proteins was previously associated with improved survivability and higher GSH content in a *Pseudomonas aeruginosa* murine model of cystic fibrosis (CF), the anti-inflammatory role of low molecular weight peptides (< 1kDa) generated from enzymatic hydrolysis of native and pressurized whey protein isolates (WPI) was examined. Pressure treatment of WPI was associated with an enhanced protein digestibility and an altered peptide profile following *in vitro* digestion. The whey peptides were tested CF and non-CF lung epithelial cells to identify for their effects on GSH metabolism. The impact of the combined treatment of fenretinide and WPH was also tested in terms of apoptosis and cytokine release in cell culture. As opposed to non-CF cells, CF cells showed a strong downtrend in release of IL-8 following the combined fenretinide and whey peptide treatment. In addition, whey peptides protected wild type epithelial cells from the apoptotic effect of fenretinide. Our results suggest the usefulness of these agents as a pharmacological treatment in CF.

RÉSUMÉ

Des études sur culture de cellules épithéliales de la trachée du type sauvage (non FC) et mutant ΔF508 CFTR (FC) ont été réalisées pour mésurer l'impact des agents qui peuvent changer le métabolisme de céramide et de glutathion (GSH). Les cellules FC ont démontré des niveaux anormalement elévés de GSH et de glutathion disulfure (GSSG), ce qui pourrait diminuer la production intracellulaire du céramide, un modulateur clé dans l'inflammation et l'apoptose. Par conséquent, des études additionnelles de culture de cellules ont été effectuées avec un connu inducteur de la synthèse du céramide *in situ*, N-4 (4-hydroxyphenyl) retinamide (fenrétinide), sur la libération d'interleukine (IL)-8, contenu intracellulaire de céramide, et sur la prolifération cellulaire tant au état basal ainsi qu'après les stimulus inflammatoires du facteur de nécrose de tumeur-α (TNF-α). Le traitement avec le fenrétinide a été associé à l'augmentation dose-dépendant dans le contenu cellulaire de céramide, tant sur les cellules FC que sur lesquelles non FC. Une inhibition de la sécrétion d'IL-8 en état inflammatoire induite par TNF-α a été aussi observée après le traitement de fenrétinide dans les cellules FC. Comme le traitement hyperbare des protéines du lactosérum était précédement associée à un taux de survie amélioré et au contenu du GSH dans un modèle murin de fibrose cystique (FC) avec *Pseudomonas aeruginosa*, le rôle anti-inflammatoire des peptides à faible poids moléculaire (< 1kDa) qui était généré par l'hydrolyse enzymatique d'isolats de protéines du lactosérum (WPI) natives et pressurisées a été examiné. Traitement de pression de WPI a été associé à une digestibilité augmentée et à un changement du profil des peptides après la digestion *in vitro*. Les peptides des protéines du lactosérum étaient testés en cellules épithéliales pulmonaires FC et non FC, pour que leur effets sur le métabolisme de GSH soient identifiés. L'impact de traitement combiné de fenrétinide et de WPH a été également testé en termes d'apoptose et de sécrétion de cytokines en culture de cellules. Par opposition aux cellules non FC, les cellules FC ont montré une forte tendance a réduire la sécrétion d'IL-8 après un traitement combiné de fenrétinide et des peptides des protéines du lactosérum. En

outre, des peptides des protéines du lactosérum a protégé les cellules épithéliales du type sauvage contre l'effet apoptotique du fenrétinide. Nos résultats suggèrent l'utilisation de ces agents comme traitement pharmacologique de la FC.

PREFACE

In this thesis, cell culture studies were carried out with a known inducer of *in situ* ceramide synthesis, *N*-4(4-hydroxyphenyl) retinamide (fenretinide), on cytokine release, intracellular ceramide content, cellular proliferation and apoptosis in cultured immortalized wild type and mutant ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) tracheal epithelial in both the basal state and following stimulation with the pro-inflammatory agent, tumor necrosis factor-α (TNF-α). The effect of hyperbaric pressure treatment on the digestibility of whey protein isolates (WPI) and the resultant peptide profiles was studied using in vitro enzymatic hydrolysis, capillary zone electrophoresis, and high performance liquid chromatography. Low molecular weight (< 1 kDa) peptides isolated from the in vitro enzymatic hydrolysis of native and pressurized WPI were tested in wild type (normal) and mutant CFTR tracheal epithelial cells to identify their possible effects on glutathione metabolism and cell proliferation. Finally, the impact of the combined treatment of fenretinide and peptides obtained from pressurized WPI was tested in terms of apoptosis and the inflammatory response of CFTR-deficient and normal tracheal epithelial cells following stimulation of TNF-α.

The present thesis is submitted in the form of original papers prepared for journal publication. The thesis is adjusted according to all other requirements of the Guidelines for Thesis Preparation in addition to the manuscripts. , Chapter 1 provides a general introduction in terms of rationale, hypothesis and objectives of the study. In Chapter 2, pertinent literature review is presented for the topics discussed in the body of the thesis. The next three sections contain the main core of the thesis and each chapter represents a completed paper for publication. The connecting texts provide the logical and scientific correlation between the different papers. The section following the manuscripts is a final summary of the major conclusions drawn from all aspects of the research.

ACKNOWLEDGMENTS

I would not have accomplished my goals if it was not for many people who supported me in so many ways. During my stay in Canada I received all the emotional, technical, financial and scientific support I needed to produce this thesis. Taking the risk of omitting people who have made positive contributions towards my graduate studies, I would like to thank to my supervisor, Dr. Stan Kubow for his patience, dedication, kindness and especially for giving me the opportunity of learning from his example of ethical behaviour, positive attitude, and exceptional scientific skills that drove my work throughout my graduate studies. I would like to thank my co-supervisor Dr. Larry Lands for his constructive critical analysis of my work, his suggestions and for his dedication during the revision of my thesis. I would like to thank my co-supervisor Dr. Larry Chan for the peptide analysis via MALDI-TOF and for the revision of my thesis.

Thanks also goes to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasília, D.F.-Brazil; Federal University of Paraná, and Tuiuti University of Paraná, Curitiba, Paraná, Brazil for funding my studies and my stay in Canada as well as the funds to develop the research via grants to SK and LL by the National Sciences and Engineering Council of Canada (NSERC) and the Canadian CF Foundation.

I could not thank the School of Dietetics and Human Nutrition enough and I would like to acknowledge this impressive organization, the quality of teaching and scientific research at this unit. Thanks go to all the staff, especially to Lise Grant, Francine Tardif and Donna Leggee for their great professional and technical support that were important to orient me during all my graduate studies. I would like to thank also Dr. Larry Lands' team at the Montreal Childrean's Hospital, Division of Respiratory Medicine for their technical support and advice.

There are also a few persons that I would like to mention because due to their help I chose to study in Canada and remain there to finish my thesis work: Ronaldo Machado Babiak, Marcos e Maria Lúcia Klüppel, João Henrique Faryniuk, André Ferraresso Piccolomini, Cláudia Seely Rocco, Deise Baptista Mendonça, Maria Eliana Madalozzo Schieferdecker, Maria Emília von der Heyde, Rosana Bento Radominski, and Salete Francisca Basso.

Thanks to my friends Connie and Luigi Migliaccio, who first received me in Montreal and made me part of their family, giving me love and encouragement.

Thanks to the friends that I have made at the School and who will be always in my heart because they gave me this gift of having an exciting and refreshing multicultural experience. I know that my beloved friends Lindiwe Sibeko, Jiun-Ni Liu, Chia-Chien Chang (Carole), Sean Mark and so many others will make a difference in people's lives not only through their professional competency, but especially through their pure intent to work towards a more fair society.

Thanks to Canada and to McGill University for receiving me as an international student.

Finally and most importantly, I would like to thank my grandmother who first taught me how to write and read, my beloved and supportive parents, Newton Vilela and Zilda Barros Vilela, and my siblings and best friends Denise Maria Vilela, Newton Vilela Júnior and Cristina Maria Vilela.

CONTRIBUTIONS OF AUTHORS

The present thesis involved the collaboration of Dr. Stan Kubow, School of Dietetics and Human Nutrition, McGill University, Macdonald Campus; Dr. Larry C. Lands, Paediatric Respiratory Medicine, Montreal Children's Hospital, Montreal, Quebec, Canada H3H 1P3 and Dr. H.M. Chan, The Center for Nutrition and the Environment of Indigenous Peoples (CINE), McGill University, Macdonald Campus.

Dr. Kubow, the candidate's primary supervisor, was the originator of the research project and was closely involved in the study design, providing ongoing guidance and feedback in all aspects of the thesis. In addition, Dr. Kubow conducted weekly meetings with the candidate to monitor progress of the work and set milestones for future accomplishments. Each manuscript draft was extensively critiqued and edited by Dr. Kubow, including the literature review. The candidate along with Dr. Kubow developed the concept of modulating ceramide metabolism in CF cells as a mechanism of anti-inflammatory action and the use of fenretinide treatment.

Dr. Larry Lands provided feedback on the manuscripts and provided helpful comments and suggestions on the thesis project. Dr. Chan provided the facilities to carry out capillary zone electrophoresis, HPLC fluorescent detection and lyophilization and feedback on the whey peptide manuscript.

As the primary author I was responsible for writing the manuscripts, creating the figures and the tables, and running statistical analyses. I performed all the experiments used in this project, including the basal cell culture maintenance, cell viability determination, trypan blue exclusion cell counting, protein and peptide assays, cell culture studies involving treatment with TNF-α, fenretinide and whey peptides, in vitro enzymatic digestion, glutathione and glutathione disulfide measurements, peptide separation via capillary zone electrophoresis, cellular lipid

extraction and derivatization and analysis of cell ceramide content using HPLC. Brian McMeehan provided technical assistance to measure IL-8 release and *in situ* end labelling of DNA strand breaks and to analyse whey peptide amino acid profiles. Behnam Azadi provided technical assistance with the digestibility assay and the pressurization of whey protein isolates. Nurlan Dauletbaev provided training regarding the specific basal maintenance of normal and CFTR deficient human tracheal epithelial cells and the cell proliferation assay using MTT, which added to my previous M.Sc. research experience in cell culture involving HeLa cells from uterus adenocarcinoma. Chia-Chien Chang provided technical assistance to determine peptide profiles via HPLC. Jiun-Ni Liu provided technical assistance to prepare the slides for the *in situ* end labelling of DNA strand breaks assay. I also received advice in CZE equipment use from Chujian Chen who oriented me with the software and basic sets.

CONTRIBUTIONS TO KNOWLEDGE/STATEMENT OF ORIGINALITY

1. Claims of Original Research

This thesis has demonstrated for the first time that:

1- Low doses of fenretinide can act to attenuate pro-inflammatory conditions in mutant ΔF508 CFTR human tracheal epithelial cells by decreasing TNF-α stimulated IL-8 release.

2- Wild type and mutant ΔF508 CFTR human tracheal epithelial cells increase intracellular ceramide content in response to fenretinide.

3- ΔF508 CFTR human tracheal epithelial cells present constitutively high levels of GSH and a GSH/GSSG ratio as compared to wild type cells.

4- ΔF508 CFTR human tracheal epithelial cells are resistant to the apoptotic effects of fenretinide.

5- Hyperbatric treatment of whey protein isolates is associated with enhanced *in vitro* digestibility, which results in an altered profile of low molecular weight peptides (< 1 kDa) as well as an altered amino acid composition.

6- Low molecular weight peptides (< 1kDa) peptides isolated from hydrolysates of pressurized whey protein isolates induce apoptosis in wild type human tracheal epithelial cells.

7- Low molecular weight peptides (< 1kDa) peptides isolated from hydrolysates from both native and pressurized whey protein isolates protect wild type human tracheal epithelial cells from the apoptotic effects of fenretinide.

2. Research Manuscripts in Preparation to Publication and Patent Application:

i) Inhibition of the Inflammatory Response of CFTR-Deficient Lung Epithelial Cells Following Pre-Treatment with Fenretinide.

ii) The Impact of High Hydrostatic Pressure on the Digestibility and Production of Bioactive Peptides from Whey Protein Isolates.

iii) Inhibition of the Inflammatory Response of CFTR-Deficient Lung Epithelial Cells Following Pre-Treatment with Whey Peptides and Fenretinide.

iv) Vilela R. M., Kubow S., and Lands L.C. 2003. Compositions and Methods for Preventing or Treating an Inflammatory Response. International Patent Application No. PCT/CA2005/000323 filed February 3, 2005.

LIST OF TABLES

CHAPTER 2

LITERATURE REVIEW

CHAPTER 4

CHAPTER 5

LIST OF FIGURES

CHAPTER 2

LITERATURE REVIEW

CHAPTER 3

CHAPTER 4

CHAPTER 5

FINAL CONCLUSION AND SUMMARY

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABC	ATP binding cassette
ACE	Enzyme-converting angiotensin I
α-LA	Alpha lactalbumin
ANOVA	Analysis of variance
AOM	Azoxymethane
ASF	Airway surface fluid
ATP	Adenosine triphosphate
ATRA	All-trans-retinoic acid
BALF	Bronchial lavage fluid
β-LG	Beta-lactoglobulin
BSA	Bovine serum albumin
BSO	L-buthionine-(S,R)-sulfoximine
CAPK	Ceramide activated protein kinase
CAPP	Ceramide-activated protein phosphatase
CCPs	Caseinophosphopeptides
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COX	Cyclooxygenase
CRP	C-reactive protein
Cys	Cysteine
CZE	Capillary zone electrophoresis
DHA	Docosahexaenoic acid
DISC	Death-inducing signaling complex
DTNB	5,5'-Dithiobis-2-nitobenzoic acid
ELF	Epithelial lining fluid
ENaC	Epithelium Na^+ channel
EP	Ethyl pyruvate
ERK	Extracellular-regulated kinase

ESI-MS	Electrospray ionization-mass spectrometry
EtOH	Ethanol
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in one second
γ-Glu-L-Cys	γ-Glutamylcysteine
Glu	Glutamate
Gly	Glycine
$GS^{\bullet}$	Oxidized form of GSH
GSH	Glutathione (γ-glutamyl-cysteinyl-glycine)
GSSG	Glutathione disulfide
GTPases	Guanosine triphosphatases
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IκB	Inhibitory kappa B
Ig	Immunoglobulin
IL	Interleukin
JNK	c-jun N-terminal kinase
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted-Laser-Desorption-Ionization/Time-of-Flight spectrometry
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MCHI	Major histocompatibility complex
MDA	Malondialdehyde
MEM	Minimum essential medium
MTT	3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	*N*-acetyl L-cysteine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NEAPC	Neutrophil elastase α 1 antiprotease complexes
NEM	*N*-ethylmaleimide

NF-κB	Nuclear factor-kappa B
N-SMase	Neutral sphingomyelinase
nWPH	Native whey protein hydrolysates
OPA	*o*-Phthaldialdehyde
P. aeruginosa	*Pseudomonas aeruginosa*
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCL	Perciliary liquid
PGE_2	Prostaglandin E_2
PEP	Positive expiratory pressure
PKA	Protein kinase A
PKC	Protein kinase C
PTPs	Protein tyrosine phosphatases
PVP	Polyvinyl-pyrrolidone
pWPH	Pressurized whey protein hydrolysates
ROS	Reactive oxygen species
RP-HPLC	Reversed phase HPLC
RT	Respiratory tract
S-1P	Sphingosine 1-phosphate
SAAs	Sulphur-containing amino acids
SAPK	Stress activated protein kinase
-SH	Sulfhydryl
SM	Sphingomyelin
SMase(s)	Sphingomyelinase(s)
SphK	Sphingosine phosphate kinase
TBARS	Thiobarbituric acid reactive substances
Tdt	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor-beta
Th2	T helper cell 2
TNF-α	Tumor necrosis factor alpha
WPC	Whey protein concentrate(s)

WPH	Whey protein hydrolysate(s)
WPI	Whey protein isolate(s)
pWPI	Pressurized WPI

CHAPTER 1

INTRODUCTION

1.1 Rationale and Statement of Purpose

Cystic fibrosis (CF) is a multisystem autosomal recessive disease characterized by a biochemical abnormality in the cystic fibrosis transmembrane conductance regulator (CFTR) protein which couples adenosine triphosphate (ATP) hydrolysis with transmembrane chloride (Cl^-) conductance across apical epithelial surfaces. (van der Vliet *et al.*, 1997).The defect in CF impairs normal movement of water and electrolytes across various epithelial surfaces, most notably in the respiratory tract (RT), but also in the pancreatohepatobiliary system, the gastrointestinal tract, and the sweat excretion system (van der Vliet *et al.*, 1997). Abnormalities of the RT in CF lead to chronic lung infection and to inadequate hydration of RT secretions making them more tenacious and difficult to clear (Boucher, 2004, Dinwiddie, 2000). Viscous secretions cause poor airway clearance and persistent infections, primarily with pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Conese *et al.*,2003) The chronic inflammatory immune response in the lungs leads to progressive tissue destruction and elevated pulmonary morbidity and mortality (van der Vliet *et al.*, 1997; Roum *et al.*, 1999; Lands *et al.*, 2000). There is no definite treatment for pulmonary inflammatory conditions in CF *per se* although drug treatment and other therapies are used to treat conditions secondary to the lung inflammation. Such treatment modalities include antibiotics for the treatment of *Pseudomonas* infection, physiotherapy, oxygen therapy and lung transplantation (Koehler *et al.*, 2004).

The respiratory manifestations of CF are characterized by a dense airway accumulation of neutrophils which are not able to clear the bacterial infections. Such neutrophil accumulation has been suggested to result in a prolonged primary acute-like inflammatory response driven by products of the local environment

(Conese *et al.*, 2003). Dysregulation of the mucosal immune system and pathological T-cell responses, however, could also play a role in the hyperinflammatory response (Conese *et al.*, 2003). The redox activation of the transcriptional factor nuclear factor-κB (NF-κB) protein via increased generation of reactive oxygen species (ROS) is also the focus of such inflammatory conditions because of the consequent expression of proinflammatory cytokines (Rahman and MacNee, 1998; Baeuerle, 2000). The situation could be more complex in cells expressing mutant CFTR as they are resistant to apoptosis triggered by hydrogen peroxide (H_2O_2) (Jungas *et al.*, 2002). There is suggestion that such resistance to apoptosis in mutant CFTR cells could be mediated by inherently high intracellular glutathione (GSH) concentrations (Jungas *et al.*, 2002). Intracellular GSH inhibits sphingomyelinase activity, which is normally induced under pro-inflammatory stimuli such as tumor necrosis factor-α (TNF-α) (Liu *et al.*, 1998; Lavrentiadou *et al.*, 2001). Hence, in the case of CFTR deficient cells, when the intracellular levels of GSH are high, intracellular ceramide production could be decreased via an inhibition of sphingomyelinase activity. As ceramide inhibits NF-kB activation and apoptosis (Signorelli *et al.*, 2001), we suggest that exacerbation of inflammatory response in CF might be secondary to a lowered response in terms of intracellular ceramide production. As a result, apoptosis would be delayed and NF-κB inhibition mediated by ceramide would be less evident under pro-inflammatory conditions. In addition, it is conceivable that the redox status (i.e., GSH/GSSG) of mutant CFTR cells is sub-optimal despite high intracellular GSH concentrations as enhanced oxidative stress has been implicated in CF (van der Vliet *et al.*, 1997). Although the redox status of CF cells has not been previously examined, low intracellular GSH/GSSG ratios could also lead to enhanced activation of NF-κB (Rahman and MacNee, 1998), which could also play a role in the hyper-inflammatory condition of CF cells.

As limited apoptosis and increased NF-κB activation via diminished ceramide production might be important factors related to chronic inflammation in CF, supplementation of ceramide or ceramide up-regulating drugs might decrease the

pro-inflammatory state in CF. Fenretinide, i.e., *N*-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic derivative of retinoic acid, has been shown to increase endothelial ceramide by *de novo*, non-sphingomyelinase-mediated synthesis, resulting in caspase-dependent endothelial apoptosis (Erdreich-Epstein *et al.*, 2002). Accordingly, fenretinide is suggested for use in cancer prevention and tumor inhibition (Ferrari *et al.*, 2003; Wu *et al.*, 2001); however, the potential anti-inflammatory effects of fenretinide on airway epithelial cells from CF patients are unknown.

Most of the benefits attributed to the tri-peptide GSH are related to its antioxidant properties conferred by the cysteine thiol group (-SH) (Papas, 1999). Whey protein isolates (WPI) are rich in cysteine and have been shown to induce/increase cellular concentrations of GSH in both *in vivo* and *in vitro* studies (Bounous *et al.*, 1989; Bounous and Gold, 1991). In addition, WPI have been proposed to have immunomodulatory effects in cell culture models (Cross and Gill, 1999; Bounous and Gold, 1991) and anticancer properties in animal models (Hakkak *et al.*, 2001; Eason *et al.*, 2004; Rowlands *et al.*, 2002). In the case of pulmonary disease, there is only one case report showing that supplementation with WPI increased whole blood GSH levels concomitantly with pulmonary function improvement (Lothian *et al.*, 2000). This result is encouraging considering that peripheral blood lymphocyte GSH concentrations were inversely correlated with airflow limitation in children with CF (Lands *et al.*, 1999a). As tissue GSH-inducing agents such as *N*-acetyl-L-cysteine (NAC) decrease NF-κB activation and down-regulate immune responsiveness (Baeuerle, 2000), it is possible that improvement of the intracellular GSH/GSSG ratio in CF via whey protein supplementation could likewise decrease the immune response under inflammatory conditions. As in the case of cancer prevention and treatment, irrefutable data correlating increased tissue GSH concentrations induced by WPI intake and positive disease outcome has still to be demonstrated. Although the bioactive component of WPI that is involved in GSH induction has not been identified, peptides or peptide fractions obtained from WPI have been implicated (Kent *et al.*, 2003). In addition to GSH

modulating properties, WPI hydrolysates or peptides have been associated with cytoprotection in response to oxidative stress (Li *et al.*, 1994), as well as hormone-like opioid (Teschemacher, 2003), antimicrobial and antiviral (Florisa *et al.*, 2003; Kilara and Panyam, 2003), antihypertensive (FitzGerald *et al.*, 2004), hypocholesterolemic (Pfeuffer and Schrezenmeir, 2000), and immunomodulatory (Cross and Gill, 1999; Gill *et al.*, 2000) effects. Interestingly, Penttila *et al.* (2001) showed that whey proteins could down-regulate the immune reaction in rat pups following sensitization with the antigen ovalbumin. The authors suggested that purified whey protein extracts have the potential therapeutic benefit in disease states where there is an excessive inflammatory reaction. The impact of peptides generated from whey proteins has not been studied previously in either normal or CFTR-deficient lung epithelial cells, particularly in relation to the impact on inflammation and GSH status.

Kubow and colleagues have developed a patent pending hyperbaric methodology of processing WPI to modify whey protein conformation (Kubow *et al.*, 2005). This approach was theorized to improve the digestibility of whey proteins and thereby provide an increased bioavailability of amino acids and small peptides. Hyperbaric treatment was thus demonstrated to increase the feed efficiency of whey protein feeding in rats and mice (Jing, 2005) and to improve their antioxidant and GSH status (Hosseini-nia, 2000); however, the specific impact of pressurization on the digestibility of whey proteins has not been examined previously. In addition, there was no information regarding whether there are differences in either content or profiles of peptides generated via the digestion of native versus pressurized WPI, particularly the low molecular weight peptides less than 1 kilodaltons (kDa) presumed to be the peptides that are primarily absorbed and most bioactive *in vivo* (Qiao *et al.*, 2004).

1.2 Project Objectives

In the light of the above, the overall goals of this thesis were to explore the effect of fenretinide and/or peptides obtained from hydrolysates of native and pressurized whey proteins on cellular proliferation, apoptosis and cytokine release in wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells. The thesis also examined whether the profile and content of low molecular weight peptides less than 1 kDa generated enzymatically from WPI are affected by pressurization and whether whey-derived peptides could modulate cellular GSH concentrations and interleukin (IL)-8 release in mutant and wild-type CFTR cells as the role of such peptides has not been previously examined. Additionally, the impact of fenretinide on cellular ceramide content was investigated in order to relate the functional effects of fenretinide on apoptosis, immune response and cellular proliferation to cellular ceramide concentrations.

The thesis is separated into four specific objectives:

a) To assess the effect of fenretinide treatment on cellular proliferation, IL-8 release, apoptosis, and cellular ceramide content in CFTR-deficient and normal human tracheal epithelial cells in non-inflammatory conditions and in the pro-inflammatory state following TNF-α stimulation.

b) To evaluate the impact of hyperbaric pressure treatment on the *in vitro* digestibility of WPI as assessed by peptide profiles and the amino acid, peptide and protein content.

c) To determine the effect of low molecular weight (<1 kDa) peptides isolated from *in vitro* hydrolysates of native and pressurized WPI on GSH and GSSG content, cell proliferation, apoptosis and cytokine release (IL-8) in immortalized

wild-type and mutant ΔF508 CFTR human tracheal epithelial cells.

d) To study the impact of the combined treatment of fenretinide and peptides (<1 kDa) from hydrolysates of pressurized WPI on cellular proliferation, IL-8 release, and apoptosis in CFTR-deficient and normal human bronchial epithelial cells in the pro-inflammatory state induced by TNF-α stimulation.

1.3 Hypotheses

1) Pressurization will enhance the digestibility of whey protein isolates leading to an altered peptide and amino acid profile of peptides less than 1 kDa.

2) The intracellular redox GSH/GSSG ratio will be depressed in human CFTR deficient tracheal epithelial cells as compared to human wild-type tracheal epithelial cells

3) Peptides less than 1 kDa from pressurized and native whey protein hydrolysates will improve the intracellular redox GSH/GSSG ratio and decrease IL-8 release in CFTR-deficient and CFTR-normal epithelial cells.

4) Fenretinide treatment will enhance cellular ceramide concentrations in CFTR-deficient and normal epithelial cells that, in turn, will lead to a diminished cytokine release, decreased cell proliferation and pro-apoptotic effects under inflammatory conditions in CFTR-deficient cells.

5) The anti-inflammatory effects of fenretinide on IL-8 release in CFTR-deficient and normal epithelial cells will be enhanced in the presence of whey peptides less than 1 kDa from pressurized and native whey protein hydrolysates.

CHAPTER 2

LITERATURE REVIEW

2.1 Cystic Fibrosis: An Overview on Pathophysiology and Epidemiology

Cystic fibrosis (CF) is an autosomal recessively inherited disease caused by a mutation in a gene on chromosome 7 that codes for a transmembrane–regulating protein called cystic fibrosis transmembrane regulator (CFTR) (Dinwiddie, 2000). The deletion of the amino acid phenylalanine at position 508 (ΔF508) accounts for > 70% of CF alleles in Caucasians and is an example of a class II mutation (Scheid *et al.*, 2001). CFTR belongs to the superfamily of membrane proteins called ATP-binding cassette (ABC) transporter that utilizes ATP to translocate different substrates across cell membranes. CFTR is expressed in the apical membrane of epithelial cells and mutations in the CFTR gene impair chloride (Cl^-) ion secretion causing severe dysfunction of various organs, particularly lungs, small and large intestine, pancreas, testis and sweat duct (Galietta and Moran, 2004).

The most devastating clinical sequelae of CF occur in the pulmonary system. CF patients are born with apparently normal lungs with unremitting bacterial infections of the airways (bronchi) occurring in the first few years of life reflecting the failure of the innate defense mechanisms of the lungs to inhaled bacterial organisms (Knowles and Boucher, 2002). Shortly after birth, many patients have endobronchial colonization with a number of microorganisms including *Staphylococcus aureus*, *Haemophilus influenzae*, and gram negative enteric organisms (*Klebsiella pneumoniae* and *Escherichia coli*). Eventually, nearly all patients become infected with *Pseudomonas (P.) aeruginosa*, which is closely associated with progressive pulmonary deterioration, although the rate of progression is highly variable and multifactorial (Bals *et al.*, 1999).

Over 1000 mutations in the CF gene have been identified and it has been difficult to identify correlations between genotype and phenotype of lung disease, making it difficult to predict severity of lung disease related to CF. Although there has been progress in the elucidation of the genetic and molecular basis of CF, the pathogenesis of the lung inflammation remains obscure (Boucher, 2004).

Because of the complexity of the disease there is still no effective treatment for CF. According to the American Cystic Fibrosis Foundation (2005), CF affects 30,000 children and adults in the United States and more than 10 million Americans are unknowing, symptom-less carriers of the defective gene. CF occurs in approximately one in every 3,200 live Caucasians births, 1/15,300 black births, and 1/32,000 Asian-American births in USA and about 1,000 new cases are diagnosed each year (Cystic Fibrosis Foundation, 2005). In Canada it is estimated that one in every 2,500 children are born with CF with a median survival over 36 years of age. Approximately 3,400 children, adolescents, and adults attend specialized CF clinics (Canadian Cystic Fibrosis Foundation, 2005).

Much has been learned from epidemiologic and clinical research and the knowledge about the pathophysiology and modifiers of the disease course such as household income, ambient air pollutants, genetic, and microbiologic factors has helped to improve the survival rates of CF patients. The survival improvement has occurred primarily amongst patients between the ages of 2 to 15 years over the past several decades but minimally in patients older than 15 years of age. This difference among age groups has been attributed to the improvement in nutritional management and the introduction of new therapies that has taken place since 1985, which have had a lower impact in older age groups (Goss and Rosenfeld, 2004). This improved survival is primarily the product of early diagnosis, close clinical observation of patients, and early implementation of prophylactic measures including attention to nutrition, chest physical therapy, the use of oral and inhaled antibiotics to decrease bacterial burden, and mucolytic agents.

Approximately 92% of patients are considered pancreatic insufficient and require pancreatic enzyme replacements to aid digestion (Bronstein *et al.*, 1992).

Malfunction of the CFTR channel has been suggested as a factor that perturbs the innate airway defense. Recently, Boucher (2004) presented a proposal to explain how the CFTR channel deficiency could be linked with inefficient innate defense. Two general hypotheses would explain the impact of the epithelial ion transport system on the efficient innate defense of airways against inhaled bacterial pathogens namely – the isotonic volume hypothesis involving mucus-dependent mechanical clearance, and the low salt/defensin hypothesis that refers to a chemical shield effect. In the first hypothesis it is postulated that an optimal mucus and perciliary mucus layer ratio and an isotonic environment is necessary to an adequate bacterial clearance. As a result of the CF defect it is postulated that due to the accelerated Na^+ through the epithelial Na^+ channel (ENaC) and the failure to secrete Cl^- through the CFTR channel in epithelial cells, the volume of perciliary liquid (PCL) needed for effective ciliary beating and cell surface lubrication would decrease and becomes hypertonic. As a result, mucus would donate water to the PCL layer that, in turn, would continue to decrease in volume. Thus, mucus would adhere to airway surfaces and a thickened environment would impair mechanical bacterial clearance favouring infection. Although it has been postulated that the normal CFTR channel regulates ENaC activity, the putative molecular interaction between CFTR and ENaC has not been elucidated. The inability of CFTR to inhibit Na^+ absorption through ENaC and the low Cl^- ions secretion in CF are suggested to be the main factors driving cells to decrease PCL volume. In the second hypothesis, previously described by Smith et al. (1996), it is postulated that a low salt airway surface fluid (ASF) environment is needed to promote antimicrobial activity of defensins. In CF the salt-sensitive antimicrobial peptides (defensins, cathelicidins) released by the epithelial cells in the presence of bacteria would lose antimicrobial activity as a result of an elevated salt concentration in the ASF environment.

In addition to the decreased volume, CF mucus secretions show increased concentrations of fractions that increase their viscosity such as sphingomyelin (SM) and phosphatidylserine. The increased phospholipid concentrations are thought to be a result of intracellular acidification, and a decrease in phosphatidylglycerol and phosphatidylcholine possibly caused by bacterial enzyme activity. Apart from phospholipids, bacteria and polymorphonuclear cells release DNA into the airway mucus, which in turn binds to proteins, mucin and lipids thereby inducing an increased viscosity and leading to an inefficient mucus transport (Puchelle *et al.*, 2002).

In the advent of an infection, as part of the innate immune response, it is expected that there is a neutrophil influx into the airways in response to chemokines and cytokines released by epithelial cells and macrophages. In fact, there is a massive neutrophil influx into the airway in CF; however, they fail to clear bacterial infections, especially in the case of *P. aeruginosa*. The high influx of neutrophils may also occur because there is a T helper cell 2 (Th2)-dominated response in CF lung disease that is characterized by interleukin (IL)-4, IL-5 or IL-10 production and a high antibody response (Bals *et al.*, 1999).

Among host and bacterial chemoattractants, IL-8 is the major neutrophil chemoattractant in the CF lung. IL-8 is released by alveolar macrophages, bronchial epithelial cells and fibroblasts in response to either an exogenous stimulus, i.e., bacterial-derived cell wall lipopolysaccharide (LPS) or endogenous stimuli such as tumour necrosis-α (TNF-α) and IL-1β (Conese *et al.*, 2003). There are reports that lung inflammation in CF patients might precede infection due to a defect in epithelial cells. In fact, it has been demonstrated that primary cultures of freshly obtained bronchial epithelial cells from normal patients secrete the anti-inflammatory cytokine IL-10 but little or no IL-8 or IL-6. In contrast, cells from clinically stable CF patients did not secrete detectable IL-10 but produced considerable amounts of IL-8 and IL-6 (Bonfield *et al.*, 1999). On the other hand, cell culture studies using cells with CFTR mutations have shown

conflicting results in terms of cytokine secretion likely due to diverse experimental conditions. Most cell culture studies have confirmed exacerbated IL-8 release in CF cells. For example, exaggerated IL-8 responses were noted when CF bronchial cells were stimulated with TNF-α (10 and 30 ng/mL) (Stecenko *et al.*, 2001, Venkatakrishnan *et al.*, 2000), or when tracheal epithelium ΣCFTE29o- cell lines and nasal epithelial cells were stimulated with *P. aeruginosa* (Scheid *et al.*, 2001), or when tracheal gland serous cells were stimulated with LPS (Kammouni *et al.*, 1997). The basal levels of IL-8, however, vary in different CF cell lines. They were either lower or similar to normal bronchial cells in some studies (Stecenko *et al.*, 2001; Venkatakrishnan *et al.*, 2000) but exacerbated in CF tracheal gland serous cells (Kammouni *et al.*, 1997). Schwiebert *et al.* (1999) demonstrated that IL-8 expression in CF and non-CF airway epithelial cells (bronchial, tracheal and alveolar) is not consistent either in basal conditions or following stimulation with TNF-α for 18 h. They suggested that there are no differences in the expression of IL-8 molecules between CF and non-CF cells.

The reasons for the contrasting findings in terms of IL-8 secretion observed among research studies when using different paired CF and non-CF cell lines is unclear. There could be inherent differences between cell lines related to altered gene expression or differences in signaling transduction pathways produced in the generation of specific cell lines. It is also possible that such differences accumulate due to factors other than CFTR status as cell lines can be unstable after many passages due to the transforming oncogenes used in their development (Becker *et al.*, 2004). Another possible factor in terms of IL-8 secretion that differentiated cells expressing wild-type CFTR or CFTR mutations was noted when these cells were incubated with TNF-α for more prolonged periods of time (24 and 48 h). IL-6 and IL-8 generation in wild-type CFTR cells ceased by 24 h despite continued presence of the stimulation whereas CF cells failed to terminate the stimulated cytokine generation. These latter results thus suggest that the mechanism used by normal cells to switch off the immune response is defective in CF cells (Stecenko *et al.*, 2001). Supporting the hypothesis that CF patients may

have such a defect in IL-8 release in epithelial cells, Muhlebach and Noah (2002) demonstrated that although broncheoalveolar lavage fluids (BALF) from CF and non-CF children presented similar levels of endotoxin activity associated with gram-negative organisms, CF patients had significant higher levels of IL-8 expressed as a function of endotoxin activity.

IL-8 is released by epithelial cells in response to TNF-α, which is the principal mediator of the acute inflammatory response to gram-negative bacteria and other infections (Abbas *et al.*, 2000). TNF-α release has been correlated with an increased activation of NF-κB in bronchial CF cells (Venkatakrishnan *et al.*, 2000). The transcriptional factor NF-κB is responsible for a coordinated induction of proinflammatory genes in response to proinflammatory cytokines such as TNF-α and IL-1β, in addition to growth factors, bacteria and viruses, immunoreceptors, protein kinase C activators, and oxidants among others. It is present in cytoplasm in an inactive form by association with inhibitory κB (IκB), an inhibitory unit. In response to a large number of proinflammatory stimuli, IκB is inactivated by phosphorylation and proteasome-mediated degradation and the released NF-κB translocates into the nucleus and binds to regulatory promoter DNA elements of genes to induce transcription (Baeuerle, 2000; Rahman and MacNee, 1998). In addition to TNF-α and IL-1β, NF-κB can be activated by oxidants or conditions that cause pro-oxidant state in cells. Hydrogen peroxide (H_2O_2) released by neutrophils induces IκB phophorylation and degradation followed by NF-κB induction. GSH through its antioxidant activity is known to prevent NF-κB activation through this mechanism (Baeuerle, 2000).

The research literature to date appears to indicate that the CFTR malfunction affects the ASF environment by impairing mechanical clearance of microorganisms and thereby favouring CF lung inflammation (Boucher, 2004). In addition, it seems that CF epithelial cells respond to the infection with prolonged secretion of IL-8 in response to TNF-α (Stecenko *et al.*, 2001); however, the link

between the abnormal epithelial cell response to infection and mutations of the CFTR channel is not known. It is also still unclear whether prolonged inflammation in CF relates predominantly to bacterial infection, or occurs as a direct consequence of the mutant CFTR protein. The metabolic adaptation to inflammation in CF and the influence of CFTR channel on this process is a complex subject, especially considering that many factors influence the immune response such as cytokines, growth factors, bacteria and viruses, oxidants, and LPS among others (Rahman and MacNee, 1998). Although it is recognized that experimental *in vitro* cellular models do not ideally reproduce the *in vivo* environment, strategies to decrease IL-8 release in cultured epithelial cells in response to either an exogenous stimulus, i.e., LPS, or endogenous stimuli such as TNF-α and IL-1β, would be a relevant approach to identify for agents that decrease CF lung deterioration, particularly since IL-8 secretion is an important chemoattractant for neutrophils (Conese *et al.*, 2003).

As described above, while the cause of the lung deterioration in CF is not clearly established, palliative treatment has been used to alleviate the symptoms and improve the quality of life of the patients. Many alternatives have been tested to treat CF airway inflammation such as corticosteroids (Kennedy, 2001), and other non-steroidal anti-inflammatory drugs such as ibuprofen (Konstan *et al.*, 1995) with improvement in lung function and decreased in the frequency of pulmonary exacerbations; however, adverse side-effects with only a modest therapeutic impact on the CF lung deterioration are still important concerns (Kennedy, 2001). More targeted treatments are currently being tested such as modulation of immune response through modulation of cytokines and the NF-κB-signaling pathway, but the risks and the efficacy against the CF-related inflammation are still to be shown (Koehler *et al.*, 2004).

2.2. Antioxidant Therapy: Is it Relevant in Cystic Fibrosis?

As described by van der Vliet *et al.* (1997), oxidative stress has been considered to be a consequence of persistent inflammation via metabolic events at the site of the inflammation in CF. Thus, infectious inflammatory – immune processes in the lungs of CF patients would be the cause of severe oxidative stress that would in turn lead to tissue damage. Increased activity of nicotinamide adenine dinucleotide phosphate ($NADP^+$) oxidase by macrophages and neutrophils and expression of inducible nitric oxide (NO) synthase by macrophages are considered important mechanisms of overproduction of reactive oxygen species (ROS) such as the superoxide anion ($O_2^{-\bullet}$), the nitrogen dioxide (NO_2) radical and peroxynitrite ($ONOO^-$) (a product of the combination of superoxide and NO) that can lead to lung inflammation. As a consequence of increased ROS, lung cells would be depleted in intracellular antioxidants especially GSH, leading to activation of redox sensitive transcriptional factors such as NF-κB and expression of genes involved in inflammation such as pro-inflammatory cytokines IL-1β, IL-8, TNF-α (Rahman and McNee, 2000). A vicious cycle would take place and the logical approach, considering the sequence of events proposed, would be to provide the patients with antioxidant therapy (Fig. 1). As shown in Figure 1, exposure of lung epithelial cells to bacteria would initiate the innate inflammatory response by the release of cytokines and chemokines into the airway surface area and the resultant recruitment of phagocytes to the site of the inflammation. During the process of phagocytosis to eliminate the foreign agent, macrophages and neutrophils would release ROS that would reach epithelial cells and decrease intracellular GSH (Abbas *et al.*, 2000). As a result of decreased antioxidant GSH, redox-sensitive transcriptional factors such as NF-κB would be activated and increase the expression of pro-inflammatory cytokines. Antioxidant therapies could protect cells from activation of pro-inflammatory cytokines through an increase in intracellular GSH status as well as via decreased ROS.

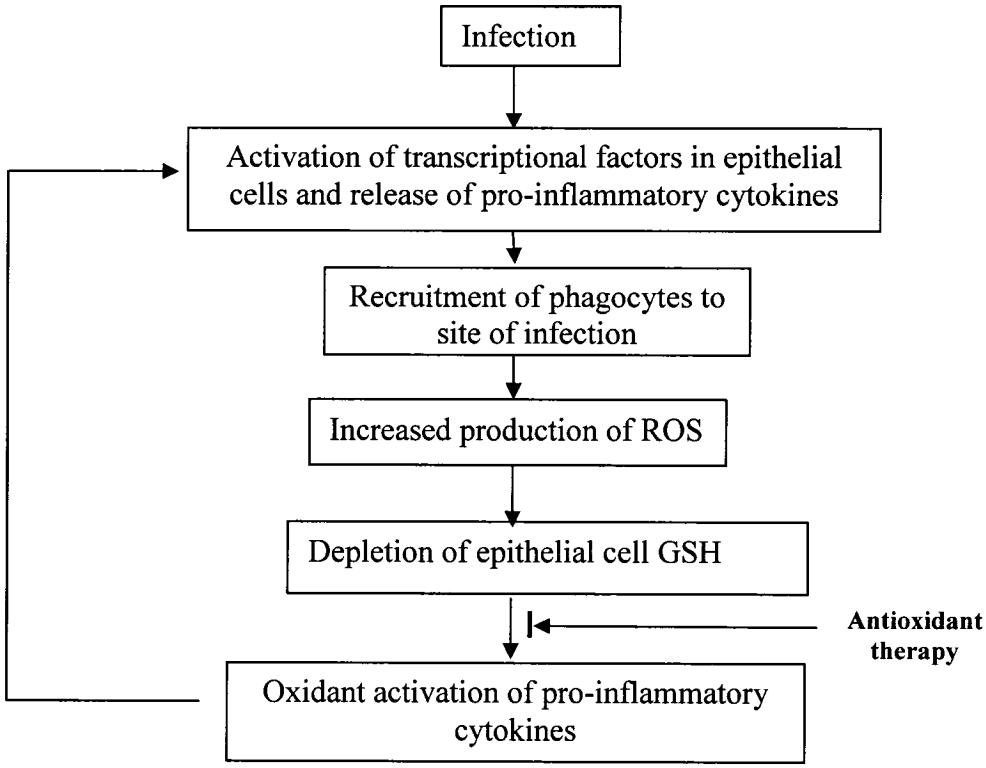


Figure 1. Mechanism of oxidant-mediated lung inflammation

Adapted from: van der Vliet *et al.* (1997)

Many papers have been published suggesting that antioxidant therapy could be useful for CF. McGrath *et al.* (1999) conducted a clinical research study comparing analysis of peripheral blood and pulmonary function tests of adult CF patients and healthy controls. Blood samples collected from CF patients at the moment of symptomatic respiratory exacerbation (reduction in forced expiratory volume in one second - FEV1) showed increased inflammatory markers such as C-reactive protein (CRP), total white blood cell count and neutrophil elastase $\alpha 1$ antiprotease complexes (NEAPC) as well as decreased levels of hydrosoluble free radical scanvengers (plasma ascorbate, total protein sulphydryls), lipid phase scavengers (retinol, lutein, lycopene), and red blood cell polyunsaturated fatty acids. Antibiotic therapy improved some of the parameters used as predictors of antioxidant status although the healthy subjects maintained superior antioxidant

status relative to the CF patients. They concluded that there is a need to provide protection from inflammation and free radical damage in CF, although the success of using antioxidant supplementation was not tested in their research.

Back *et al.* (2004) used the same rationale to suggest antioxidant therapy after conducting a cross-sectional study that showed elevated markers of oxidative stress and diminished antioxidant molecules in four different age groups of CF patients (< 6 y, 6-11 y, 12-17 y, ≥18 y) when compared with healthy control subjects. Plasma concentrations of β-carotene, β-cryptoxanthin, and total lycopene were lower in CF patients. In addition, all indicators of oxidative stress studied (i.e., plasma thiobarbituric acid-reactive substances and protein carbonyls, $F_2\alpha$-isoprostane concentrations in the breath condensate) were higher than those indicators in control subjects. They suggested use of antioxidants beginning at young age but again antioxidant therapy was not tested clinically.

A longitudinal study conducted by Lagrange-Puget *et al.* (2004) involving 312 CF patients (from 6 mo to 45 y) both in a stable state and during bronchial exacerbation showed that antioxidant activity was severely deficient in CF. Levels of vitamin A, vitamin E, carotenoids, the reduced form of GSH, and malondialdehyde (MDA) were significantly lower in the blood plasma of CF patients than in controls. In addition, acute bronchial exacerbation was associated with a significant decrease in vitamin A, lutein, and MDA levels. Surprisingly, when FEV1 was taken as an independent predictor, total and reduced GSH levels were negatively correlated to better lung function although antioxidant levels (vitamin A, vitamin E, and carotenoids) were positively correlated. The oxidative markers (MDA and lipid peroxides) were not significantly modified by FEV1. The authors suggested that plasma GSH measurement is probably not a relevant indicator of airway GSH status as CF patients have decreased levels of GSH in the lung lining fluid indicating impaired antioxidant protection of the airway epithelium.

Lands *et al.* (1999a) also showed the same inverse relation between lung function and PBMC (peripheral blood mononuclear cells) concentrations of GSH but also demonstrated a positive correlation between GSH concentrations in PBMC and nutritional status. On the basis of this latter finding, it is reasonable to suggest that in response to lung inflammation an increased synthesis of GSH can occur provided there is adequate nutritional status and availability of GSH biosynthetic precursors.

Taken together, the above studies have shown that CF patients generally have poor antioxidant status when compared with healthy subjects and also that respiratory exacerbations can decrease antioxidant protection even further. It is not clear, however, if poor plasma antioxidant status reflects pulmonary function or if antioxidant therapy would improve lung function. In terms of plasma GSH status, it is uncertain whether the inverse correlation of lung function with plasma GSH indicates that this index is not a good predictor of lung function or whether decreased GSH plasma may have functional role in the improvement of lung function due to properties of GSH independent from antioxidant function, i.e., immune modulation.

To date, the relevance of using GSH or GSH precursors as a therapeutic approach in CF is still unclear although a number of different interventions to enhance the GSH status in CF have been tested. For example, when GSH provided in aerosol format was delivered to seven CF patients, an antioxidant effect was noted as assessed by a decreased $O_2^{-\bullet}$ release from inflammatory cells that were collected by bronchial lavage from the epithelial lining fluid (ELF) (Roum *et al.*, 1999). On the other hand, pulmonary function tests were unchanged from baseline after GSH aerosol therapy. This research thereby indicated that despite effective delivery of GSH directly into the airway cells to improve GSH status of ELF, the beneficial impact of such intervention could not be proven. In addition, although the total GSH concentrations increased after the treatment, GSSG also increased to a level

corresponding to 66% of the total GSH pool in the ELF (Roum *et al.*, 1999). Elevation in the intracellular content of GSSG can change cellular redox status and may result in interference in biological status either through oxidative stress and cell damage or through oxidant-dependent signal transduction affecting protein function (Rahman *et al.*, 2005). Proteins with cysteine residues have been shown to exhibit redox sensitivity such as transcriptional factors, caspases, protein tyrosine phosphatases (PTPs), small guanosine triphosphatases (GTPases) and cytoskeleton components (Rahman *et al.*, 2005). In contrast to the Roum *et al.* study (1999), Griese *et al.* (2004) showed that GSH inhalation in CF patients significantly increased GSH levels in BALF and improved lung function; however, reversal of markers of oxidative injury such as oxidized proteins and lipids was not observed when assessed 14 days after daily inhalation of GSH via either 300 or 450 mg thrice-daily doses. More recently, Hartl *et al.* (2005) indicated that inhaled GSH improves lung function without having any effect on markers of oxidative stress in BALF from CF patients. Moreover, GSH affected primarily the pulmonary immune response by decreasing prostaglandin E_2 (PGE_2) in the BALF of CF patients showing an inverse correlation between lung function and PGE_2. This latter study suggests that improvement of lung function may be due to immunomodulatory properties of GSH independent from antioxidant function; however, GSSG levels were not measured and the GSH levels were exclusively analysed in the extracellular environment.

Administration of cysteine in the form of whey protein isolates (WPI) as a dietary precursor for induction of GSH synthesis in respiratory epithelial tissue and blood cells has also been proposed for treatment of CF. In a double-blind randomized controlled clinical trial, Grey *et al.* (2003) compared the therapeutic impact of ingestion of 10 g of either WPI or casein taken twice a day by 24 stable CF patients during a three month period. They detected a 30% increase in lymphocyte GSH levels in the whey-treatment group (11 subjects); however, no difference in lung function was observed between the two groups of patients. The authors

suggested that increases in lymphocyte GSH did not reverse the tissue damage in CF but it could still have the potential to arrest further damage.

Most of the work supporting the use of antioxidants for CF treatment assumes that inflammation leads to increased ROS, activating redox-sensitive NF- κB that, in turn, contributes to persistent inflammation. The results with antioxidant therapy to date, however, do not seem to provide support for this concept considering that only indirect measurements of antioxidant status have been used such as GSH levels in peripheral blood and lymphocytes and that there are ethical and technical difficulties in making measurement of epithelial intracellular GSH *in vivo*, especially in damaged lungs. Considering the above limitations, the use of wild-type and CFTR deficient cultured cells is a useful approach.

The CFTR channel is permeable to GSH (Lindsdell and Hanrahan, 1998; Kogan *et al.*, 2003) and has been shown to be unable to adequately export GSH to the ELF in CF under basal conditions (Gao *et al.*, 1999) and during inflammation (Day *et al.*, 2004). The fact that the ELF in CF patients has a low GSH content, however, does not necessarily indicate a low intracellular content of GSH. In fact, low intracellular lung epithelial GSH content has not been shown in studies with CFTR deficient cells to date. Two studies have compared the intracellular levels of GSH between CFTR mutant and wild-type lung epithelial cells. Gao *et al.* (1999) measured GSH levels in the apical environment as well as the intracellular GSH content by seeding immortalized human tracheal epithelial cells from homozygous ΔF508 CF patients on coated permeable supports. They confirmed a decreased apical medium GSH content in CF cells; however, intracellular GSH concentrations were not adversely affected by the defect in CFTR function in unstimulated conditions. More recently, Jungas *et al.* (2002) showed that HeLa cells expressing mutant CFTR demonstrated higher basal intracellular concentrations of GSH when compared with the cells expressing wild-type CFTR. In addition, they showed that the CFTR-deficient cells were more resistant to apoptosis induced by H_2O_2, an important ROS released by neutrophils in response

to TNF-α released by macrophages and lymphocytes during inflammation (Nathan, 1987). It is important to note that Gao *et al.* (1999) did not investigate GSH trafficking and intracellular content under inflammatory conditions. Also, when comparing the Gao *et al.* (1999) and Jungas *et al.* (2002) studies, it should be considered that different cell culture conditions were applied as a serum-free supplemented medium was used to grow the cells in Gao *et al.* (1999) study. Serum-free conditions lack albumin that can act as an amino acid ligand to deliver nutrients to the cells (Peters, 1996) and serum starvation leads to decreased intracellular GSH content (Shaw and Chou, 1986; Kang and Enger, 1991). Moreover, serum-free or serum-starved (i.e., 0.5% fetal bovine serum [FBS]) conditions increase intracellular ROS levels and apoptosis in cultured cells and these are inhibited by both GSH and NAC (Satoh *et al.*, 1996; Lee and Piedrahita, 2002). It is thus conceivable that serum-free conditions in the Gao *et al.* (1999) study led to depressed levels of intracellular GSH in both mutant CFTR and wild-type cells thereby obviating GSH differences between the two cell types. An important limitation to both of the above studies, however, is that the GSSG content was not assessed. The GSH/GSSG ratio is an important intracellular index of oxidative stress and it is possible that lower GSH/GSSG ratios occur in CF cells, which would lead to redox activation of NF-κB during inflammation (Rahman and MacNee, 1998).

Considering the above, one can suggest that redox-activation of NF-κB might occur in CF lung epithelial cells as a result of a low intracellular GSH/GSSG due to increased ROS under inflammatory conditions. In this context, treatment with antioxidants or GSH precursors could play a role in decreasing inflammation through inhibition of the redox activation of NF-κB. However, the effect of antioxidants and GSH precursor agents on the intracellular molecular modulation of inflammation in CF still needs to be investigated.

2.3 Emerging Information on Molecular Events in CF. Does the Impact of GSH on Gene Transcription Overcome its Importance as an Antioxidant in Inflammatory Response in CF?

It is controversial whether excessive pulmonary inflammation is an intrinsic property of the CFTR defect or whether it is secondary to the unique environment of the CF lung. It has been shown in studies using both transformed and primary cell cultures that CFTR deficient human airway epithelial cells are hyper-responsive to some proinflammatory stimuli such as LPS, TNF-α and IL-1β (Stecenko *et al.*, 2001; Kammouni *et al.*, 1997). The authors suggest that as a result of infection with *P. aeruginosa*, alveolar macrophages produce TNF-α and IL-1β, which could then trigger the exaggerated inflammatory response by CFTR deficient cells. Stecenko *et al.* (2001) compared IL-8 and IL-6 release after incubating transformed and primary normal and deficient CFTR cells with TNF-α for different periods of time. When cells were incubated for short periods (one or four hours) the cytokine generation was not excessive in CF cells implying that the mechanisms for the exaggerated response is not due to a constitutive increase in the number or binding affinity of TNF-α receptors in CF cells. On the other hand, when cells expressing wild-type CFTR were incubated for prolonged periods of time (24 and 48 h), IL-6 and IL-8 generation ceased by 24 h despite continued presence of the stimulation. In contrast, CF cells failed to terminate the stimulated cytokine generation. These results suggest that the mechanism used by normal cells to switch off the immune response is defective in CF cells. In accordance to the hypothesis that CF cells fail to terminate stimulated cytokine generation, Venkatakrishnan *et al.* (2000) demonstrated that CF bronchial epithelial cells had markedly elevated NF-κB activation and production of IL-8 compared with normal cells when stimulated with TNF-α. They showed that both in unstimulated and TNF-α-stimulated conditions, there were no differences in processing and degradation of IκB-α in CF and non-CF cells; however, basal levels of IκB-β were increased in CF cells. Moreover, treatment with TNF-α in

CF cells resulted in increased formation of hypophosphorylated IκB-β as well as increased nuclear localization of IκB-β. Unlike IκB-α and the basal phosphorylated IκB-β, hypophosphorylated IκB-β is unable to mask the nuclear localization signal and the DNA binding domain of NF-κB. The authors suggested that NF-κB bound to hypophosphorylated IκB-β in mutant CFTR cells is protected from inactivation by IκB-α and thus can enter or remain in the nucleus to mediate persistent transcriptional activation of proinflammatory genes.

Apart from the work of Kammouni *et al.* (1997), the research presented above seems to agree that normal and CF epithelial cells demonstrate little NF-κB activation and IL-8 production in the absence of an inflammatory stimulus. More recently, Becker *et al.* (2004) tested the response of primary airway epithelial cells to various stimuli and showed that CF-derived cells did not present more activated NF-κB or secrete more IL-8 than non-CF cells, either in their basal state or when stimulated with IL-1β, TNF-α or *P. areuginosa* at 8, 24 or 48 h in the absence of fetal bovine serum. When *P. areuginosa* was tested in the presence of 10% human serum, however, CF cells responded with an exaggerated and sustained IL-8 response. Taken together, these results show that exaggerated immune response depends on the cell lines and culture conditions studied and that intrinsic basal differences in inflammation due to mutant CFTR *per se* are not a primary cause for the hyper-inflammatory condition of the CF lung. More likely, CF cells are less capable of downregulating the proinflammatory response. As discussed above, CF dysregulated cytokine production has been related to increase in NF-κB activation but the mechanisms by which the transcriptional factor is activated or how CF cells fail to downregulate the proinflammatory responses are not known. More recently, attention has been addressed regarding the influence of oxidative stress and GSH levels on inflammatory gene transcription. In particular, it seems that the influence of GSH on the inflammatory response goes beyond its antioxidant effects. In that regard, a recently published study examined RAW 264.7 murine macrophage-like cells

stimulated with LPS following incubation with either ethyl pyruvate (EP) or NAC, a precursor for endogenous GSH synthesis and oxidant scavenger (Song *et al.*, 2004). Intriguingly, incubation with EP, which caused a decreased in intracellular GSH showed an inhibition of LPS-induced NF-κB DNA binding to a much greater extent than NAC, which stimulated endogenous GSH concentrations (Song *et al.*, 2004). Two complementary hypotheses were proposed to explain why NAC and EP had anti-inflammatory effects despite opposite effects on intracellular GSH. Firstly, NAC-induction of cellular GSH could decrease activation of IκB by decreasing its ROS oxidative damage, thus keeping NF-κB in its inactive state (Baeuerle, 2000). On the other hand, depletion of GSH via EP may downregulate the inflammatory response by interfering with the binding of the activated form of NF-κB to DNA via oxidation of the cysteine residue in the DNA-binding region required for NF-κB binding (Song *et al.*, 2004). If intracellular GSH promotes cellular resistance to apoptosis in CF cells stimulated by H_2O_2 (Jungas *et al.*, 2002), this environment might therefore favour NF-κB binding to DNA after its pro-inflammatory activation and thereby interfere with downregulation of the inflammatory response.

It is also possible that downregulation of the ceramide pathway in CF lung cells could be related to the persistent inflammation in CF. Ceramide is a sphingosine-based lipid signaling molecule important in regulating diverse cellular processes in response to extracellular stimulus in the advent of metabolic and/or oxidative stress (Andrieu-Abadie *et al.*,2001). It is a hydrophobic molecule that tends to remain within the membrane bilayer and under different circumstances can regulate cellular differentiation, proliferation, apoptosis and inflammatory response through direct or indirect regulation of a number of enzymes and signaling components, including mitogen-activated protein (MAP) kinases, phospholipases and transcriptional factors such as NF-κB (Hannun and Obei, 2002). Apoptosis is an important physiologic event during processes such as embryogenesis differentiation, wound healing and immune response (Kolesnick

and Krönke, 1998). It had been described as a controlled form of cell death, where the integrity of the plasma membrane is preserved until late in the process, thus enabling packing of disintegrating organelles into membrane-bound vesicles without leakage of toxic intracellular components (Kolesnick and Krönke, 1998). Apoptotic cells are thus removed by phagocytosis without inducing or exacerbating inflammation and producing tissue damage (Kolesnick and Krönke, 1998).

Two main routes have been defined for the generation of ceramide: (1) hydrolysis of sphingomyelin (SM), an abundant sphingolipid species in cell membranes, by the action of sphingomyelinases (SMases); and (2) by *de novo* biosynthesis catalyzed by ceramide synthase (Levade and Jaffrezou, 1999). The hydrolytic pathway, however, is the major source for ceramide in cellular responses to extracellular signaling (i.e., TNF-α, LPS, gamma-interferon, and interleukins). TNF-α and H_2O_2 have been shown to modulate apoptosis through the SM pathway, which is initiated by hydrolysis of SM in the cell membrane to generate the second messenger ceramide (Hannun and Obei, 2002).

The activation of SMase is the first step of the sphingolipid ceramide-signalling pathway that can flow through different downstream effectors, for modulation of different cellular responses (Pyne and Pyne, 2000). Many molecular events related to apoptosis can be affected by SM and related sphingolipids such as ceramides, sphingosine and sphingosine-1phosphate (S-1P). SM is a major component in plasma membranes and might be directly implicated in intracellular signalling pathways. Upon cellular stimulation, several enzymes, including SMases, ceramide synthase, neutral ceramidase and sphingosine kinase, can participate in the production of bioactive sphingolipids. A single activation can have more than one target enzyme, even in the same cell (Riboni *et al.*, 1997).

As a general rule, ceramide induces apoptosis via caspases and C-Jun N-terminal kinase (JNK) activation, however, the conversion of ceramide into sphingosine and S-1P determines cell fate (Fig. 2).

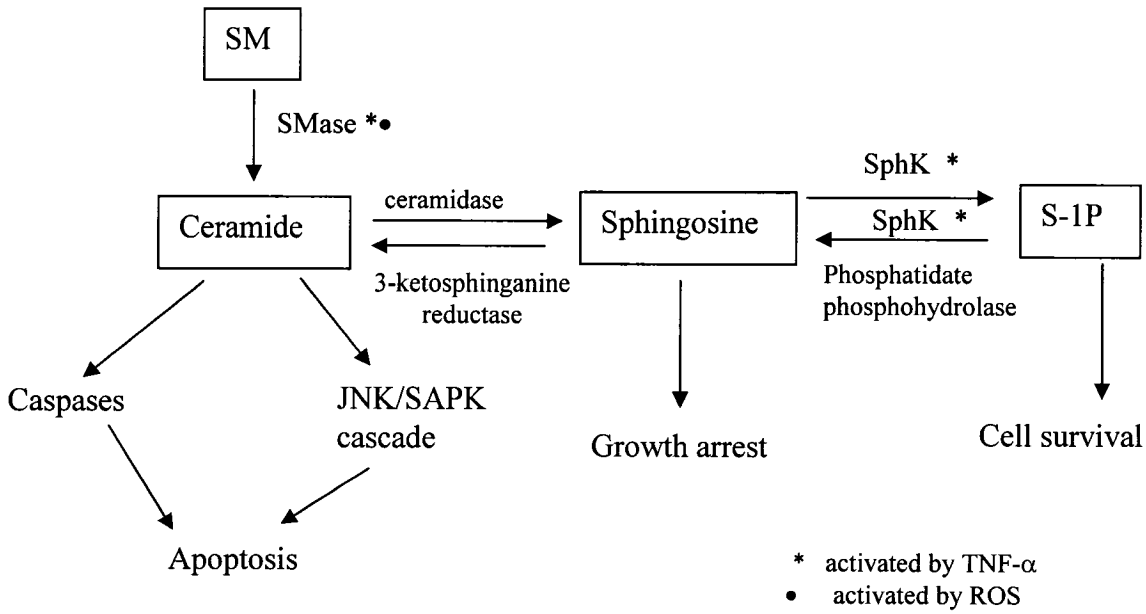


Figure 2. Proposed relationship between apoptosis and sphingolipid metabolism Ceramide acts as a second messenger and signals apoptosis through the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) cascade and also by direct effects on mitochondrial homeostasis through caspases. The SAPK/JNK pathway stimulates activity of AP-1 nuclear factors (e.g., c-Jun) that promote transcriptional activation of various genes that appear necessary to apoptosis. SM (sphingomyelin), SMase (sphingomyelinase), SphK (sphingosine phosphate kinase), S-1P (sphingosine-1 phosphate), ROS (reactive oxygen species).
Sources: Andrieu-Abadie *et al.* (2001); Pyne and Pyne (2000); Riboni *et al.* (1997)

Ceramide-mediated apoptosis has not been completely understood. It seems to require the stress activated protein kinase (SAPK) cascade since interruption of

this cascade abolishes the apoptotic response to ceramide. One proximal target for ceramide is ceramide activated protein kinase (CAPK), which possibly recruits mitogen activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) kinase (MEKK1). MEKK1 activates SAPK-kinase (SEK 1), which engages JNK. Another proximal target for ceramide is ceramide-activated protein phosphatase (CAPP), which inhibits the cytoprotective MAPK cascade through inactivation of protein kinase C (PKC) (Ariga *et al.*, 1998; Kolesnick and Krönke, 1998; Andrieu-Abadie *et al.*, 2001; Levade *et al.*, 2002). Other direct targets for ceramide have been identified such as ceramide-activated protein phosphatases PP1 and PP2A that cause dephosphorylation of several cellular proteins including PKCα, c-Jun, Bcl-2, lysosomal protease cathepsin D and mitochondrial CDase (Hannun and Obei, 2002). More recently, Miyaji *et al.* (2005) demonstrated that generation of ceramide in lipid rafts facilitates efficient Fas clustering, death-inducing signalling complex (DISC) formation and caspase-8 activation leading to downstream cascade of caspase-3 activation and apoptosis.

When normal human airway epithelial cells are exposed to H_2O_2, neutral sphingomyelinase (N-SMase) is activated; SM is hydrolysed leading to the generation of ceramide that can induce the apoptotic process (Chan and Goldkorn, 2000). This would be one of the mechanisms by which normal epithelial cells respond to H_2O_2 and TNF-α released by phagocytes during inflammation to suppress inflammatory processes and limit the survival of disturbed or necrotic cells. As opposed to apoptotic cells, necrotic cells release their cytoplasm content of toxic enzymes into the surrounding tissue, which would exacerbate inflammation (Kolesnick and Krönke, 1998). Similar to airway epithelial cells, astrocytes prepared from rat cerebral tissue, respond to TNF-α by rapid degradation of SM to ceramide. Decreased GSH content was also observed following TNF-α exposure and NAC prevented the cytokine-induced decrease in GSH content and the degradation of SM to ceramide (Singh *et al.*, 1998). These results suggest that GSH may play a critical role in the regulation of generation of ceramide.

Membrane associated N-SMase is inhibited by GSH as demonstrated by Liu and Hannun (1997). They treated SMase obtained from Molt-4 human leukemia cells membranes with physiologic concentrations of GSH (1-20 mM) and showed that N-SMase was inhibited in dose-dependent manner. The reducing agents dithiothreitol or β-mercaptothanol, GSSG and the thiol-modified GSHs, S-ethyl and S-methyl GSH did not affect the enzyme activity, suggesting that the sulfhydryl group of GSH is not required for inhibition of N-SMase. They also showed that depletion of GSH results in SM hydrolysis and generation of ceramide. Based on these results it was speculated that under stable conditions with normal intracellular GSH, the N-SMase exists as an inactive enzyme. Thus, depletion of GSH would result in activation of SMase. Liu *et al.* (1998) showed that GSH reversibly inhibits N-SMase from human mammary carcinoma MCF-7 cells. Treatment of MCF-7 cells with TNF-α induced a decrease in cellular GSH, which was followed by hydrolysis of SM and generation of ceramide. The TNF-α induced SM hydrolysis and ceramide generation was followed by cell death that was inhibited when the cells were pretreated with GSH, GSH-methylester, or NAC. The same concept was tested in lung cells by Lavrentiadou *et al.* (2001), showing that depletion of GSH is needed to induce intracellular ceramide generation and apoptosis in alveolar epithelial cells. The addition of H_2O_2 to the culture decreased GSH levels, increased ceramide content and promoted apoptosis. Pre-incubation of the cells with GSH prevented the H_2O_2-mediated decrease in intracellular GSH levels, and induction of ceramide generation. They also showed that thiol-containing antioxidants other than NAC did not prevent apoptosis mediated by H_2O_2, reinforcing that the role of GSH in apoptosis does not depend only on its antioxidant thiol group. Thus, according to the above cited literature, there may be a link between the antiapoptotic property of GSH and the resistance of CF cells to apoptosis (Lavrentiadou *et al.*, 2001; Liu *et al.* 1998; Jungas *et al.*, 2002). Lavrentiadou *et al.* (2001) showed that depletion of GSH is needed to induce intracellular ceramide generation and apoptosis in alveolar epithelial cells and Jungas *et al.* (2002) showed that the high intracellular GSH content in mutant CF cells allowed these cells to be more resistant to H_2O_2-

mediated apoptosis than non-CF cells. Additionally, as GSH inhibits SMase (Liu and Hannun, 1997, Lavrentiadou *et al.*, 2001), the resistance of CF cells to decreasing intracellular GSH following H_2O_2 treatment could exacerbate their inflammatory response due to an inhibition of intracellular ceramide production since sphingomyelin hydrolysis and ceramide release has been shown to inhibit NF-κB (Luberto *et al.*, 2000; Signorelli *et al.*, 2001). In that regard, Signorelli *et al.* (2001) working with Jurkat leukemia and MCF-7 breast cancer cells, suggested a dual role of ceramide in terms of NF-κB function. Ceramide on its own was suggested to act as an inhibitor of NF-κB; however, ceramide could also serve as a substrate for SM synthase by inducing diacyl glycerol production, which could lead to NF-κB activation.

2.4 The Therapeutic Potential of Fenretinide

Fenretinide, i.e., *N*-(4-hydroxyphenyl)retinamide (4-HPR) a synthetic derivative of retinoic acid that has been shown to increase endothelial ceramide by *de novo*, non-SMase-mediated synthesis (Erdreich-Epstein *et al.*, 2002) resulting in caspase-dependent endothelial apoptosis of human brain microvascular endothelial cells. Accordingly, fenretinide is suggested as a potential drug for ceramide-based therapy to be used in cancer prevention (Kolesnick, 2002). Zou *et al.* (1998) compared the effects of 4-HPR and all-trans-retinoic acid (ATRA) on the growth and apoptosis of human non-small cell lung cancer cell lines and normal bronchial epithelial cells and demonstrated that 4-HPR is more potent than ATRA in inducing apoptosis in cancer cells. 4-HPR has also been shown to induce apoptosis in ovarian cancer cell lines (Cuello *et al.*, 2004), MCF-7 breast cancer cells (Poot *et al.*, 2002) and human prostate cancer (Webber *et al.*, 1999). Fenretinide is reported to have fewer side-effects compared to naturally-occurring retinoids including vitamin A (Ulukaya and Wood, 1999). The safety profile for fenretinide is excellent as minimal side-effects have been noted in a variety of clinical trials using fenretinide on a prophylactic basis (Ulukaya and Wood 1999). Clinical trials have shown that fenretinide does not induce generalized vascular

damage in humans (Reynolds and Lemons 2001). Fenretinide has also been used to treat subjects (2-21 years of age) with neuroblastoma to define fenretinide pharmacokinetics and maximal tolerated dose in children, and to assess short- and mid-term toxicity in this age range (Garaventa *et al.*, 2003). Fenretinide was given orally once a day in 28-day courses. Liver and renal functions and clinical evaluation were assessed weekly. The side effects that occurred in 15 of the 45 subjects tested were the same as those observed in adult subjects. The side effects were noted to be tolerable and readily reversible within 7 days following discontinuation of the treatment. As discussed above, as compared to normal cells, CF cells are resistant to apoptosis when treated with H_2O_2, which has been related to high intracellular GSH concentrations (Jungas *et al.*, 2002). As GSH inhibits SMase (Liu and Hannun, 1997, Lavrentiadou *et al.*, 2001), a drop in intracellular GSH would be needed for ceramide release and inhibition of NF-κB activation (Signorelli *et al.*, 2001). Based on the discussion above, we proposed the treatment of CFTR-deficient cells with fenretinide as an alternative strategy to increase intracellular ceramide, bypassing SM pathway, and thus decrease expression of pro-inflammatory cytokines. To our knowledge, fenretinide has not been tested in CF cells or used with the purpose of decreasing inflammation in any condition so far.

2.5 Whey Proteins, Glutathione Status, and Immunomodulatory Properties

Whey proteins can be described as non-casein proteins in milk that remain soluble in liquid milk after casein precipitation at pH 4.6 and 20°C. Whey proteins are globular proteins and include: β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin, lactoperoxidase and immunoglobulin (Ig) proteins such as IgA, IgM, and IgG (Holt, 1985). The quantitatively most important proteins in whey are β-LG and α-LA, which represent 70 to 80% of the total whey protein content (Smithers *et al.*, 1996). Numerous studies have indicated many important nutritional and immune benefits to human breast milk feeding, which have largely been attributed to the biological properties of whey proteins (Labbok *et al.*, 2004).

An enhancement of a wide variety of biological functions has been attributed from intake of whey proteins including: improved absorption of iron, retinol and calcium; immune modulation; and enhanced antioxidant and antimicrobial functioning (Shah, 2000). In particular, the immune enhancing and antimicrobial properties of whey proteins have been suggested to provide important immune protection for infants during their first months of life (Shah, 2000). Whey proteins are present in higher proportions relative to casein in human milk whereas bovine milk contains casein as the major protein (Table 1). The predominance of whey proteins in human breast milk has been associated with improved immune function and protein digestibility as well as decreased allergenicity relative to bovine milk feeding (Cordle, 1994; van Odijk *et al.*, 2003; Hanson *et al.*, 2003). In order to provide more favourable nutritional properties, the casein to whey protein ratio in certain infant formulas has been adjusted from a 80:20 ratio as found in bovine milk to a 40:60 ratio seen in human breast milk (Okamoto *et al.*, 1991; Wit, 1998).

Table 1. Protein composition of human and bovine milk (% of total content)

	Human	**Bovine**
Caseins	35	79
Whey Proteins		
α-LA	17	3.5
β-LG	Not detectable	9
Lactoferrin	17	Trace
Serum Albumin	6	1
Lysozyme	6	Trace
IgA	11	3
IgG	Trace	Trace
IgM	Trace	Trace
Others	8	4.5

Source: Hambraeus (1977).

During the commercial manufacture of cheese, the separation of whey proteins from casein occurs because whey proteins do not coagulate in the presence of acid and are resistant to the action of chymosin (Shah, 2000). Whey proteins, which correspond to approximately 20% of the total protein content in bovine milk, separate from the curd as a yellowish liquid containing 100% of the milk lactose and a variety of minerals (Morr and Ha, 1993). Whey proteins can be further concentrated to obtain whey protein concentrates (WPC) with a protein content ranging from 50 to 75%, or whey protein isolates (WPI) with protein content ≥ 90% (Morr and Ha, 1993). Whey processing methods such as membrane fractionation, precipitation, and ion exchange chromatography can lead to significant differences in protein composition and conformation that can, in turn, alter the functional properties of the whey protein products (Schmidt *et al.*, 1984).

An important nutritional difference between human and bovine milk is the proportionality of individual whey proteins, which differ greatly in their digestibility. α-LA is the primary whey protein in human milk and it is readily digestible (Shah, 2000). Moreover, α-LA, Ig and serum albumin, which are all present in higher concentrations in human relative to bovine milk (Table 1), are readily digested by pepsin, trypsin, chymotrypsin and pancreatin both *in vitro* and *in vivo* (Mutilangi *et al.*, 1995; Kitabatake and Kinekawa, 1998). In contrast, β-LG, which is absent in human milk but represents about half the total whey protein content in bovine milk, is a more resistant protein to digestion (Shah, 2000). β-LG is not easily hydrolyzed by pepsin and trypsin and is only partly digested by pancreatin (Kitabatake and Kinekawa, 1998; Kinekawa and Kitabatake, 1996). This resistance to digestion has been related to the globular structure of β-LG due to the simultaneous presence of two disulphide bridges and one free sulfhydryl group (-SH), which gives a rigid structure to this protein (Pantako *et al.*, 2001; Schimdt and Poll, 1991). This stability is relevant to the transport of retinol from the cow to the young calf, but it is less important for

human infants, who can develop an allergenic reaction due to the presence of this intact protein in the gut (Wit, 1998).

In addition to their use in infant formulas, whey proteins isolated from bovine milk are used as ingredients in processed foods due to their ability of modify the texture, appearance and organoleptic characteristics of foods and thus improve consumer acceptance (Mahaan Group, 2005). Some functional properties of whey proteins in foods include high solubility, good hydration and viscosity when thermally denatured, good emulsifying and gelling characteristics, and excellent foam stability (Linden and Lorient, 1999). More recently, whey proteins have been considered as promising functional foods, i.e., as foods having additional health benefits other than the already well-established nutritional benefits (Balagtas *et al.*, 2003). In particular, due to their rich content of sulphur-containing amino acids (SAAs), particularly the thiol amino acids cysteine and methionine (Table 2), whey proteins have been indicated in human, animal and cell culture trials to exert important antioxidant and immune modulatory properties via induction of tissue GSH content (Table 3).

Table 2. Cysteine thiol amino acid content of whey proteins

	Molecular Mass (kDa)	Amino acid residues	Cysteine residues per molecule	Cysteine disulfide $(Cys)_2$	Glu-$(Cys)_2$
β-Lactoglobulin	18,400	162	5	2	0
α-Lactalbumin	14,200	125	8	4	0
Serum albumin	66,000	582	35	17	6
Lactoferrin	77,000	708	40	17	4

Source: Bonous and Gold (1991).

In addition to its role as a critical substrate for protein synthesis, cysteine plays important biological functions as an extracellular reducing agent, and as the rate-limiting precursor for the synthesis of GSH (γ-glutamyl-cysteinyl-glycine). GSH

is a tripeptide thiol that is ubiquitously found within mammalian cells. GSH synthesis requires two ATP-dependent enzymatic reactions: (1) formation of γ-glutamylcysteine (γ-Glu-L-Cys) from glutamate (Glu) and cysteine (Cys); and (2) synthesis of GSH from γ-glutamylcysteine and glycine (Gly).

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \longleftrightarrow \text{L-}\gamma\text{-Glu-L-Cys} + \text{ADP} + \text{Pi} \quad (1)$$

$$\text{L-}\gamma\text{-Glu-Gys} + \text{Gly} + \text{ATP} \longleftrightarrow \text{GSH} + \text{ADP} + \text{Pi} \quad (2)$$

Due to the presence of a reactive sulfhydryl group, GSH can readily form glutathione disulfide (GSSG) via reactions between the oxidized forms of GSH ($GS^{\bullet}$) or by reactions of $GS^{\bullet}$ with other thiol compounds (GSSR) (DeLeve and Kaplowitz, 1991). GSH can also be regenerated from GSSG via the action of GSSG reductase in the presence of NADPH.

As a non protein-thiol, GSH can detoxify free radicals and peroxides, regulate cell growth and protein function and maintain immune function (DeLeve and Kaplowitz, 1991). In addition, GSH is a substrate for GSH transferases and peroxidases, enzymes that catalyze the reactions for detoxification of xenobiotics and reactive oxygen species (Parcell, 2002). Cellular GSH status (i.e., total GSH and its redox state) has been accepted as an important index of oxidative stress and plays a major role in the redox buffering of the cell environment (Rahman *et al.*, 2005). A variety of disease states such as pulmonary and hepatic diseases, cancer and heart disease are associated with lower tissue GSH concentrations, which has suggested that approaches to enhance tissue GSH concentrations could be therapeutically advantageous (Bounous *et al.*, 1989). More recently, new biological roles for GSH have been identified including protein glutathionylation as an element of intracellular signal transduction, gene expression, intracellular nitric oxide storage and transport, and apoptosis (Wu *et al.*, 2004).

The provision of adequate amounts of sulfydryl-containing amino acids (i.e, cysteine) as well as glutamate (or glutamine), glycine and serine has been

indicated to be important for maximal GSH synthesis (Wu *et al.*, 2004). In particular, since cysteine is the limiting amino acid for GSH synthesis, strategies have been utilized to increase intracellular GSH via an increased dietary supply of cysteine via cysteine rich proteins such as whey. As shown in Table 3, intake of whey proteins has been shown to increase the GSH content in human and animal tissues. The mechanism(s) of action whereby whey proteins can induce tissue GSH concentrations, however, is still not clear.

Augmentation of tissue GSH with whey protein intake has been shown in both human and animal feeding trials. Bounous *et al.* (1989) demonstrated enhanced liver and heart GSH concentrations and increased longevity from the feeding of whey protein-based diets (20% w/w) relative to either Purina mouse chow or casein-rich (20% w/w) diets in older male mice. A comparison between WPI-containing diet versus a casein diet containing cysteine at equivalent amounts to the WPI diet showed that the WPI-containing diet was associated with greater and more consistent increases in splenic GSH in immunized mice than the cysteine-supplemented casein diet (Bounous *et al.*, 1989). Similarly, Zommara *et al.* (1998) showed in a rat feeding trial that a free amino acid mixture duplicating the amino acid composition of whey protein was not associated with a significant increase in hepatic GSH concentrations as opposed to a whey protein-containing diet. The authors suggested that the GSH-inducing effects of cysteine were limited by the feedback inhibition of γ-glutamylcysteine synthetase in rats fed with the amino acid mixture. Conversely, it was suggested that γ-glutamylcysteine could act as an efficient and bioavailable substrate for GSH biosynthesis, since γ-glutamylcysteine can bypass the feedback inhibition of γ-glutamylcysteine synthetase (Meister, 1991) and is readily transported into cells (Meister *et al.*, 1986).

Bounous and Gold (1991) proposed that the release of an intact γ-glutamylcysteine peptide following digestion of whey proteins containing undenatured, intramolecular disulfides is crucial for optimal GSH synthesis. γ-Glutamylcysteine is commonly found in the primary sequences of cysteine-rich

whey proteins such as β-LG, α-LA and bovine serum albumin (BSA), in which γ-glutamylcysteine residue accounts for 20%, 25% and 17.6% of total cysteine residues, respectively (Eigel *et al.*, 1984). Bounous and Gold (1991) also suggested the glutamic acid-cysteine segments are preserved during the digestion of undenatured dietary whey proteins that were not exposed to heat treatment during their isolation whereas they would be broken down during exposure to heat treatment. In support of his contention, a WPI (Immunocal™) that was isolated under low temperature conditions, showed the greatest hepatic GSH concentrations and highest immune response in comparison to other whey protein products in healthy mice fed 20% dietary whey proteins (Bounous and Gold, 1991).

The GSH-inducing properties of Immunocal™, which is a commercial WPI product indicated to be an undenatured whey protein preparation, have been demonstrated clinically. For example, Bounous and colleagues fed Immunocal™ at 39.2 g per day for a period of three months to three HIV-seropositive individuals who frequently exhibit a systemic GSH deficiency (Bounous *et al.*, 1993). In subjects who maintain an adequate total caloric intake, the supplementation of Immunocal™ was associated with an increased body weight and an increased GSH content of mononuclear cells. A case report of a patient who had obstructive lung disease responsive to corticosteroids, and low whole blood GSH levels showed that one month of supplementation with Immunocal™ was associated with increased whole blood GSH levels and dramatic improvement in pulmonary function (Lothian *et al.*, 2000).

Long term supplementation of Immunocal™ was demonstrated to induce lymphocyte GSH concentrations in healthy adults, which was associated with increased skeletal muscle performance, as well as decreased percent body fat without weight change, thereby suggesting muscle mass accumulation (Lands *et al.*, 1999b). Dietary supplementation with Immunocal™ has also been demonstrated to increase plasma GSH levels in patients with chronic hepatitis C, but not in patients with hepatitis B (Watanabe *et al.*, 2000). Conversely, a

significant GSH inducing effect of Immunocal™ was not demonstrated in the plasma of HIV-infected patients, whereas a heat denatured WPI, Protectamin™, showed a significant enhancement in plasma GSH levels in these patients (Micke *et al.*, 2001). The higher cysteine content in Immunocal (4.17 wt%) versus Protectamin (2.28 wt%) however, could have led to a greater difficulty in digestibility since proteins high in cysteine content are more difficult to digest (Pantako *et al.*, 2001; Schimdt and Poll, 1991). Since malabsorption is common among HIV-infected patients, the larger amount of cysteine-rich proteins may have led to a lower digestibility thereby decreasing the GSH-inducing properties of Immunocal™. In contrast to the Bounous hypothesis that the glutamic acid-cysteine dipeptide would be preserved in undenatured dietary whey proteins and crucial for optimal GSH synthesis, Kent *et al.* (2003) demonstrated that buthionine sulfoximine (BSO) treatment inhibited by 50% the intracellular increase in intracellular GSH content induced by WPI hydrolysates with cultured RWPE-1 cells. As BSO irreversibly inhibits the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase, it appears that free cysteine and glutamate present in whey protein hydrolysates could play a role in GSH synthesis via the γ-glutamyl-cysteine synthesis pathway.

Table 3. Effects of WPI on GSH status in human, animal and cell culture trials

Authors	Object of the study	Treatment	Overall results
Baruchel and Viau (1996)	Normal peripheral blood mononuclear cells from healthy volunteers and MATB 13672 cell line (rat mammary carcinoma) Jurkat T cells.	WPI added at concentrations of 0.1 μg/mL to 1.0 mg/L to the cell cultures.	Normal lymphocytes had intracellular GSH increased when treated with the 100 μg/mL WPI treatment, which was associated with an increase in cell proliferation. Tumor cells showed a decrease in GSH concentrations and an inhibition in cellular proliferation with the 100 μg/mL WPI treatment.

Hakkak *et al.* (2001)	Male Sprague-Dawley rats.	WPI and casein control diets given to pregnant Sprague- Dawley rats and their offspring. Offspring had lifetime exposure to the diets and also received tumor inducer AOM (azoxymethane).	WPI dietary treatment was associated with a lower tumour incidence than the control diet although no difference in hepatic GSH concentrations was observed.
Bounous *et al.* (1989)	Aged (17-20 months) male C57BL/6NIA mice.	Three groups of 18 mice fed for 6.3 months with undenatured WPI versus casein (20 g/100g diet) or Purina mouse chow control groups.	Undenatured WPI dietary treatment was associated with an increased immune response and increased hepatic and cardiac GSH levels relative to the casein and Purina chow controls.
Bounous and Gold (1991)	Immunized C3H/HeJ mice (seven weeks old).	Mice fed during three weeks with undenatured WPI versus casein (20 g/100g diet) or denatured WPI (20 g/100g diet).	Undenatured WPI dietary treatment was associated with a higher humoral immune response and increased hepatic GSH levels relative to casein and denatured WPI controls.
Watanabe *et al.* (2000)	Open study with 25 patients with chronic hepatitis B or C.	Whey (12 g twice/day) for 12 weeks with an induction period with casein for two weeks prior to the whey treatment and a follow up period with casein for four weeks after the whey protein.	↑ GSH levels (plasma) – hepatitis B. ↓ Serum lipid peroxide levels. No change in hepatitis C patients (17 patients).
Lands *et al.* (1999b)	20 healthy adults.	Casein (placebo) or whey (Immunocal) (20 g/day) for three months.	↑ Total GSH (lymphocyte). ↑ Muscle performance.
Grey *et al.* (2003)	Double-blind randomized clinical trial with 24 CF patients.	10 g of WPI or 10 g casein taken twice a day by stable patients during three months.	Increase (30%) in lymphocyte GSH levels in the whey-treatment group (11 subjects). No improvement in pulmonary function tests.
Micke *et al.* (2001)	Double blind clinical trial with 30 HIV-patients.	45 g of WPI for two weeks.	Increase in plasma GSH levels from the baseline values.
Micke *et al.* (2002)	Patients with advanced HIV-infection.	WPI (45 g/day for six months).	Increased plasma GSH levels from the baseline values during a six month intake of WPI.

Lothian *et al.* (2000)	Case report 40-year-old woman with obstructive airway disease.	Use of 10 g WPC per day during one month on her own accord.	Improvement of pulmonary function tests from the baseline including an ↑ in total lung capacity and a decrease in residual volume/total lung capacity. ↑ In blood lymphocyte GSH levels.
Kennedy *et al.* (1995)	Free clinical trial with five patients with metastatic carcinoma.	Fed 30 g of WPC daily for six months.	Six patients had initially high blood GSH levels and two patients had these levels droped, which was sustained over the six month treatment period.
Kent *et al.* (2003)	*In vitro* cell culture study (human prostate epithelial cells).	Effect of WPH on intracellular GSH and resistance to *t*-butyl hydroperoxide.	WPH ↑ GSH and protected the cells from oxidant insult as compared to undigested WPI.

As mentioned above, it is conceivable that an increase in GSH content in CF cells could help to protect lungs from ROS damage produced during inflammation and slow the course of the disease. Grey *et al.* (2003) failed to show clinical improvement in CF patients from whey protein treatment, perhaps because whey protein intake may not be capable of improving the functionality of already damaged lungs in the advanced stages of the disease. The authors also suggested that an increase in lymphocyte GSH levels might not be reflective of CF airway epithelial cells GSH status.

Similar to the findings of certain CF cell lines (Jungas *et al.*, 2002), intracellular GSH concentrations have been noted to be several-fold higher in human cancer cells in comparison to normal cells (Russo *et al.*, 1986). In that regard, the relatively high GSH concentrations have been suggested to protect tumor cells from immune system attack (Russo *et al.*, 1986). Paradoxically, WPI treatment was shown to cause GSH depletion in cultured human mammary carcinoma cells whereas GSH concentrations in normal cells were enhanced by WPI exposure (Baruchel and Viau, 1996). In addition, WPI treatment was associated with an inhibition of tumor cell growth, which appeared to be mediated by an inhibition of

GSH metabolism (Baruchel and Viau, 1996). An important limitation of the cell culture studies of Baruchel and Viau (1996), however, is that the cells were exposed to intact whey proteins, which is unlikely to happen *in vivo* due to the digestive breakdown of whey proteins in the gastrointestinal tract. In support of the findings of Baruchel and Viau (1996), high circulating blood levels of GSH were shown to be depleted in cancer patients receiving WPI treatment, which was correlated with tumor regression (Kennedy *et al.*, 1995). Hence, there is a suggestion that a selective enhancement in GSH via the use of WPI in cancer therapy could protect normal tissue against the deleterious effects of chemotherapy and, at the same time, render tumor cells more vulnerable to treatment (Baruchel *et al.*, 1995). This selective effect was proposed as a consequence of a change in GSH metabolism in tumor cells (Rouse *et al.*, 1995). Although Bounous and Gold (1991) have suggested that GSH induction via the γ-glutamylcysteine peptide was the key factor that mediated the protective action of whey protein against colon cancer development in mice, other work has shown that whey protein dietary treatment was associated with decreased tumor incidence in an animal model of colon cancer despite unaltered tissue GSH concentrations (Hakkak *et al.*, 2001). Similarly, Zommara *et al.* (2002) suggested that the whey protein IgG might modulate immune responses *in vivo* via mechanisms independent of GSH as antiperoxidative effects were noted from the supplementation of IgG to rats fed vitamin E deficient diets with no change in tissue GSH status. Hence, more mechanistic studies are needed to clarify the relationship of whey protein treatment with GSH status and positive clinical outcomes such as remission of symptoms of diseases such as CF and cancer. As opposed to cancer cells, non-tumor prostate epithelial cells have been shown to increase GSH when exposed to whey protein hydrolysates (WPH) as compared to undigested WPI (Kent *et al.*, 2003). An important limitation of Kent *et al.* (2003), however, is the use of WPH without excluding non-digested proteins and large molecular weight peptides from the hydrolysates as well as the use of undigested WPI as a control, which is not applicable to the *in vivo* context.

In addition to enhancement of cellular GSH concentrations, whey protein intake has also been suggested to play a role in immune modulation (Table 4), which could provide further insight regarding the link between whey protein treatment and disease outcomes. The majority of studies described in Table 4 show that whey proteins can up-regulate the humoral immune responsiveness as exhibited by elevated levels of antibodies against diverse antigens and an increased proliferation of immune cells (Low *et al.*, 2003; Ford *et al.*, 2001; Mercier *et al.*, 2004; Bounous and Gold, 1991).

Apart from actions of whey proteins in their native state, peptides cleaved from whey proteins have also been demonstrated to exert bioactive immune system effects. The role of peptides as obtained from enzymatic hydrolysates of whey proteins is contradictory as Cross and Gill (1999) showed no effect of 10 mg/mL of whey protein hydrolysates (< 10 kDa) on murine splenic lymphocyte proliferation whereas Mercier *et al.* (2004) demonstrated enhanced lymphocyte proliferation at 2 mg/mL (< 10 kDa) dose. The apparent differences between the two studies are likely due to differences in experimental conditions such as the enzymes used to digest the whey proteins as well as the enzyme: substrate ratio and duration of the digestion process that could produce different peptide fractions with different effects on cell proliferation. In addition, Cross and Gill (1999) induced lymphocyte proliferation by adding diverse mitogens and cytokines to the leucocyte culture as opposed to Mercier *et al.* (2004) that analysed lymphocyte proliferation in nonstimulated condition. Mercier *et al.* (2004) also demonstrated that lymphocyte stimulation was observed with diverse fractions of low molecular weight peptides resultant from enzymatic hydrolysis at 0.5 μg/mL and 500 μg/mL concentrations. Apart from these latter two studies, the impact of peptides present in WP digests has not been examined although peptide fractions generated through enzymatic hydrolysis of whey proteins have demonstrated antibacterial (Florisa *et al.*, 2003), opioid (Shah, 2000), and antihypertensive (i.e., angiotensin converting enzyme [ACE]) inhibitory properties (FitzGerald *et al.*, 2004).

Table 4. Immunomodulatory effects of whey proteins

Authors	Object of study	Treatment	Overall results
Bounous and Gold (1991)	*Ex vivo* study to verify the response of spleen cells of C3H/HeJ mice that were fed WPC to sheep red blood cells.	Mice fed during three weeks with undenatured WPC versus control casein (20 g/100g diet).	Higher number of plaque forming cells and increased heart GSH concentrations in WPC-fed animals.
Cross and Gill (1999)	*In vitro* effect of whey proteins on male spleen cells extracted from BALB/c mice.	Cells treated with 400 μg/mL of whey protein and 10mg/mL of whey protein hydrolysates (< 10 kDa) versus BSA treatment.	Whey proteins but not whey protein hydrolysates suppressed T and B lymphocyte proliferative response to either mitogens (LPS, Concavalin A) or an alloantigen obtained from Swiss mice. Cytokine (IL-2)-induced proliferation was unaffected.
Low *et al.* (2003)	*In vivo* study to verify the effect of WPC on the humoral and intestinal tract immune response of male BALB/c mice primed to vaccine antigens.	Animals fed with WPC or chow diet.	WPC-fed mice produced elevated levels of antigen-specific intestinal tract (mucosal) and serum antibodies to all tested antigens in comparison to the control-fed mice.
Ford *et al.* (2001)	*In vivo* study to verify the effect of whey protein on female C57BL/6J mice contaminated with sporulated oocysts of *Eimeria vermiformis.*	Animals were fed with whey protein, casein or soy protein.	The whey protein group had increased total white blood cells, total lymphocytes, $CD4^+$ and $CD8^+$ as compared with the casein and soy fed groups. Whey > casein > soy in terms of IFN-γ production in stimulated splenic cells.
Penttila *et al.* (2001)	Study with rat pups to verify the effect of growth factor enriched whey protein on ovoalbumin oral tolerance.	Animals fed (oral gavage) with 200 μg of growth factor, enriched whey protein /day or casein or PBS only from days 4 to 9 *post-utero* and concurrently received ovoalbumin (900 μg) via oral gavage on days four and	Following a second exposure to ovoalbumin *in vitro*, whey protein treatment down-regulated splenic cell and mesenteric lymph cell proliferative responses to ovalbumin as compared to casein control. TGF-β secretion was increased before and

		five of life. Sacrificed on day 10 to collect spleen cells and small intestine tissue to assess immune response to second ovoalbumin exposure.	after treatment with ovoalbumin in the whey protein group whereas intestinal MHCI (major histocompatibility complex I) expression was decreased with whey protein treatment.
Mercier *et al.* (2004)	Study of the impact of whey protein hydrolysates on in vitro non mitogen stimulated lymphocyte proliferation.	Cells were treated with whey protein enzymatic digests (2mg/mL of <10 kDa peptides) and 0.5 or 500 μg/mL of peptides fractions (mostly < 5 kDa) derived from the whey protein enzymatic digests and compared to the control BSA treatment.	Increase lymphocyte proliferation with the whey protein enzymatic digest treatments and peptide fractions.

As opposed to other studies described in Table 4, Penttila and colleagues (2001) showed a down-regulation of splenic cell and mesenteric lymph cell proliferative responses to antigen stimulation when rat pups were fed with whey proteins as opposed to phosphate buffered saline (PBS) or casein treatment. Penttila *et al.* (2001) study utilized a model system that mimics oral tolerance to proteins as rats were sensitized with ovoalbumin and had a second exposure to this antigen. This latter approach differs from studies showing an upregulation of immune responsiveness with whey protein treatment as the other studies examined only the initial sensitization of the immune response to antigens or mitogens as opposed to the immune response that proceeds following a secondary antigenic exposure. As TGF-β is a cytokine involved in the downregulation of the immune response to antigens, the authors suggested that the observed increase in TGF-β secretion by the whey protein-treated immune cells is due to a mechanism by which whey protein could down-regulate the immune system and thus also decrease excessive inflammatory responses observed in certain disease states.

Based on the above findings, it could be suggested that peptides present in whey protein hydrolysates could have immunomodulatory properties that could also conceivably play a role to down-regulate inflammation in diseases that present an excessive inflammatory response such as CF.

2.6 Bioactive Bovine Milk Peptides

The two major factors in determining protein quality are the amino acid profile and protein digestibility. Although the amino acid profile is important in evaluating the nutritive quality of a protein, the digestibility of that protein is the primary determinant of the bioavailability of its amino acids and peptides and its nutritive value (Hsu *et al.*, 1977). Digestibility involves protein hydrolysis, which cleaves proteins into peptides and amino acids via proteolytic enzymes. Approximately 30-50% of dietary nitrogen is absorbed in the form of small peptides (Roberts and Zaloga, 1994) via specific intestinal transport carriers for dipeptides and tripeptides (Leibach and Ganapathyand, 1996). There is also transport of specific peptides of larger molecular weights via pinocytosis or through paracellular channels (Walker *et al.*, 1976).

Bioactive peptides or functional peptides can be defined as peptides derived from food that exert systemic physiological effects, or produce local effects in the gastrointestinal tract in addition to their nutritional value (Yoshikawa *et al.*, 2000). Food-derived peptides may thus act as bioactive ingredients in functional foods and nutraceuticals (Clare and Swaisgood, 2000). On the other hand, most major bovine milk proteins have relatively little bioactivity in their native state. These concepts are important because bioactive peptides are inactive within the sequence of parent protein and can only be made available when released during gastrointestinal digestion or through specific food processing (Korhonen and Pihlanto, 2003). Proteolytic digestion of bovine milk proteins is a major source of a wide variety of food-derived bioactive peptides although many plant and animal proteins also contain potential bioactive sequences (Dziuba *et al.*, 1999).

Bioactive peptides can contain 2 to 20 amino acid residues per molecule and numerous peptides present in bovine milk proteins have been found to have specific bioactivities that include opiate, mineral binding, immunomodulatory, ACE (angiotensin I converting enzyme) inhibitory, anti-thrombotic and antimicrobial effects (Clare and Swaisgood, 2000; Korhonen and Pihlanto, 2003) (Table 5). Certain regions in the primary amino acid sequence of milk proteins contain overlapping sequences of multifunctional bioactive peptides that have been categorized as 'strategic zones', which are partially protected from proteolytic breakdown (Meisel and Bockelmann, 1999). Bioactive peptides contain a partial or total resistance to enzymatic hydrolysis and due to their low molecular size they can be absorbed intact to produce biological effects. The potency of the food-derived peptides, however, decreases as the chain-length increases (Roberts *et al.*, 1999). *In vitro* assays that involve the addition of proteolytic enzymes to protein substrates at the optimum incubation conditions of the enzymes are considered to be reliable approaches for the estimation of true protein digestibility and thus the release of food peptides from the parent protein (FAO/WHO, 1991).

Digestion of proteins begins in the stomach via the action of endopeptidase pepsin, which has broad substrate specificity and an optimal pH in the range of 1-2 (Charman *et al.*, 1997). In the more alkaline pH of the small intestine, the polypeptides are further cleaved by the pancreatic proteases such as trypsin, chymotrypsin, elastase, and carboxypeptidases A and B, which have an optimal activity in the range of 7-8. The resulting digestate contains primarily oligopeptides as well as free amino acids. The free amino acids are absorbed via specific amino acid transport systems across the brush border membrane. Peptide transport is favoured as small peptides are absorbed more rapidly than free amino acids (Webb, 1990). At the brush border membrane, the oligopeptides are further cleaved by amino-, di-, and endopeptidases, resulting in a mixture of amino acids and small peptides, which can be absorbed by the enterocytes. The oligopeptides undergo further hydrolysis via a variety of brush border peptidases including

aminopeptidases, endopeptidases and dipeptidases, resulting in products consisting primarily of free amino acids and di- and tripeptides (Ganapathy and Leibach, 1999).

There is increasing evidence that significant amounts of small molecular weight peptides can avoid digestion to amino acids to enter the circulation in intact form (Vermeirssen *et al.*, 2004). The amino acid composition of the peptides play a role in their resistance to digestive enzyme as proline- and hydroxyproline-containing peptides have been shown to be resistant to degradative action by digestive enzymes. In that regard, C-terminal proline-proline tripeptides are resistant to proline-specific peptidases (FitzGerald and Meisel, 2000). Peptide transporters are present in the intestinal basolateral membrane that can allow entry of hydrolysis-resistant small peptides into the portal circulation from the enterocyte (Gardner, 1984). Passage of intact peptides across the intestinal mucosa can also occur via other mechanisms such as paracellular routes for large water-soluble peptides via the tight junctions between cells and transcellular routes for highly lipid-soluble peptides. Thus, the molecular size and structural properties of peptides, such as hydrophobicity, can determine their major transport route (Shimizu *et al.*, 1997). After reaching the systemic circulation, however, peptides are exposed to peptidase enzymes, which can limit the half-life of certain peptides in plasma to the order of one minute (Vermeirssen *et al.*, 2004).

It is important to acknowledge, however, that the component responsible for a specific biologic effect of a protein is not always clear as demonstrated by the *in vivo* and in *vitro* studies shown in Tables 3 and 4. Whey proteins have been recognized as an important source of biologically active peptides from bovine milk (Shah, 2000; Yoshikawa *et al.*, 2000; Meisel and Fitzgerald, 2003). It is also possible; however, that incomplete protein digestion may not lead to release of bioactive peptides thereby making the peptides unavailable for absorption in the gut. Alternatively or in addition, inefficient digestion may release bioactive peptides in insufficient amounts to exert a biologic function when such proteins

are ingested *in natura*. In that regard, both *in vivo* and *in vitro* digestibility studies have shown that bovine whey proteins, especially α-LA and β-LG, are not readily digested in the stomach (Kitabatake and Kinekawa, 1998; Mutilangi *et al.*, 1995; Kinekawa and Kitabatake, 1996; Qiao *et al.*, 2004; Pantako *et al.*, 2001). Moreover, in the case of β-LG, even following prolonged contact with pancreatic enzymes, there is incomplete digestion of this protein in the lumen (Reddy *et al.*, 1988). This resistance of these whey proteins to digestion has been associated with their globular conformation as well as the simultaneous presence of disulfide bridges and free thiol groups in β-LG, which gives a rigid structure to this protein (Pantako *et al.*, 2001; Schimdt and Poll, 1991). Most peptide bonds are located in the interior of the globular proteins making them inaccessible to digestive enzymes that need to bind to their substrates to perform proteolysis (Adler-Nissen, 1997). The inability to completely digest bovine α-LA and β-LG make them potential food allergens and enzymatic hydrolysis been used to decrease the allergenicity of whey proteins used in infant formulas (Cordle, 1994; Peng *et al.*, 2004). Commercial enzymatic hydrolysis treatment is used to break down milk proteins into free amino acids and small peptides so that intact proteins will no longer be present in the lumen and thus the interaction with antibody binding site regions will not occur (Cordle, 1994; Peng *et al.*, 2004). Different techniques have been used to increase bovine milk protein digestibility in order to increase protein hydrolysis and thereby obtain more bioactive peptides. Heat treatment has been used to increase susceptibility of milk proteins to proteolysis as it alters the conformation of the proteins to provide greater exposure of the peptide bonds to digestive enzymes. As a consequence, further protein digestion is facilitated and more peptides are available for absorption (Reddy *et al.*, 1988).

Table 5. Bioactive peptides isolated from bovine milk proteins

Parent protein	Peptide	Bioactivity
α-, β-casein	Casomorphins	Opioid agonist
	Casokinins	ACE inhibitory
	Phosphopeptides	Mineral binding
α-, β-casein, lactoferrin	Immunopeptides	Immunomodulatory
κ-casein	Casoxins	Opioid antagonist
	Casoplatelins	Antithrombotic
κ-casein hydrolysate	Glycomacropeptides	Anti-stress
α_{s2}-casein	Casocidin	Antimicrobial
α_{s1}-casein	Isracidin	Antimicrobial
α-LA, β-LG	Lactorphins	Opioid agonist
α-LA	Lactokinin	ACE inhibitory
BSA	Serorphin	Opioid agonist
β-LG, BSA	Immunopeptides	Immunomodulatory
Lactoferrin	Lactoferroxins	Opioid antagonist
	Lactoferricin	Antimicrobial

ACE: angiotensin I converting enzyme. Opioid effects may include: prolonged gastrointestinal time, antidiarrheal action by electrolyte absorption, possible stimulation of insulin and somatostatin, sleep induction, respiratory depression, hypotension, bradycardia and mood influence.

Adapted from: Korhonen and Pihlanto (2005) and Modler (2000)

Higher activity of caseinophosphopeptides (CPPs) has been obtained when casein is successively digested with pepsin and trypsin as opposed to acid-precipitated casein or casein exposed to tryptic digestion alone. The systems used to separate and concentrate the peptides can also differ and thus affect the profile of peptides produced. For example, peptides with molecular weights < 1 kDa produced by

selective membrane ultrafiltration of hydrolysed α-LA or β-LG exhibit greater ACE-inhibitory activity than other higher molecular weight fractions (Korhonen and Pihlanto, 2003). In that respect, it is important to note that only low molecular weight peptides (lower than 1 kDa) can be readily absorbed into the bloodstream (Qiao *et al.*, 2004; Pantako *et al.*, 2001). As described above, it is important to emphasize that the effect observed *in vitro* does not always imply the same effect *in vivo*, mainly due to the bioavailability of these peptides after oral ingestion. In the case of the ACE inhibitory peptides, they have to reach the cardiovascular system in an active form after overcoming gastrointestinal digestion, intestinal absorption, enterocyte, and serum degradation (Vermeirssen *et al.*, 2004). It should be noted that studies involving human adults have demonstrated the appearance of bioactive peptides in the stomach, small intestine and blood following ingestion of either milk or yoghurt (Vermeirssen *et al.*, 2004). For example, smaller peptides derived from casein and lactoferrin have been recovered from the small intestine. Moroever, casein glycomacropeptide fragments called casoplatelines are released during gastrointestinal digestion and absorbed intact into the blood, supporting their putative *in vivo* anti-thrombotic effects (Vermeirssen *et al.*, 2004).

Although an important aspect to study, *in vivo* digestion of proteins to assess the bioavailability of peptides is limited by the high cost and technical difficulties of such analyses. Hence, *in vitro* digestibility tests are preferred as the initial step as they offer a rapid and simple way to identify potential bioactive components. A great variety of *in vitro* digestion tests have been reported in the literature (Multilangi *et al.*, 1995; Kitabatake and Kinekawa, 1998; Vermeirssen *et al.*, 2003). The differences among *in vitro* assays involve such factors as the type of proteolytic enzymes and incubation conditions used and the methods involved to recover and define specific peptide by-products. Several food products contain peptides identified via *in vitro* digestibility assays and subsequently these peptides were demonstrated to reach the systemic circulation to exert bioactive effects such as anti-hypertensive action (Vermeirssen *et al.*, 2004).

2.7 Pressurized Whey Proteins: a New Perspective

An approach used to improve whey protein digestibility is heat treatment. Heat treatment is also used as a sterilization method and it is one of the most important processes involved in the industrial processing of milk and dairy products. The use of temperature at 80°C for 1 h has been shown to denature and change the conformation of proteins such as β-LG by disrupting the hydrophobic interactions that are the main molecular force holding monomers together. This approach has been shown to facilitate increased *in vitro* and *in vivo* proteolysis of β-LG (Mutilangui *et al.*, 1995; Kitabataki and Kinekawa, 1998). In the case of infants, however, heat treatment of milk does not seem to result in increased *in vivo* digestion efficiency, possibly due to the fact that the conditions that prevail in the stomach during infancy are not optimal for pepsin activity (pH 3-4) (Alting *et al.*, 1997; Sakai *et al.*, 2000). As discussed above, extensive enzymatic hydrolyses *in vitro* is needed to generate whey protein hydrolysates for use in infant formulas. Also, some studies have indicated that at temperatures higher than 75-80°C or at prolonged heating times, covalent intermolecular cross-links are generated as a result of disulphide exchange reactions leading to the formation of whey protein aggregates that are poorly digestible (Hurley *et al.*, 1993; Carbonaro *et al.*, 1998). Such aggregates were also noted at the temperatures used in UHT-treatment and sterilization of milk and the *in vitro* protein digestibility of total whey extracts of heat treated milks was increasingly decreased with greater intensity of milk heat treatment (Carbonaro *et al.*, 1998). Disulphides in protein structure are well known to decrease the susceptibility to proteolysis, which can be improved in β-LG and other proteins by the chemical cleavage of disulphide bonds (Reddy *et al.*, 1988). Heat treatment could also have unfavourable effects relative to WPI bioactivity as Bounous and Gold (1991) indicated that undenatured WPI (Immunocal™) isolated under low temperature conditions showed higher GSH biosynthesis and immune modulation as compared to heat-denatured WPI. Other major disadvantages of heat treatment include the thermal degradation and losses

of heat sensitive components such as vitamins, pigments, color, and aromas (Noguchi, 2000).

High pressure processing, known also as high hydrostatic or hyperbaric pressure processing, is the process by which a food protein in either liquid or solid state is subjected to pressures of several hundred MPa. Similar to thermal treatment, high pressure treatment can also denature proteins and kill microorganisms with the additional advantages of maintenance of taste and flavour characteristics as this process occurs at low temperatures without any physical damage (Noguchi, 2000). In addition to the pressure level, i.e., the degree of pressure applied to a certain food, two modes of pressure can be used by the food industry: static (pressure hold) or dynamic (pressure pulse). The number of pulses used refers to successive series of pressurization and depressurization, and the holding time refers to the length of time a given food is kept under a certain amount of pressure before depressurization (Heremans, 1997). The effects of high pressure on proteins have shown that low-pressure treatment can induce reversible changes such as dissociation of protein-protein complexes, the binding of ligands, and protein conformational changes. In general, protein denaturation occurs when the pressures are higher than about 500 MPa, which is, in most cases, irreversible (Heremans, 1997).

It is difficult to establish an overall standard pressurization condition to achieve protein denaturation since the sensitivity of different proteins to high pressure varies and also because different conditions used during high-pressure treatment such as temperature and pH can also change the protein response to the hyperbaric treatment. In the case of whey proteins, β-LG seems to be more sensitive towards pressure than BSA and α-LA. It appears that differences in the secondary structure and differences in the number of disulphide bonds in whey proteins play an important role in terms of sensitivity of the protein to pressure treatment. β-LG contains only two disulphide bonds whereas α-LA is stabilized by four disulphide bonds and BSA by 17 disulphide bonds (Heremans, 1997;

Tanaka and Kunugi, 1996). Additionally, although β-LG has fewer disulphide bonds than the monomeric protein α-LA, it has one –SH group per monomer, which accounts for approximately 90% of the free –SH groups in milk (Modler, 2000). Tanaka *et al.* (1996a) showed that, at neutral pH and dansylated (prepared at atmospheric pressure), α-LA structural changes were reversible up to pressures of 400 MPa whereas β-LG lost reversibility of structural changes at 150 MPa or lower. In another study, Tanaka *et al.* (1996b) showed that 400 MPa pressure treatment for 1 h induced the denaturation of β-LG and exposed its single unpaired buried –SH group to the protein surface. They verified this finding, by treating the protein with *N*-ethylmaleimide (NEM), an agent which chemically modifies –SH, and analysing the ability of the –SH group to react with 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB). The authors concluded that exposure of –SH by pressurization makes the thiol group more reactive and dimerization through intermolecular reaction of –SH occurs.

High pressure is one of the most successful techniques that have been used to non-thermally sterilize foods; however, the nutritional implications have received much less attention than those relevant to processing for microbiological safety and preservation. Whether important nutritional implications will arise from new combination uses of physical, natural and conventional food processing techniques is still relatively unexplored (Gould, 2001).

Dr. Kubow and collaborators have produced a series of animal studies testing the bioactivity of whey proteins as affected by pressure processing (Kubow *et al.*, 2005). The initial studies utilized whey proteins treated by repeated pulse cycling, which caused changes in protein conformation leading to altered exposure of –SH groups to DTNB (Hosseini-nia, 2000). Hyperbaric pressure treatment of WP was presumed to decrease the surface polarity of the molecule thereby exposing the free sulfhydryl groups in the hydrophobic regions to the polar environment. They analysed the nutritional, antioxidative and antigenic effects of feeding the WPI subjected to repeated pulse cycling to newly weaned male rats. Briefly, WPI was

submitted to a pressure of 400 MPa in a combination of two pulse cycles plus 10 min holding time followed by an additional pulse cycle at 25°C (i.e., a total of 3 cycles). The rats were fed semi-purified diets containing either pressurized WPI (pWPI) or native WPI at a concentration of 20 wt% for either 17 or 35 days. The pWPI fed animals had decreased serum IgG and IgE, decreased levels of plasma thiobarbituric acid reactive substances (TBARS), increase hepatic GSH concentrations, greater weight gain and feed efficiency ratios when compared with the controls fed native WPI (Hosseini-nia, 2000). It was suggested that the enhanced weight gain and tissue GSH concentrations could be due to an increase in the digestibility of the pWPI; however, effect of pressurization on protein digestibility was not tested.

Recent electrospray ionization-mass spectrometry (ESI-MS) studies on whey proteins, however, have shown that holding time and pressure levels can be modified to achieve comparable results between pressures exceeding 500 MPa holding time and multiple cycle pressurization at 400 MPa (Alvarez, 2004). In these studies, high hydrostatic pressure treatment of whey proteins resulted in changes in the tertiary structure allowing groups buried in the hydrophobic core of the proteins that were previously inaccessible to the solvent to be charged thereby increasing the charge-state-distribution. Thus, pressure treatment of InPro™ showed that 550 MPa treatment without holding time and 3-cycle pressurization at 400 MPa produced similar partial unfolding of β-LG, allowing more groups within the interior of the protein to be susceptible to solvation. In addition, the changes in the charge-state-distribution following pressurization were greater for β-LG in InPro™ (Innovatech Inc.) than those observed for the β-LG molecule in BiPro™ (Davisco Inc.). The findings thus indicated that high hydrostatic pressure induced changes in the tertiary structure of whey proteins that were indicative of a relaxation of the native structure of the protein molecules, which could improve the digestibility of the proteins.

Jing (2005) compared the effect of pWPI *in vivo* using two different modes of treatment: the 3-cycle pressure treatment as described above and a 1-cycle pressurization using 550 MPa without holding time. Newly weaned male Sprague Dawley rats were fed a diet containing native WPI, 1-cycle or 3-cycle pWPI for 40 days. The 1-cycle pressure treatment group showed significantly greater growth than the group fed native whey protein suggesting that 1-cycle pressure treatment could be used to increase feed efficiency. No differences, however, were observed among the dietary groups in terms of tissue lipid peroxidation indices, liver peroxides, or tissue GSH concentrations, which conflicts with the results of Hosseini-nia (2000). These contradictory findings could be a result of the different commercial sources of the native WPI used in the two studies as Hosseini-nia (2000) used Provon 190™ (Glambia Inc.) whereas InPro™ (Innovatech Inc.) was used in the study of Jing (2005). Many variables associated with commercial WPI production could have led to the differential impact of pressurization in these two animal feeding trials. Functional effects of WPI can be affected by factors such as the choice of isolation processes (membrane filtration or ion exchange) and spray drying techniques used by the manufacturer, whether acid or rennet coagulation was used, as well as batch to batch differences (Hurley *et al.*, 1990). In support of this contention, Jing (2005) noted that mice fed 1-cycle pressurized InPro™ showed a higher feed efficiency ratio, apparent nitrogen digestibility and muscle GSH content as compared to mice fed 1-cycle pressurized BiPro™. These two commercial sources of WPI have major differences in protein profiles despite similar sulfur amino acid levels, such as the cysteine and methionine content. For example, the β-LG content of BiPro™ is nearly double (80%) the β-LG concentration noted in InPro™ (48%). On the other hand, the content of glycomacropeptides may reach up to 20% of the total protein content of InPro™, which is produced via cross-flow microfiltration. BiPro™ whey protein isolate is produced using an ion exchange resin, which leads to a much lower content of glycomacropeptides. Also, as noted above, the β-LG in InPro™ is more susceptible to the tertiary structural changes induced by 1-cycle pressurization than BiPro™.

In another study, following lung infection with *P. aeruginosa* as a model of lung infection in CF, mice fed with pWPI (InPro^{TM}) produced via 3-cycle pressure treatment had a significantly lower weight loss and mortality relative to mice fed either native WPI or casein (Hosseini-nia *et al.*, 2002). Similar to the findings of Jing (2005), uninfected mice fed pWPI demonstrated no improvement in tissue GSH content as compared to native WPI feeding when InPro^{TM} was used as the commercial source of WPI; however, *P. aeruginosa*-infected mice fed pWPI did show higher pulmonary levels of GSH when compared to infected mice fed either native WPI or casein fed mice. These findings thus suggest that pWPI using InPro^{TM} could be an effective protein source to diminish infective and inflammatory processes associated with *P. aeruginosa* infection in CF.

The research to date presents potentially interesting applications for the development of new functional foods based on pressure treatment of WPI to produce products with more potent nutritional and nutraceutical properties. More studies, however, are needed to confirm the antioxidant and immunomodulatory potential of pWPI. It is also conceivable that pWPI would be more accessible to digestive enzymes, which would allow for a more rapid proteolysis of the proteins and an increased or altered release of small bioactive peptides. The altered profile of peptides released from WP digestion could thus play a role in the modulation of the immune response and tissue GSH concentrations.

References

Abbas A. K., Lichtman A. H., Pober J. S. 2000. *Cellular and Molecular Immunology*. 4th Ed. Philadelphia: W.B. Saunders Company. pp. 1-553.

Alder-Nissen J. 1997. Enzymatic hydrolysis of food proteins. *Process Biochemistry*. 12: 18-24.

Alting A. C., Meijer R. J., van Beresteijn E. C. 1997. Incomplete elimination of the ABBOS epitope of bovine serum albumin under simulated gastrointestinal conditions of infants. *Diabetes Care.* 20: 875-80.

Alvarez P. 2004. Effect of applied hydrostatic pressure on the structure and rheological properties of whey proteins. M.Sc. Thesis, McGill University, Montreal, QC, Canada.

Andrieu-Abadie N., Gouazé V., Salvayre R., Levade T. 2001. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radical Biology and Medicine.* 31: 717-728.

Ariga T., Jarvis W. D., Yu R. K. 1998. Role of sphingolipid-mediated cell death in neurodegenerative diseases. *Journal of Lipid Research.* 39: 1-16.

Back E., Frindt C., Nohr D., Frank J., Ziebach R., Stern M., Ranke M., Biesalski H. K. 2004. Antioxidant deficiency in cystic fibrosis: when is the right time to take action? *American Journal of Clinical Nutrition.* 80: 374-384.

Baeuerle P. A. Reactive oxygen species as costimulatory signals of cytokine-induced NF-κB activation pathways. In: Chandan K. S., Sies H., Baeuerle P. A. 2000. *Antioxidant and Redox Regulation of Genes.* San Diego: Academic Press. pp. 181-201.

Balagtas J. V., Hutchinson F. M., Krochta J. M., Summer D. A. 2003. Anticipating market effects of new uses for whey and evaluating returns to research and development. *Journal of Dairy Science.* 86: 1662-1672.

Bals R., Weiner D. J., Wilson J. M. 1999. The innate immune system in cystic fibrosis lung disease. *The Journal of Clinical Investigation.* 103: 303-307.

Baruchel S., Wang T., Farah R., Jamali M., Batist G. 1995. *In vivo* selective modulation of tissue glutathione in a rat mammary carcinoma model. *Biochemical Pharmacology*. 50: 1505-1508.

Baruchel S., Viau G. 1996. *In vitro* selective modulation of cellular glutathione by a humanized native milk protein isolate in normal cells and rat mammary carcinoma model. *Anticancer Research*. 16: 1095-1100.

Becker M. N., Sauer M. S., Muhlebach M. S., Hirsh A. J., Wu Q, Verghese M. W., Randell S. H. 2004. Cytokine secretion by cystic fibrosis airway epithelial cells. *American Journal Respiratory and Critical Care Medicine.* 169: 645-653.

Bonfield T., Konstan M. W., Berger M. 1999. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *Journal of Allergy and Clinical Immunology.* 104: 72-78.

Boucher R. C. 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. *European Respiratory Journal.* 23: 146-158.

Bounous G., Batist G., Gold P. 1989. Immunoenhancing property of dietary whey protein in mice: role of glutathione. *Clinical Investigation Medicine.* 12: 154-161.

Bounous G., Gervais F., Amer V., Batist G., Gold P. 1989. The influence of dietary whey protein on tissue glutathione and the diseases of aging. *Clinical Investigation Medicine.* 12: 343-349.

Bounous G., Gold P. 1991. The biological activity of undenaturated dietary whey proteins: role of glutathione. *Clinical Investigation Medicine.* 14: 296-309.

Bounous G., Baruchel S., Fahetz J., Gold P. 1993. Whey proteins as a food supplement in HIV-seropositive individuals. *Clinical and Investigatigative Medicine*. 16: 204-209.

Bronstein M. N., Sokol R. J., Abman S. H., Chatfield B. A., Hammond K. B., Hornbridge K. M. 1992. Pancreatic insufficiency, growth and nutrition in infants identified by newborn screening as having cystic fibrosis. *The Journal of Pediatrics*. 120:533-540.

Canadian Cystic Fibrosis Foundation. 2005. *About cystic fibrosis: What is cystic fibrosis?* (Online). http://www.ccff.ca/page.asp?id=1. August, 28.

Carbonaro M., Bonomi F., Iametti S., Cappelloni M., Carnovale E. 1998. Aggregation of proteins in whey from raw and heat-processed milk: Formation of soluble macroaggregates and nutritional consequences. *Lebensmittel-Wissenschaft and Technologie.* 31: 522-529.

Chan C., Goldkorn T. 2000. Ceramide path in human lung cell death. *American Journal of Respiratory Cell and Molecular Biology.* 22: 460-468.

Charman W. N., Porter C. J. H., Mithani S., Dressman J. B. 1997. Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. *Journal of Pharmaceutical Sciences.* 86: 269-282.

Clare, D. A., Swaisgood, H. E. 2000. Bioactive milk peptides: A prospectus. *Journal of Dairy Science.* 83: 1187-1195.

Conese M., Copreni E., Di Gioia S., De Rinaldis P., Fumarulo R. 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *Journal of Cystic Fibrosis*. 2: 129-135.

Cordle T. C. 1994. Control of food allergies using protein hydrolysates. *Food Technology*. 48: 72-76.

Cross M. L., Gill H. S. 1999. Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology*. 77: 345-350.

Cuello M., Coats A. O., Darko I., Ettenberg S. A., Gardner G. J., Nau M. M., Liu J. R. Birrer M. J., Lipkowitz S. 2004. *N*-(4-hydroxyphenyl)retinamide(4HPR) enhances TRAIL-mediated apoptosis through enhancement of a mitochondrial-dependent amplification loop in ovarian cancer cell lines. *Cell Death and Differentiation*. 11: 527-541.

Cystic Fibrosis Foundation. 2005. *Persons with cystic fibrosis: About cystic fibrosis.* (Online). http://www.cff.org/about_cf/what_is_cf/. August, 28.

Day B. J., van Heeckeren A. M., Min E., Velsor L. W. 2004. Role for cystic fibrosis transmembane conductance regulator protein in a glutathione response to bronchopulmonary *Pseudomonas* infection. *Infection and Immunity*. 72: 2045-2051.

DeLeve L., Kaplowitz N. 1991. Glutathione metabolism and its role in hepatotoxicity. *Pharmacology and Therapeutics*. 52: 287-305

Dinwiddie R. 2000. Pathogenesis of lung disease in cystic fibrosis. *Respiration*. 67: 3-8.

Dziuba J., Minkiewicz P., Nalecz D. 1999. Biologically active peptides from plant and animal proteins. *Polish Journal of Food and Nutritional Science*. 8: 3-16.

Eason R .R., Velarde M. C., Chatman L. Jr., Till S. R., Geng Y., Ferguson M., Badger T. M., Simmen R. C. 2004. Dietary exposure to whey proteins alters rat

mammary gland proliferation, apoptosis, and gene expression during postnatal development. *Journal of Nutrition.* 134: 3370-3377.

Eigel W. N., Butler J. E., Ernstrom C. A., Farrell H. M., Harwalkar V. R., Jenness R., Whitney R. M. 1984. Nomenclature of proteins of cow's milk: fifth revision. *Journal of Dairy Science.* 67: 1599-1631.

Erdreich-Epstein A., Tran L. B., Bowman N. N., Wang H., Cabot M. C., Durden D. L., Vlckova J., Reynolds C. P., Stins, M. F., Groshen S., Millard M. 2002. Ceramide in fenretinide-induced endothelial cell apoptosis. *Journal of Biological Chemistry.* 277: 49531-49537.

FAO/WHO. 1991. Protein quality evaluation. Report of Joint FAO/WHO Expert Consultation. Food and Agriculture Organization of United Nation, Rome, Italy, pp. 1–34.

Ferrari N., Morini M., Pfeffer U., Minghelli S., Noonan D. M., Albini A. 2003. Inhibition of Kaposi's sarcoma in vivo by fenretinide. *Clinical Cancer Research.* 9: 6020-6029.

Fitzgerald R. J., Meisel H. 2000. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition.* 84: S33-S37.

FitzGerald R. J., Murray B. A., Walsh D. J. 2004. Hypotensive peptides from milk proteins. *Journal of Nutrition.* 134: 980S-988S.

Florisa R., Recio I., Berkhout B., Visser S. 2003. Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Current Pharmaceutical Design.* 9: 1257-1275.

Ford T. F., Wong C. W., Colditz I. G. 2001. Effects of dietary protein types on immune responses and levels of infection with *Eimeria vermiformis* in mice. *Immunology and Cell Biology.* 79: 23-28.

Galietta L. J. V., Moran O. 2004. Identification of CFTR activators and inhibitors: chance or design? *Current Opinion in Pharmacology.* 4: 497-503.

Ganapathy V., Leibach F. H. Protein digestion and assimilation. In: Yamada T. 1999. *Textbook of Gastroenterology.* Philadelphia: Lippincott Williams and Wilkins. pp. 456-467.

Garaventa A., Luksch R., Lo Piccolo M. S., Cavadini E., Montaldo P. G., Pizzitola M. R., Boni L., Ponzoni M., Decensi A., De Bernardi B., Bellani F. F., Formelli F. 2003. Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. *Clinical Cancer Research.* 9: 2032-2039.

Gao L., Kim K. J., Yankaskas J. R., Forman H. J. 1999. Abnormal glutathione transport in cystic fibrosis airway epithelia. *American Journal of Physiology.* 277: L113-18.

Gardner M. L.G. 1984. Intestinal assimilation of intact peptides and proteins from the diet – a neglected field? *Biological Reviews.* 59: 289–331.

Gill H. S., Doull F., Rutherfurd K. J., Cross M. L. 2000. Immunoregulatory peptides in bovine milk. *British Journal of Nutrition.* 84: S111-117.

Goss C. H., Rosenfeld M. 2004. Update on cystic fibrosis epidemiology. *Current Opinion in Pulmonary Medicine.* 10: 510-514.

Gould G. W. 2001. Symposium on nutritional effects of new processing technologies: an overview. *Proceedings of the Nutrition Society.* 60: 463-474.

Grey V., Mohammed S. R., Smountas A. A., Bahlool R., Lands L. C. 2003. Improved glutathione status in young adult patients with cystic fibrosis supplemented with whey proteins. *Journal of Cystic Fibrosis*. 2: 195-198.

Griese M., Ramakers J., Krasselt A., Starosta V., von Konisngsbruggen S., Fischer F. R., Müllinger B., Huber R. M., Maier K., Rietschel E., Scheuch G. 2004. Improvement of alveolar glutathione and lung function but not oxidative state in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*. 169: 822-828.

Groff J. L., Gropper S. S. 2000. *Advanced Nutrition and Human Metabolism.* Belmont: West/Wadsworth. pp. 1-584.

Hakkak R., Korourian S., Ronis M. J. J., Johnston J. M., Badger T. M. 2001. Dietary whey protein protects against azoxymethane-induced colon tumors in male rats. *Cancer Epidemiology Biomarkers and Prevention*. 10: 555-558.

Hambraeus, L. Proprietary milk versus human breast milk in infant feeding. 1997. *Pediatric Clinics of North America*. 24: 17-36.

Hannun Y. A., Obei L. M. 2002. The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *The Journal of Biological Chemistry*. 277: 25847-25850.

Hanson L. A., Korotkova M., Telemo E. 2003. Breast-feeding, infant formulas, and the immune system. *Annals of Allergy, Asthma and Immunology*. 90: 59-63.

Hartl D., Starosta V., Maier K., Beck-Speier C., Rebhan R. M., Rietschel E., Krauss-Etschmann S., Griese M. 2005. Inhaled glutathione decreases PGE_2 and increases lymphocytes in cystic fibrosis lungs. *Free Radical Biology and Medicine*. 39: 463-472.

Heremans K. High pressure effects on proteins. In: Damodaran S., Paraf A. 1997. *Food proteins and their applications*. New York: Marcel Dekker, Inc. pp. 473-502.

Holt C. 1985. The milk salts: their secretion, concentrations and physical chemistry. In: Developments in Dairy Chemistry. London: Applied Science Publishers. pp. 143-181.

Hosseini-nia T. 2000. Structural and nutritional properties of whey proteins as affected by hyperbaric treatment. PhD. Thesis. McGill University, Montreal, QC, Canada.

Hosseini-nia T., Kubow S., Grey V., Mohammed S., Bahlool R., Lands L. C. 2002. Inhibition of *Pseudomonas aeroginosa*-mediated mortality and weight loss via ingestion of whey treated by hyperbaric pressure. *Pediatric Pulmonology.* Suppl. 24: A283.

Hsu H. W., Vavak D. L., Satterlee L. D., Miller G. A. 1977. A multienzyme technique for estimating protein digestibility. *Journal of Food Science.* 42: 1269-1273.

Hurley W. L., Ventling B. L., Ma M., Larson B. L. 1990. Variability in physicochemical properties of dried wheys from commercial sources. *Journal of Food Quality.* 13: 119–127.

Hurley W. L., Zou S., Magura C. E., Jimenez-Flores R. 1993. Heating skim milk alters the migration of immunoreactive milk proteins in acrylamide gels. *Journal of Agricultural and Food Chemistry*. 41: 280-282.

Jing Y. 2005. Effects of pressurization on the digestibility and glutathione inducing property of whey protein isolates in rats and mice. M.Sc. Thesis. McGill University, Montreal, QC, Canada.

Jungas T., Motta I., Duffieux F., Fanen P., Stoven V., Ojcius D. M. 2002. Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry.* 277: 27912-27918.

Kammouni W., Figarella C., Marchand S., Merten M. 1997. Altered cytokine production by cystic fibrosis tracheal gland serous cells. *Infection and Immunity.* 65: 5176-5183.

Kang Y-J., Enger M. D. 1991. Increased glutathione levels in quiescent, serum-stimulated NRK-49F cells are associated not with a response to growth factors but with nutrient repletion. *Journal of Cellular Physiology.* 148: 197-201.

Kennedy M. J. 2001. Inflammation and cystic fibrosis pulmonary disease. *Pharmacotherapy.* 21: 593-603.

Kent K. D., Harper W. J., Bomser J. A. 2003. Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. *Toxicology in Vitro.* 17: 27-33.

Kennedy M. J. 2001. Inflammation and cystic fibrosis pulmonary disease. *Pharmacotherapy.* 21: 593-603.

Kennedy R. S., Konok G. P., Bounous G., Baruchel S., Lee T. D. G. 1995. The use of whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I-II clinical study. *Anticancer Research.* 15: 2643-2650.

Kilara A., Panyam D. 2003. Peptides from milk proteins and their properties. *Critical Reviews in Food Science and Nutrition*. 43: 607-633.

Kinekawa Y., Kitabatake N. 1996. Purification of β-lactoglobulin from whey protein concentrate by pepsin treatment. *Journal of Dairy Science*.79: 350-356.

Kitabatake N., Kinekawa Y. 1998. Digestibility of bovine milk whey protein and β-lactoglobulin *in vitro* and *in vivo*. *Journal of Agricultural and Food Chemistry*. 46: 4917- 4923.

Knowles M. R., Boucher R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways ("Perspective"). *Journal of Clinical Investigation*. 109: 571-577.

Koehler D. R., Downey G. P., Sweezey N. B., Tanswell A. K., Hu J. 2004. Lung inflammation as a therapeutic target in cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology*. 31: 377-381.

Kogan I., Ramjeesingh M., Li C., Kid J. F., Wang Y., Leslie E. M., Cole S. P. C., Bear C. E. 2003. CFTR directly mediates nucleotide-regulated glutathione efflux. *European Molecular Biology Organization*. 22: 1981-1989.

Kolesnick R. N., Krönke M. 1998. Regulation of ceramide production and apoptosis. *Annual Review of Physiology*. 60: 643-665.

Kolesnick R. 2002. The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *The Journal of Clinical Investigation*. 110: 3-8.

Konstan M. W., Byard P. J., Hoppel C. L., Davis P. B. 1995. Effect of high-dose ibuprofen in patients with cystic fibrosis. *New England Journal of Medicine*. 332: 848-854.

Korhonen H., Pihlanto A. 2003. Food-derived bioactive peptides – opportunities for designing future foods. *Current Pharmaceutical Design*. 9: 1297-1308.

Korhonen H., Pihlanto A. Milk-derived bioactive peptides: formation and prospects for health promotion. In: Shortt C. 2005. *Handbook of Functional Dairy Products*. New York: CRC Press. pp.109-124.

Kubow S., Lands L. Rohlicek C., Chan H. M., Ismail A. A., Ramaswamy H., Grey V, Alvarez P. 2005. Ultra high pressure modified proteins and uses thereof. *U.S. Patent Application*. filed October 21, 2005.

Labbok M. H., Clark D., Goldman A. S. 2004. Breastfeeding: maintaining an irreplaceable immunological resource. *Nature Reviews Immunology*. 4: 565-572.

Lagrange-Puget M., Durieu I., Ecochard R., Abas-Chorfa F., Drai J., Steghens J-P., Pacheco Y., Vtal-Durand D., Bellon G. 2004. Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. *Pediatric Pulmonology*. 38: 43- 49.

Lands L. C., Grey V. L., Grenier C. 2000. Total plasma antioxidant capacity in cystic fibrosis. *Pediatric Pulmonology*. 29: 81-87.

Lands L. C., Grey V., Smountas A. A. 1999a. Lymphocyte glutathione levels in children with cystic fibrosis. *Chest*. 116: 1-9. *Erratum in: Chest*. 2000. 117: 296.

Lands L. C., Grey V. L., Smountas A. A. 1999b. Effect of supplementation with a cysteine donor on muscular performance. *Journal of Applied Physiology*. 87: 1381-1385.

Lavrentiadou S. N., Chan C., Kawcak T., Ravid T., Tsaba A., van der Vliet A., Rasooly R., Goldkorn T. 2001. Ceramide-mediated apoptosis in lung epithelial

cells is regulated by glutathione. *American Journal of Respiratory Cell and Molecular Biology*. 25: 676-684.

Lee C. K., Piedrahita J. A. 2002. Inhibition of apoptosis in serum starved porcine embryonic fibroblasts. *Molecular and Reproductive Development.* 62: 106-12.

Levade T., Jaffrezou J. P. 1999. Signalling sphingomyelinases: which, where, how and why? *Biochimica et Biophysica Acta.* 1438: 1-17.

Levade T., Malagarie-Cazenave S., Gouazé V., Ségui B., Tardy C., Betito S., Andrieu-Abadie N., Cuvillier O. 2002. Ceramide in apoptosis: a revisited role. *Neurochemical Research*. 27: 601-607.

Leibach F. H., Ganapathy V. 1996. Peptide transporters in the intestine and the kidney. *Annual Review of Nutrition.* 16: 99-119.

Li L., Clark K., Lau B. H. S. 1994. Thymic peptides increase glutathione level and glutathione disulfide reductase activity in vascular endothelial cells. *Biotechnology Therapeutics*. 51: 87-97.

Linden G., Lorient D. New ingredients in food industry: biochemistry and agriculture. In: Linden G. 1999. *The Dairy Industry*. England: Woodhead Publishing. pp. 100-120.

Lindsdell P., Hanrahan J. W. 1998. Glutathione permeability of CFTR. *The American Physiological Society*. 275: C323-326.

Liu B., Hannun Y. A. 1997. Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. *The Journal of Biological Chemistry*. 272: 16281-16287.

Liu B., Andrieu-Abadie N., Levade T., Zhang P., Obeid L .M., Hannun Y. A. 1998. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. *Journal of Biological Chemistry*. 273: 11313-11320.

Lothian B., Grey V., Kimoff R. J., Lands L. C. 2000. Treatment of obstructive airway disease with a cysteine donor protein supplement: a case report. *Chest*. 117: 914-16.

Low P. P. L., Rutherfurd K. J., Gill H. S., Cross M. L. 2003. Effect of dietary whey protein concentrate on primary and secondary antibody responses in immunized BALB/c mice. *International Immunopharmacology*. 3: 393-401.

Luberto C., Yoo D. S., Suidan H. S., Bartoli G. M., Hannun Y. A. 2000. Differential effects of sphingomyelin hydrolysis and resynthesis on the activation of NF-κB in normal and SV40-transformed human fibroblasts. *The Journal of Biological Chemistry*. 275: 1470-14766.

Mahaan Group., 2005. (Online). http://www.mahanfoods.com. April, 19.

McGrath L. T., Mallon P., Dowey L., McClean E., McDonnell M., Devine A., Copeland S., Elborn S. 1999. Oxidative stress during acute respiratory exacerbations in cystic fibrosis. *Thorax*. 54: 518-523.

Meisel, H., Bockelmann W. 1999. Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek*. 76: 207-215.

Meisel H., Fitzgerald R. 2003. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design*. 9: 1289-1295.

Meister A., Anderson M. E., Hwang O. 1986. Intracellular cysteine and glutathione delivery systems. *Journal of the American College of Nutrition.* 5: 137-51.

Meister A. 1991. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacology and Therapeutics*. 51: 155-194.

Mercier A., Sylvie F. G., Fliss I. 2004. Immunomodulating effects of whey proteins and their enzymatic digests. *International Dairy Journal*. 14: 175-183.

Micke P., Beeh K. M., Schlaak J. F., Buhl R. 2001. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *European Journal of Clinical Investigation*. 31: 171-178.

Micke P., Beeh K. M., Buhl R. 2002. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. *European Journal of Nutrition*. 41: 12-18.

Miyaji M., Jin Z-X., Yamaoka S., Amakawa R., Fukuhara S., Sato S. B., Kobayashi T., Domae T. M., Bloom E. T., Okazaki T., Umehara H. 2005. Role of membrane sphingomyelin and ceramide in platform formation for Fas-mediated apoptosis. *Journal of Internal Medicine*. 202: 249-259.

Modler H. W. Milk processing. In: Nakai S. 2000. *Food Proteins: Processing Applications*. New York: Willey-VCH. pp. 1-88.

Morr C. V., Ha E. Y. 1993. Whey protein concentrates and isolates: processing and functional properties. *Critical Reviews in Food Science and Nutrition.* 33: 431.

Muhlebach M., Noah T. L. 2002. Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine.* 165: 911-915.

Mutilangui W. A. M., Panyam D., Kilare A. 1995. Hydrolysates from proteolysis of heat-denatured whey proteins. *Journal of Food Science.* 60: 1104-1109.

Nathan C. F. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *Journal of Clinical Investigation.* 180: 1550-1560.

Noguchi A. 2000. Modern Processing and Utilization of Legumes - Recent Research and Industrial Achievements in Soybean Foods in Japan. In: Asian Productivity Organization. *Processing and Utilization of Legumes.* Tokyo: Asian Productivity Organization. pp. 63-75.

Okamoto M., Hayashi R., Enomoto A. 1991. High-pressure Proteolytic digestion of food proteins: Selective elimination of β-Lg in bovine milk whey. *Agricultural and Biological Chemistry.* 55: 1253-1257.

Pantako O. T., Lemieux L., Amiot J. 2001. The effects of α-lactalbumin and whey protein concentrate on dry matter recovery, TCA soluble protein levels, and peptide distribution in the rat gastrointestinal tract. *Canadian Journal of Physiology and Pharmacology.* 79: 320-28.

Papas A. M. 1999. *Antioxidant status, diet, nutrition, and health.* New York: CRC Press. pp. 1- 650.

Parcell S. W. 2002. Sulfur in human and applications in medicine. *Alternative Medicine Review*. 7: 22-23.

Peng H. J., Su S. N., Tsai J. J., Tsai L. C., Ku H. L., Kuo S. W. 2004. Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naïve and sensitized mice. *Clinical and Experimental Allergy*. 34: 663-670.

Penttila I. A., Zhang M. F., Bates E., Regester G., Read L. C., Zola H. 2001. Immune modulation in suckling rat pups by a growth factor extract derived from milk whey. *Journal of Dairy Research*. 68: 587-599.

Peters T. Jr. 1996. *All About Albumin: Biochemistry, Genetics, and Medical Applications*. San Diego: Academic Press. pp. 51-124.

Pfeuffer M., Schrezenmeir J. 2000. Bioactive substance in milk with properties decreasing risk of cardiovascular diseases. *British Journal of Nutrition*. 84 (Suppl. 1): S155-159.

Poot M., Hosier S., Swisshelm K. 2002. Distinct patterns of mitochondrial changes precedes induction of apoptosis by all-trans-retinoic acid and *N*-(4-hydroxyphenyl) retinamide in MCF7 breast cancer cells. *Experimental Cell Research*. 279: 128-140.

Puchelle E., Bajolet O., Abély M. 2002. Airway mucus in cystic fibrosis. *Paediatric Respiratory Reviews*. 3: 115-119.

Pyne S., Pyne N. J. 2000. Sphingosine 1-phosphate signalling in mammalian cells. *Biochemical Journal*. 349: 385-402.

Qiao Y., Lin X., Odle J., Whitaker A., van Kempen T. A. T. G. 2004. Refining in vitro digestibility assays: Fractionation of digestible and indigestible peptides1. *Journal of Animal Science.* 82: 1669-1678.

Rahman I., MacNee W. 1998. Role of tramscription factors in inflammatory lung diseases. *Thorax.* 53: 601-612.

Rahman I., MacNee W. 2000. Oxidative stress and regulation of glutathione in lung inflammation. *The European Respiratory Journal.* 16: 534-554.

Rahman I., Biswas S. K., Jimenez L. A., Torres M., Forman H. J. 2005. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxidants and Redox Signaling.* 7: 42-59.

Reddy I. M., Kella N. K. D., Kinsella J. E. 1988. Structural and conformational basis of the resistance of β-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry.* 36: 737-741.

Reynolds C. P., Lemons R. S. 2001. Retinoid therapy of childhood cancer. *Hematology/oncology Clinics of North America.* 15: 867-910.

Roberts P. R., Zaloga G. P. 1994. Dietary bioactive peptides. *New Horizons.* 2: 237-243.

Roberts P. R., Burnery J. D., Black K. W., Zaloga G. P. 1999. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion.* 60: 332-337.

Riboni L., Viani P., Bassi R., Prinetti A., Tettamanti, G. 1997. The role of sphingolipids in the process of signal transduction. *Progress in Lipid Research.* 36: 153-195.

Roum, J. H., Borok Z., McElvaney N. G., Grimes G. J., Bokser A. D., Buhl R., Crystal R. G. 1999. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. *Journal of Applied Physiology*. 87: 438-443.

Rouse K., Nwokedi E., Woodlife J. E., Epstein J., Klimberg S. V. 1995. Glutamine enhances selectivity of chemotherapy through changes in glutathione metabolism. *Annals of Surgery*. 221: 420-426.

Rowlands J. C., Hakkak R., Ronis M. J., Badger T. M. 2002. Altered mammary gland differentiation and progesterone receptor expression in rats fed soy and whey proteins. *Toxicological Sciences*. 70: 40-45.

Russo A., DeGraff W., Friedman N., Mitchell J. B. 1986. Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs. *Cancer Research*. 46: 2845-2848.

Sakai K., Yoshino K., Satter M. A., Ota F., Nii Y., Fukuta K., Ueda N., Shimizu Y., Yamamoto S. 2000. Effects of pH variation and NaCl on in vitro digestibility of cow's milk proteins in commercially available infant formulas. *Journal of Nutritional Science and Vitaminology*. 46: 325-328.

Satoh T., Sakai N., Enokidi Y., Uchiyama Y., Hataanaka H. 1996. Survival factor-insensitive generation of reactive oxygen species induced by serum deprivation in neuoronal cells. *Brain Research*. 733: 9-14.

Saxelin M., Korpela R., Mäyra-Mäkinen A. Classifying functional dairy products. In: Mattila-Sandholm, T. 1999. *Functional Dairy Products*. Cambridge, England: Woodhead Publishing. pp. 1-16.

Scheid O., Kempster L., Griesenbach U., Davies J. C., Dewar A., Weber P. P., Colledge W. H., Evans M. J., Geddes D. M., Alton E. W. 2001. Inflammation in cystic fibrosis airways: relationship to increased bacterial adherence. *European Respiratory Journal.* 17: 27-35.

Schmidt, R. H., Packard, V. S., Morris, H. A. 1984. Effect of processing on whey protein functionality. *Journal of Dairy Science.* 67: 2723-2733.

Schimdt D. G., Poll J. K. 1991. Enzymatic hydrolysis of whey proteins. Hydrolysis of alpha-lactalbumin and beta-lactoglobulin in buffer solutions by proteolytic enzymes. *Netherlands Milk Dairy Journal.* 45: 240-255.

Schwiebert L. M., Estell K., Propst S. M. 1999. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *American Journal of Physiology.* 276 (Cell Physiol.45): C700-C710.

Shah N. P. 2000. Effects of milk-derived bioactives: an overview. *British Journal of Nutrition.* 84(Suppl.1): S3-S10.

Shaw J. P., Chou I. N. 1986. Elevation of extracellular glutathione content associated with mitogenic stimulation of quiescent fibroblast. *Journal of Cell Physiology.* 129: 193-198.

Shimizu M., Tsunogai M., Arai S. 1997. Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides.* 18: 681–687.

Signorelli P., Luberto C., Hannun Y.A. 2001. Ceramide inhibition of NF-κB activation involves reverse translocation of classical protein kinase C (PKC) isoenzymes: requirement for kinase activity and carboxyl-terminal phosphorylation of PKC for the ceramide response. *The FASEB Journal.* 15: 2401-2414.

Singh I., Pahan K., Khan M., Singh A. K. 1998. Cytokine-mediated induction of ceramide production is redox-sensitive. *The Journal of Biological Chemistry.* 273: 20354-20362.

Smith J. J., Travis S. M., Greenberg E. P., Welsh M. J. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell.* 85: 229-236.

Smithers G. W., Ballard F. J., Copeland A. D., Silva K. J. D., Dionysius D. A., Francis G. L., Goddard C., Grieve P. A., McIntosh G. H., Mitchell I. R., Pearce R. J., Regester G. O. 1996. New opportunities from the isolation and utilization of whey proteins. *Journal of Dairy Science.* 79: 1454-1459.

Song M., Kellum J. A., Kaldas H., Fink M. P. 2004. Evidence that glutathione depletion is a mechanism responsible for the anti-inflammatory effects of ethyl pyruvate in cultured lipopolysaccharide-stimulated RAW 264.7 cells. *The Journal of Pharmacology and Experimental Therapeutics.* 308: 307-316.

Stecenko A. A., King G., Torii K., Breyer R. M., Dworski R., Blackwell T. S., Christman J. W., Brigham K. L. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation.* 25: 145-155.

Tanaka N., Koyasu A., Kobayashi I., Kunugi S. 1996a. Pressure-induced change in proteins studied through chemical modification. *International Journal of Biological Macromolecules.* 18: 275-280.

Tanaka N., Tsurui Y., Kobayashi I., Kunugi S. 1996b. Modification of single unpaired sulphydryl group of β-lactoglobulin under high pressure and the role of intermolecular S-S exchange in the pressure denaturation [single SH of β-lactoglobulin and pressure denaturation]. *International Journal of Biological Macromolecules.* 19: 63-68.

Tanaka N., Kunugi S. 1996. Effect of pressure on the deuterium exchange reaction of α-lactalbumin and β-lactoglobulin. *International Journal of Biological Macromolecules*. 18: 33-39.

Teschemacher H. 2003. Opioid receptor ligants derived from proteins. *Current Pharmaceutical Design*. 9: 1331-1344.

Ulukaya E., Wood E. J. 1999. Fenretinide and its relation to cancer. *Cancer Treatment Reviews*. 25: 229-235.

van der Vliet A., Eiserich J. P., Marelich G. P., Halliwell B., Cross C. E. Oxidative stress in cystic fibrosis: Does it occur and does it matter? *Advances in Pharmacology*. 38: 491-513.

van Odijk, Kull I., Borres M. P, Brandtzaeg P., Edberg U., Hanson L. A., Host A., Kuitunen M, Olsen S. F., Skerfving S., Sundell J., Wille S. 2003. Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infany and its impact on later atopic manifestations. *Allergy*. 58: 833-843.

Venkatakrishnan A., Stecenko A. A., King G., Blackwell T. R., Brigham K. L., Christman J. W., Blackwell T. S. 2000. Exaggerated activation of nuclear factor-κB and altered IκB-β processing in cystic fibrosis bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*. 23: 396-403.

Vermeirssen V., Van Camp J., Devos L., Verstraete W. 2003. Release of angiotensin I converting enzyme (ACE) inhibitory activity during in vitro gastrointestinal digestion: from batch experiment to semi-continuous model. *Journal of Agricultural Food Chemistry*. 51: 5680-5687.

Vermeirssen V., Camp J. V., Verstraete W. 2004. Bioavailability of angiotensin I converting enzyme inhibitor peptides. *British Journal of Nutrition.* 92: 357-366.

Walker W. A., Abel S. N., Wu M., Bloch K. J. 1976. Intestinal uptake of macromolecules. V. Comparison of the in-vitro uptake by rat small intestine of antigen-antibody complexes prepared in antibody and antigen excess. *Journal of Immunolology.* 117: 1028-1032.

Watanabe A., Okada K., Shimizu Y., Wakabayashi H., Higuchi K., Niiya K., Kuwabara Y., Yasuyama T. Y., Ito H., Tsukishiro T., Kondon Y., Emi N., Kohri H. 2000. Nutritional therapy of chronic hepatitis by whey protein (non-heated). *Journal of Medicine.* 31: 283-302.

Webb K. E. Jr. 1990. Intestinal absorption of protein hydrolysis products: a review. *Journal of Animal Science.* 68: 3011-3022.

Webber M. M., Bello-Deocampo D., Quader S., Deocampo N. D., Metcalfe W. S., Sharp R. M. 1999. Modulation of the malignant phenotype of human prostate cancer cells by *N*-(4-hydroxyphenyl) retinamide (4HPR). *Clinical and Experimental Metastasis.* 17: 255-263.

Wit J. N. 1998. Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science.* 81: 597-608.

Wu J. M., DiPietrantonio A. M., Hsieh T. C. 2001. Mechanisms of fenretinide (4-HPR)-induced cell death. *Apoptosis.* 6: 377-388.

Wu G., Fang Y. Z., Yang S., Lupton J. R., Turner, N.D. 2004. Glutathione metabolism and its implications for health. *Journal of Nutrition.* 134: 489-492.

Yoshikawa M., Fujita H., Matoba N., Takenaka Y., Yamamoto T., Yamauchi R., Tsuruki H., Takahata K. 2000. Bioactive peptides derived from food proteins preventing lifestyle-related diseases. *Biofactors*. 12: 143-146.

Zommara M., Toubo H., Sakono M., Imatzumi K. 1998. Prevention of peroxidative stress in rats fed on a low vitamin E-containing diet by supplementing with a fermented bovine milk whey preparation: Effects of lactic acid and β-lactoglobulin on the antiperoxidative action. *Bioscience Biotechnology and Biochemistry*. 62: 710-717.

Zommara M. A., Toubo H., Imaizumi K. 2002. Supplementing bovine milk immunoglobulin G prevents rats fed on a vitamin E-deficient diet from developing peroxidation stress. *Annals of Nutrition and Metabolism*. 46: 97-102.

Zou C. P., Kurie J. M., Lotan D., Zou C. C., Hong W. K., Lotan R. 1998. Higher potency of *N*-(4-hydroxyphenyl)retinamide than all-trans-retinoic acid in induction of apoptosis in no-small cell lung cancer cell lines. *Clinical Cancer Research*. 4: 1345-1355.

CHAPTER 3

Inhibition of the Inflammatory Response of CFTR-Deficient Lung Epithelial Cells Following Pre-Treatment with Fenretinide

Regina Maria Vilela[1], Larry C. Lands[2], Brian Meehan[2] and Stan Kubow[1,3]

[1]School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
[2]Montreal Children's Hospital
Division of Respiratory Medicine, Room D-380
2300 Tupper Street, Montreal, Quebec H3H 1P3

[3]Corresponding Author: Dr. Stan Kubow
School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
E-mail: stan.kubow@mcgill.ca

Abstract

Cystic Fibrosis (CF) is characterized by a biochemical abnormality in the cystic fibrosis transmembrane conductance regulator (CFTR) channel. CFTR-deficient lung epithelial cells may have high constitutive glutathione (GSH) levels that could decrease the intracellular content of the second sphingolipid messenger, ceramide. Altered ceramide levels in CF cells could, in turn, lead to their resistance to apoptosis and an immune hyper-responsiveness. As fenretinide is a ceramide up-regulating drug that inhibits the activation of the pro-inflammatory transcriptional factor, nuclear factor (NF)-κB, the impact of fenretinide on unstimulated and tumor necrosis factor (TNF)-α stimulated production of NF-κB-dependent interleukin (IL)-8 was studied in immortalized wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) tracheal epithelial cells. Despite higher constitutive levels of GSH in CF cells, their intracellular ceramide content was not different from non-CF cells. Clinically relevant concentrations of fenretinide (1.25, 2.5 and 5 μM) inhibited TNF-α-induced IL-8 production of CF cells but increased the inflammatory response in non-CF cells at 2.5 μM. Although fenretinide treatment was associated with a higher intracellular ceramide content in the mutant ΔF508 CFTR cells, the fenretinide-mediated decrease in IL-8 secretion was not consistently explained by changes in the intracellular content of this sphingolipid. Fenretinide was ineffective in increasing the susceptibility to apoptosis in CF cells whereas non-CF cells were sensitive to the apoptosis induced by both fenretinide and cisplatin exposure. These results present the possibility that the lung inflammation in CF could be attenuated via low dose fenretinide treatment.

3.1 Introduction

Cystic Fibrosis (CF) is the most common lethal hereditary disease among Caucasians and it is characterized by a biochemical abnormality in the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Dinwiddie, 2000). The most common clinical manifestation in CF is chronic lung infection that leads to progressive tissue destruction and elevated pulmonary morbidity and mortality (Bals *et al.*, 1999; Knowles and Boucher, 2002). Lung tissue damage in CF has been related to an abnormally exacerbated immune response in CF respiratory epithelial cells. This exacerbation has been related to an exaggerated activation of the pro-inflammatory transcriptional factor nuclear factor (NF)-κB (Venkatakrishnan *et al.*, 2000), possibly due to a lower capacity of down-regulating the proinflammatory response (Stecenko *et al.*, 2001; Becker *et al.*, 2004).

One molecule that is known to inhibit NF-κB activation is ceramide (Signorelli *et al.*, 2001), an intermembrane sphingolipid recognized as a second messenger in the molecular modulation of apoptosis. The sphingomyelin (SM) cycle, with the conversion of SM to ceramide by sphingomyelinase (SMase), is a key signaling pathway in many cell systems (Hannun, 1996). Two main routes have been defined for the generation of ceramide: (1) hydrolysis of SM, an abundant sphingolipid species in cell membranes, by the action of SMase; and (2) by *de novo* biosynthesis catalyzed by ceramide synthase (Levade and Jaffrezou, 1999). The hydrolytic pathway, however, is the major source for ceramide in cellular responses to extracellular signaling (i.e., tumor necrosis factor (TNF)-α, hydrogen peroxide (H_2O_2), lipopolysaccharides (LPS) and γ-interferon (Chan and Goldkorn, 2000).

Cellular treatment with TNF-α has emerged as one of the best-characterized models of cytokine-induction and of ceramide function. TNF-α induces activation of SMase in these cells and this activation has been related to a drop of

glutathione (GSH) that follows the activation of the death receptor and caspase 8 (Liu *et al.*, 1998; Luberto *et al.*, 2000). This would be one of the mechanisms by which normal epithelial cells respond to H_2O_2 and TNF-α released by phagocytes during inflammation to suppress inflammatory processes and limit the survival of disturbed or necrotic cells. Conversely, extracellular supplementation of GSH or *N*-acetylcysteine (NAC), a known precursor of GSH, inhibit ceramide generation induced via oxidative stressors such as TNF-α, IL-1-β, hypoxia, and doxorubicin (Beaver and Waring, 1995; Liu *et al.*, 1998; Singh *et al.*, 1998; Lavrentiadou *et al.*, 2001).

GSH inhibits the activation of the neutral, magnesium-dependent N-SMase and inhibits ceramide generation induced by TNF-α in human mammary carcinoma cells (Liu *et al.*, 1998). In addition, Lavrentiadou *et al.* (2001) have shown that low intracellular GSH levels in lung cells are required for ceramide production, whereas high GSH levels inhibit the generation of ceramide. GSH concentrations have been suggested to be relatively high in CF cells (Linsdell and Hanrahan, 1998; Jungas *et al.*, 2002). Jungas *et al.* (2002) have shown that CF cells are less sensitive to oxidative stress induced by H_2O_2 than normal cells, in part due to inherently high constitutive GSH levels in the CF cells.

Due to the inhibitory action of GSH on SMase, we hypothesized that cellular ceramide production could be decreased in mutant ΔF508 CFTR lung epithelial cells exposed to physiological or non-physiological stressors (i.e., TNF-α, heat stress, bacterial infections), which act to induce cellular ceramide concentration via the sphingomyelinase pathway (Levade and Jaffrezou, 1999). In addition to modulation of NF-κB activation, ceramide regulates/mediates pro-apoptotic signaling involved in the cell death (Hannun, 1996). Hence, blockage of *in situ* ceramide production could also render CF cells more resistant to pro-apoptotic stimuli. CFTR-associated defects in apoptosis has been suggested to contribute to the pathogenesis of the lung disease in CF as apoptosis of infected epithelial cells

in CF may be critical for clearance of *Pseudomonas* (*P.*) *aeruginosa* (Cannon *et al.*, 2003).

Fenretinide, *N*-(4-hydroxyphenyl) retinamide, is a synthetic derivative of all-trans-retinoid acid, which up-regulates ceramide and has been used widely for anti-cancer treatment and prophylaxis (Ulukaya and Wood, 1999) but the potential effects of fenretinide on airway CF epithelial cells are unknown. Since fenretinide increases intracellular ceramide levels by *de novo*, non-sphingomyelinase-mediated ceramide synthesis (Erdreich-Epstein *et al.*, 2002), fenretinide could act downstream of SMase to enhance cellular ceramide levels and thereby decrease the pro-inflammatory condition in CF. Alternatively, or in addition, fenretinide could provide additional benefits to CFTR deficient cells, as decreased activation of NF-κB (Shimada *et al.*, 2002) and enhanced apoptosis (Cuello *et al.*, 2004) has been observed in tumor cells treated with fenretinide. As exacerbations in lung inflammation in CF may be initiated at the epithelial level by secretion of cytokines, a primary objective was to investigate this aspect *in vitro* in relation to fenretinide supplementation. The human epithelial cells used in this study secrete cytokines upon stimulation with TNF-α. IL-8 was chosen for analysis, as this is the primary cytokine responsible for proliferation and recruitment of neutrophils in airway inflammation.

3.2 Materials and Methods

Materials

Immortalized wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells were gifts obtained from Dr. D. Gruenert (University of California at San Francisco). The supplies for the maintenance of cell culture such as minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, L- glutamine, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Burlington,

Ontario, Canada). Trypsin-EDTA solution (0.25%) and C6 ceramide were obtained from Sigma-Aldrich Co (Oakville, Ontario, Canada). The solution used to coat the T-75 flasks and 24-well plates was prepared with collagen type I bovine, and human fibronectin obtained from BD Biosciences (Oakville, Ontario, Canada); bovine serum albumin (BSA), and LHC basal medium obtained from Biosource-Biofluids Division (Camarillo California, USA). Human recombinant TNF-α was obtained from BD Pharmigen (Oakville, Ontario Canada) and prepared with 0.1% bovine serum albumin (BSA). To determine IL-8 release ELISA kits (Pharmigen, OptEIA Human IL-8 Set, catalog # 550999) were obtained from BD Bioscience (Oakville, Ontario, Canada). Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma-Aldrich Co. The synthetic retinoid, fenretinide [*N*-(4-hydroxyphenyl) retinamide], was obtained from Sigma-Aldrich Co. The column used for the HPLC analysis was Nucleosil 100 (C18) 5 μm, 150 mm x 4.6 mm obtained from Alltech Associates Inc. (Deerfield, Illinois, USA).

Cells Culture

General Procedures

Cells were grown in pre-coated T-75 flasks in Eagle's MEM containing 10% FBS and re-fed every 2-3 days until confluent. The confluent, adherent monolayers were then released from the plastic surface after treatment with polyvinyl-pirrolidone (PVP)-trypsin-EDTA and were seeded to 24-well plates or 60 mm dishes for 24 h before receiving the treatments.

Treatment with Fenretinide and TNF-α

Cells were treated with fenretinide at doses established previously in cell culture studies (Wang *et al.*, 2001; Erdreich-Epstein *et al.*, 2002) to effectively increase cellular ceramide content. Wild-type (9HTEo-) and mutant ΔF508 CFTR human

epithelial cells (CFTE29o-), seeded at 0.4 and 0.6 x 10^6 cells/mL in 24-well plates, respectively, were grown in Eagle's MEM containing 10% FBS for 24 h until nearly confluent. The MEM was replaced with fresh medium containing 2% FBS and 1.25 μM, 2.5 μM or 5 μM of fenretinide (reconstituted in ethanol) and the cells were allowed to grow for 24 h at 37°C in 5% CO_2. After 24 h the medium was replaced with fresh MEM 2% FBS containing the same initial concentrations of fenretinide in order to characterize the impact of fenretinide on IL-8 release in an unstimulated condition after an additional 24 h. To assess the effect of fenretinide on IL-8 production in a stimulated state, after the initial 24 h incubation with fenretinide, cells were treated with MEM 2% FBS containing 1.25 μM, 2.5 μM or 5 μM fenretinide and concurrently stimulated with human recombinant TNF-α (10 $ng.mL^{-1}$) for an additional 24 h. All experiments included unstimulated negative control wells and had vehicle ethanol controls.

IL-8 Release and Cell Viability Assays

After the treatment described above, the supernatant was collected to determine IL-8 released using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates were coated with capture antibody (anti-IL-8) overnight, washed with 0.05% Tween-20 in PBS and coated with phosphate buffer (PBS) 10% FBS in order to block non-specific binding. Known concentrations of IL-8 (standard) and the samples containing the IL-8 released by the cells after treatment (supernatant) were added as aliquots into appropriate wells, incubated for 2 h and decanted from the wells. Anti-IL-8 plus enzyme reagent (biotinylated detection antibody conjugated to Streptavidin-Horseradish) were added and incubated for 1 h. After washing the plate, a solution containing a substrate for the enzyme (TMB-peroxide chromogen) present in the anti-IL-8 + enzyme reagent mixture was added and the plate was incubated for 30 min. The reaction was stopped using a 2N H_2SO_4 solution and the absorbance was read at 450 nm using a Titertek II Multiscan MCCB40 (Labsystems, Finland). The

absorbances were then used to calculate the IL-8 concentration from the standard curve and adjusted by their dilution factor.

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). Briefly, after collecting the supernatant to determine IL-8 release, the cells were gently washed with PBS and MTT solution (MTT, 0,5mg/mL culture medium free of phenol red) was added and the cells were incubated for 3 h at 37°C. After incubation, the supernatant was aspirated, HCl-isopropanol solution (0,04N HCl in isopropanol) was added and after 5 min the optical densities were measured at 540 nm using a series 750 microplate reader from Cambridge Technology, Inc. (Cambridge, MA, USA). The optical density (O.D.) values were converted into cell numbers by using a cell proliferation standard curve with a cell seeding concentration ranging from 0.4 to 1 x 10^6/mL cells (Loodsdrech *et al.*, 1994).

Intracellular Ceramide Content

Adherent epithelial cell lines grown on coated 60 mm dishes for 24 h were treated with fenretinide with the same concentrations used to determine IL-8 release as described above. At 24 h after the incubation with fenretinide and 24 h before the experiment, the cells were stimulated with TNF-α. Cells were washed twice with PBS, scraped in 1.5 mL of PBS and centrifuged at 500 x g for 10 min. The cell concentrates underwent lipid extraction, alkaline hydrolysis and derivatization with *o*-phthaldialdehyde (OPA) reagent for HPLC fluorescence detection as described elsewhere (Zimmermann *et al.*, 2001; Ginis *et al.*, 1999; Dbaibo *et al.*, 1997). Derivatized samples (15 μL) were injected into a C18 5 μm column (150 mm x 4.6 mm) and eluted isocratically at a flow rate of 0.6 mL/min using a Shimadzu C-R4A Chromatopac HPLC with the fluorescence detected at excitation/emission wavelengths 340/454 nm using a Shimadzu spectrofluorometric detector LC-6A (Shimadzu Scientific Instruments, Inc.,

Kyoto, Japan). The internal standard, C6 ceramide, showed a retention time of 12 min. The standard curve for ceramide was linear from 30 pmoles to 2 nmoles. Ceramide values were normalized on a per lipid phosphate basis.

Measurement of Intracellular Glutathione

Quantitative determination of the total intracellular GSH and glutathione disulfide (GSSG) was performed according to a modification of the method of Anderson (1985). Adherent epithelial cell lines, grown on coated 60 mm dishes with MEM 10% FBS during the first 24 h after seeding and for additional 48 h with MEM 2% FBS were washed twice with 5 mL PBS containing 25 mg% BSA. The cells were treated with 1.5 mL of 25 mg% BSA in PBS solution, scraped, and then subjected to centrifugation at 500 x g for 10 min (Microlite Microcentrifuge OM 3580 from Thermo IEC, Needham Heights, MA, USA). The supernatant was discarded and the cells were resuspended in 1 mL of PBS followed by another centrifugation at 400 x g for 6 min. The supernatant was discarded and the cells were washed once with 0.45 mL PBS and re-suspended with 0.45 mL PBS. Samples were diluted with 0.1 mL 10 mM HCl and sonicated for two cycles of 20 s. The cell suspension was filtered using a centrifugal Millipore filter (catalog #42407, Millipore, Nepean, Ontario, Canada) at 14,000 x g for 60 min at 4°C. The protein concentration of the unfiltered portion of the samples was determined by the Bradford assay (catalog #500-0006, Bio-Rad, Mississauga, Ontario, Canada). One aliquot of the filtrates were transferred to a 96-well microplate for measurement of total GSH using the GSH reductase recycling method of Anderson *et al.* (1985) following treatment of the samples with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). To measure intracellular GSSG, exclusive of GSH, an additional step was included to the protocol as follow: after deproteinization, 1 M solution of 2-vinylpyridine in ethanol was added to a second aliquot of the samples and incubated during 60 min to derivatize GSH. The standards received the same treatment. After the derivatization, GSSG was measured following the same GSH reductase recycling method. The microplate was placed on the series

750 microplate reader from Cambridge Scientific (Cambridge, MA) using a 420-nm filter and the absorbance was read for 25 min. Samples were compared with the GSH and GSSG calibration curves to determine the GSH and GSSG concentrations in each well. The linearity of the GSH and GSSG calibration curves ranged from 0.1 to 16 μM and from 0.25 to 8 μM, respectively.

In Situ end Labelling of DNA Strand Breaks

Adherent epithelial cell lines grown on coated coverslips within 24 well plates for 24 h were treated with fenretinide with the same concentrations used to determine IL-8 release as described above. At 24 h after the incubation with fenretinide and 24 h before the experiment, the cells were stimulated with TNF-α (experimental group) or with cisplatin at 25 μM or 12.5 μM (as a positive control). After a 48 h period of cell growth exposed to fenretinide (24 h after stimulated with TNF-α), cells were fixed using 4% paraformaldehyde in PBS at pH of 7.4 for one h at room temperature, washed, and then made permeable using 0.1% Triton X-100 in 0.1% citrate buffer for 2 min on ice. After washing in PBS, the coverslips were individually removed from 24 well plates and then cells were incubated with Terminal deoxynucleotidyl transferase (Tdt) enzyme conjugated to fluorescein (catalog #11684795910, Roche Molecular Biochemicals, Indianopolis, IN) for 1.0 h at 37°C. A negative control slide was produced by incubating slides of both cell lines with buffer minus the Tdt enzyme. The Tdt enzyme binds to the 3-OH free end of DNA strand breaks. Following this procedure, cells were washed twice with PBS. Coverslips were placed on slides using the VectaShield Anti Fade media (catalog # H-1000, Vector Laboratories Canada Inc., Burlington, Ontario). Slides were labelled individually with numbers to keep the observer blinded to the outcome until after the slides were reviewed. The slides were observed at 400X (total magnification) using a fluorescent capable microscope (Axiophot Microscope, Zeiss, Germany) and photographs were taken using a camera RT slides and Spot RT Image Analysis System software (Diagnostic Instruments,

Inc., MI, USA). Nuclei that appeared brightly fluorescent were considered positive for apoptosis.

Microscopic Cell Morphology

Adherent epithelial cell lines grown on coated coverslips within 24-well plates for 24 h were treated with fenretinide with the same concentrations used to determine IL-8 release as mentioned above. At 24 h after the incubation with fenretinide and 24 h before the experiment, the cells were stimulated with TNF-α (experimental group). After a 48 h cell growth period in the presence of fenretinide (24 h after stimulated with TNF-α), cells were fixed using 4% paraformaldehyde in PBS at pH of 7.4. Slides were labelled individually with numbers to keep the investigator blinded and observed at 400 x (total magnification) using an inverted microscope (Nikon Eclipse TE2000-U, Nikon Inc.Melville, NY). Photographs were taken using the software Simple PC version 5.2 (Comprix Inc., USA) for visualization of cell confluence and morphology.

Statistics

Data were expressed as means ± standard error of the mean. The minimum number of replicate experiments for all measurements was at least three. Differences among groups were examined by analysis of variance (ANOVA) and Tukey's *post hoc* comparisons for values of cytokines, cell numbers, and intracellular ceramide concentrations. Student's *t*-test was performed on the GSH and GSSG data. The partial Pearson's correlation test was used to assess the correlation between IL-8 levels and cell numbers after controlling for fenretinide dose levels. SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois) was used in all statistical analyses. Level of significance was set at $P < 0.05$.

3.3 Results and Discussion

IL-8 Production in Response to Fenretinide

Unstimulated non-CF and CF control cultures produced similar low levels of IL-8 (146 ± 14 and 291 ± 22 pg.mL^{-1}, respectively) under basal conditions with a trend for higher levels in association with CF cells. Sham treatment (ethanol) did not increase IL-8 secretion in either cell type (data not shown). The present results are similar to other cell culture studies that indicate small or no differences in basal levels of inflammation in CF epithelial cells (Schwiebert *et al.*, 1999; Becker *et al.*, 2004; Venkatakrishnan *et al.*, 2000).

Exposure of cells to 10 ng.mL^{-1} TNF-α resulted in more than a 90-fold increase (27,406 ± 1,724 pg·mL^{-1}) in secreted IL-8 levels in CF cells whereas non-CF cells exhibited greater than 420-fold increase (62,358 ± 6,810 pg·mL^{-1}) in IL-8 concentrations relative to the unstimulated MEM controls (291 ± 32 pg·mL^{-1} and 146 ± 14 pg·mL^{-1} respectively). A similar increase in IL-8 expression in the presence of TNF-α has been observed in previous work using the CF cell line used in the present study (CFTE29o- homozygous for the ΔF508 CFTR mutation) (Schwiebert *et al.*, 1999). Studies are controversial regarding the inflammatory responses that occur when CF cells are exposed to a cell stress response such as TNF-α. *In vitro* studies performed with lung epithelial cells from CF individuals or with immortalized cell lines in which CFTR status has been manipulated give conflicting results, ranging from increased production of IL-8 (Tabary *et al.*, 1998) to no changes (Bedard *et al.*, 1993; Schwiebert *et al.*, 1999) or decreased (Massengale *et al.*, 1999) levels of inflammatory cytokines as observed in the present study. In comparison to the 93.9 and 424.7-fold increase in IL-8 production following TNF-α stimulation as compared to unstimulated MEM 2% FBS controls, fenretinide treatment in the unstimulated condition was associated

with only a slight increase (i.e., maximal 4.5 and 29.8-fold) in IL-8 release in both CF and non-CF cultures over basal conditions (Figs. 1A and 1B).

The major finding from this study was that CF human tracheal epithelial cells treated with fenretinide under TNF-α stimulated conditions showed a significant reduction in IL-8 release. The release of IL-8 from CF cells showed a dose-dependent reduction by fenretinide supplementation (Fig. 2 A). At the lowest dose (1.25 μM), a significant decrease of 37% in IL-8 release relative to the control culture was observed following fenretinide treatment. The higher doses (2.5 and 5 μM) were associated with more potent decreases in IL-8 secretion (55.75 and 73.3%, respectively). As opposed to CF cells, IL-8 concentrations were not responsive to fenretinide treatment in the normal epithelial cells studied (9HTEo-) at the lowest and highest doses. The intermediate dose promoted a 77% increase in the IL-8 secretion (Fig. 2 B). This result might be due to inherent differences in the responsiveness of the ceramide pathway to fenretinide in non-CF cells.

Relevant fenretinide concentrations were chosen in the present study to mimic the *in vivo* situation as the effective concentrations of 2.5 and 5.0 μM are in the range of peak plasma levels in neuroblastoma patients receiving fenretinide therapy for 28 days (Garaventa *et al.*, 2003). The 2.5 μM fenretinide dose has been observed to effectively induce anti-cancer effects in tumor cell lines (Maurer *et al.*, 1999). These results thereby show that low dose fenretinide treatment of CF human tracheal epithelial cells can act to attenuate a pro-inflammatory condition.

Intracellular Ceramide Content

We postulated that anti-inflammatory, anti-proliferative and apoptotic effect of fenretinide would be exerted in CF cells as mediated via changes in intracellular ceramide. Ceramide and sphingosine, along with their phosphorylated derivatives, play an important role as second messengers involved in regulating inflammation, cell proliferation, differentiation and apoptosis (Hannun and Bell, 1989).

Importantly, both cell lines respond to the fenretinide treatment with increase in intracellular ceramide content (Figs. 3A and 3B); however, a significant increase was detected only at the higher dose (2.5 μM). Moreover, higher levels of ceramide were detected in CF vs. non-CF cells in association with fenretinide treatment. Our results do not explain why ceramide content was higher in CF cells following fenretinide stimulation; however, it can be postulated that an imbalance in sphingosine-1P (S-1P) in CF cells may have an impact on the higher accumulation of ceramide observed. It has been demonstrated that the CFTR channel enhances uptake of transport of S-1P and that this process is impaired in CF epithelial cells (Boujaoude *et al.*, 2001). Presumably, CF cells would therefore have more intracellular S-1P available (Boujaoude *et al.*, 2001), which would limit the possibility of conversion of ceramide into S-1P, thereby leading to the observed higher ceramide concentrations in CF cells following fenretinide treatment. This effect would not be observed in normal epithelial cells because CFTR channel would be able to pump out excessive amounts of S-1P.

We postulated that the increase in intracellular ceramide following fenretinide treatment could inhibit IL-8 release since ceramide has been shown to inhibit NF-κB (Signorelli *et al.*, 2001); however, low dose (1.25 μM) fenretinide treatment was associated with a decreased IL-8 release despite unchanged intracellular ceramide concentrations. The implication of this latter finding is that other mechanisms apart from increased ceramide concentrations are involved in the anti-inflammatory effect of fenretinide. One possibility is that fenretinide could downregulate IL-8 release via direct inhibition of NF-κB activation. Fenretinide has been shown in prostate cancer cells to inhibit activation of inhibitory κBα (IκBα) degradation, which can lead to inhibition of NF-κB activation (Shimada *et al.*, 2002). According to Shimada *et al.* (2002), inhibition of NF-κB, which is one of the major transcriptional molecules of the cyclooxygenase (COX) gene, would explain the effect of fenretinide in the downregulation of the expression of COX-2 and the decreased production of prostaglandin E_2 as previously described by

Merritt *et al.* (2001). Dowregulation of COX-2 through fenretinide treatment could provide benefits to CF patients by decreasing the pro-inflammatory effects associated with higher levels of arachidonic acid and lower levels of docosahexaenoic acid in mucosal and submucosal tissues observed in CF patients (Freedman *et al.*, 2004).

Intracellular Glutathione Concentrations

To evaluate whether GSH and GSSG concentrations could correlate with sensitivity to fenretinide-mediated inhibition of IL-8 release in the CF vs. non-CF cells, the relative GSH and GSSG concentrations were measured in both cell lines. The basal GSH and GSSG concentrations were 8 and 21-fold higher (Figs. 4 A and 4B), respectively, in CF cells which suggests that non-CF cells lose intracellular GSH and GSSG due to CFTR-dependent transport. These findings, which are the first to demonstrate enhanced GSH and GSSG concentrations in human CF lung epithelial cells, are in concert with the findings of Jungas *et al.* (2002). These authors noted significantly higher GSH concentrations in HeLa epithelial cells expressing the most common CFTR mutation associated with CF. The relatively high GSH concentrations found in the mutant CFTR lung epithelial cell line was not observed previously by Gao *et al.* (1999) who used different cell culture conditions from those used in the present study and by Jungas *et al.* (2002). In particular, Gao *et al.* (1999) did not use fetal bovine serum (FBS) to supplement the cell culture medium. Serum starved culture conditions could alter cellular metabolism to a catabolic state as FBS contains albumin that is necessary for amino acid uptake in cell culture (Groff and Gropper, 2000; Peters, 1996) and serum starvation leads to depressed intracellular levels of GSH (Shaw and Chou, 1986; Kang and Enger, 1991). Hence, it is possible that differences in GSH content between wild-type and CF cells were obviated via low basal GSH concentrations induced in the serum-starved cells. An important aspect not assessed in the Gao *et al.* (1999) or Jungas *et al.* (2002) studies, was the measurement of the basal redox GSH/GSSG ratio. The present study showed that

despite the higher intracellular GSH content in CF cells, the basal redox GSH/GSSG ratio was considerably lower as compared to this ratio in non-CF cells (3.08 ± 0.25 S.E and 10.86 ± 2.5 S.E, respectively; $P < 0.05$). Our finding thus supports previous *in vivo* studies that detected GSSG as a large proportion of the total GSH pool detected in the epithelial lining fluid of CF patients (Rahman *et al.*, 2005). Rahman *et al.* (2005) suggested that the lowered redox ratio could be the result of oxidative stress or an outcome of oxidant-dependent effects on signal transduction that affects protein function (Rahman *et al.*, 2005).

Numerous studies have demonstrated that changes in intracellular redox play a key role in regulation of neutral SMase (N-SMase), which is the primary route of generation of intracellular ceramide via hydrolysis of SM in cell membranes (Andrieu-Abadie *et al.*, 2001). In particular, both GSH and GSSG are well known potent inhibitors of the N-SMase (Liu *et al.*, 1998). As GSSG lacks the antioxidant properties of GSH, it thus appears that the prevention of activation of sphingomyelinase is due to GSH and GSSG functioning as specific allosteric regulators of the enzyme. Since ceramide can inhibit NF-κB activation that stimulates the release of pro-inflammatory cytokines such as IL-8 (Signorelli *et al.*, 2001), high intracellular GSH concentrations in CF could lead to decreased ceramide synthesis via SMase and thereby decrease the capability to down-regulate pro-inflammatory responses. Due to limitations in the measurement of intracellular GSH and ceramide content within the same culture wells, a link between these two indices could not be directly established. We hypothesized that the well characterized effect of fenretinide in increasing ceramide by *de novo* non-SMase-mediated ceramide synthesis (Erdreich-Epstein *et al.*, 2002) may be a mediator in the inhibition of TNF-α stimulation of IL-8 in CF epithelial cells. Ceramide upregulation via fenretinide in this manner would overcome a block in ceramide synthesis via SMase pathway mediated by high constitutive levels of GSH and GSSG in CF cells. This mechanism of anti-inflammatory action of fenretinide can only be a partial explanation since intracellular ceramide

concentrations were unchanged in CF cells at the lowest fenretinide dose that showed an inhibitory effect on IL-8 release.

Cell Proliferation and Apoptosis

Except for the lower dose (1.25 μM), fenretinide decreased CF epithelial cell numbers under both stimulated and unstimulated conditions (Fig. 5A). Moreover, a negative correlation ($r = -0.86$, $P = 0.01$) was observed between fenretinide dose levels and cell numbers. The wild-type cell line showed a similar statistically significant decrease in cell numbers under the stimulated condition with fenretinide treatment (Fig. 5B). The anti-proliferative effects of fenretinide could be mediated via changes in intracellular ceramide as ceramide and sphingosine, along with their phosphorylated derivatives, play an important role as second messengers involved in regulating cell proliferation (Hannun and Bell, 1989). In that regard, both cell lines respond to the fenretinide treatment with increase in intracellular ceramide content (Figs. 3A and 3B); however, a significant increase in ceramide content was detected only at the higher dose (2.5 μM), in accordance with the observation that the cell numbers only decreased significantly with the higher fenretinide dose.

The present results showed CF epithelial cells as opposed to wild-type epithelial cells were resistant to the apoptotic effects of the cisplatin, which was used as a positive control for apoptosis (Figs. 6 and 7). There is increasing evidence that apoptosis is important in the remodeling of lung tissue after acute lung injury for the clearance of excess epithelial stem cells after repair (Bardales *et al.*, 1997) and for the normal removal of excess mesenchymal cells from resolving lesions (Polunovsky *et al.*, 1993). There is indication that more rapid apoptosis of infected epithelial cells in CF is needed for clearance of *P. aeruginosa* and that CFTR-associated defects in apoptosis could be an important contributor to the pathogenesis of the lung disease in CF (Cannon *et al.*, 2003). Although cisplatin induces cell apoptosis, as observed with 9HTEo- cells, the mechanism of action is

not fully understood. Cisplatin generates reactive oxygen species and triggers cellular responses involving multiple pathways, including DNA repair, transcription inhibition, cell cycle arrest, and apoptosis involving the c-Jun N-terminal kinase enzyme (JNK; stress-activated protein kinase) pathway (Siddik, 2003). The JNK enzyme is a member of the mitogen-activated protein (MAP) kinase family, which also involves extracellular signal-regulated kinase and p38-MAPK. The JNK pathway is particularly important in apoptosis signaling (Ip and Davis, 1998). Antioxidants such as the endogenous GSH can protect cells from cisplatin-induced cytotoxicity (Muldoon *et al.*, 2001). NAC is an antioxidant thiol that is a potent inducer of intracellular GSH concentrations and may also directly bind to and inactivate platinum agents (Zafarullah *et al.*, 2003). The GSH enhancing activity of NAC can also prevent apoptosis and promote cell survival by activating extracellular-regulated kinase (ERK) MAP kinase (Li *et al.*, 2000) that, in turn, may lead to enhanced cell growth. Thus, the inherently high GSH content of CFTR-deficient cells could also be a mechanism of action for both their resistance to cisplatin-induced apoptosis and their increased cell proliferation. In concert with previous studies, our results showed an increased GSH levels and resistance to apoptosis in CFTR deficient cells relative to normal epithelial bronchial cells that showed classic signs of apoptosis in response to cisplatin. The apoptosis and cell morphology data indicate that despite similar response to TNF-α in terms of decreased cell numbers under the stimulated condition, non-CF cells respond to fenretinide in a different manner when compared to CF cells.

Fenretinide induced apoptosis in the non-CF cells with positive fluorescence *in situ* end labeling of DNA showing strand breaks with classic peripheral nuclear condensation of chromatin (Fig. 7). Also, apoptosis was confirmed with the observation of morphologic signs of apoptosis such as cytosol shrinkage and plasma membrane blebbing using a phase contrast microscopy (Fig. 8). Conversely, CF cells did not show positive fluorescence with no signs of nuclear fragmentation in response to the same doses of fenretinide (Fig. 6). Fenretinide

has been demonstrated to induce apoptosis in several cell types; however, not all cells are sensitive to the apoptotic effects of fenretinide (Chen *et al.*, 1999). Recently, Harris *et al.* (2005) demonstrated an overexpression of the antiapoptotic protein Bcl-2 among the CF patients. Overexpression of Bcl-2 could thus be one of the mechanisms that might explain the resistance to apoptosis in CF cells, in addition to the apoptotic BAX inhibition through high intracellular GSH concentrations (Jungas *et al.*, 2002). Additionally, it is conceivable that excessive amounts of S-1P associated with CF cells (Boujaoude *et al.*, 2001) could also play a role in increasing the resistance of CFTR deficient cells to apoptosis due to anti-apoptotic effects of S-1P (Pyne and Pyne, 2000). Fenretinide has been demonstrated to promote apoptosis via hydroperoxide production followed by mitochondrial permeability transition, caspase activity, and DNA fragmentation in human cutaneous squamous carcinoma cells (Hail and Lotan, 2001). Interestingly, HeLa cells with CFTR ΔF508 mutation were shown to be resistant to apoptosis induced by hydroperoxide and depended on chemical GSH depletion to allow for the proapoptotic effects of Bax protein activation (Jungas *et al.*, 2002). Moreover, as the JNK pathway has been implicated in fenretinide-induced apoptosis (Chen *et al.*, 1999), the relatively high intracellular GSH content of CF cells could have mediated their apoptotic resistance to fenretinide in a similar manner to cisplatin as discussed above.

Taking these results together, a diminution in cell proliferation was likely responsible for the lower cell numbers in the fenretinide-treated CF cells rather than the apoptotic cell death observed in non-CF cells exposed to fenretinide. In accordance with our findings, fenretinide has been demonstrated to suppress cell proliferation in several cancer cell lines (Ulukaya and Wood, 1999). Fenretinide treatment could thus be advantageous by decreasing the rate at which epithelial cells proliferate, which could lead to a generally more mature and differentiated epithelium. These events could, in turn, result in enhanced mucin biosynthesis and secretion to improve the capability of combating bacterial infection.

To our knowledge, this is the first demonstration that fenretinide could be an effective agent to reduce inflammation and decrease proliferation in CF cells. Although a positive correlation ($r = 0.91$, $P < 0.001$) was noted between decreased cell numbers and the IL-8 levels, partial correlation analysis showed that this relationship was not significant after controlling for fenretinide treatment ($r = 0.5$, $P = 0.07$), indicating that fenretinide accounts for the IL-8 decrease regardless of decrease in cell numbers. In addition, low dose of fenretinide significantly decreased IL-8 release without affecting cell viability.

As fenretinide is one of the synthetic retinoids that has shown low toxicity and side-effects in humans, the potential use of this drug for treatment of CF is relevant. Fenretinide is a synthetic retinoid that is reported to have fewer side-effects compared to naturally occurring retinoids including vitamin A (Ulukaya and Wood, 1999). This drug has been used on a prophylactic basis to decrease cancer risk in the general population. The safety profile for fenretinide is excellent as minimal side-effects have been noted in a variety of clinical trials using fenretinide (Ulukaya and Wood, 1999). It is the subject of a multitude of clinical trials, including use to prevent cancer recurrence in patients (Kim, 2005). Hence, this drug is an interesting candidate as a potential primary treatment to ameliorate acute lung inflammation in CF, which is a primary cause of mortality and morbidity in CF.

In the present work, results were obtained using only immortalized human respiratory tracheal epithelial cells. Additional studies need to be done to better understand the cytokine response to fenretinide using different cell lines from the entire spectrum of cells in the human respiratory tract. As the experimental model used in our studies represents an *in vitro* environment, conclusions about CF pathogenesis should be made with caution. Further work is now required to determine whether the *in vitro* observations found in this study can be translated into a clinically meaningful effect in CF patients.

Acknowledgments

The authors thank Nurlan Dauletbaev and Serge Dernovici for their technical advice and help. The first author was sponsored scholarships by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Process n. 200026/93-3 (RN), Brasília, D.F.-Brazil; Federal University of Paraná, and Tuiuti University of Paraná, Curitiba, Paraná, Brazil. The research was funded via grants to SK and LL by the National Sciences and Engineering Council of Canada (NSERC) and the Canadian CF Foundation.

References

Anderson M. E. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology.* 113: 548-551.

Andrieu-Abadie N., Gouazé V., Salvayre R., Levade, T. 2001. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radicals in Biology and Medicine.* 31: 717-728.

Bals R., Weiner D. J., Wilson J. M. 1999. The innate immune system in cystic fibrosis lung disease. *The Journal of Clinical Investigation.* 103: 303-7.

Bardales R. H., Xie S. S., Schaefer R. F., Hsu S. M. 1997. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. *The American Journal of Pathology.* 149: 845-852.

Becker M. N., Sauer M. S., Muhlebach M. S., Hirsh A. J., Wu Q., Verghese M. W., Randell S. H. 2004. Cytokine secretion by cystic fibrosis airway epithelial cells. *American Journal Respiratory and Critical Care Medicine.* 169: 645-653.

Bedard M., McClure C. D., Schiller N. L., Francoeur C., Cantin A., Denis M. 1993. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology*. 9: 455–462.

Beaver J. P., Waring, P. 1995. A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. *European Journal of Cell Biology*. 68: 47-54.

Boujaoude L. C., Bradshaw-Wilder C., Mao C., Cohn J., Ogretmen B., Hannun Y. A., Obeid L. M. 2001. Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid. Modulation of cellular activity of sphingosine 1-phosphate. *Journal of Biological Chemistry*. 276: 35258-35264.

Cannon C. L., Kowalski M. P., Stopak K. S., Pier G. B. 2003. *Pseudomonas aeruginosa*-induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. *Respiratory Cell and Molecular Biology*. 29: 188-197.

Chan C., Goldkorn T. 2000. Ceramide path in human lung cell death. *American Journal of Respiratory Cell and Molecular Biology*. 22: 460-468.

Chen Y., Zhou G., Tan Tse-Hua1. 1999. c-Jun N-terminal kinase mediates apoptotic signaling induced by *N*-(4-Hydroxyphenyl) retinamide. *Molecular Pharmacology*. 56: 1271-1279.

Cuello M, Coats A. O., Darko I., Ettenberg S. A., Gardner G. J., Nau M. M., Liu J. R., Birrer M. J., Lipkowitz S. 2004. *N*-(4-hydroxyphenyl) retinamide (4HPR) enhances TRAIL-mediated apoptosis through enhancement of a mitochondrial-

dependent amplification loop in ovarian cancer cell lines. *Cell Death and Differentiation*. 11: 527-541.

Dbaibo G. S., Perry D. K., Gamard C. J., Platt R., Poirier G. G., Obeid L. M, Hannun Y. A. 1997. Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF) -α: CrmA and Bcl-2 target distinct components in the apoptotic pathway. *The Journal of Experimental Medicine*. 185: 481-490.

Dinwiddie R. 2000. Pathogenesis of lung disease in cystic fibrosis. *Respiration*. 67: 3-8.

Erdreich-Epstein A., Tran L. B., Bowman N. N., Wang H., Cabot M. C., Durden D. L., Vlckova J., Reynolds C. P., Stins, M. F., Groshen S., Millard M. 2002. Ceramide signaling in fenretinide-induced endothelial cell apoptosis. *The Journal of Biological Chemistry*. 277: 49531-49537.

Freedman S. D., Blanco P. G., Zaman M. M., Shea J. L., Ollero M., Hopper I. K., Weed D. A., Gelrud A., Regan M. M., Laposata M., Alvarez J. G., O'Sullivan B. P. 2004. Association of cystic fibrosis with abnormalities in fatty acid metabolism. *The New England Journal of Medicine*. 350: 560-569.

Garaventa A., Luksch R., Lo Piccolo M. S., Cavadini E., Montaldo P. G., Pizzitola M. R., Boni L., Ponzoni M., Decensi A., De Bernardi B., Bellani F. F., Formelli F. 2003. Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. *Clinical Cancer Research*. 9: 2032-2039.

Ginis I., Schweizer U., Brenner M., Liu J., Azzam N., Spatz M., Hallenbeck J. M. 1999. TNF-α pretreatment prevents subsequent activation of cultured brain cells with TNF-α and hypoxia via ceramide. *American Journal of Physiology*. 276 (*Cell Physiology* 45): C1171-C1183.

Hail N. Jr., Lotan R. 2001. Mitochondrial respiration is uniquely associated with prooxidant apoptotic effects of *N*-(4-hydroxyphenyl) retinamide. *Journal of Biological Chemistry.* 276: 4514-45621.

Harris J. F., Fisher M. J., Hotchkiss J. R., Monia B. P., Randell S. H., Harkema J. R., Tesfaigzi Y. 2005. Bcl-2 sustains increased mucuous and epithelial cell numbers in metaplasic airway epithelium. *American Journal of Respiratory and Critical Care Medicine.* 171: 764-772.

Hannun Y. A. 1996. Functions of ceramide in coordinating cellular responses to stress. *Science.* 274: 1855-1859.

Hannun Y. A., Bell R. M. 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science.* 243: 500–507.

Ip Y. T., Davis R. J. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. *Current Opinion in Cell Biology.* 10: 205-219.

Jungas T., Motta I., Duffieux F., Fanen P., Stoven V., Ojcius D. M. 2002. Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry.* 277: 27912-27918.

Kang Y. J., Enger M. D. 1991. Increased glutathione levels in quiescent, serum-stimulated NRK-49F cells are associated not with a response to growth factors but with nutrient repletion. *Journal of Cellular Physiology.* 148: 197-201.

Knowles M. R., Boucher R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways ("Perspective"). *The Journal of Clinical Investigation.* 109: 571-577.

Lavrentiadou S. N., Chan C., Kawcak T., Ravid T., Tsaba A., van der Vliet A., Rasooly R., Goldkorn T. 2001. Ceramide-mediated apoptosis in lung epithelial cells is regulated by glutathione. *American Journal of Respiratory Cell and Molecular Biology*. 25: 676-684.

Levade T., Jaffrezou J. P. 1999. Signalling sphingomyelinases: which, where, how and why? *Biochimica et Biophysica Acta*. 1438: 1-17.

Li W. Q., Dehnade F., Zafarullah M. 2000. Thiol antioxidant, *N*-acetylcysteine, activates extracellular signal-regulated kinase signaling pathway in articular chondrocytes. *Biochemical and Biophysical Research Communications*. 275: 789-794.

Linsdell P., Hanrahan J. W. 1998. Glutathione permeability of CFTR. *American Journal of Physiology*. 275: C323-326.

Liu B., Andrieu-Abadie N., Levade T., Zhang P., Obeid L. M., Hannun Y. A. 1998. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. *The Journal of Biological Chemistry*. 273: 11313-11320.

Loosdrecht van de A. A., Beelen R. H. J., Ossenkoppele G. J., Broekhoven M. G., Langenhuijsen M. M. A. C. A. 1994. Tetrazolium-based mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of Immunological Methods*. 174: 311-320.

Luberto C., Yoo D. S., Suidan H. S., Bartoli G. M., Hannun Y. A. 2000. Differential effects of sphingomyelin hydrolysis and resynthesis on the activation of NF-kappa B in normal and SV40-transformed human fibroblasts. *The Journal of Biological Chemistry*. 275: 14760-14766.

Massengale A. R., Quinn F. Jr, Yankaskas J., Weissman D., McClellan W. T., Cuff C., Aronoff S. C. 1999. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*. 20: 1073-1080.

Maurer B. J., Metelitsa L. S., Seeger R.C., Cabot M. C., Reynolds C. P. 1999. Increase of ceramide and induction of mixed apoptosis/necrosis by *N*-(4-hydroxyphenyl)-retinamide in neuroblastoma cell lines. *Journal of the National Cancer Institute*. 91: 1138-1146.

Merritt G., Aliprandis E. T., Prada F., Rigas B., Kashfi K. 2001. The retinoid fenretinide inhibits proliferation and downregulates cyclooxygenase-2 gene expression in human colon adenocarcinoma cell lines. *Cancer Letters*. 164: 15-23.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65: 55-63.

Muldoon L. L., Walker-Rosenfeld S. L., Hale C., Purcell S. E., Bennett L. C., Neuwelt E. A. 2001. Rescue from enhanced alkylator-induced cell death with low molecular weight sulfur-containing chemoprotection. *The Journal of Pharmacology and Experimental Therapeutics*. 296: 797-805.

Kim E. S. Fenretinide and lonafarnib in treating patients with advanced or recurrent head and neck cancer. In: National Institutes of Health (NIH). 2005. (Online). http://www.clinicaltrials.gov/ct/show/NCT00103090. October, 26.

Polunovsky V. A., Chen B., Henke C., Snover D., Wendt C. 1993. Role of mesenchymal cell death in lung remodeling after injury. *The Journal of Clinical Investigation*. 92: 388-397.

Pyne S., Pyne N. J. 2000. Sphingosine 1-phosphate signalling in mmmmalian cells. *The Biochemical Journal*. 349: 385-402.

Schwiebert L. M., Estell K., Propst S. M. 1999. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *American Journal of Physiology*. 276: C700–C710.

Shaw J. P., Chou I. N. 1986. Elevation of extracellular glutathione content associated with mitogenic stimulation of quiescent fibroblast. *Journal of Cell Physiology*. 129: 193-198.

Shimada K., Nakamura M., Ishida E., Kishi M., Yonehara S., Konishi N. 2002. Contributing of mitogen-activated protein kinase and nuclear factor kappa B to *N*-(4-hydroxyphenyl) retinamide-induced apoptosis in prostate cancer cells. *Molecular Carcinogenesis*. 35: 127-137.

Siddik Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*. 22: 7265-7279.

Signorelli P., Luberto C., Hannun Y.A. 2001. Ceramide inhibition of NF-kappaB activation involves reverse translocation of classical protein kinase C (PKC) isoenzymes: requirement for kinase activity and carboxyl-terminal phosphorylation of PKC for the ceramide response. *The FASEB Journal*. 15: 2401-2414.

Singh I., Pahan K., Khan M., Singh A. K. 1998. Cytokine-mediated induction of ceramide production is redox-sensitive. Implications to proinflammatory cytokine-mediated apoptosis in demyelinating diseases. *Journal of Biological Chemistry*. 273: 20354-20362.

Stecenko A. A., King G., Torii K., Breyer R. M., Dworski R., Blackwell T. S., Christman J. W., Brigham K.L. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation*. 25: 145-155.

Tabary O., Zahm J. M., Hinnrasky J., Couetil J. P., Cornillet P., Guenounou M., Gaillard D., Puchelle E., Jacquot J. 1998. Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells *in vivo* and *in vitro*. *The American Journal of Pathology*. 153: 921–930.

Ulukaya E. and Wood E. J. 1999. Fenretinide and its relation to cancer. *Cancer Treatment Reviews*. 25: 229-35.

Venkatakrishman A., Stecenko A. A., King G., Blackwell T. R., Brigham K. L., Christman J. W., Blackwell T. S. 2000. Exaggerated activation of nuclear factor-κB and altered IκB-β processing in cystic fibrosis bronchial epithelial cells. *Respiratory Cell and Molecular Biology*. 23: 1-16.

Wang H., Maurer B. J., Reynolds C. P, Cabot, M. C. 2001. *N*-(4-hydroxyphenyl) retinamide elevates ceramide in neuroblastoma cell lines by coordinate activation of serine palmitoyltransferase and ceramide synthase. *Cancer Research*. 61: 5102-5105.

Zafarullah M., Li W. Q., Sylvester J., Ahmad M. 2003. Molecular mechanisms of N-acetylcysteine actions. *Cellular and Molecular Life Sciences*. 60: 6-20.

Zimmermann C., Ginis I., Furuya K., Klimanis D., Ruetzler C., Spatz M. 2001. Lipopolysaccharide-induced ischemic tolerance is associated with increased levels of ceramide in brain and in plasma. *Brain Research*. 895: 59-65.

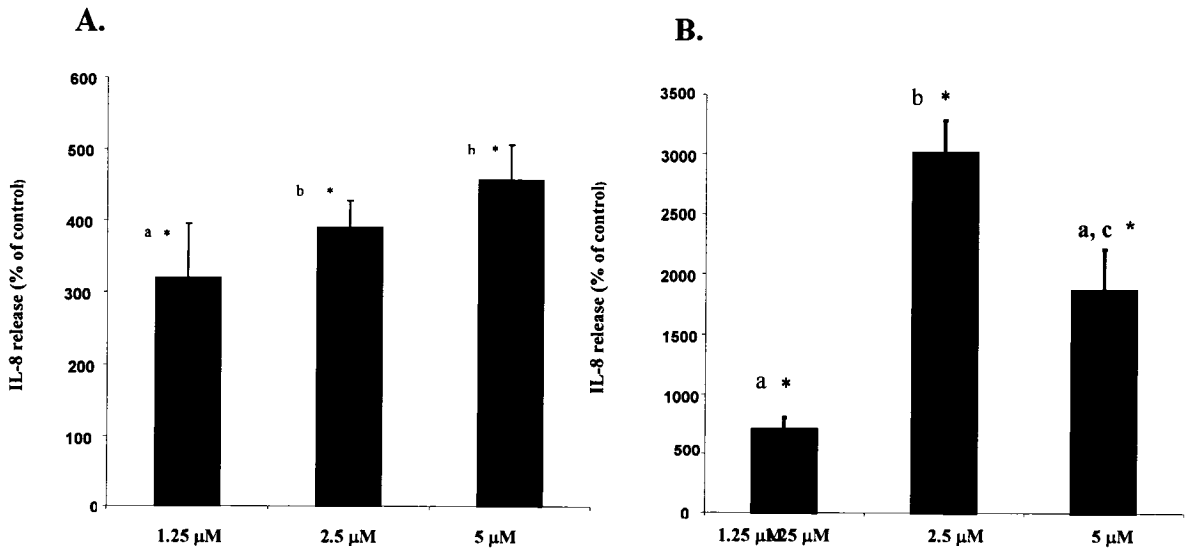


Figure 1. Effect of fenretinide on IL-8 release under basal conditions in: (A) CFTE29o- cells; and (B) 9HTEo- cells. The cells were seeded in 24-well plates at 0.6 x 10^6/mL and 0.4 x 10^6/mL, respectively, and incubated in MEM containing 10% FBS for 24 h. After 24 h the medium was replaced by MEM containing 2% FBS and 1.25 µM, 2.5 µM or 5 µM of fenretinide (HPR) for an additional 24 h (pre-incubation). After 24 h, the medium was replaced by MEM 2% FBS and the cells were incubated with the same concentrations of fenretinide for another 24 h. The supernatant was collected to determine IL-8 release using an ELISA kit (Pharmigen, OptEIA Human IL-8 Set). Vehicle control wells contained ethanol (EtOH) at concentrations equivalent to those used with fenretinide treatments. Results are means ± S.E. of 3-7 independent experiments. Columns not sharing common letters represent means that differed significantly ($P < 0.05$) by Tukey's *post hoc* comparison **A.** 100% (291 ± 32 pg·mL^{-1}) corresponds to IL-8 release by untreated control cells (MEM 2% FBS). Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated (MEM 2% FBS) controls by ANOVA. **B.** 100% (146 ± 14 pg·mL^{-1}) corresponds to IL-8 release by untreated control cells (MEM 2% FBS). Asterisks (*) indicate significant differences ($P <$ 0.05) as compared to untreated controls by ANOVA.

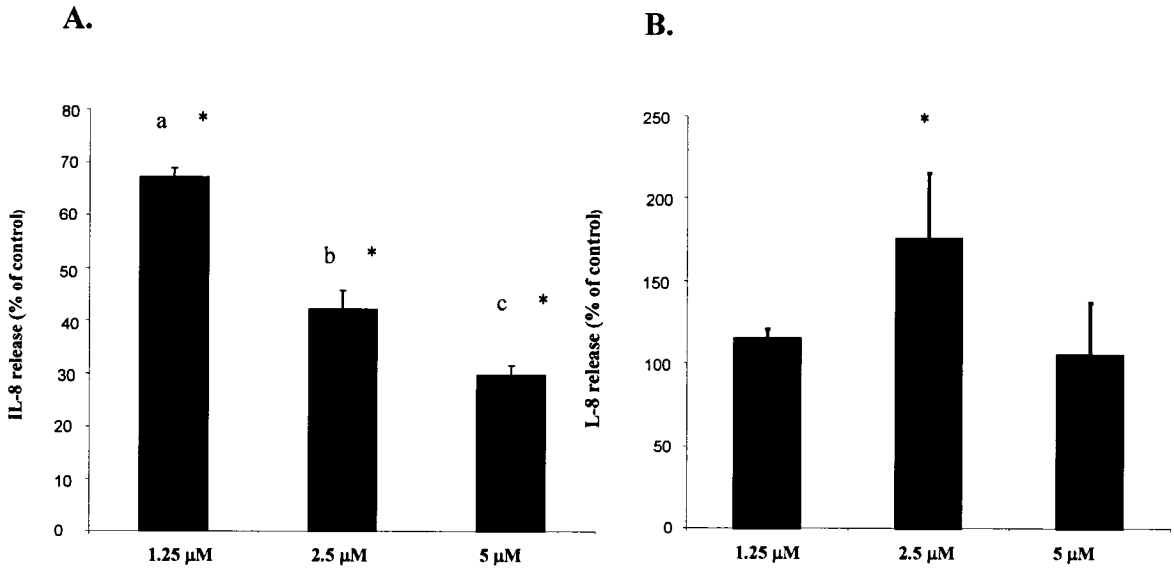


Figure 2. Effect of fenretinide on IL-8 release under stimulated conditions in: (A) CFTE29o- cells; and (B) 9HTEo- cells. The cells were seeded and kept during 24 h as described in Fig. 1. After 24 h the medium was replaced by MEM containing 2% FBS and 1.25 µM, 2.5 µM or 5 µM of fenretinide (HPR) for additional 24 h (pre-incubation). After 24 h, the medium was replaced by MEM 2% FBS and the cells were incubated with the same concentrations of fenretinide and stimulated with human recombinant TNF-α for another 24 h. The supernatant was collected to determine IL-8 release using an ELISA kit (Pharmigen, OptEIA Human IL-8 Set). Vehicle control wells contained ethanol (EtOH) at concentrations equivalent to those used with fenretinide treatments (data not shown). Results are means ± S.E. of 3 - 7 independent experiments. Columns not sharing common letters represent means that differed significantly ($P < 0.05$) by Tukey's *post hoc* comparison. **A.** 100% (27,406 ± 1,724 $pg \cdot mL^{-1}$) corresponds to IL-8 release by untreated control cells (MEM 2% FBS) stimulated with TNF-α. Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated controls (MEM 2% FBS) by ANOVA. **B.** 100% (62,358.85 ± 6,810 $pg \cdot mL^{-1}$) corresponds to IL-8 release by untreated control cells (MEM 2% FBS) stimulated with TNF-α. Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated controls (MEM 2% FBS) by ANOVA.

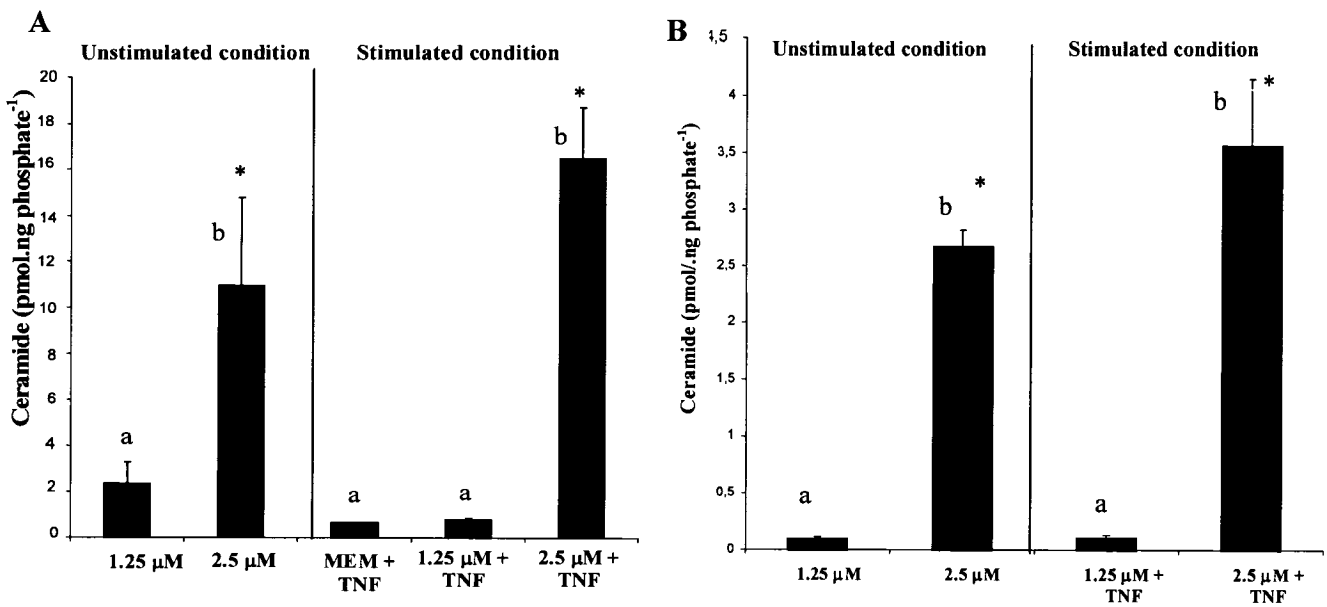


Figure 3. Effect of fenretinide on intracellular ceramide concentrations in: (A) CFTE29o- cells; and (B) 9HTEo- cells. CFTE29o- and 9HTEo- cells were treated as described in methods. **A.** Basal levels of ceramide (MEM 2% untreated cells) did not reach the minimal detectable values (30 pmoles). **B.** Stimulated and non-stimulated basal levels of ceramide (MEM 2% untreated cells and MEM 2% + TNF-α untreated) did not reach the minimal detectable values (30 pmoles). Results are means ± S.E. of 3 independent experiments. Columns not sharing common letters represent means that differed significantly ($P < 0.05$) by Tukey's *post hoc* comparison. Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated controls (MEM 2% FBS for non inflammatory condition and MEM 2% + TNF-α for inflammatory condition) by ANOVA.

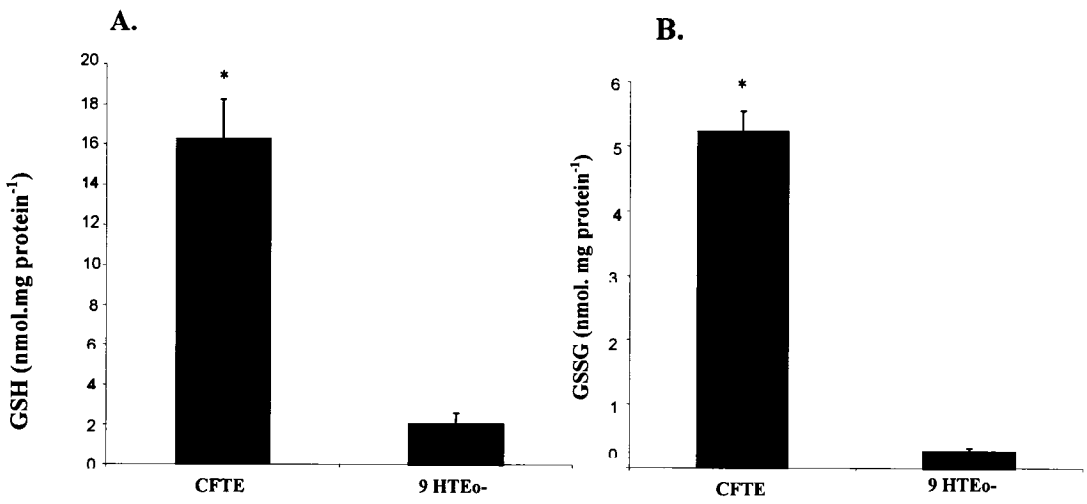


Figure 4. Intracellular basal GSH and GSSG content. Concentrations of intracellular (**A**) GSH and (**B**) GSSG in normal (9HTEo-) and CFTR deficient (CFTE29o-) human tracheal epithelial cells. Results are means ± SEM of three independent experiments. *Significantly different ($P < 0.05$) by Student's *t-test*.

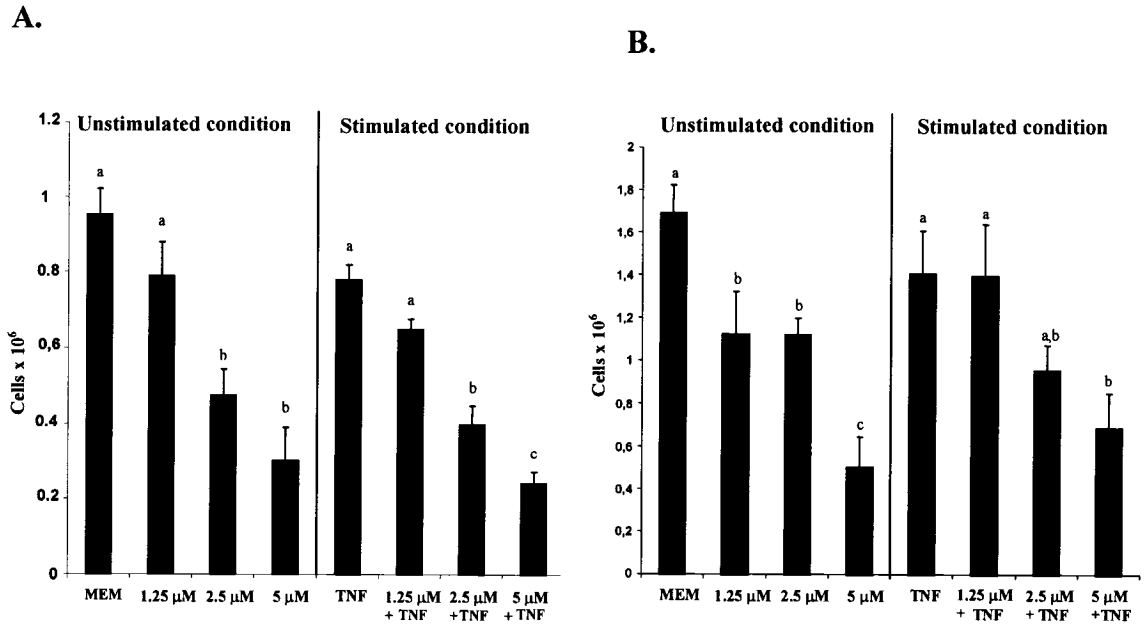


Figure 5. Effect of fenretinide on CFTE29o-(A) and 9HTEo- (B) cell viability. CFTR deficient (CFTE29o-) and normal human epithelial cells (9HTEo-) were treated as described on Fig. 1A and 2A (non-inflammatory condition), and 1B and 2B (inflammatory condition), respectively, for non-inflammatory and inflammatory conditions. After the supernatant was collected to determine IL-8 release, the cells were treated with MTT solution, incubated for 3 h at 37°C and made permeable with HCl-isopropanol. Optical densities were measured at 540 nm and the values were converted to cell numbers by using a standard curve previously established as described in methods. Results are means ± S. E. Columns not sharing common letters represent means that differed significantly ($P < 0.05$) by Tukey's *post hoc* comparison.

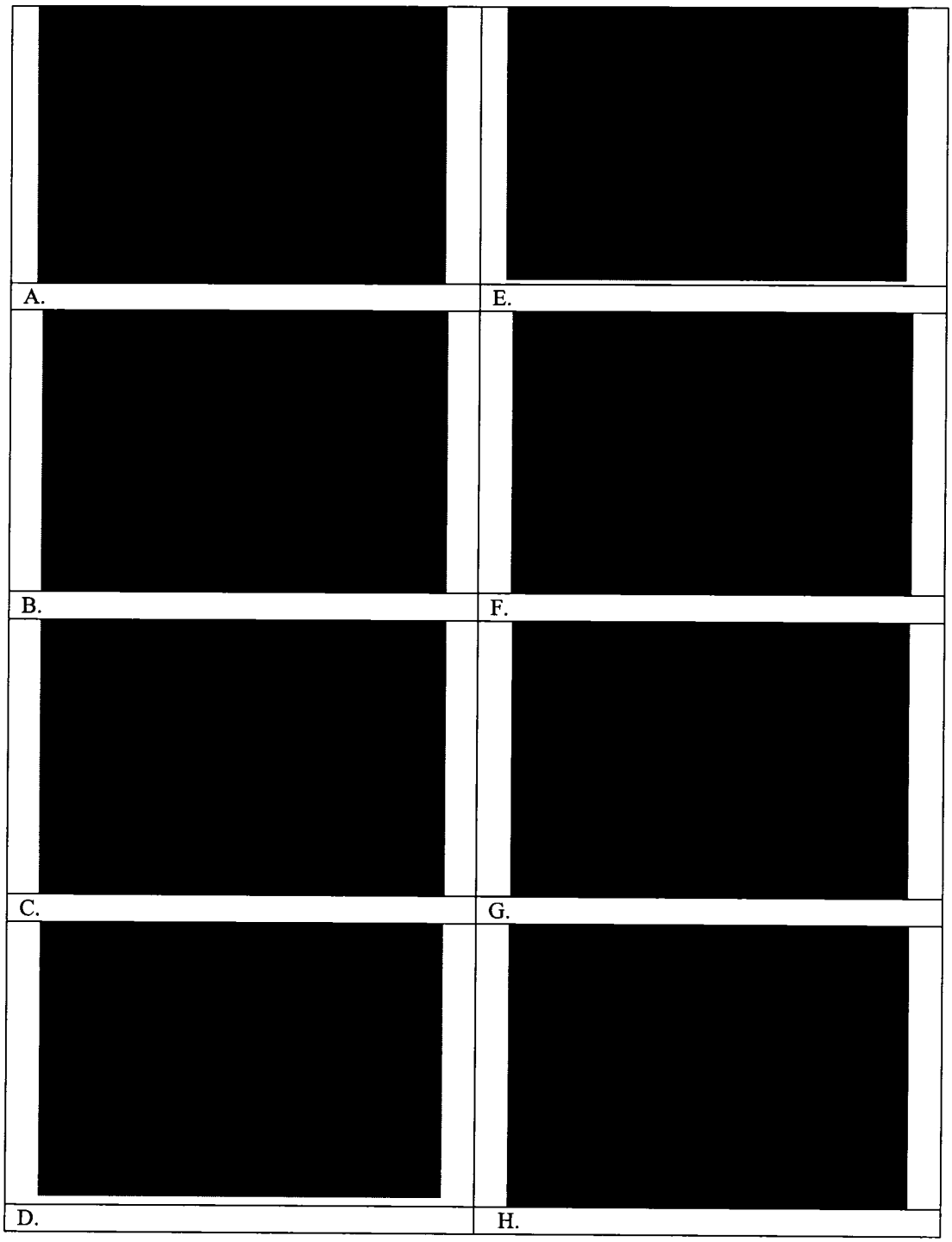

A.
E.
B.
F.
C.
G.
D.
H.

Figure 6. Images of CFTE26o- cells under inflammatory and non-inflammatory conditions treated with fenretinide: **A**. Negative control (see methods for details), **B**. MEM 2%, **C**. MEM 2% + fenretinide 1.25 μM, **D**. 2% + fenretinide 2.5 μM, **E**. Positive control (cisplatin 25 μM), **F**. MEM 2% TNF-α stimulated, **G**. MEM 2% TNF-α stimulated and fenretinide treated (1.25 μM), **H**. MEM 2% TNF-α stimulated and fenretinide treated (2.5 μM). Apoptotic nuclei are identified by the fluorescence produced by the binding of Tdt enzyme binds to the 3-OH free end of DNA strand breaks as described in methods. In contrast, normal nuclei showed no fluorescence.

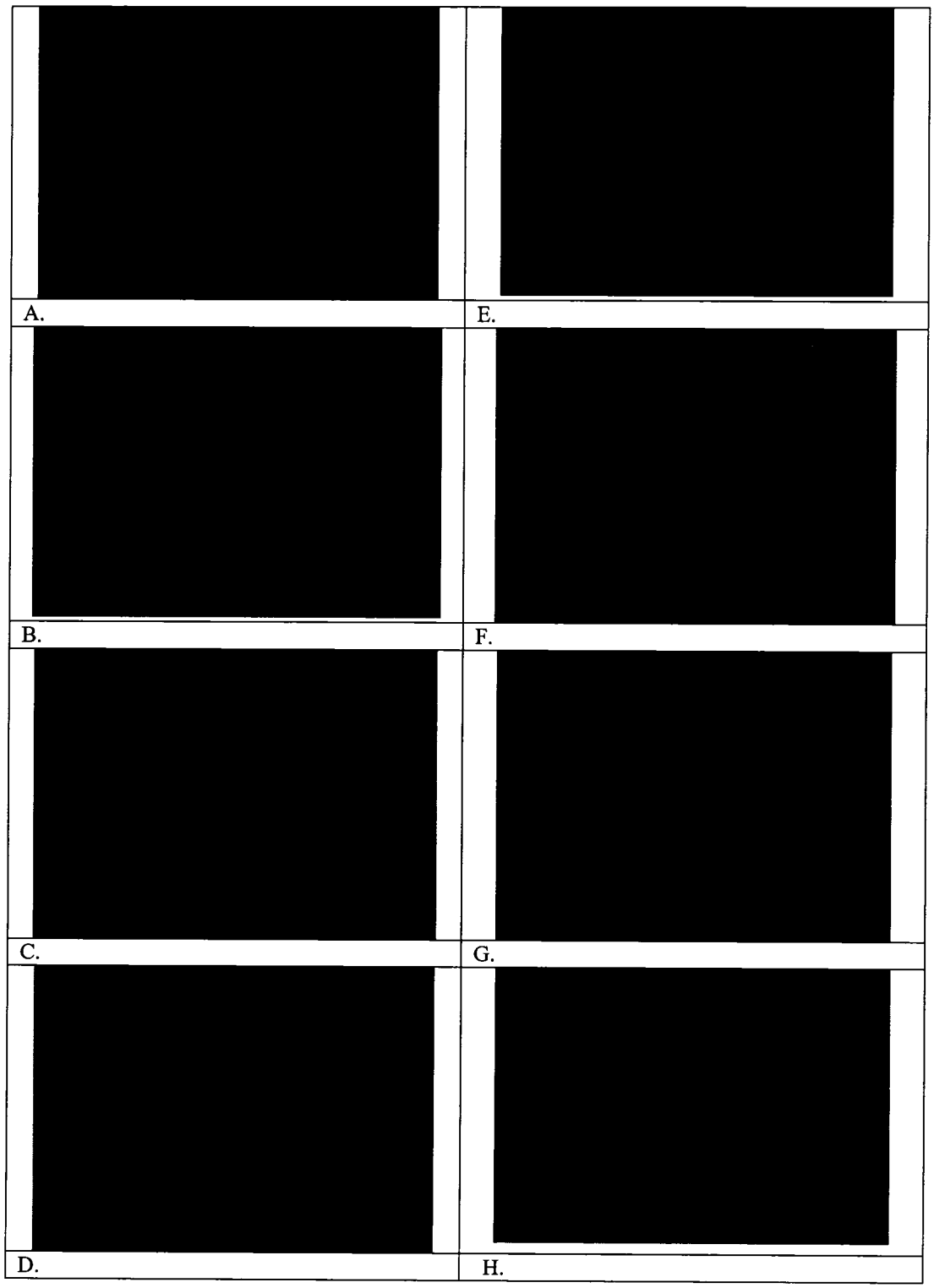

A.
E.
B.
F.
C.
G.
D.
H.

Figure 7. Images of 9HTEo- cells under inflammatory and non-inflammatory conditions treated with fenretinide: **A**. Negative control, **B**. MEM 2%, **C**. MEM 2% + fenretinide 1.25 μM, **D**. 2% + fenretinide 2.5 μM, **E**. Positive control (cisplatin 25 μM), **F**. MEM 2% TNF-α stimulated, **G.** MEM 2%, TNF-α stimulated and fenretinide treated (1.25 μM), **H.** MEM 2% TNF-α stimulated and fenretinide treated (2.5 μM). Apoptotic nuclei are identified by the fluorescence produced by the binding of Tdt enzyme binds to the 3-OH free end of DNA strand breaks as described in methods. In contrast, normal nuclei showed no fluorescence.

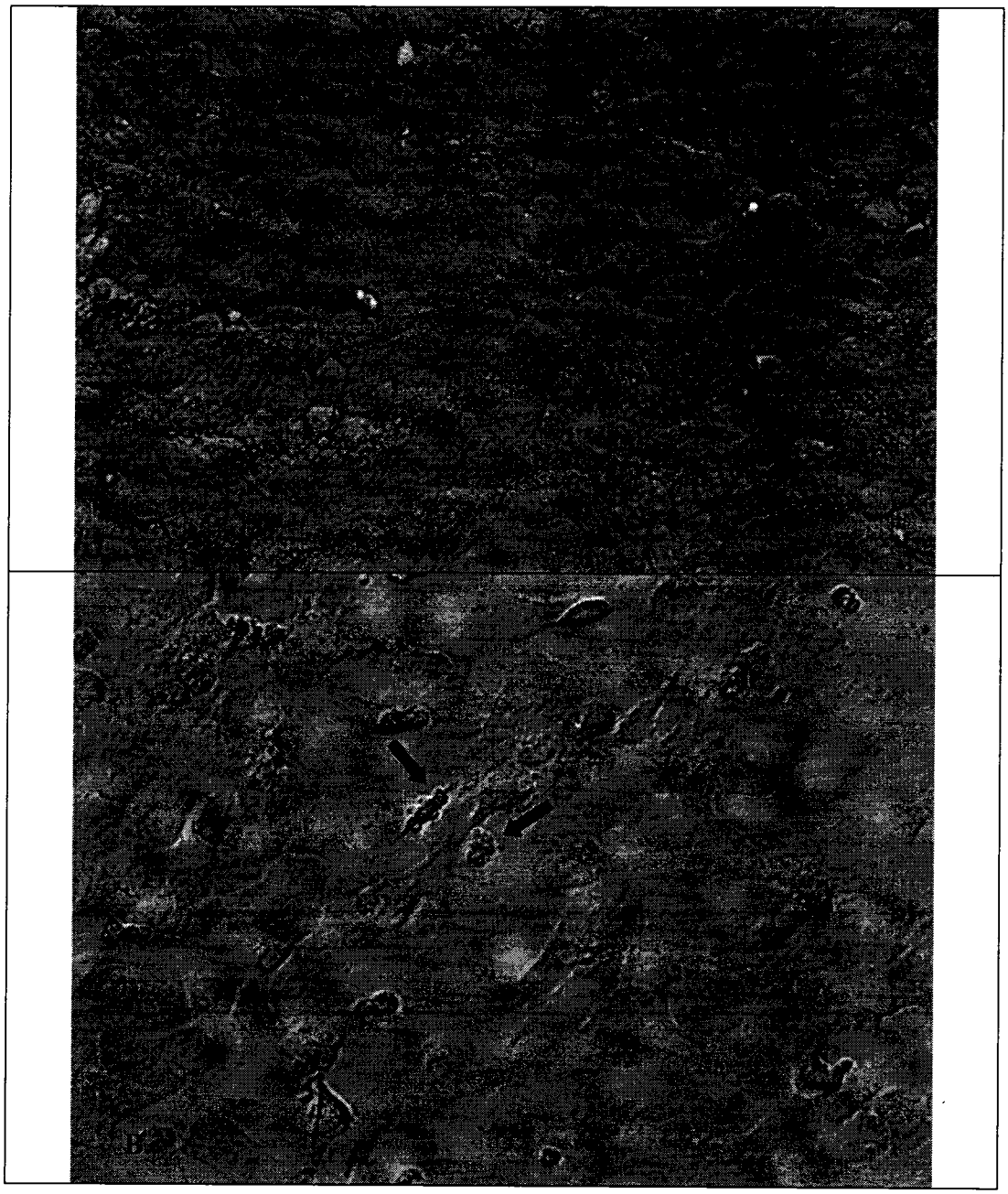


Figure 8. Morphologic comparison between normal epithelial cells under TNF-α stimulation (MEM 2% FBS + TNF-α) and after treatment with fenretinide (2.5 μM) under TNF-α stimulation. A. 9HTEo- cells stimulated with TNF-α untreated (control), **B.** 9HTEo- cells stimulated with TNF-α and treated with fenretinide (2.5 μM). Arrows indicate morphologic signs of apoptosis (cytosol shrinkage and plasma membrane blebbing).

CONNECTING STATEMENT

The results obtained in Chapter 3 showed that fenretinide inhibited TNF-α-induced IL-8 production of mutant ΔF508 CFTR cultured cells in a dose response manner. These results thus present the possibility that the lung inflammation in CF could be attenuated via low dose fenretinide treatment. In addition, CF cells showed constitutively high levels of GSH; however, the redox GSH/GSSG ratio was several-fold lower as compared to wild-type cells, which indicates the presence of oxidative stress in CF cells. Whey protein based diets and whey protein hydrolysates have been shown to improve the intracellular redox GSH/GSSG ratio as well as to exert immunomodulatory activity in *in vitro* and *ex-vivo* studies. There is little knowledge, however, concerning the bioactive components in whey proteins in terms of enhancing GSH status although peptides have been proposed. Moreover, pressure processing has recently been shown to cause partial unfolding of whey proteins, which coud lead to enhanced digestibility and greater release of bioactive peptides involved in GSH synthesis. Hence, *in vitro* digestibility studies were performed to determine if native and pressurized whey proteins differ in terms of digestibility and their peptide profiles. As enhancement of the redox status of cells in terms of GSH/GSSG has been associated with decreased IL-8 release, subsequent cell culture studies to test the impact of whey peptides derived from hydrolysed native or pressurized whey proteins on the intracellular levels of GSH, apoptosis and IL-8 release were performed using tracheal epithelial CF and non-CF cells.

CHAPTER 4

The Impact of High Hydrostatic Pressure on the Digestibility and Production of Bioactive Peptides from Whey Protein Isolates

Regina Maria Vilela[1], Larry C. Lands[2], H.M. Chan[1,3], Behnan Azadi[1] and Stan Kubow[1,4]

[1]School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
[2]Montreal Children's Hospital
Division of Respiratory Medicine, Room D-380
2300 Tupper Street, Montreal, Quebec H3H 1P3
[3]Centre for Indigenous Peoples' Nutrition and Environment (CINE), Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9

[4]Corresponding Author: Dr. Stan Kubow
School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
e-mail: stan.kubow@mcgill.ca

Abstract

Whey protein isolate (WPI) treatment has been proposed to provide anti-inflammatory benefits to cystic fibrosis (CF) via induction of tissue glutathione (GSH), which could be mediated via whey protein-derived peptides. Peptides such as γ-glutamyl-cysteine as well as proteolytic digests of whey proteins have been shown to enhance intracellular concentrations of GSH. An objective of this study was to investigate the potential of high hydrostatic pressure to enhance the *in vitro* digestibility of WPI and to generate bioactive low molecular weight (<1 kDa) peptides that could exert GSH-enhancing and anti-inflammatory properties in cell cultures of wild type and mutant CF transmembrane conductance regulator (CFTR) tracheal epithelial cells. An *in vitro* methodology described herein demonstrated that hydrostatic pressure processing enhanced the digestibility of WPI to proteolytic enzymes and resulted in altered peptide profile as assessed by capillary zone electrophoresis, reverse-phase high performance chromatography and gas chromatography-mass spectrometry. The exposure of mutant CFTR cells to a dose of peptides (12.5 μg/mL) with molecular weights < 1 kDa isolated from hydrolysates of whey proteins exposed to high hydrostatic pressure treatment (pressurized whey protein hydrolysates, pWPH), was associated with increased intracellular levels of reduced glutathione (GSH) and total GSH as compared to treatment with peptides obtained from native whey protein hydrolysates (nWPH). No significant effects on cellular total or reduced GSH content were observed following the treatment of wild type epithelial cells with either pWPH or nWPH although an increase in GSSG was noted with the 500 μg/mL dose of pWPH. A tendency for decreased secretion of interleukin (IL)-8 was associated with pWPH and nWPH treatment in the defective CFTR cells, which was not observed in wild type cells. The results of the present study indicate that hydrostatic pressure processing has the potential to enhance cellular GSH status and anti-inflammatory properties of whey proteins in cells with the mutant CFTR condition.

4.1 Introduction

There is increasing use of bovine whey proteins in either native or predigested forms in infant formulas and medical foods for nutritional and therapeutic applications (de Ferrer, 1995; Hays and Wood, 2005; Poullain *et al.,* 1989). In that regard, whey proteins contain bioactive peptides in their primary sequence that can be released during gastrointestinal protein hydrolysis to exert physiological effects. Bioactive peptides in whey protein hydrolysates have demonstrated cytoprotective effects towards oxidative stress (Bartfay *et al.*, 2003), and to induce levels of the major intracellular antioxidant, glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) in cultured prostatic cells (Kent *et al.*, 2003). Human trials have demonstrated that intake of whey proteins, which are cystine-rich, can increase GSH levels in lymphocytes (Grey *et al.*, 2003) and plasma (Kennedy *et al.*, 1995; Micke *et al.*, 2001). Whey proteins such as bovine serum albumin (BSA), β-lactoglobulin (β-LG) and immunoglobulins contain glutamic acid-cysteine segments in their primary sequences that form disulfide bridges. It has been postulated that during digestion of undenatured whey protein isolates (WPI), low-molecular-weight peptides such as γ-glutamylcysteine or cystine are released and absorbed intact to stimulate GSH biosynthesis (Bounous and Gold, 1991). Cystine, the disulfide form of cysteine, can be used towards intracellular synthesis of GSH (Anderson, 1995) as cystine is reduced to two moles of cysteine, which is the rate-limiting amino acid in GSH biosynthesis. The GSH molecule is a ubiquitous cellular non-protein sulfhydryl that functions as a potent cellular antioxidant since the reduced form of GSH can readily donate its sulfhydryl proton to quench reactive oxygen species (ROS). The oxidized form GSH forms a disulfide linkage with a second oxidized GSH molecule to yield glutathione disulfide (GSSG). A high intracellular GSH/GSSG ratio provides strong protection against cell damage induced by oxidative stress (Rahman and MacNee, 2000).

Major developments in the isolation and processing of whey proteins have improved their physicochemical and sensory properties as ingredients within foods (Haeusel *et al.*, 1990). There is relatively little knowledge, however, regarding the impact of food processing on the digestibility and antioxidant activity of whey proteins. The sensitivity

of proteins to digestion as well as the type of peptides formed from the proteolysis is dependent on the amino acid sequence and three-dimensional structure of proteins. In that regard, whey proteins have a globular conformation that makes them more resistant to digestive enzyme proteolysis (Mutilangui *et al.*, 1995). High hydrostatic pressure processing is a food processing technique used as an alternative to heat treatment for food sterilization whereby the proteins are subjected to pressures of several hundred MPa (Camp and Huyghebaert, 1995). Although the primary structure of proteins remains intact during pressure treatment (Mozhaev *et al.*, 1994), high-pressure treatment above 200 MPa can cause changes in the secondary and tertiary structure that can lead to irreversible denaturation of the protein (Hendrickx *et al.*, 1998). Several studies have demonstrated that *in vitro* proteolytic digestion of individual whey proteins such as β-LG and α-lactalbumin is enhanced when carried out under elevated hydrostatic pressures (Hayashi *et al.*, 1987; Stapelfeldt *et al.*, 1996). It is unclear; however, whether the prior exposure of whey proteins to high hydrostatic pressure can enhance their subsequent *in vitro* digestibility under ambient conditions or whether hydrostatic pressure processing could alter the profile of bioactive peptides released upon hydrolysis.

The impact of peptides generated from whey protein hydrolysis has not been studied previously in lung epithelial cells, particularly in relation to their impact on inflammation and GSH status. High concentrations of GSH are present in lung epithelial lining fluid and GSH is important in maintenance of the integrity of the lung airspace epithelia *in vitro* and *in vivo* (Li *et al.*, 1997). There is abundant evidence that GSH also plays a major role in regulating inflammatory mediators such as cytokines increased by oxidative stress (Meister, 1988; Haddad., 2000). The ROS signaling regulating the transcription of interleukin (IL)-4 (Jeannin *et al.*, 1995), IL-6, IL-8 (Gosset *et al.*, 1999), and tumor necrosis factor (TNF)-α (Gosset *et al.*, 1999) involve thiol-dependent mechanisms. Depletion of lung epithelial lining fluid GSH has been described in lung inflammatory disorders such as cystic fibrosis (CF), adult respiratory distress syndrome (Bunnell and Pacht, 1993), and idiopathic pulmonary fibrosis (Cantin *et al.*, 1989). Cellular GSH depleting agents such as L-buthionine-(S, R)-sulfoximine (BSO) can enhance cytokine secretion by up-regulating ROS (Gosset *et al.*, 1999). Conversely, the GSH precursor N-

acetyl-L-cysteine (NAC) inhibits TNF-α-induced activation of nuclear factor (NF)-κB activity and IL-8 promoter-mediated reporter gene expression in airway epithelial cells (Harper *et al.*, 2001).

The CF condition is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene that encodes a cAMP-dependent chloride channel located in the apical membrane of epithelial cells. Lung damage due to chronic inflammation, mucus accumulation and chronic infection is the major cause of death in CF. Chronic inflammation is also associated with increased oxidative stress in CF patients (van der Vliet *et al.*, 1997). Antioxidant therapy such as supplementation of WPI to induce tissue GSH has been proposed to combat chronic inflammation in the CF lung (Grey *et al.*, 2003). CFTR deficiency is known to result in decreased GSH export leading to a reduction of GSH in the CF apical fluid (Gao *et al.*, 1999); however, relatively little is known regarding GSH status of airway epithelial cells or whether intervention with peptides derived from whey proteins can stimulate GSH status and diminish IL-8 secretion in mutant CFTR lung epithelial cells.

The first part of this study utilizes an *in vitro* enzymatic digestion protocol to study the effect of high hydrostatic pressure on the digestibility and peptide profile of whey protein subjected to different forms of high hydrostatic pressure. A combination of ultrafiltration techniques and enzymic hydrolyses were used to produce a hydrolysate in which peptide fragments > 1 kDa were removed, as these latter peptides are considerably less bioavailable and are not observed in the ileal juices in the pig model of human digestion following protein feeding (Qiao *et al.*, 2004). Treatment of wild type tracheal epithelial cells (9HTEo-) and mutant CFTR cells (CFTE29o-) with the low molecular weight peptides obtained from hydrolysates of native and pressurized whey proteins was undertaken to examine whether such peptides could lead to differential effects on GSH homeostasis. The present study also tested the hypothesis that an induction of intracellular GSH via whey peptide treatment would antagonistically affect the release of the cytokine and chemokine, IL-8, under pro-inflammatory conditions as raised secretory

levels of this cytokine is recognized as a major contributory factor in lung pathophysiology (Hirani *et al.*, 2001).

4.2 Materials and Methods

Materials

InPro^{TM} WPI was obtained from Volac Nutrition, Inc. (England) and contains a 92% protein content obtained from cross-flow microfiltration. To perform *in vitro* protein digestion, pepsin (catalog # P-7012) from porcine stomach mucosa and pancreatin (catalog # P-1625) from porcine pancreas were obtained from Sigma-Aldrich Co (Oakville, Ontario) and prepared in 0.01M HCl and phosphate buffer pH 7.0, respectively. Dulbecco's phosphate-buffered saline was obtained from Gibco BRL (Burlington, Ontario). To determine protein content the Bio-Rad protein assay dye reagent was obtained from ICN Biomedicals (Aurora, OH). Determination of the α-amino group content required preparation of an OPA (*o*-phthaldialdehyde) solution, which used the reagents sodium tetraborate, sodium dodecyl sulfate and 2-mercaptoetanol, which were obtained from Sigma-Aldrich (Oakville, Ontario). Amicon ultrafiltration regenerated cellulose membranes (NMWL: 1 kDa) and ultrafiltration stirred units for isolation of low molecular weight peptides from enzymic hydrolysates were obtained from Millipore (Nepean, Ontario).

Wild type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells were gifts obtained from Dr. D. Gruenert (University of California). The supplies for the maintenance of cell culture such as minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, L- glutamine, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Burlington, Ontario). Trypsin-EDTA solution (0.25%) was obtained from Sigma-Aldrich (Oakville, Ontario). The solution used to coat the T-75 flasks and 24-well plates was prepared with collagen type I bovine, and human fibronectin was obtained from BD Biosciences (Oakville, Ontario); bovine serum albumin (BSA), and LHC basal medium were obtained from

Biosource-Biofluids Division (Camarillo, California). Cell viability was determining by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma-Aldrich Co. To determine IL-8 release ELISA kits (catalog # 550999, OptEIA Human IL-8 Set, Pharmigen) were obtained from BD Bioscience (Oakville, Ontario).

Methods

Hydrostatic Pressure Treatment and Sample Preparation for Enzymatic Hydrolyses

Pressure treatment was applied to whey protein isolate solutions at 1 cycle of 550 MPa or 3 cycles of 400 MPa. Briefly, a 15% solution (w/v) of WPI was prepared using double distilled water and inserted into sealed plastic bags. Pressurization of the WPI solutions was undertaken using a high pressure machine from GEC Alstom ACB (Nantes, France) with a maximum capacity of 690 MPa. The pressure chamber was filled with water as the hydrostatic fluid and sealed plastic bags containing the WPI solution were submerged to receive the pressure treatment. During the pressure cycles, the temperature was kept below 26-27°C and after reaching 550 MPa (within three to four min) the pressure was released within one min (1 cycle pressure treatment). For the 3-cycle pressure treatment, the process of pressurization at 400 MPa was repeated for three times, i.e., increasing pressure to 400 MPa and then releasing it to ambient pressure for three consecutive times. The native 15% (w/v) WPI solution and the pressurized 15% (w/v) WPI solutions were lyophilized by placement into vacuum chambers of Flexi-Dry MP Lyophilizer (FTS Systems, Stone Ridge, NY) and freeze-dried under -80°C and 90 MT vacuum in preparation for future *in vitro* digestibility studies. The time needed to freeze-dry the samples depended on the volume used and varied from 48 to 72 h.

In Vitro Enzymatic Digestion

The enzymatic digestion method was a modification of the methods of Multilagi *et al.* (1995) and Kitabataki and Kinekawa (1998) that were developed to simulate *in vivo*

gastrointestinal digestion of milk proteins in terms of biochemical, physical and mechanical conditions including the enzyme concentration, pH adjustment (i.e., pH 1.5 and then 7.8), temperatures for optimal enzyme activity (37°C and 40°C), shaking and incubation time. The 30 min incubation period with pepsin corresponded to the *in vivo* gastric half-emptying time described by Mah *et al.* (1994). After the lyophilization treatment described above, native and pressurized whey proteins were diluted in double distilled water at a concentration of 3 mg protein/mL and the pH of the solution was adjusted to 1.5 with HCl. Digestion experiments were performed using triplicate solutions at 37°C in 50 mL Erlenmeyer flasks placed in a shaking water bath. Digestion was initiated by the addition of freshly prepared enzyme stock solution (5 mg pepsin/mL in 0.01M HCl) to the protein solutions to reach an enzyme to substrate ratio of 1:100. The peptidic digestion was interrupted after 30 min by adding 1.0 M NaOH solution, which raised the pH to approximately 6, thereby inactivating pepsin irreversibly. After pepsin inactivation, the digests were put on ice and the pH was adjusted to 7.8 with 1.0 M NaOH and kept at -80°C until the next day for further digestion with pancreatin or for future analytical analyses. The peptidic digests were thawed at room temperature and placed in water bath at 40°C. Freshly prepared pancreatin stock solution (5 mg/mL in sodium phosphate buffer pH 7.0) was added to the pepsin pre-digested mixture to reach an enzyme to substrate ratio of 1:30. After 60 min incubation, 150 mM Na_2CO_3 solution was added to stop the reaction. The pancreatic digests were stored at –20°C until subsequent isolation of low molecular weight mass peptides.

Peptide Isolation

After the pepsin-pancreatin digestion, the resulting hydrolysates from native, single cycle and triple cycle pressurized WPI were subject to ultrafiltration to remove the low molecular weight mass peptides via cellulose membranes with a molecular weight cut-off (MWCO) of 3 or 1 kDa (Millipore) in a stirred ultrafiltration membrane reactor (Model 8050, Millipore, Nepean, Ontario) under nitrogen gas pressure of 40 psi. The process was performed under a refrigerated environment at 4°C and the peptides in the permeate were freeze-dried in a vacuum concentrator (Flexi-Dry MP Lyophilizer, FTS Systems Inc.,

Stone Ridge, NY) at -80°C and 90 MT vacuum for further analytical analyses and cell culture experiments. The freeze-dried peptides were stored in sealed tubes flushed with N_2 under -20°C if not used immediately.

Protein Content Determination

For the pepsin digestion experiments, the protein content of the whey protein solutions was determined at 5 min intervals for 30 min after starting the digestion with pepsin. Protein content was determined via the Bradford (1976) method according to the instructions of the kit manufacturer (catalog #500-0006, Bio-Rad, Mississauga, Ontario). Briefly, aliquots taken from the samples during the experiment were mixed with the dye reagent according to the manufacturer's recommendations and, after incubation for 5 min at room temperature; the optical densities (O.D.) at 540 nm were measured. The results were expressed as % of control (time 0), which corresponds to the total of whey protein in the solution before digestion. Considering that the protein content decreases as the digestion time progress, the volume of the aliquot to take was determined at time 0 and was based on the maximum linear absorbance obtained from the standard curve using BSA with the concentration ranging from 0.2 to 0.9 mg/mL.

α-Amino Group Analysis

The amount of released α-amino groups can be measured using reagents that react specifically with amino groups, yielding derivatives that can be detected spectrophotometrically via OPA. The α-amino group content was determined according to a spectrophotometric assay, which is based on the OPA reaction with α-amino groups released by hydrolysis as described by Church *et al.* (1983). Briefly, 50 mL of OPA solution was freshly prepared as follow: 25 mL of 100 mM sodium tetraborate solution in water; 2.5 ml of 20% (wt/wt) SDS; 40 mg of OPA (dissolved in 1 mL of ethanol); 100 μL of β-mercaptoethanol, and water to complete the volume. Aliquots of each sample were collected before and after digestion with pepsin and pancreatin and added to 1 mL of OPA solution and incubated for precisely 2 min. Considering that the α-amino group

content increases as the digestion time progresses, the volume of the aliquot to be taken was determined at time 0 and was based on the minimum linear absorbance obtained from the standard curve using Phe-Gly with the concentration ranging from 25 to 150 μM. The O.D. was measured two min later at 340 nm wavelength since OPA absorption is stable only after 20 min (Panasiuk *et al.*, 1998). Because the absorbance was sensitive to the pH, the efficiency of the digestion was determined by measuring the O.D. at 0 and 30 min at pH 1.5 for pepsin digestion and at 0 and 60 min at pH 7.8 for pancreatin digestion. The O.D. was also determined after the ultrafiltration to detect the peptides with molecular weight less than 1 kDa. The efficiency of the digestion was determined taking into consideration the net α-amino groups detected (O.D. before filtration less O.D. after filtration) after digestion with pepsin and after digestion with pancreatin. The results were expressed as μM of Phe-Gly.

Capillary Zone Electrophoresis (CZE) Analysis

Following pancreatin treatment, the lyophilized permeate containing < 1 kDa peptides obtained from hydrolysates of native WPI and 1-cycle and 3-cycle pressure treated WPI were analyzed by capillary zone electrophoresis (CZE). Prior to CZE analysis, the peptide solutions were filtered through a 0.2 μm low binding cellulose acetate membrane (Nalgene-nalge®, Nunc International Corporation, Rochester, NY). The running sodium phosphate buffer (0.1 M, pH 2.5) was obtained from Sigma-Aldrich. The sample stock peptide solutions were prepared by dissolving 50 mg of the freeze-dried filtered hydrolysates in 1.0 mL of the running buffer that was diluted (1:10) with nanopure water. The solutions were either kept on ice for immediate CZE analysis or stored at -80°C for later CZE analyses. The separation buffer solution was degassed before use. The CZE analysis was carried out using a Beckman P/ACE^{TM} 2200 HPAC instrument (Beckman Instruments, Inc., Fullerton, CA) coupled to an IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated (57 cm × 50 μm, the length from intake to detector was 50 cm) fused silica capillary column was assembled in a P/ACE cartridge (Polymicro Technologies, Phoenix, Arizona) for the

capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest run time. The CZE analyses were performed for a total duration of 30 min at a constant voltage (27 kV) and temperature (30°C) using a UV-detection at 214 nm. Before the first sample application, the capillary column was pre-rinsed with nanopure water for 2 min and separation buffer for additional 2 min or until the baseline line was stable. Between sample runs, the capillary was flushed with NaOH 1M for two min, and HCL 1N for additional two min followed by the pre-rinse. A mixture of peptide standards (peptide standard for CZE, catalog # P2693; Sigma-Aldrich Co, Oakville, Ontario) was used to verify the precision and accuracy of the method. The best peak resolution was achieved by injecting 40 μL of samples containing 2 mg/mL of lyophilized peptide extracts obtained following the dual enzyme digestion of pepsin and pancreatin digestion described above. The migration time of the individual peptides were compared relative to the migration time of BSA and results were expressed as % of the internal standard (BSA; 400 μg/mL).

Reversed Phase (RP)-HPLC Peptide Analysis

The lyophilized MWCO fractions of less than 1 kDa from native WPI and 1-cycle pressure treated WPI (50 mg) were dissolved in nanopure water (1:10), filtered through 0.22 μm filters (Millex GV; Millipore, Bedford, Massachusetts) and loaded on an analytical C18-reverse phase (RP) column (5 μm; 250 mm × 50 mm) (Vydac Series-218TP54, Vydac Company, Herperia, CA) and separated with a Waters HPLC system. The injection volume was 100 μL. Mobile phase A was prepared by adding 1.0 mL trifluoroacetic acid (TFA) to 1000 mL HPLC grade water. Mobile phase B was prepared by adding 1.0 mL TFA to 1000 mL acetonitrile. Separation of peptide components was performed using a binary gradient of 0.1% trifluoroacetic acid (TFA) in water (phase A) and 0.1% TFA in acetonitrile (phase B) on a gradient from 5% phase B at 5 min to 60% phase B at 60 min at a flow rate of 1.0 mL/min and the column temperature was maintained at 25 °C. The column was washed for 5 min with 100% B and re-equilibrated for 15 min between injections. The flow rate was 1.0 mL/min UV data were recorded from 210 nm to 285 nm.

Free Amino Acid Content Analysis

The free amino acid content of the lyophilized MWCO fractions of less than 1 kDa from the hydrolysates of native WPI and 1-cycle pressure treated WPI was determined using lithium ion exchange HPLC (Biochrom 30 England) with postcolumn ninhydrin detection. A 50 μL peptide solution (20 mg/mL; w/v in distilled water) was injected onto and the separation of the amino acids was accomplished through an optimal combination of pH and cation strength (i.e., lithium citrate concentrations ranged from 0.2-1.65 M and pH ranged from 2.8 - 3.5 depending on the amino acids eluted) with a temperature gradient to enhance separation. A constant flow rate of 25 mL/h was used during 135 min run and the postcolumn reaction between ninhydrin and eluting amino acids from the column was monitored at 440 and 570 nm. These wavelengths detect the characteristic purple color resulting from the reaction between the free amino and carboxyl groups of the amino acids with ninhydrin. The identification and quantitation of the amino acids in the samples was obtained using a standard curve based on an external standard, which contained of all the known amino acids. To assure precision between runs, an internal standard composed by D-glucosaminic acid was used and to ensure the correct identification of the amino acids in the chromatograms, samples were spiked with the external standard.

Peptide Analysis via Matrix-Assisted-Laser-Desorption-Ionization/Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption ionization-mass spectrometry was carried out on a time-of-flight mass spectrometer (MALDI-TOF) with the the Voyager™ Biospectrometry™ Workstation with delayed Extraction™ technology (Applied Biosystems, Foster City, CA). The lyophilized MWCO fractions of less than 1kDa from native WPI and 1-cycle pressure treated WPI were dissolved in 0.5 mM ammonium acetate in methanol. In order to break disulphide bridges, dithiotrietol (DTT; 10 μL) (Sigma, St Louis, MO) was added was added to each tube of the lyophilized peptidic mixtures for reduction.

Cell Culture

General Procedures

Cells were grown in pre-coated T-75 flasks in Eagle's minimal essential medium (MEM) containing 10% FBS, re-fed every 2-3 days until confluent. The confluent, adherent monolayers were then released from the plastic surface after treatment with polyvinyl-pyrrolidone (PVP)-trypsin-EDTA and were seeded to 24-well plates or 60 mm dishes for 24 h before receiving the treatments.

Cell Culture Treatment with Whey Peptides and TNF-α

Wild type and mutant ΔF508 CFTR cells seeded at 0.4 and 0.6 x 10^6 cells/mL respectively, were grown in MEM containing 10% FBS for 24 h until nearly confluence. Cells were treated with MWCO fractions of less than 1 kDa from the hydrolysates of native WPI (nWPH) and 1-cycle pressure treated WPI (pWPH) at doses of 12.5 and 500 μg/mL for GSH experiments, and 12.5 μg/mL for the IL-8 release experiments. These doses of whey peptides have been demonstrated previously to exert bioactive effects on immune function and GSH status in cell culture. For example, the 12.5 μg/mL dose was shown to stimulate lymphocyte proliferation of whey peptides with molecular weights < 1 kDa (Mercier *et al.*, 2004). Doses of thymic peptides of 12.5 μg/mL were effective in stimulating intracellular GSH concentrations in vascular endothelial cells (Li *et al.*, 1994). Kent *et al.* (2003) demonstrated that a dose of hydrolyzed WPI of 500 μg/mL increased intracellular GSH in prostatic epithelial cells. Following the first 24 h incubation, the MEM 10% FBS was replaced with fresh medium containing 2% FBS and filtered sterilized nWPH and pWPH solutions. The cells were allowed to grow for 24 h at 37°C in 5% CO_2 and the medium was replaced with fresly prepared peptides solution in MEM 2%. After an additional 24h of incubation, either the cells or the supernatant were collected to assess the impact of nWPH and pWPH on intracellular GSH production, and IL-8 release, respectively. Intracellular GSH and IL-8 release were performed in independent experiments using 60 mm dishes and 24-well plates respectively. To assess

the effect of whey protein hydrolysates on IL-8 production in a stimulated state, after the initial 24 h incubation with nWPH and pWPH, cells were treated with MEM 2% FBS containing 12.5 μg/mL of nWPH or pWPH and concurrently stimulated with human recombinant TNF-α (10 ng/mL) for an additional 24 h. All experiments included unstimulated negative control wells. The viability of the peptide-treated and untreated cells was determined by the MTT assay.

IL-8 Release and Cell Viability Assays

After the peptide treatments described above, the supernatant was collected to determine IL-8 released using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates were coated with capture antibody (anti-IL-8) overnight, washed with 0.05% Tween-20 in PBS and coated with phosphate buffer (PBS) 10% FBS in order to block non-specific binding. Known concentrations of IL-8 (standard) and the samples containing the IL-8 released by the cells after treatment (supernatant) were added as aliquots into appropriate wells, incubated for 2 h and decanted from the wells. Anti-IL-8 plus enzyme reagent (biotinylated detection antibody conjugated to streptavidin-horseradish) were added and incubated for 1 h. After washing the plate, a solution was added which contained a substrate for the enzyme (TMB-peroxide chromogen) present in the anti-IL-8 + enzyme reagent mixture and the plate was incubated for 30 min. The reaction was stopped using a 2 N H_2SO_4 solution and the absorbance was read at 450 nm using a Titertek II Multiscan MCCB40 (Labsystems, Finland). The O.D. were then used to calculate the IL-8 concentration from the standard curve and adjusted by their dilution factor.

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). Briefly, after collecting the supernatant to determine IL-8 release, the cells were gently washed with PBS. Cells were incubated with MTT (Sigma) at 0.5 mg/mL in culture medium free of phenol red for 3 h at 37°C. After incubation, the supernatant was aspirated and HCl-isopropanol solution (0.04 N HCl in isopropanol) was added to

dissolve the formazan crystals formed in viable metabolic active cells. Solubilization of the formazan crystals results in a coloured solution allowing the quantification of viable cells. After 5 min of treatment with isopropanol, the optical densities were measured at 540 nm using an automated series 750 microplate spectrophotometer (Cambridge Technology, Inc., Cambridge, MA, USA). The optical density values were converted into cell numbers by using a cell proliferation standard curve with a cell seeding concentration ranging from 0.4 to 1 x 10^6/mL cells (Loodsdrech *et al.*, 1994).

Analysis of Cellular Glutathione

Quantitative determination of the total intracellular GSH and glutathione disulfide (GSSG) was performed spectrophotometrically by the glutathione reductase recycling assay according to a modification of the method of Anderson (1985). Adherent epithelial cell lines, grown on coated 60 mm dishes with MEM 10% FBS during the first 24 h after seeding and for additional 48 h with MEM 2% FBS were washed twice with 5 mL PBS containing 25 mg% BSA. The cells were treated with 1.5 mL volume of 25 mg% BSA in PBS solution, scraped, and then subjected to centrifugation at 500 x g for 10 min (Microlite Microcentrifuge OM 3580, Thermo IEC, Needham Heights, MA) in order to remove any extracellular GSH and GSSG. The supernatant was discarded and the cells were resuspended in 1 mL of PBS followed by another centrifugation at 400 x g for 6 min. The supernatant was discarded and the cells were washed once with 0.45 mL PBS and re-suspended with 0.45 mL PBS. Samples were diluted with 0.1 mL 10 mM HCl and sonicated for 2 cycles of 20 s in order to disrupt cell membranes. The cell suspension was filtered using a centrifugal Millipore filter (catalog #42407, Millipore, Nepean, Ontario) at 14,000 x g for 60 min at 4°C. The protein concentration of the unfiltered portion of the samples was determined by the Bradford protein assay, according to the manufacturer's instructions (catalog #500-0006, Bio-Rad, Mississauga, Ontario). From the filtrate, two aliquots of each sample were taken to determine GSH and GSSG, respectively. In one aliquot, GSH was derivatized with 1 M solution of 2-vinylpyridine in ethanol for 60 min to quantify GSSG exclusive of GSH, while the second aliquot was ready to assay for GSH. In 96-well microplates GSH and GSSG concentrations were measured by using

the GSH reductase recycling method of Anderson (1985) following treatment of the samples with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The microplate was placed on the series 750 microplate reader from Cambridge Scientific (Cambridge, MA) using a 420-nm filter and the absorbance was read for 25 min.

The filtered samples were compared with the GSH and GSSG calibration curves to determine the GSH and GSSG concentrations in each well. The linearity of the GSH and GSSG calibration curves ranged from 0.1 to 16 μM and from 0.25 to 8 μM, respectively. The concentration of GSH and GSSG for each sample was determined from a standard curve and expressed as nmoles of GSH. The amount of the reduced form of GSH was calculated as the difference between the total GSH and oxidized GSH (GSSG), (i.e. total GSH - oxidized GSH = reduced GSH). The GSH content was expressed as nmol/mg protein or as the GSH redox ratio (reduced GSH/GSSG).

Statistics

Statistical analysis was performed using an ANOVA with Tukey's *post hoc* test to determine significant statistical differences between groups in the digestibility studies and LSD's *post hoc* comparisons was applied for values of cytokines, cell numbers, and intracellular GSH concentrations. Two tailed Student's *t* test was used to determine the significance of the comparison between intracellular GSH and GSSG content in the comparison of CF *vs.* non-CF cells and in peptide content between native *vs.* 3-cycle pressure treated WPI. All digestions and experiments were carried out in triplicate and data were expressed as mean ± standard error of the mean (SEM). SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois) was used in all statistical analyses. $P < 0.05$ was taken as statistically significant.

4.3 Results and Discussion

Hydrostatic pressure treatment increases WPI digestibility and alters peptide profiles generated from enzymatic hydrolyses

Figure 1 shows the *in vitro* protein digestibility with pepsin of native, 1-cycle and 3-cycle pressure-treated WPI. Analysis of variance showed significant ($P < 0.05$) main effects of both length of time of enzyme exposure and type of protein treatment. Native whey proteins were more resistant to pepsin hydrolysis as a 20 min digestion was needed to show a significant ($P < 0.05$) decrease in protein content and only a 30.9% decrease in protein content was observed after the 30 min pepsin digestion (Fig. 1). In contrast, WPI that had undergone 1-cycle or 3-cycle pressure treatment showed significant ($P < 0.05$) decreases in protein content by 5 min of pepsin treatment and demonstrated decreases in protein content of 51% and 68%, respectively following 30 min pepsin digestion. The Bradford protein assay used for protein determination does not detect peptides smaller than 3 kDa as these low molecular weight peptides do not form the dye-protein complex needed for colorimetic detection (Sapan *et al.*, 1999). It thus appears that 1-cycle and 3-cycle hydrostatic pressure treatment increases the digestibility of whey proteins to an extent that there is greater release of low molecular weight peptides of < 3 kDa from pepsin digestion. At all incubation times with pepsin, 1-cycle and 3-cycle pressure treated WPI showed a significantly ($P < 0.05$) greater degree of proteolysis than native WPI. Likewise, 3-cycle pressure treated WPI demonstrated a significantly ($P < 0.05$) greater degree of digestibility with pepsin at all incubation times as compared to 1-cycle pressure treated WPI. In preliminary tests, lyophilized pressurized proteins could be stored at -20°C for periods up to 12 months, without significant changes in their digestibility profiles (data not shown).

The low digestibility of native WPI is likely due is high resistance of β-LG, which constitutes the major protein in WPI, to pepsin-mediated hydrolysis. Numerous studies have demonstrated that there is a low accessibility to peptide bonds localized in the interior of the globular structure of β-LG to hydrolytic action of pepsin (Mutilangi, 1995;

Kitabatake and Kinekawa, 1996; Kinekawa and Kitabatake, 1998; Pantako *et al.*, 2001; Qiao *et al.*, 2004) and large oligopeptides are produced as pepsin hydrolysis is limited to peptide bonds involving only phenylalanine or tyrosine (Biesalski and Grimm, 2005). Enzyme-substrate binding is required for protein hydrolysis to occur; however, most peptide bonds are located in the interior of globular proteins such as β-LG and thus are not accessible to proteolytic enzymes. For globular proteins, denaturation of the protein is required for protein breakdown since a greater number of peptide bonds are exposed after denaturation. Recent electrospray ionization mass spectrometry (ESI-MS) studies have indicated that both single cycle pressure treatment at 550 MPa as well as 3-cycle pressure treatment at 400 MPa caused a partial unfolding of genetic variants of β-LG as exhibited by a higher proportion of charges in the charge-state-distribution of pressurized β-LG relative to the native forms (Alvarez, 2004). Hence, it is likely that the partial unfolding of β-LG induced by pressure treatment exposed the hydrophobic amino acids buried in the interior of the molecule to the proteolytic enzymic action thus increasing the rate of hydrolysis as only the unfolded molecules are susceptible to degradation by proteolytic enzymes (Stauffer, 1989).

Further confirmation of the increased digestibility of pressure-treated whey proteins is shown by the significantly ($P < 0.05$) greater release of peptides with molecular weights < 3 kDa from pressure-treated WPI hydrolysates relative to native WPI hydrolysates following pepsin and pancreatin digestion and ultrafiltration (Fig. 2). As peptides with molecular weights larger than 1 kDa have not been observed in digestive juices following protein feeding in animal models (Qiao *et al.*, 2004), peptides under molecular weight of 1 kDa were isolated by ultrafiltration following pepsin as well as pepsin followed by pancreatin digestion. After 30 min pepsin digestion, enzymic hydrolysates from 1-cycle pressurized WPI showed a significantly ($P < 0.05$) higher content of peptides smaller than 1 kDa as compared to native WPI hydrolysates (Fig. 3). Interestingly, 3-cycle pressure treated whey proteins did not show an enhanced release of peptides smaller than 1 kDa following pepsin digestion relative to 1-cycle treated or native WPI despite the significantly greater drop in protein content in the 3-cycle pressure treated WPI during pepsin digestion (Fig. 3). As the Bradford protein assessment method does not detect

polypeptides less than 3 kDa (Sapan *et al.*, 1999), the present results thus indicate that 1-cycle pressurization of WPI was relatively more effective in generating peptides < 1 kDa following pepsin digestion as compared to 3-cycle pressurization that appeared to generate more peptides with molecular weights < 3 kDa. In contrast to the finding of a greater content of < 3 kDa peptides in digests of 3-cycle pressure treated *vs.* native WPI following digestion with pepsin-pancreatin (Fig. 2), no differences in total content of < 1 kDa peptides were observed among all the treatment groups (Fig. 3). Pancreatin enzymes including the endopeptidase enzymes, trypsin, chymotrypsin and elastase and the exopeptidase enzymes, aminopeptidase and carboxypeptidases, are highly efficient in cleaving proteins and peptides into low molecular peptides, which could account for the similar < 1 kDa peptide content among the treatment groups (Biesalski and Grimm, 2005). Hydrolysates showing a similar extent of hydrolysis or in the release of small molecular weight peptides, however, may differ in their peptide composition as measured by mass spectrometry, CZE and RP-HPLC (Mutilangi *et al.*, 1995; Smyth and Fitzgerald, 1998).

Characterization of the wide variety of low molecular weight peptides released from hydrolysis of native and pressure-treated WPI following pepsin and pancreatin digestion was carried out to further define whether these fractionated hydrolysates could differ in terms of their peptide profiles. Peptides released from the pepsin digests of native and 3-cycle pressurized whey proteins were analyzed by MALDI-TOF. Although it is difficult to distinguish between the different peptide mixtures due to the large number of peptide molecular ions present, the MS analysis of the end products after 30 min of pepsin digestion demonstrated that different peptide profiles were observed when pressure treatment was applied (Fig. 4). In particular, the peaks at mass 2429 and 2430 were absent in the pressure-treated WPI (Fig. 5). These peptides were also removed by DTT treatment of the native WPI suggesting that the peptides were likely to be dimers with disulfide bonds. Extensively disulfide-linked proteins such as whey proteins may be resistant to specific proteolysis such as trypsin (Brown and Hartley, 1966; Smyth, 1967); however, more aggressive proteolysis with nonspecific proteases such as pepsin can generate cysteinyl peptides whose mass is affected by reducing agents such as DTT

(Brown and Hartley, 1966; Smyth, 1967; Wallis *et al.*, 2001). The present findings thereby indicate that pressure treatment can facilitate the breakage of disulphide bonds in association with pepsin digestion, which could enhance pepsin-mediated whey protein digestion. In support of this latter concept, previous studies have shown a dramatic enhancement of *in vitro* pepsin-mediated digestion of β-LG after treatment with thioredoxin to break the intramolecular disulfide bonds (del Val *et al.*, 1999).

Protein hydrolysates of native and pressured treated whey proteins were characterised by CZE, which is based on size and charge and is complementary to peptide separation obtained via RP-HPLC (Madsen *et al.*, 1997). Figure 6 represents typical electropherograms of the profiles of peptides less than 1 kDa that were isolated from pepsin-pancreatin hydrolysates of native, single and 3-cycle pressure-treated WPI. The number of peaks and the peak areas relative to the internal BSA standard differed among the hydrolysates as 1-cycle and 3-cycle pressure treated whey proteins generated a greater number of peptide fragments than native whey proteins as well as a different migration pattern and peak areas relative to BSA. These latter findings suggest that despite similar total content of small molecular weight peptides among the three hydrolysates, different profiles of peptides were generated following pancreatin digestion of pressurized whey proteins as compared to native whey proteins. Similar peptide profiles were observed in the electropherograms of peptides obtained from single and 3-cycle pressure-treated WPI although there was decreasing number of peptide fragments in single cycle as compared triple cycle pressure-treated whey protein hydrolysates. The larger number of small peptides associated with pressure treatment also implicates a greater degree of hydrolysis as an increased variety of small peptides is produced with a greater extent of hydrolysis as reported for enzymic hydrolysates generated under various process conditions (Gonzalez-Tello *et al.*, 1994).

The electropherograms of peptides resulting from pancreatin digestion of pressurized whey protein showed a larger number of fragments eluting later than BSA (Fig. 6), which was not observed in the chromatograms obtained from the peptides obtained from native protein hydrolysates. The additional peaks observed with 1-cycle and 3-cycle pressure

treatment could possibly have been the result of cleavage of original peptides found in hydrolysates of native WPI, as some of the peptide fragments associated with native whey samples were not observed with pressure treatment. As the migration time in CZE depends on the charge to mass ratio, it is expected that large molecules with isoelectric points higher than 2.5 would have a slow migration speed considering that the running buffer used was adjusted to pH 2.5. Since the peptides and amino acids isolated from the hydrolysates had molecular weights lower than BSA, it is reasonable to assume that peptides that eluted earlier than BSA are more positively charged than the peptides that eluted later than BSA, which supposedly have negative or neutral net charges. Hence, it appears that pressure treatment of whey proteins allows digestive enzymes to produce peptides with different molecular weights and charges as compared to peptides derived from native whey proteins.

The typical chromatograms of RP-HPLC analysis of < 1 kDa peptides isolated from hydrolysates of native WPI and 1-cycle pressure treated WPI following *in vitro* pepsin-pancreatin digestion are presented in Figures 7 and 8. Chromatographic elution patterns of peptides via RP-HPLC are usually recorded at wavelengths ranging from 200 and 220 nm as absorption at these wavelengths is mainly caused by peptide bonds whereas at higher wavelengths only aromatic amino acids are detected (Woods and O'Bar, 1970; Becklin and Desiderio, 1995). Most of the peaks were eluted in 5-14 min and 18-37 min intervals, although other peaks were visible on the chromatogram in the 39-49 min interval. Although the RP-HPLC peptide profile of pressure-treated whey did not differ markedly from peptide profiles of native whey, HPLC chromatograms obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm showed the presence of peptide peaks at 35 min and 48 min at approximate wavelengths of 214 to 220 nm, which were not observed in the peptide profiles of native whey protein hydrolysates (Fig. 7). Likewise, the HPLC peptide map at 214 nm showed the presence of peptide peaks at 35.1 min and 48.5 min in the hydrolysates of 1-cycle treated WPI, which were not observed in peptide profiles obtained from native whey hydrolysates (Fig. 8). The nature of the eluted peptides associated with pressure treatment is unclear since several characteristics can influence the elution time of peptides on a reversed phase column

such as amino acid composition including the hydrophobicity of the amino acid side chains in small peptides (< 15 residues) (Hearn *et al.*, 1988) or the peptide length and molecular weight for larger peptides (Chabanet and Yvon, 1992).

The amino acid analysis demonstrated that the permeates from the filtered native and 1-cycle pressure-treated whey protein hydrolysates obtained following pancreatin digestion differed in terms of the profiles and content of free amino acids and their derivatives (Table 1). The profile of free amino acids also differed as a higher content of all amino acids was observed in the pressure-treated sample apart from cystine and proline. The most evident differences between the native and pressure-treated samples were observed in terms of the amino acid derivatives. Markedly higher concentrations of argininosuccinic acid and sarcosine were observed in the filtered native protein hydrolysates whereas WPH, on the other hand, γ-aminobutyric acid, a known neuropeptide involved in the modulation of corticotropin-releasing hormone from the hypothalamus (Eskandari *et al.*, 2003), and *o*-phosphoethanolamine, a molecule that is part of the structure of glycerolipids and sphingolipids such as phosphatidylethanolamine and phosphorylceramide (Malgat *et al.*, 1986) were detected only in the pressure-treated whey samples. These results are in concert with the CZE and HPLC analyses showing differing peptide and amino acid profiles following *in vitro* enzymatic hydrolysis of pressure treated *vs.* native whey proteins.

Although hyperbaric pressure processing has been demonstrated to increase whey protein digestibility *in vitro* in previous studies, such pressure treatment was carried out in the presence of proteolytic enzymes as pressurization was shown to increase the catalytic effect of proteolytic digestive enzymes such as pepsin (Stapelfeldt *et al.*, 1996). The present work extends such previous studies to indicate that after exposure to higher hydrostatic pressures, whey proteins subsequently show enhancement of *in vitro* digestibility, without the need of pressure treatment of the proteolytic enzymes. In this regard, it is noteworthy that the lyophilized pressurized proteins could be stored at -20°C for periods up to 12 months, without significant changes in their digestibility profiles

(data not shown) suggesting that the pressure-induced conformational changes were relatively stable, at least under cold storage.

Effect of peptides isolated from whey protein hydrolysates on cell viability and IL-8 secretion

In view of the above findings that pressure treatment increased digestion efficiency generating unique peptide fragments and that bioactive peptides present in whey protein hydrolysates have been shown to play a role in immune modulation and GSH homeostasis (Low *et al.*, 2003; Ford *et al.*, 2001; Mercier *et al.*, 2004; Bounous and Gold, 1991), peptides isolated from native and pressurized whey proteins hydrolysates were examined for their capability to downregulate the excessive inflammatory response in CF cells. To test this possibility, cultured tracheal epithelial CF and non-CF cells were treated with peptides of MWCO < 1 kDa isolated from of native (nWPH) and 1-cycle pressure-treated (pWPH) whey protein hydrolysates and IL-8 release was analysed in both non-stimulated and TNF-α stimulated conditions. TNF-α is recognized to play an important role in disease pathogenesis during acute inflammation (Carlsen *et al.*, 2004) and is an important mediator of multiple inflammatory events in lungs (Arai *et al.*, 1990). Unstimulated CF and non-CF control cultures produced similar low levels of IL-8 release (166 ± 21 and 287 ± 43 $pg \cdot mL^{-1}$, respectively) under basal conditions. Sham treatment (ethanol) did not increase IL-8 secretion in either type of culture (data not shown). The present results are similar to our previous study (Vilela *et al.*, 2005) and other cell culture studies that indicate small or no differences in basal levels of the pro-inflammatory response in CF epithelial cells (Schwiebert *et al.*, 1999; Becker *et al.*, 2004; Venkatakrishnan *et al.*, 2000).

Exposure of cells to 10 $ng \cdot mL^{-1}$ TNF-α resulted in more than a 114-fold increase (19,009 ± 943 $pg \cdot mL^{-1}$) in secreted IL-8 levels in CF cells whereas non-CF cells exhibited greater than 230-fold increase (78,202 ± 9,940 $pg \cdot mL^{-1}$) in IL-8 concentrations relative to the unstimulated MEM controls. These latter results are in accordance to our previous work showing that non-CF cells are more sensitive to TNF-α induction of IL-8 release (Vilela

et al., 2005). Similarly, Massengale *et al.* (1999) demonstrated that non-CF cells are more sensitive to LPS-mediated IL-8 stimulation than CF cells; however, a consistent pattern of differences in the secretion of IL-8 between CF and non-CF epithelial cells under TNF-α is not always present. CF cells have also been shown to be equally sensitive to TNF-α when compared to non-CF cells (Bedard *et al.*, 1993; Schwiebert *et al.*, 1999).

The viability of the peptide treated cells as assessed by the MTT reduction assay was unaffected by peptide treatment both at the 12.5 and 500 μg/mL peptide concentrations (data not shown). This result is in concert to the findings of Kent *et al.* (2003) who noted no significant decrease in cell viability following treatment of whey protein hydrolysates in cultured human prostate epithelial cells at concentrations of 500 μg/mL. Although the MTT assay detects mitochondrial activity rather than cell viability, these phenomena are intimately related and MTT has been widely used to assess cell injury (Schaeffer *et al.*, 2003; Koenig and Meyerhoff, 2003).

Non-CF cells in either basal or stimulated conditions were unaffected by either the nWPH or pWPH treatments in terms of IL-8 release as compared with control cells (Fig. 9 and 10) although a strong tendency for decrease IL-8 secretion in response to nWPH treatment was observed ($P = 0.09$) under stimulated conditions, which was not seen with pWPH. On the other hand, CF cells were more sensitive to hydrolysate treatment as both nWPH and pWPH significantly ($P < 0.05$) stimulated IL-8 production in CF cells under basal conditions. Conversely, there was also a strong tendency for both the nWPH and pWPH treatments to decrease inflammatory response in CF cell lines under TNF-α stimulated conditions ($P = 0.08$ and 0.11, respectively) (Fig. 10). Although the P values were not significant, significance might have been achieved with a larger sample size. The low dose of whey peptides (12.5 μg/mL) used was based on previous cell culture studies demonstrating immunomodulatory effects in response to such low peptide doses (Mercier *et al.*, 2004; Li *et al.*, 1994). The present results thereby indicate the possibility that low dose pWPH treatment could have the potential as co-adjuvant treatment to

attenuate the pro-inflammatory condition of CF cells without affecting non-CF cells in this regard.

Effect of peptides isolated from whey protein hydrolysates on GSH status

As noted in our previous findings in the CFTE29o- human tracheal epithelial cell line (Vilela *et al.*, 2005), the intracellular concentrations of total GSH and reduced GSH of CFTR deficient cells seen in the present study were approximately eight to ten-fold higher than in wild-type cells ($P < 0.05$) (Fig. 11-12). Similar findings have also observed by Jungas *et al.* (2002) with mutant CFTR HeLa cells. It is possible that the relatively higher GSH levels in CFTR deficient cells may indicate a compensating mechanism to reduce excessive oxidative stress as cells utilize intracellular GSH to protect themselves against oxidants. Moreover, similar to our previous studies (Vilela *et al.*, 2005), more than three-fold lower GSH/GSSG ratios were observed in mutant CFTR epithelial cells as compared to wild-type cells under basal conditions (Fig. 11-12). As GSH and GSSG are the major redox pair involved in cellular redox homeostasis, a lower cellular GSH/GSSG ratio is regarded as a representative marker for oxidative stress, which can perturb cellular function leading to an induction of proinflammatory responses (Rahman and MacNee, 2000). The lower basal GSH/GSSG ratios in the CF as compared with mutant CFTR epithelial cells ($P < 0.05$) may thus explain the increased propensity towards oxidative stress in the CFTR-deficient epithelial cells. Oxidative stress leading to redox activation of NF-κB has been proposed as an explanation of the persistent inflammation and lung damage in CF (van der Vliet *et al.*, 1997). CF patients generally have poor antioxidant status (McGrath *et al.*, 1999; Lands *et al.*, 1999) and antioxidant therapy has been proposed (Roum *et al.*, 1999; Hartl *et al.*, 2005). As whey protein hydrolysates were shown to induce cellular GSH concentrations (Kent *et al.*, 2003), the impact of nWPH and pWPH to improve the redox status in CF and non-CF cells as measured by intracellular levels of GSH and GSSG was assessed.

The GSH modulatory activity in mutant CFTR cells from exposure of 12.5 and 500 μg/mL doses of peptides (MWCO < 1kDa) isolated from native and pressurized whey

protein hydrolysates following pepsin and pancreatin treatment is shown in Figures 11 and 12. Treatment of CF cells with pWPH at the dose of 12.5 μg/mL showed a strong tendency to increase the reduced form of GSH ($P = 0.09$), total GSH ($P = 0.15$) and the GSH/GSSG ratio ($P = 0.09$) relative to untreated controls (Fig. 11). Previous studies have shown that thymic peptides at doses of 12.5 μg/mL increase GSH concentrations in cultured bovine pulmonary artery endothelial cells (Li *et al.*, 1994). In contrast to CF cells, non-CF cells treated with pWPH at 500 μg/mL had a significant ($P < 0.05$) increase in GSSG relative to untreated controls and the nWPH and pWPH treatments at the 500 and 12.5 μg/mL dose, respectively. In addition, a decrease in the GSH/GSSG ratio was promoted at the 12.5 μ/mL of nWPH (Fig. 12). Taken together, the above findings indicate that the observed differences in peptide profiles in pWPH *vs.* nWPH resulted in differences in their biochemical properties in terms of GSH modulation. The reason for the contrasting finding for differences in the response of CF vs. non-CF cells to the WPH treatment in terms of modulation of GSH status is not explainable from this experimental set-up but could be due to intrinsic metabolic factors that affect the relative amounts of GSH and GSSG in the two cell lines. The factors that may differ between the two cell lines might include the rates of biosynthesis and utilization of GSH in oxidation/reduction reactions as well as involvement of GSH in a variety of reactions requiring reducing equivalents such as direct interaction with free radical species, enzymatic reduction of lipid hydroperoxides and the regeneration of reduced forms of redox pairs such as cysteine/cystine (Jones *et al.*, 2004).

The uptrend in intracellular GSH levels in CFTR deficient cells associated with pWPH as compared to the other whey hydrolysate treatments could be an outcome of an increase in GSH synthesis and/or a reduction in GSH efflux out of the cell (Bannai and Tateishi, 1986). There is a possibility that pWPH supplies intracellular GSH stores via provision of cell-permeant GSH precursors in the form of peptides as demonstrated previously with thymic peptides at the dose of 12.5 μg/mL (Li *et al.*, 1994). Although marked differences were observed in the amino acid profiles between nWPH and pWPH, the amino acid content of the whey protein hydrolysates are unlikely to exert GSH-modulating effects. GSH-stimulating effects of amino acid supplements such as cystine and glutamine in cell

cultures have been noted at the millimolar level (Issels *et al.*, 1988; Hammond *et al.*, 2002) as opposed to the micromolar concentrations present in the whey protein hydrolysates. The nature and mechanism of action of the GSH modulating peptides cannot be discerned with the present experimental context. A rate-limiting factor in the synthesis of GSH in hepatic cells is cystine uptake through the Xc-system, which supplies cystine and glutamate required for GSH synthesis (Deneke and Fanburg, 1989). Kent *et al.* (2003) proposed that the GSH stimulating activity of the WPH in human prostate epithelial cells could be attributed to an increased availability of cysteine. Supplementation of non-cysteine containing peptides could also play a role since the di-peptide, alanylglutamine, was shown to increase GSH concentrations in CaCo-2 cells that were depleted after H_2O_2 treatment (Alteheld *et al.*, 2005). Moreover, oxidant stress has been reported to increase both the uptake of cystine-glutamate and the synthesis of GSH in endothelial cells (Deneke and Fanburg, 1989). Hence, it is possible that the isolated peptides from whey protein hydrolysates could have exerted more potent effects on improving intracellular GSH status under conditions of oxidant stress.

The results of the present work differ from Kent *et al.* (2003) who noted that treatment of human prostate epithelial cells with hydrolyzed WPI (500 μg/mL) for 24 or 48 h was associated with significant increases in intracellular GSH as compared with control cells receiving no hydrolyzed WPI. The impact of hydrolyzed WPI on GSSG content, however, was not reported by Kent *et al.* (2003). Apart from possible metabolic differences between the cell lines used, it is possible that the divergent digestion protocols could account for the discrepancy in GSH stimulating activity of the whey protein hydrolysates tested. Kent *et al.* (2003) used trypsin, chymotrypsin and peptidase, whereas in the present study WPI was hydrolyzed initially via pepsin, followed by treatment with pancreatin that contains a mixture of trypsin, chymotrypsin and elastase. Previous literature has consistently demonstrated that the profile and bioactivity of peptides isolated from whey proteins can vary considerably depending on the digestive enzymes used (Vermeirssen *et al.*, 2003). Another possible explanation is that Kent *et al.* (2003) did not fractionate their whey protein hydrolysates to isolate the low molecular weight peptides with MWCO < 1kDa that are more bioavailable (Qiao *et al.*, 2004).

Hence, it is conceivable that the *in vitro* GSH stimulating activity in human normal epithelial cells observed by Kent *et al.* (2003) were due to relatively larger molecular weight peptides that were excluded in the present study.

The present study was unable to establish a link between intracellular GSH status and IL-8 secretion due to limitations in the measurement of these two indices within the same culture wells. Both nWPH and pWPH treatments were associated with strong decreasing trends in IL-8 release in CFTR deficient cells (Fig. 10). However, CF and non CF cells responded differently to whey proteins in terms of intracellular redox state. In general, pWPH (12.5 μg/mL) promoted a strong uptrend in CF GSH/GSSG ratio ($P = 0.095$) (Fig. 11) and both nWPH (12.5 μg/mL) and pWPH (12.5 μg/mL and 500 μg/mL) promoted a significant or a strong downtrend in non CF GSH/GSSG ratio ($P = 0.048$, $P = 0.1$, and $P = 0.06$ respectively) (Fig. 12). Further studies using larger sample numbers are necessary to determine the relationship between GSH modulation and IL-8 release in the CF cells with whey hydrolysate treatment, particularly since several studies have shown that exogenous agents such as NAC that enhance intracellular GSH concentrations abolish stimulated IL-8 release from human alveolar epithelial cell lines (Harper *et al.*, 2001; Rahman *et al.*, 2003; Antonicelli *et al.*, 2004).

In summary, the data from the present study indicate that high hydrostatic pressure processing of whey protein can enhance *in vitro* proteolysis. The altered profile of low molecular weight peptides isolated from hydrolysates of pressurized whey proteins was associated with a strong uptrend in the total and reduced GSH content in cultured mutant CFTR cells in comparison to peptides released from native whey hydrolysates. The present study results suggests that bioactive peptides produced from native and pressurized whey protein hydrolysates may have utility acting as anti-inflammatory agents via inhibition of IL-8 release.

Acknowledgments

The authors thank Brian Meehan and Chia-Chien Chang for their technical help. The first author was sponsored scholarships by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Process n. 200026/93-3 (RN), Brasília, D.F.-Brazil; Federal University of Paraná, and Tuiuti University of Paraná, Curitiba, Paraná, Brazil. The research was funded via grants to SK and LL by the National Sciences and Engineering Council of Canada (NSERC) and the Canadian CF Foundation.

References

Alvarez P. 2004. Effect of applied hydrostatic pressure on the structure and rheological properties of whey proteins. M.Sc. Thesis, McGill University, Montreal, QC., Canada.

Anderson M. E. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*.113: 548-551.

Antonicelli F., Brown D., Parmentier M., Drost E. M., Hirani N., Rahman I., Donaldson K., MacNee W. 2004. Regulation of LPS mediated inflammation *in vivo* and *in vitro* by the thiol antioxidant Nacystelyn. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 286: L1319–L1327.

Arai K. I., Lee F., Miyajima A., Miyatake S., Arai N., Yokota T. 1990. *Annual Reviews in Biochemistry*. 59: 783-836.

Alteheld M. E., Evans L. H., Gu V., Ganapathy F. H., Leibach D. P., Jones T. R., Ziegler. 2005. Alanylglutamine dipeptide and growth hormone maintain PepT1-mediated transport in oxidatively stressed Caco-2 cells. *British Journal of Nutrition*. 135: 19–26.

Bartfay W. J., Davis M.T., Medves J. M., Lugowski S. 2003. Milk whey protein decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy. *Canadian Journal of Cardiology.* 19: 1163-*8.*

Bannai S., Tateishi N. 1986. Role of membrane transport in metabolism and function of glutathione in mammals. *Journal of Membrane Biology.* 89: 1-8.

Becker M. N., Sauer M. S., Muhlebach M. S., Hirsh A. J., Wu Q, Verghese M. W., Randell S. H. 2004. Cytokine secretion by cystic fibrosis airway epithelial cells. *American Journal Respiratory and Critical Care Medicine.* 169: 645-53.

Becklin R. R., Desiderio, D. M. 1995. The amount of ultraviolet absorbance in a synthetic peptide is directly proportional to its number of peptide bonds. *Analytical Letters.* 28: 2175-2190.

Bedard M., McClure C. D., Schiller N. L., Francoeur C., Cantin A., Denis M. 1993. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology.* 9: 455-462.

Biesalski H. K., Grimm, P. 2005. *Pocket Atlas of Nutrition.* New York: Thieme. pp. 1-122.

Bounous G, Batist G., Gold P. 1989. Immunoenhancing property of dietary whey protein in mice: role of glutathione. *Clinical Investigation Medicine.* 12: 154-161.

Bounous G., Gold P. 1991. The biological activity of undenaturated dietary whey proteins: role of glutathione. *Clinical Investigation Medicine.* 14: 296-309.

Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.

Brown J. R., Hartley B. S. 1966. Location of disulphide bridges by diagonal paper electrophoresis. The disulphide bridges of bovine chymotrypsinogen A. *Biochemical Journal*. 101: 214-228.

Bunnell E., Pacht E. R. 1993. Oxidized glutathione is increased in the alveolar fluid of patients with the adult respiratory distress syndrome. *American Review of Respiratory Disease*. 148: 174–1178.

Camp J.V., Huyghebaert A. 1995. High pressure induced gel formation of whey proteins and haemoglobin concentrate. *Lebensmittel - Wissenschaft - Technologie*. 28: 111-117.

Cantin A. M., Hubbard R.C., Crystal R.G. 1989. Glutathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis. *American Review of Respiratory Disease*. 139: 370-372.

Carlsen H., Alexander G., Austenaa L. M., Ebihara K., Blomhoff R. 2004. Molecular imaging of the transcription factor NF- κB, a primary regulator of stress response. *Mutation Research*. 551: 199–211.

Chabanet C., Yvon M. 1992. Prediction of peptide retention time in reversed-phase high-performanceliquid chromatography. *Journal of Chromatography*. 599: 211-225.

Church F. C., Swaisgood H. E., Porter D. H., Catignani G. L. 1983. Spectrophotometric assay using ophthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science*. 66: 1219-1227.

de Ferrer R. P. A. 1995. Past and present status in the design of infant formulas. *Archivos Latinoamericanos de Nutricion. 45: 265-73.*

del Val G., Yee B. C., Lozano R. M., Buchanan B. B., Ermel R.W., Lee Y. M., Frick O.L. 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *Journal of Allergy and Clinical Immunology.* 103: 690-697.

Deneke S. M., Fanburg B. L. 1989. Regulation of cellular glutathione. *American Journal of Physiology.* 257: L163-73.

Eskandari F., Webster J. I., Sternberg E. M. 2003. Neural immune pathways and their connection to inflammatory diseases. *Arthritis Research and Therapy.* 5: 251-265.

Ford T. F., Wong C. W., Colditz I. G. 2001. Effects of dietary protein types on immune responses and levels of infection with *Eimeria vermiformis* in mice. *Immunology and Cell Biology.* 79: 23-28.

Gao L, Kim K. J., Yankaskas J. R, Forman H. J. 1999. Abnormal glutathione transport in cystic fibrosis airway epithelia. *American Journal of Physiology.* 277: L113-L118.

Gonzalez-Telllo P., Camacho F., Jurado E., Paez M. P., Guadix E. M. 1994. Enzymatic hydrolysis of whey proteins. II: Molecular-weight range. *Biotechnology and Bioengineering.* 44: 529-532.

Gosset P., Wallaert B., Tonnel A. B., Fourneau C. 1999. Thiol regulation of the production of TNF-α, IL-6 and IL-8 by human alveolar macrophages. *European Respiratory Journal* .14: 98–105.

Grey V., Mohammed S. R., Smountas A. A., Bahlool R., Lands L. C. 2003. Improved glutathione status in young adult patients with cystic fibrosis supplemented with whey proteins. *Journal of Cystic Fibrosis.* 2: 195-198.

Griese M., Ramakers J., Krasselt A., Starosta V., van Koningsbruggen S., Fischer R., Ratjen F., Mullinger B., Huber R. M., Maier K., Rietschel E., Scheuch G. 2004. Improvement of alveolar glutathione and lung function but not oxidative state in cystic fibrosis. *American Journal Respiratory and Critical Care Medicine.*169: 822-828.

Haddad J. J. 2000. Glutathione depletion is associated with augmenting a proinflammatory signal: evidence for an antioxidant/pro-oxidant mechanism regulating cytokines in the alveolar epithelium. *Cytokines Cellular and Molecular Therapy.* 6: 177-187.

Haeusel R., Lorenzen P. C., Reimerdes E. H., Brueckner H. 1990. Fractionation of main whey proteins and potential industrial feasibility 1. *European Dairy Magazine.* 1: 8-11.

Hammond A. H., Garle M. J., Sooriakumaran P., Fry J. R. 2002. Modulation of hepatocyte thiol content by medium composition: implications for toxicity studies. *Toxicology in Vitro.* 16: 259–265.

Harper R., Wu K., Chang M. M., Yoneda K., Pan R., Reddy S. P., Wu R. 2001. Activation of nuclear factor-κB transcriptional activity in airway epithelial cells by thioredoxin but not by *N*-acetyl-cysteine and glutathione. *American Journal of Respiratory Cell and Molecular Biology.* 25: 178–185.

Hartl D., Starosta V., Maier K., Beck-Speier I., Rebhan C., Becker B. F., Latzin P., Fischer R., Ratjen F., Huber R. M., Rietschel E., Krauss-Etschmann S., Griese M. 2005. Inhaled glutathione decreases PGE_2 and decreases lymphocytes in cystic fibrosis lungs. *Free Radical Biology and Medicine.* 39: 463-472.

Hayashi R., Kawamura Y., Kunugi S. 1987. Introduction of high pressure to food processing: preferential proteolysis of ß-lactoglobulin in milk whey. *Journal of Food Science.* 52: 1107-08.

Hays T., Wood R. A. 2005. A systemic review of the role of hydrolysed infant formulas in allergy prevention. *Archives of Pediatrics and Adolescent Medicine.* 159: 810-816.

Hearn M. T. W., Aguilar M. I., Mant C. T., Hodges R. S. 1988. High performance liquid chromatography of amino acids, peptides and proteins. LXXXV: Evaluation of the use of hydrophobicity coefficients for prediction of peptide elution profiles. *Journal of Chromatography.* 438: 197-210.

Hendrickx M., Ludikhuyze L., Van den Broek I., Weemaes C. 1998. Effects of high pressure on enzymes related to food quality. *Trends in Food Science and Technology.* 9: 197-203.

Hirani N., Antonicelli F., Strieter R. M., Wiesener M. S., Ratcliffe P. J., Haslett C., Donnelly S. C., 2001. The regulation of interleukin-8 by hypoxia in human macrophages: a potential role in the pathogenesis of the acute respiratory distress syndrome (ARDS). *Molecular Medicine.* 7: 685-697.

Issels R. D., Nagele A., Eckert K. G., Wilmanns W. 1988. Promotion of cystine uptake and its utilisation for glutathione biosynthesis induced by cysteamine and *N*-acetylcysteine. *Biochemical Pharmacology.* 37: 881-888.

Jeannin P., Delneste Y., Lecoanet-Henchoz S., Gauchat J. F., Life P., Holmes D., Bonnefoy J. Y. 1995. Thiols decrease human interleukin (IL)-4 production and IL-4-induced immunoglobulin synthesis. *Journal of Experimental Medicine.* 182: 1785-1792.

Jones D. P., Go Y. M., Anderson C. L., Ziegler T. R., Kinkade J. M., Kirlin W. G. 2004. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *The FASEB Journal.* 18: 1246-1248.

Jungas T., Motta I., Duffieux F., Fanen P., Stoven V., Ojcius D. M. 2002. Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing

wild-type and mutant cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry.* 277: 27912-27918.

Kennedy R. S., Konok G. P., Bounous G., Baruchel S., Lee T. D. G. 1995. The use of whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I-II clinical study. *Anticancer Research.* 15: 2643-2650.

Kent K. D., Harper W. J., Bomser J. A. 2003. Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. *Toxicology in Vitro.* 17: 27-33.

Kinekawa Y, Kitabatake. N. 1996. Purification of β-lactoglobulin from whey protein concentrate by pepsin treatment. *Journal of Dairy Science.* 79: 350-356.

Kitabatake N., Kinekawa Y. 1998. Digestibility of bovine milk whey protein and β-lactoglobulin *in vitro* and in *vivo*. *Journal of Agricultural and Food Chemistry.* 46: 4917-4923.

Koenig M. L., Meyerhoff J. L. 2003. *In vitro* neuroprotection against oxidative stress by pre-treatment with a combination of dihydrolipoic acid and phenyl-butyl nitrones. *Neurotoxicity Research.* 5: 265-72.

Lagrange-Puget M., Durieu I., Ecochard R., Abas-Chorfa F., Drai J., Steghens J-P., Pacheco Y., Vtal-Durand D., Bellon G. 2004. Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. *Pediatric Pulmonology.* 38: 43- 49.

Lands L. C., Grey V., Smountas A. A. 1999. Lymphocyte glutathione levels in children with cystic fibrosis. *Chest.* 116: 1-9. *Erratum in: Chest.* 2000. 117: 296.

Li W. Q., Dehnade F., Zafarullah M. 2000. Thiol antioxidant, *N*-Acetylcystene, activates extracellular signal-regulated kinase signaling pathway in articular chondrocytes. *Biochemical and Biophysical Research Communications.* 275: 789-794.

Li L., Clark K., Lau B. H. 1994. Thymic peptides increase glutathione level and glutathione disulfide reductase activity in vascular endothelial cells. *Biotechnology Therapeutics.* 5: 87-97.

Li T., Shi M. M., Forman H. J. 1997. Increased transcription of the regulatory subunit of γ-glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. *Archives of Biochemistry and Biophysics.* 342: 126-133.

Low P. P. L., Rutherfurd K. J., Gill H. S., Cross M. L. 2003. Effect of dietary whey protein concentrate on primary and secondary antibody responses in immunized BALB/c mice. *International Immunopharmacology.* 3: 393-401.

Malgat M., Maurice A., Baraud J. 1986. Sphingomyelin and ceramide-phosphoethanolamine synthesis by microsomes and plasma membranes from rat liver and brain. *Journal of Lipid Research.* 27: 251-260.

Madsen J. S., Ahmt T. O., Otte J., Halkier T., Qvist K. B. 1997. Hydrolysis of β-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. *International Dairy Journal.* 7: 399-409.

Mah S., Roos N., Benamouzig R., Sick H., Baglieri A., Huneau J. F., Tom D. 1994. True exogenous and endogenous nitrogen fractions in the human jejunum after ingestion of small amounts of 15N-labeled casein. *Journal of Nutrition.* 124: 548-555.

Massengale A. R., Quinn F. Jr., Yankaskas J., Weissman D., McClellan W. T., Cuff C., Aronoff S. C. 1999. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*. 20: 1073-1080.

McGrath L. T., Mallon P., Dowey L., McClean E., McDonnell M., Devine A., Copeland S., Elborn S. 1999. Oxidative stress during acute respiratory exacerbations in cystic fibrosis. *Thorax*. 54: 518-523.

Mercier A., Gauthier S. F., Fliss I. 2004. Immunomodulating effects of whey proteins and their enzymatic digests. *International Dairy Journal*. 14: 175-183.

Meister A., 1988. Glutathione metabolism and its selective modification. *Journal of Biological Chemistry*. 263: 17205-17208.

Micke P., Beeh K. M., Schlaak J. F., Buhl R. 2001. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *European Journal of Clinical Investigation*. 31: 171-178.

Mozhaev V. V., Heremans K., Frank J., Masson P., Balny C. 1994. Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends in Biotechnology*. 12: 493-501.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65: 55-63.

Mutilangui W. A. M., Panyam D., Kilare A. 1995. Hydrolysates from proteolysis of heat-denatured whey proteins. *Journal of Food Science*. 60: 1104-1109.

Panasiuk R., Amarowicz R., Kostyra H., Sijtsma L. 1998. Determination of alpha-amino nitrogen in pea protein hydrolysates: a comparison of three analytical methods. *Food Chemistry*. 62: 363-367.

Pantako O. T., Lemieux L., Amiot J. 2001. The effects of α-lactalbumin and whey protein concentrate on dry matter recovery, TCA soluble protein levels, and peptide distribution in the rat gastrointestinal tract. *Canadian Journal of Physiology and Pharmacology.* 79: 320-328.

Poullain M.G., Cezard J. P., Roger L., Mendy F. 1989. Effect of whey proteins, their oligopeptide hydrolysates and free amino acid mixtures on growth and nitrogen retention in fed and starved rats. *Journal of Parenteral and Enteral Nutrition.* 13: 382-6. Erratum in: *Journal of Parenteral and Enteral Nutrition.* 1989. 13: 595.

Qiao Y., Lin X., Odle J., Whitaker A., van Kempen T. A. T. G. 2004. Refining *in vitro* digestibility assays: fractionation of digestible and indigestible peptides1. *Journal of Animal Science.* 82: 1669-1678.

Rahman I., MacNee W. 2000. Oxidative stress and regulation of glutathione in lung inflammation. *The European Respiratory Journal.* 16: 534-554.

Rahman I., Gilmour P. S., Jimenez L. A., Biswas S. K., Antonicelli F., Aruoma O. I. 2003. Ergothioneine inhibits oxidative stress- and TNFα-induced NFκB activation and interleukin-8 release in alveolar epithelial cells. *Biochemical and Biophysical Research Communications.* 302: 860-864.

Roum J. H., Borok Z., McElvaney N. G., Grimes G. J., Bokser A. D., Buhl R., Crystal, R. G. 1999. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cytic fibrosis. *Journal of Applied Physiology.* 87: 438-443.

Sapan C. V., Lundblad R. L., Price N. C. 1999. Colorimetric protein assay techniques. *Biotechnology Applied Biochememistry.* 29: 99-108.

Schaeffer C., Vandroux D., Thomassin L., Athias P., Rochette L., Connat J. L. 2003. Calcitonin gene-related peptide partly protects cultured smooth muscle cells from

apoptosis induced by an oxidative stress via activation of ERK1/2 MAPK. *Biochimica et Biophysica Acta.* 1643: 65-73.

Schwiebert L. M., Estell K., Propst S. M. 1999. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *American Journal of Physiology.* 276 (*Cell Physiolology* 45): C700-C710.

Smyth D.G. 1967. Techniques in enzymic hydrolysis. *Methods Enzymology.* 11: 214-231.

Smyth M., Fitzgerald R. J. 1998. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available protease preparations. *International Dairy Journal.* 8: 819-827.

Stapelfeldt H., Petersen P. H., Kristiansen K. R., Qvist K. B., Skibsted L. H. 1996. Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin by trypsin, thermolysin and pepsin. *Journal of Dairy Research.* 63: 111-118.

Stauffer, C. E. 1989. *Enzyme Assay for Food Scientists.* Van Nostrand Reinhold, New York. USA. pp.1-317.

Venkatakrishnan A., Stecenko A. A., King G., Blackwell T. R., Brigham K. L., Christman J. W., Blackwell T. S. 2000. Exaggerated activation of nuclear factor-κB and altered IκB-β processing in cystic fibrosis bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology.* 23: 396-403.

Vermeirssen V., Van Camp J., Devos L., Verstraete W. 2003. Release of angiotensin I converting enzyme (ACE) inhibitory activity during *in vitro* gastrointestinal digestion: from batch experiment to semi-continuous model. *Journal of Agricultural Food Chemistry.* 51: 5680-5687.

Woods A. H., O'Bar P. R. 1970. Absorption of proteins and peptides in the far ultraviolet. *Science.* 167: 179-181.

Vilela R. M., Lands L. C., Meehan B., Kubow S. 2005. Inhibition of the inflammatory response of CFTR-deficient lung epithelial cells following pre-treatment with fenretinide. Submitted.

van der Vliet A., Eiserich J. P., Marelich G. P., Halliwell B., Cross C. E. Oxidative stress in cystic fibrosis: Does it occur and does it matter? In: Antioxidants in Disease Mechanisms and Therapy. 1997. *Advances in Pharmacology.* 38: 491-513.

Wallis T. T., Pitt J. J., Gorman J. J. 2001. Identification of disulfide-linked peptides by isotope profiles produced by peptic digestion of proteins in 50% ^{18}O water. *Protein Science.* 10: 2251-2271.

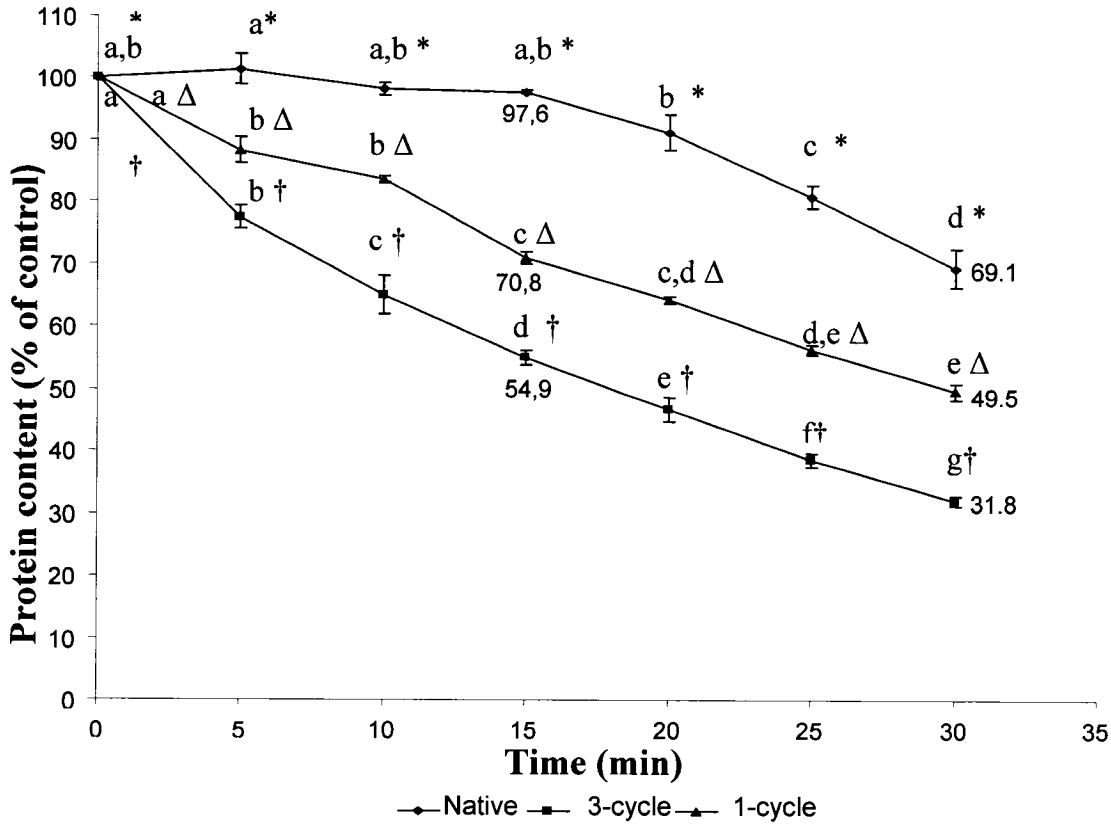


Figure 1. Effect of hydrostatic pressure treatment on *in vitro* digestion of WPI. WPI were exposed to either 3-cycle treatment at 400 MPa or 1-cycle treatment at 550 MPa and the solutions were lyophilized. Using lyophilized native WPI and pressure-treated WPI samples, 3% solutions (w/v) were prepared and digested with pepsin during 30 min at 37°C. Aliquots were taken every 5 min and the protein content was determined at 590 nm (n = 3). Error bars show 95% CI of mean. The numbers along the curves represent the percentage of proteins detected at 15 and 30 min. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. Treatments not sharing common symbols (*, Δ, †) indicate significant differences ($P < 0.05$, ANOVA) between groups at each time point by Tukey's *post hoc* comparison.

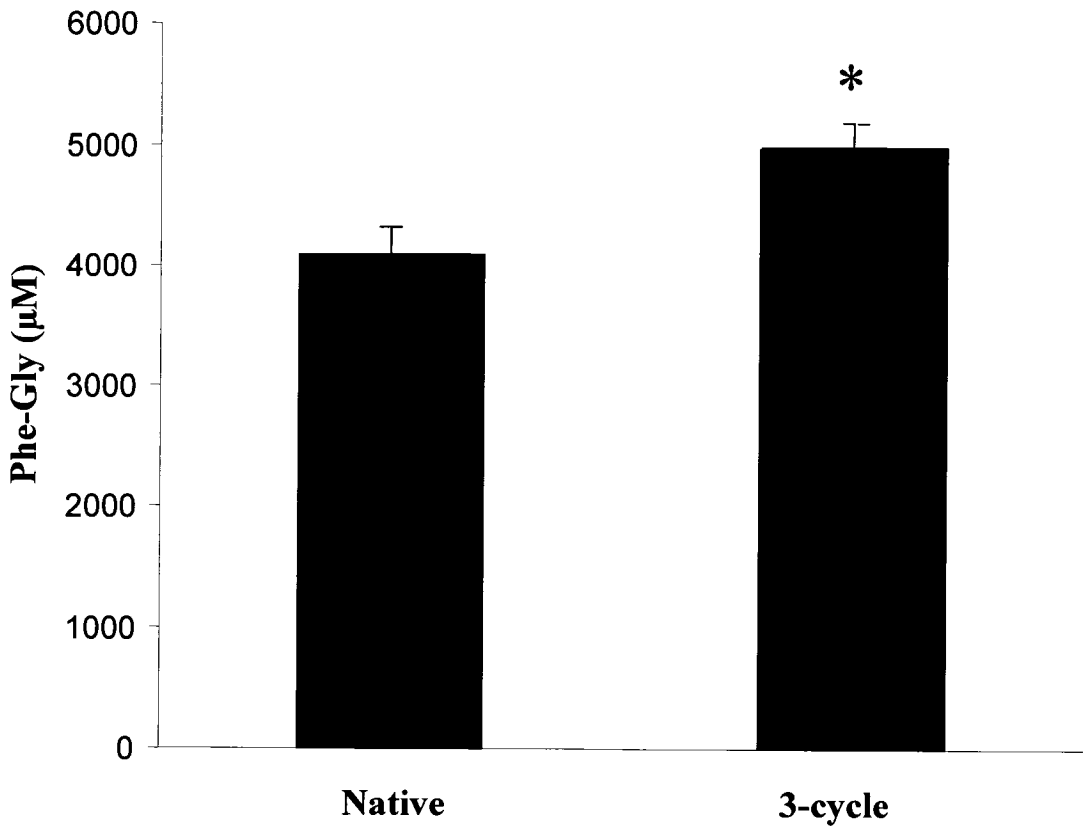


Figure 2. Effect of hydrostatic pressure treatment on *in vitro* digestion of WPI. WPI was untreated (native) or exposed to either 3-cycle treatment at 400 MPa and the solutions were lyophilized. Using lyophilized native WPI and pressure-treated WPI samples, 3% solutions (w/v) were prepared and digested with pepsin for 30 min followed by pancreatin digestion for an additional 60 min. Ultrafiltration was used to separate peptides with molecular weight lower than 3 kDa. The amino acid content of the filtrate was determined at 340 nm. n=3. Error bars show 95% CI of mean. Asterisks (*) indicate significant differences ($P < 0.05$) between the treatments by two-tailed Student's *t*-test.

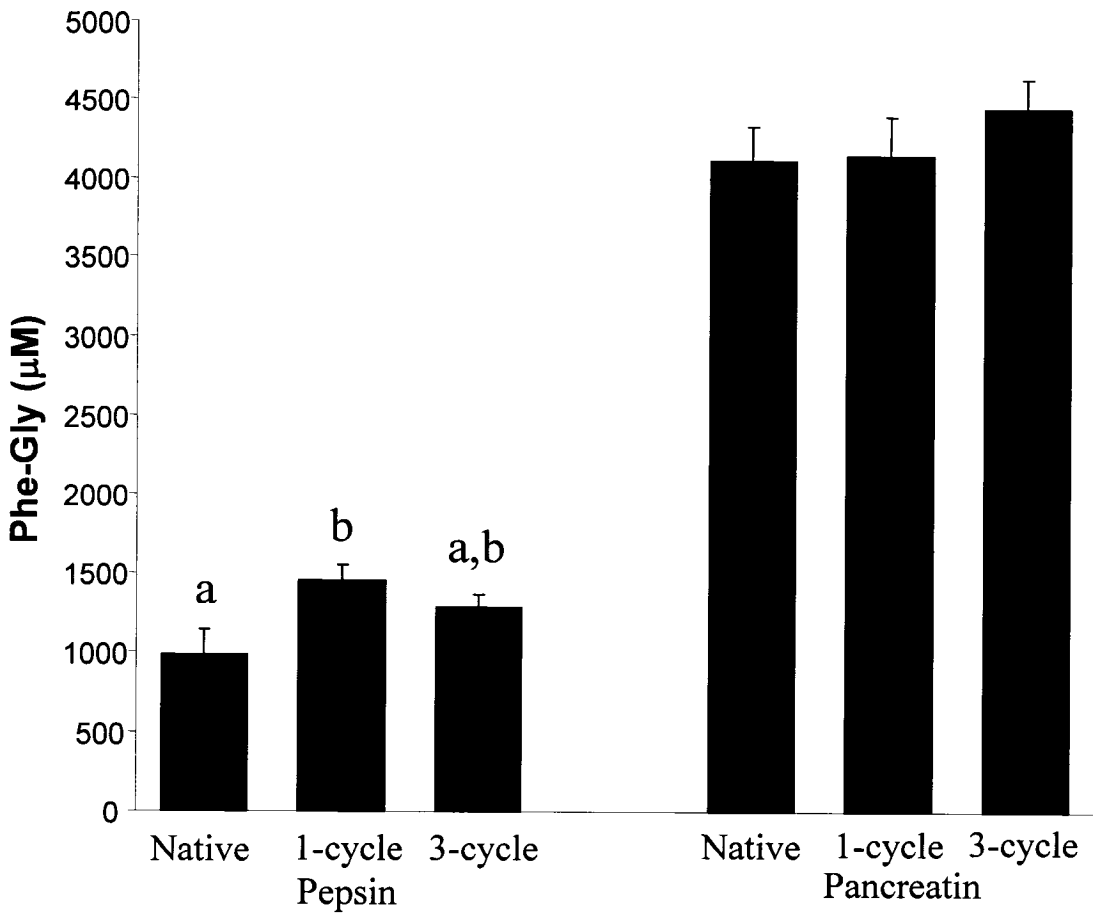
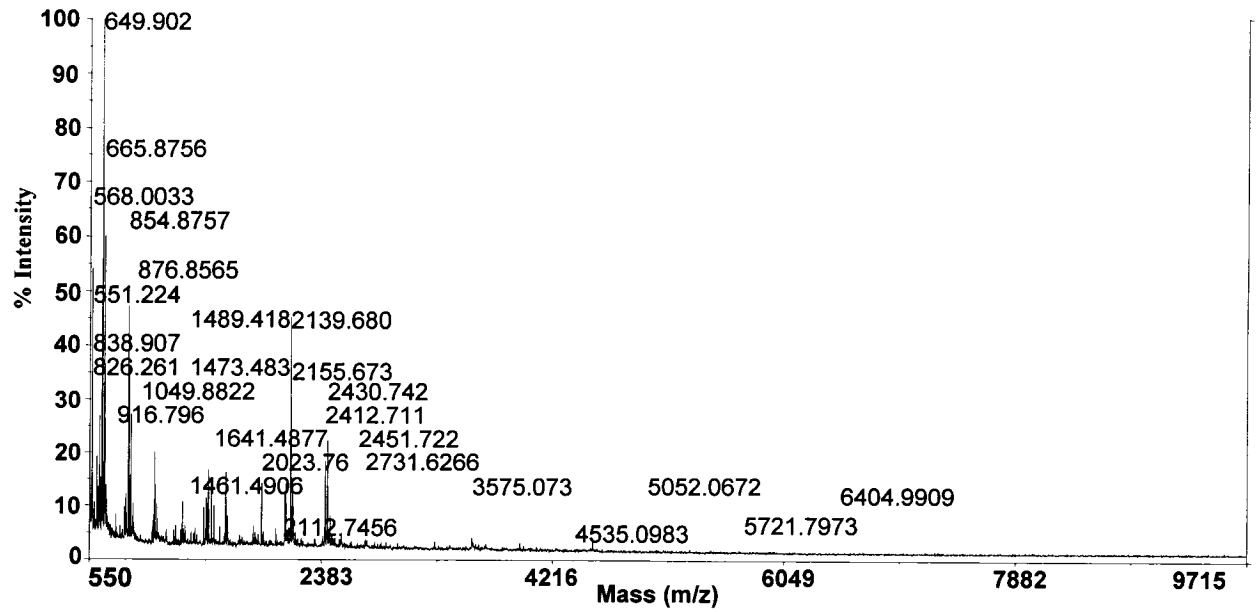
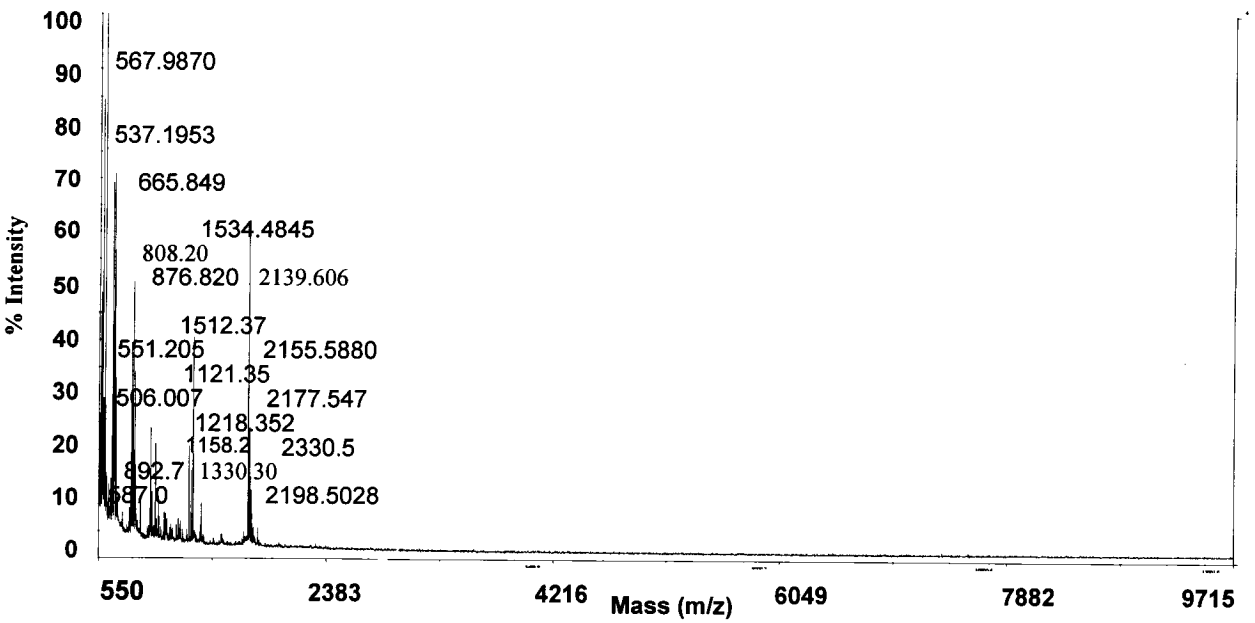


Figure 3. The content of peptides with molecular weight lower than 1 kDa in native WPI, WPI treated at 1 cycle pressure at 550 MPa and WPI treated at 3 cycle pressure at 400 MPa after *in vitro* enzymatic digestion by pepsin alone, and by pepsin followed by pancreatin. The enzyme/substrate ratio used was 1/100 for pepsin and 1/30 for pancreatin. In both experiments, peptides were separated by ultrafiltration and the amount of peptides/amino acids released at the end of the digestion with pepsin (on the left) and pancreatin (on the right) was determined at 340 nm. Experiments were performed six times. Error bars show 95% CI of mean. Statistical analysis was performed by ANOVA, followed by Tukey's *post hoc* comparison test. Columns not sharing common letters represent means that differed significantly ($P < 0.05$).

A


649.902
665.8756
568.0033
854.8757
876.8565
551.224
1489.418
2139.680
838.907
826.261
1473.483
2155.673
1049.8822
2430.742
916.796
2412.711
1641.4877
2451.722
2023.76
2731.6266
1461.4906
3575.073
5052.0672
6404.9909
2112.7456
4535.0983
5721.7973
% Intensity
Mass (m/z)
550
2383
4216
6049
7882
9715

B


567.9870
537.1953
665.849
1534.4845
808.20
876.820
2139.606
1512.37
551.205
2155.5880
1121.35
506.007
2177.547
1218.352
1158.2
2330.5
892.7
1330.30
2198.5028
% Intensity
Mass (m/z)
550
2383
4216
6049
7882
9715

C

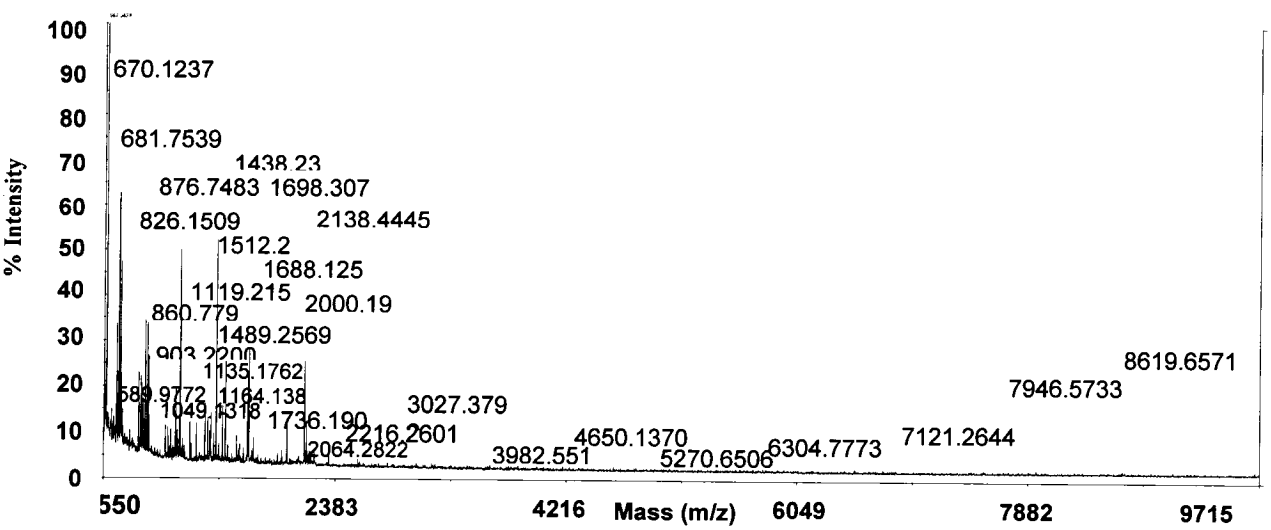
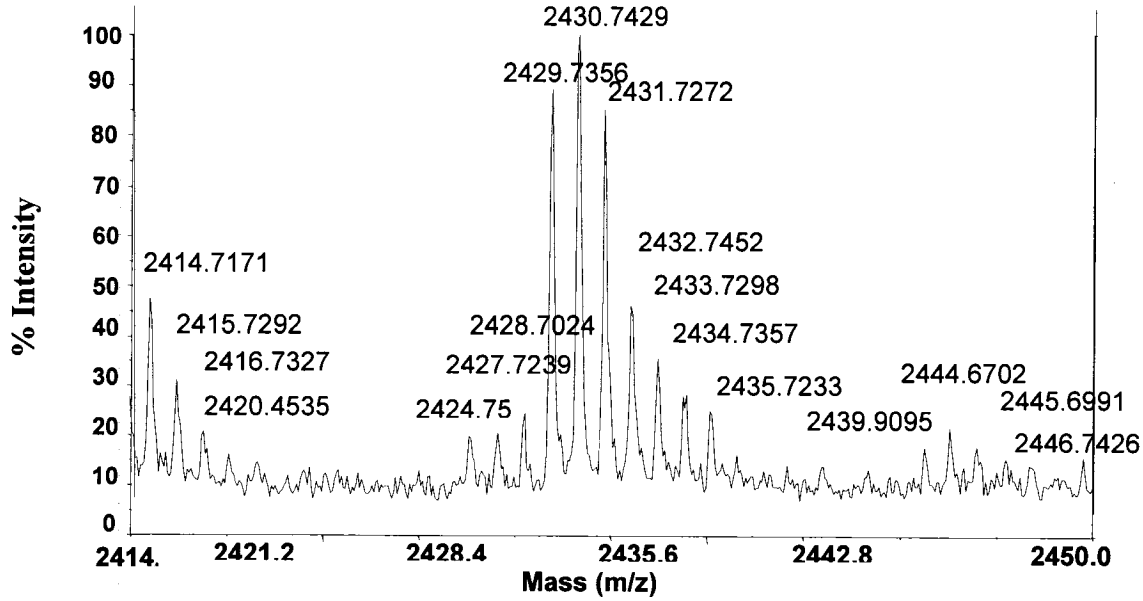
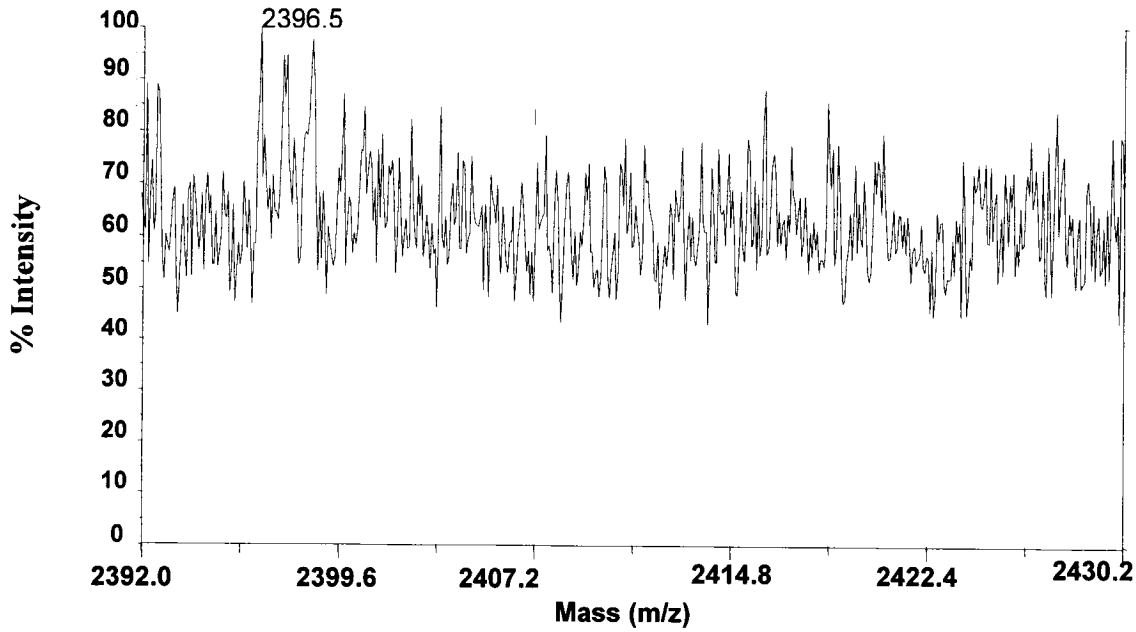

670.1237
681.7539
1438.23
876.7483
1698.307
826.1509
2138.4445
1512.2
1688.125
1119.215
2000.19
860.779
1489.2569
1135.1762
8619.6571
7946.5733
1164.138
3027.379
1736.190
2216.2601
4650.1370
7121.2644
2064.2822
3982.551
5270.6506
6304.7773
% Intensity
Mass (m/z)
550
2383
4216
6049
7882
9715

Figure 4. MALDI-MS profiles of peptide extracts with molecular weight < 1 kDa. The peptides were obtained from *in vitro* pepsin digestion and ultrafiltration of: (**A**) native WPI; (**B**) WPI treated at 3 cycle pressure at 400 MPa; and (**C**) reducing DTT treatment of < 1 kDa peptides obtained from *in vitro* pepsin digestion of native WPI.

A


2430.7429
2429.7356
2431.7272
2414.7171
2432.7452
2433.7298
2415.7292
2428.7024
2434.7357
2416.7327
2427.7239
2435.7233
2444.6702
2420.4535
2424.75
2445.6991
2439.9095
2446.7426
% Intensity
Mass (m/z)

B


2396.5
% Intensity
Mass (m/z)

C

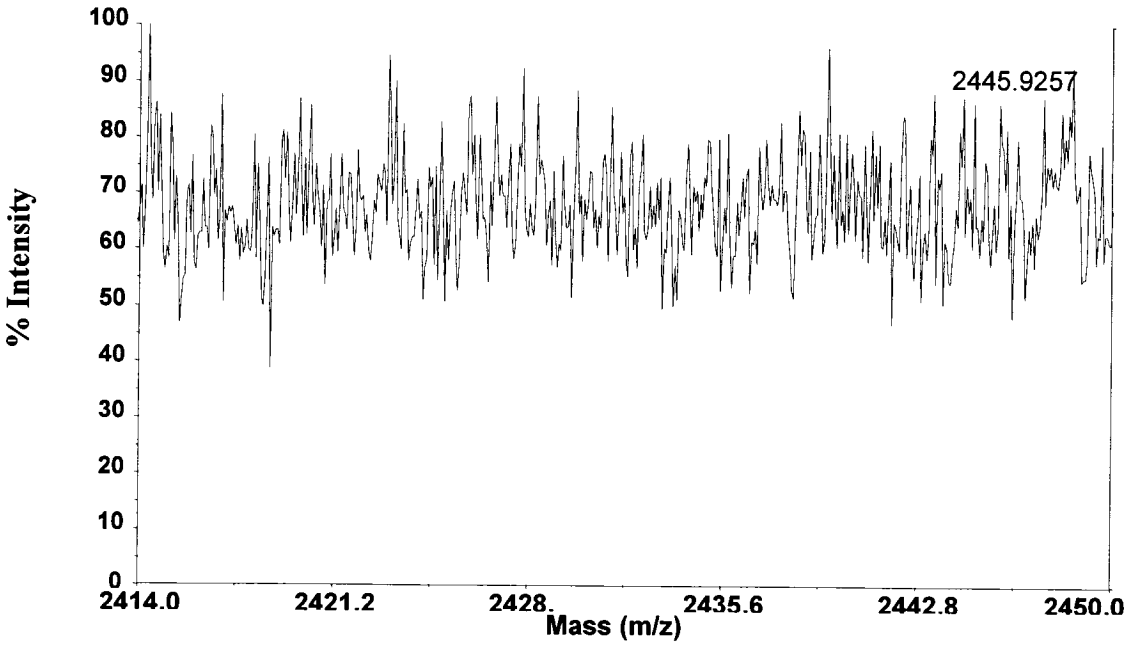

2445.9257
% Intensity
Mass (m/z)

Figure 5. MALDI-MS profiles of peptide extracts with molecular weight < 1 kDa. The peptides were obtained from *in vitro* pepsin digestion and ultrafiltration of: (**A**) native WPI; (**B**) WPI treated at 3 cycle pressure at 400 MPa; and (**C**) reducing DTT treatment of < 1 kDa peptides obtained from *in vitro* pepsin digestion of native WPI.

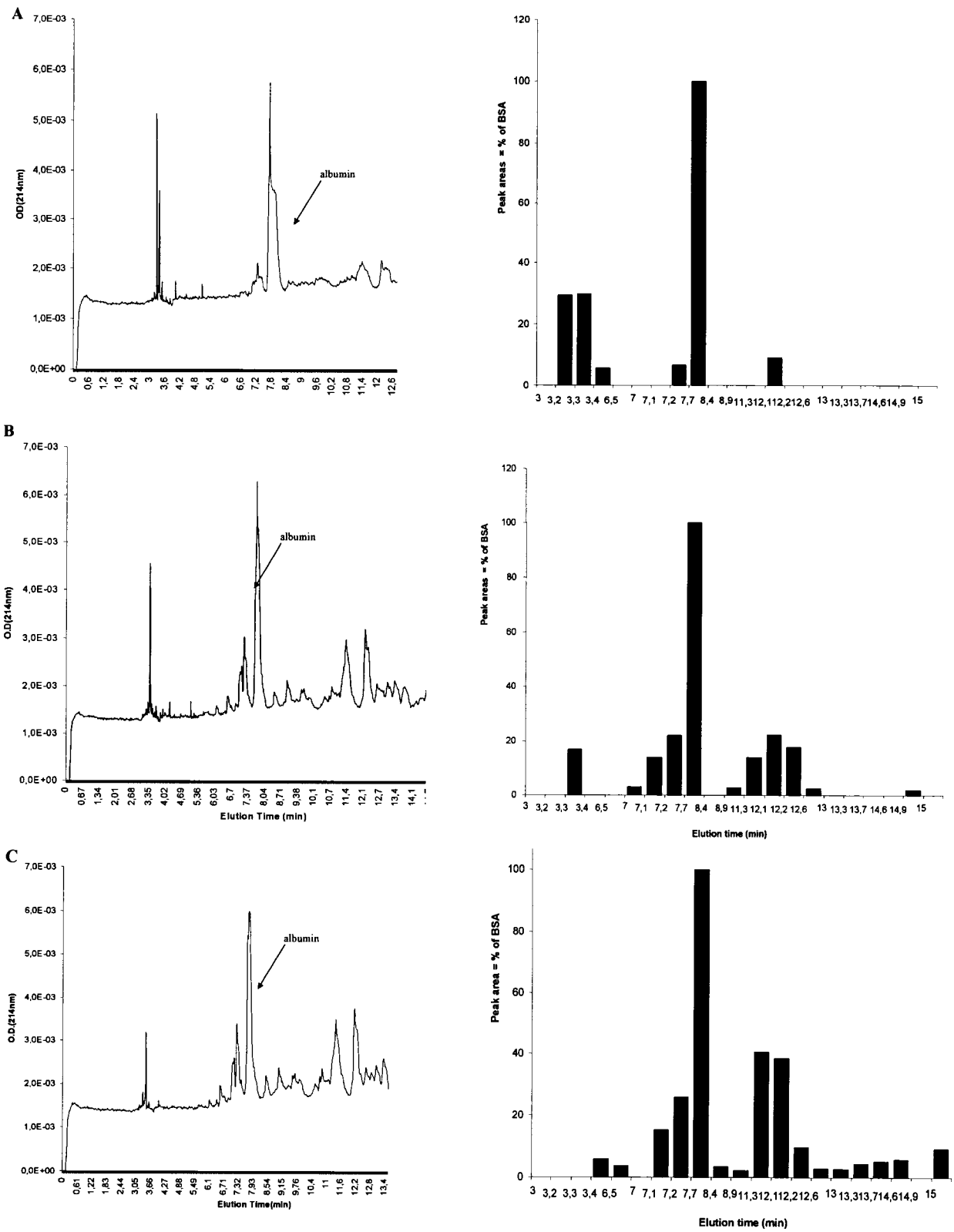
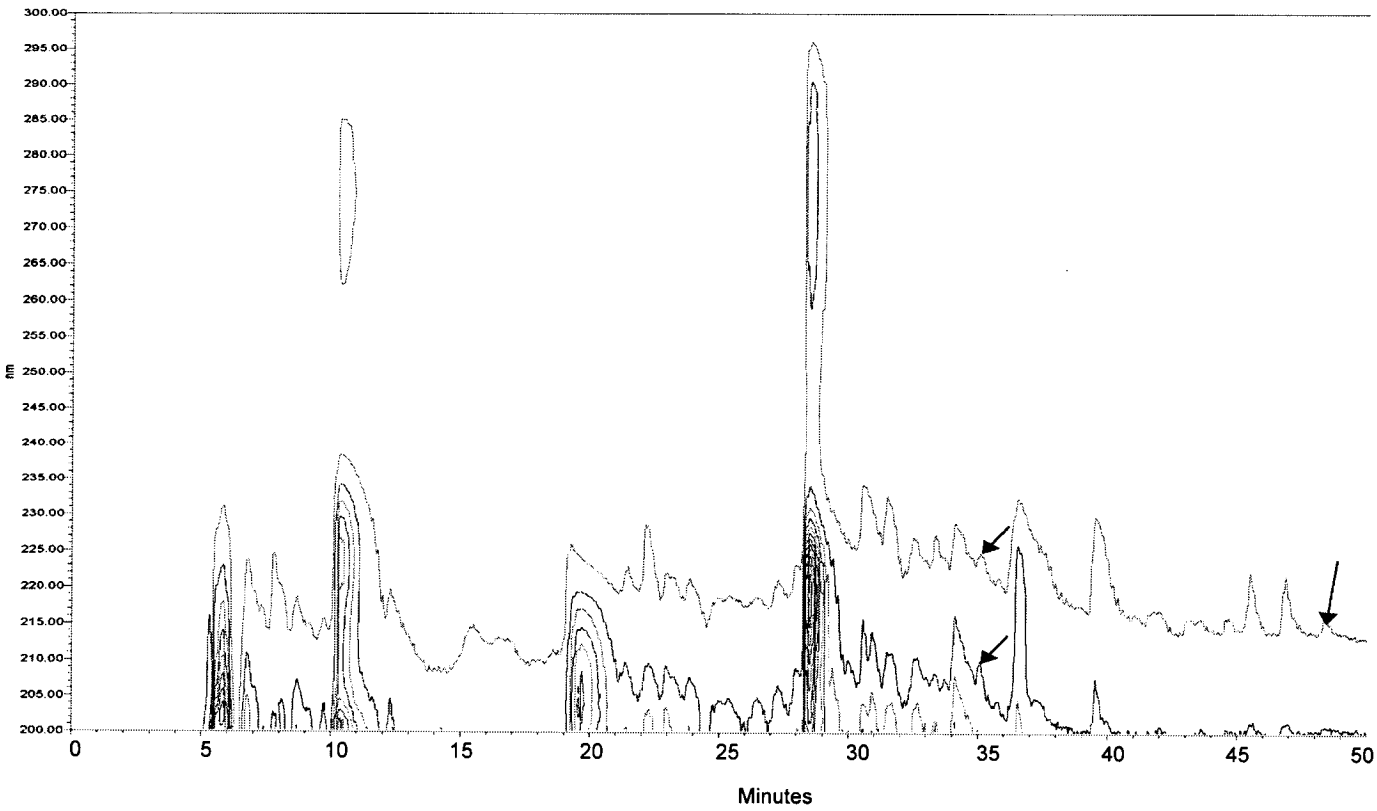

A
OD(214nm)
albumin
Peak areas = % of BSA
B
O.D(214nm)
albumin
Elution Time (min)
Peak areas = % of BSA
Elution time (min)
C
O.D.(214nm)
albumin
Elution Time(min)
Peak area = % of BSA
Elution time (min)

Figure 6. Profiles of peptide extracts with molecular weight < 1 kDa. The peptides were obtained following *in vitro* digestion and ultrafiltration of (**A**) native WPI, (**B**) WPI treated at 1cycle pressure at 550 MPa, and (**C**) WPI treated at 3-cycle pressure at 400 MPa. Capillary zone electrophoresis was used to generate the electropherograms of the separated peptides, which were detected at 214 nm. CZE conditions: 40 μL of the sample solution at a concentration of 40 mg/mL was injected and the peptides migrated from the positive to the negative pole at 27 kV and 30°C using 0.1 M phosphate buffer (pH 2.5). BSA (400 μg/mL) was used as internal standard. Graphs on the right represent the relative amounts of peptides as compared to the internal standard bovine serum albumin (small grid background) as calculated from the peak areas (100% corresponds to the albumin peak area).

A


nm
300.00
295.00
290.00
285.00
280.00
275.00
270.00
265.00
260.00
255.00
250.00
245.00
240.00
235.00
230.00
225.00
220.00
215.00
210.00
205.00
200.00
0
5
10
15
20
25
30
35
40
45
50
Minutes

B

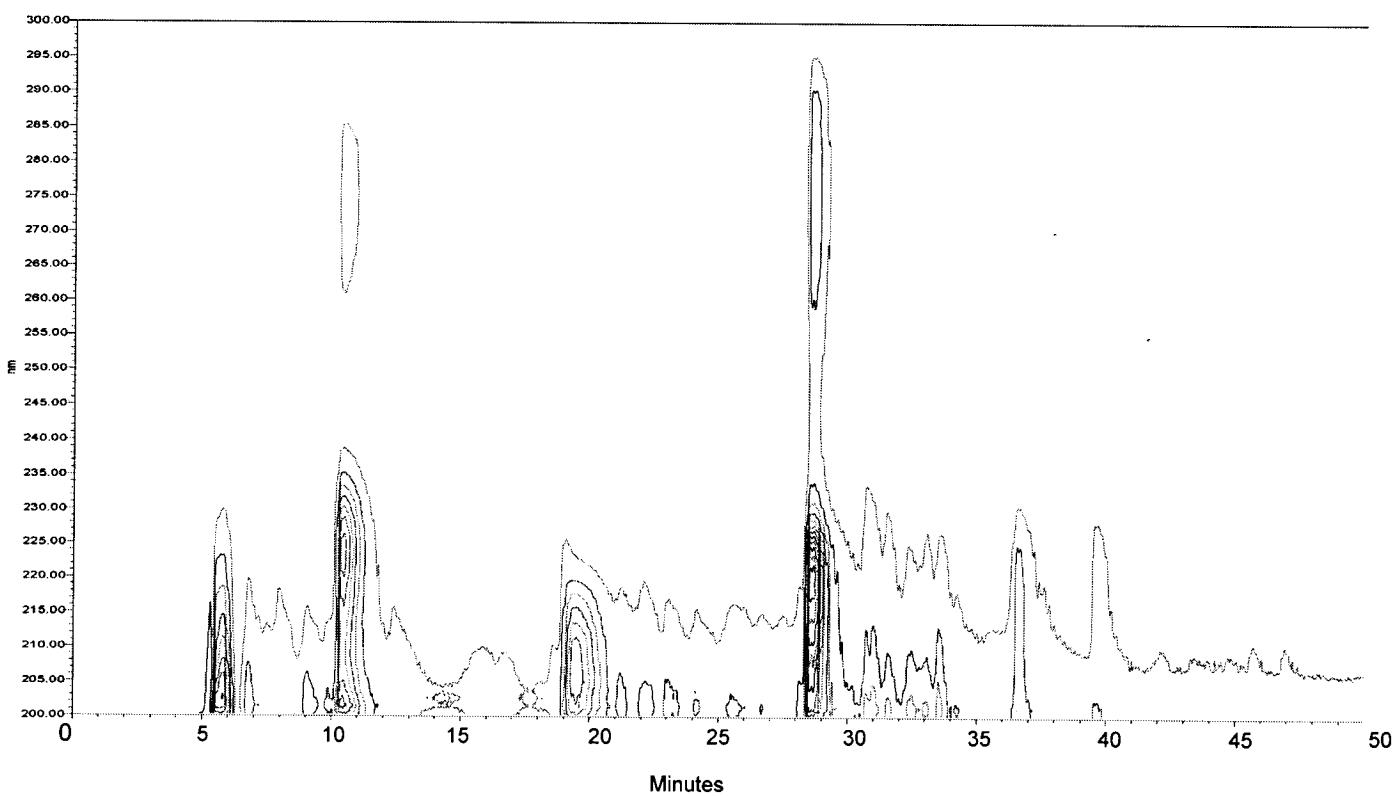
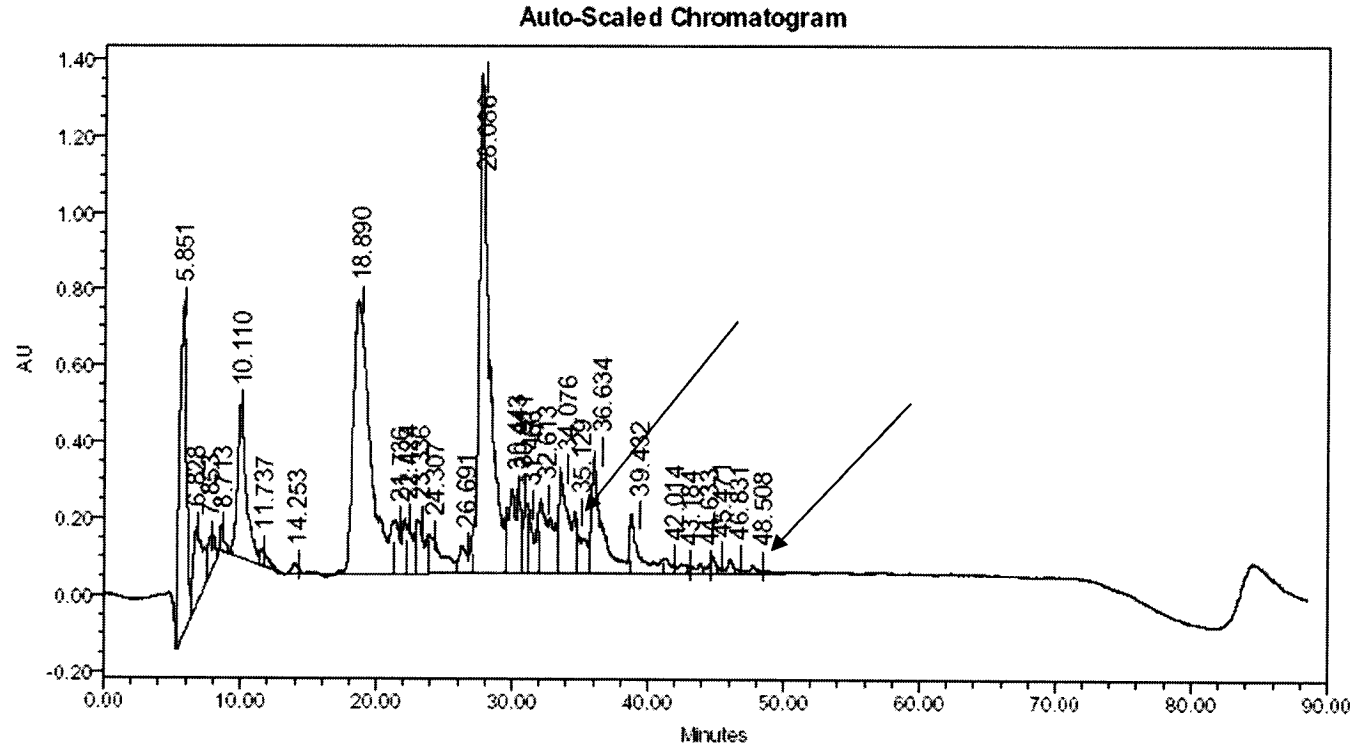

nm
300.00
295.00
290.00
285.00
280.00
275.00
270.00
265.00
260.00
255.00
250.00
245.00
240.00
235.00
230.00
225.00
220.00
215.00
210.00
205.00
200.00
0
5
10
15
20
25
30
35
40
45
50
Minutes

Figure 7. Chromatogram of peptides from reversed-phase HPLC obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm. (A) Peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of WPI exposed to hydrostatic pressure at 1-cycle of 550 MPa. **(B)** Peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of native WPI. Samples were loaded on analytical C18-reverse phase column (5 μm; 250 mm × 4.6 mm) (Vydac Series-218TP54, Vydac Company, Herperia, CA) and separated with a Waters HPLC system (Waters, Milford, MA). Conditions for RP-HPLC separation are as described in Methods section. The arrows identify some extra peaks found in native WPI as compared to presuized WPI, and indicate differences and peptide profile as a result of pressure treatment.

Run Time 90.0 Minutes



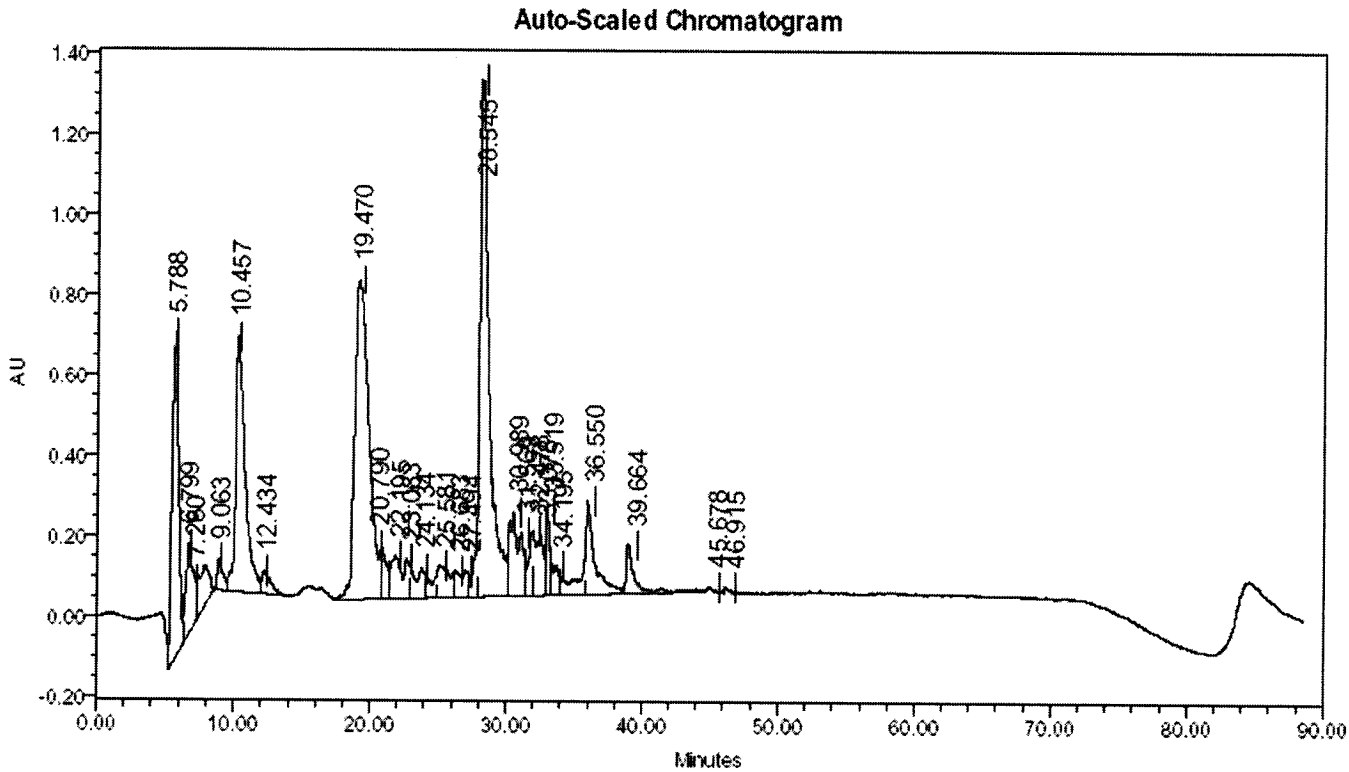


Figure 8. Chromatogram of peptides from reversed-phase HPLC chromatogram obtained via detection at 214 nm (A) Peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of WPI exposed to hydrostatic pressure at 1-cycle of 550 MPa. **(B)** Peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of native WPI. Samples were loaded on analytical C18-reverse phase column (5 μm; 250 mm × 4.6 mm) (Vydac Series-218TP54, Vydac Company, Herperia, CA) and separated with a Waters HPLC system (Waters, Milford, MA). Conditions for RP-HPLC separation are as described in Methods section. The arrows confirm the presence of peaks found in Fig. 7.

Table 1. Amino acid analysis of whey protein hydrolysates (total free amino acid content < 1 kDa)

Amino acid compounds (570 nm)	nWPH (μmol/L)	pWPH (μmol/L)
Amino acids		
alanine	135	169
arginine	688	694
asparagine	28	36
aspartic acid	24	33
cystine	5	5
glutamic acid	36	37
glutamine	530	559
glycine	93	98
histidine	200	232
isoleucine	368	380
leucine	2391	2538
lysine	1934	2086
methionine	268	296
phenylalanine	1045	1056
proline*	41	23
serine	34	36
threonine	269	290
tryptophan	369	488
tyrosine	660	691
valine	624	646

See notes at end of table

Amino acid derivatives		
argininosuccinic acid	187	61
β-alanine	213	211
α-aminoisobutyric acid	97	121
α-aminoadipic acid	14	10
γ-aminobutyric acid (GABA)	[1]N.D.	40
α-amino-n-butyric acid	6	21
citrulline	45	63
ethanolamine	43	59
hydroxyproline*	25	33
ornithine	5	10
o-phosphoethanolamine	N.D.	2
o-phosphoserine	11	23
sarcosine	127	107

*measured at 440 nm

[1]N.D.= not detectable

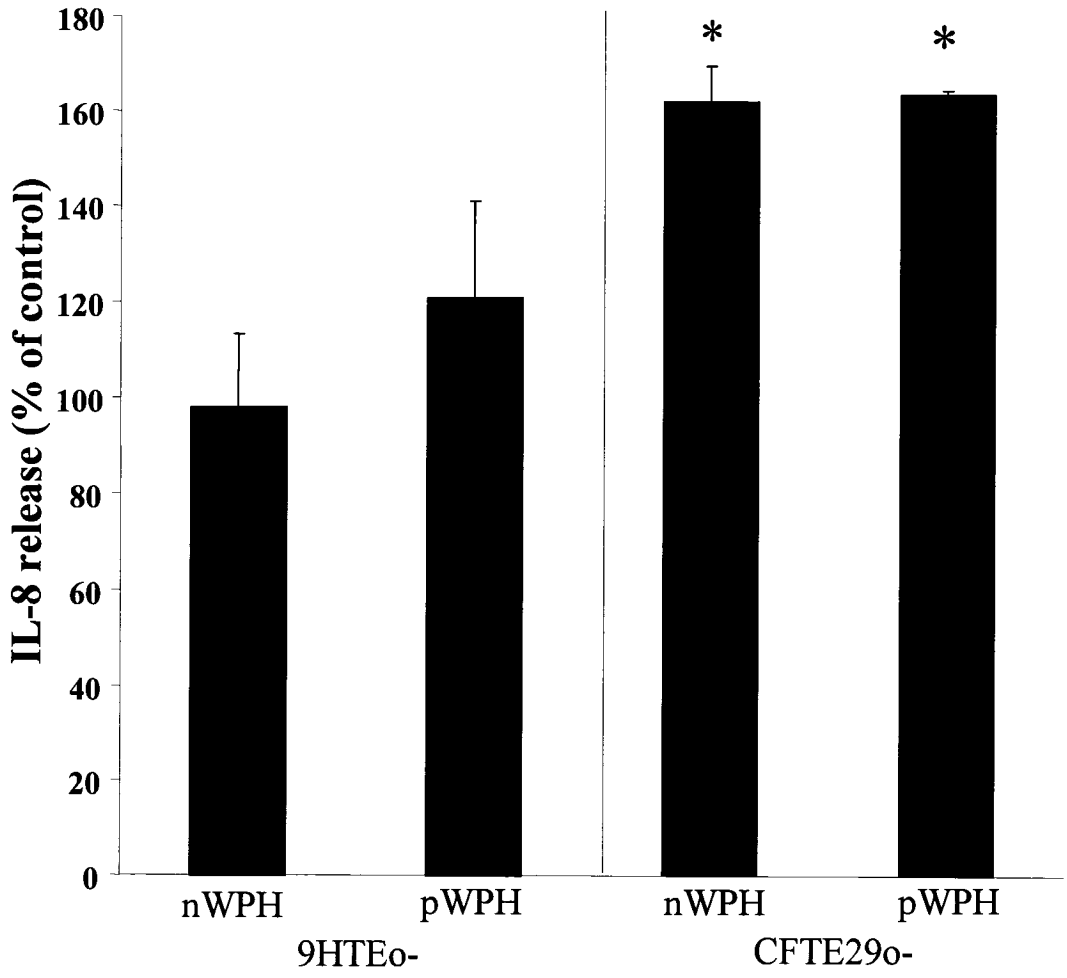


Figure 9. IL-8 release after treatment of non-CF and CF cells with pressure-treated (pWPH) and native whey protein hydrolysates (nWPH) under unstimulated conditions. As described in the Methods section, wild-type tracheal epithelial cells (non-CF 9HTEo-) and CFTR deficient cells (CF cells CFTE29o-), were treated with 12.5 μg/mL of pWPH and nWPH solution (w/v) for 48 h in order to characterize the impact of nWPH and pWPH on IL-8 release in an unstimulated basal condition. Results are means ± S.E. of three independent experiments Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated controls by ANOVA and LSD's *post hoc* analysis. The IL-8 release values for the controls were 287 ± 43 pg·mL^{-1} for non-CF cells and 166 ± 21 pg·mL^{-1} for CF cells.

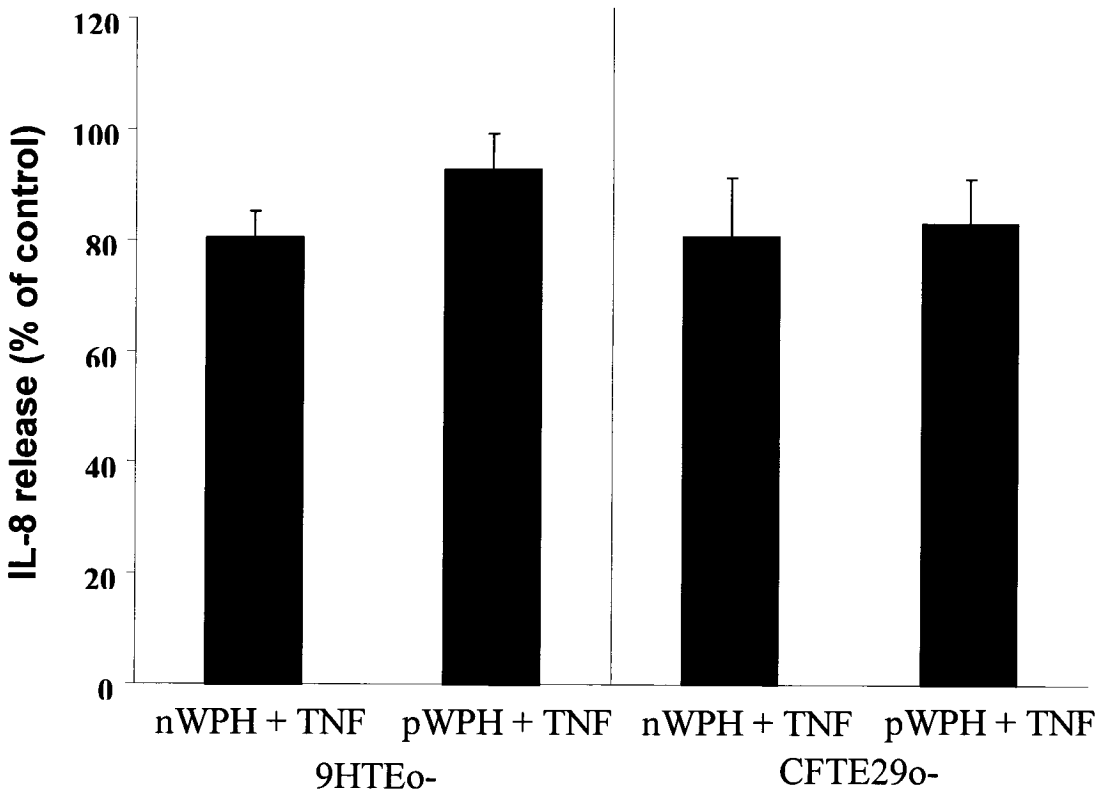
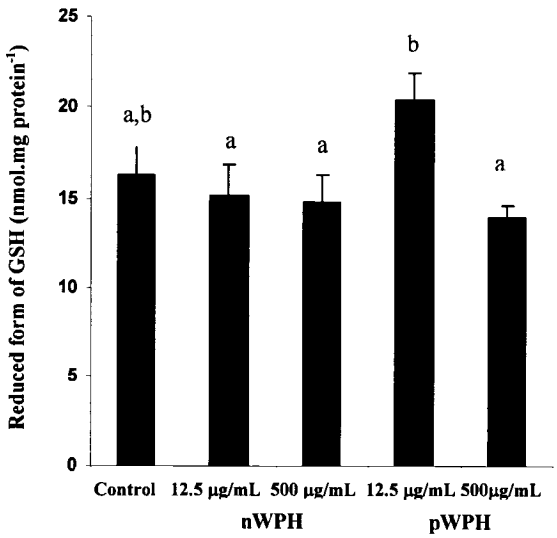
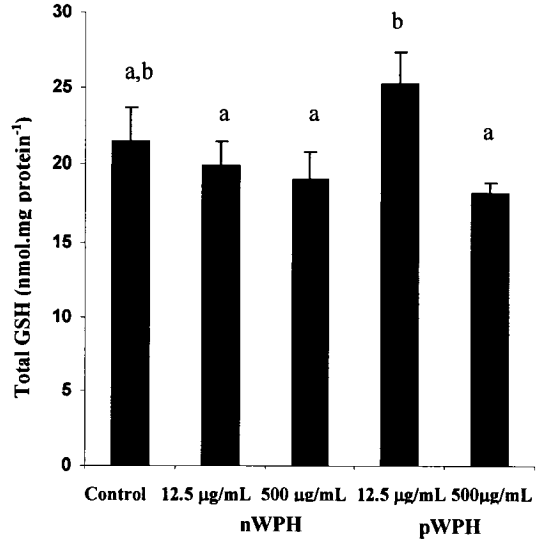
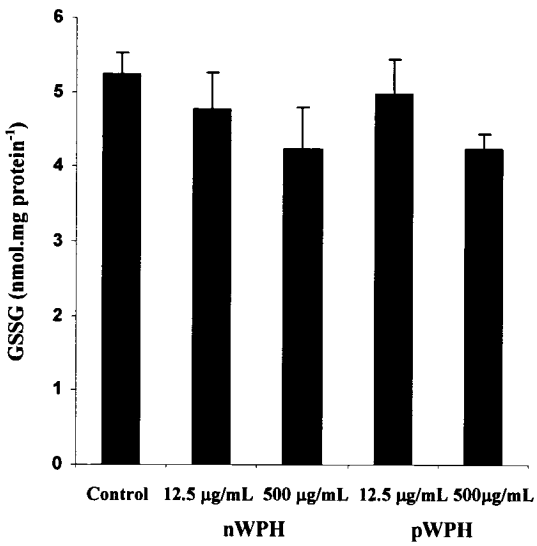


Figure 10. IL-8 release after treatment of non-CF and CF cells with pressure-treated and native whey protein hydrolysates (pWPH; nWPH) under TNF-α stimulated conditions. As described in the Methods section, wild-type tracheal epithelial cells (non-CF 9HTEo- cells), and CFTR deficient cells (CF CFTR29o- cells), were treated with 12.5 μg/mL of nWPH and pWPH in MEM 2% FBS for 24 h. After 24 h, the medium was replaced by fresh MEM 2% FBS containing 12.5 μg/mL of WPH concurrently with human recombinant TNF-α (10 ng·mL $^{-1}$) for an additional 24 h. All experiments included stimulated negative control wells. Results are means ± S.E. of three independent experiments and ANOVA and LSD's *post hoc* statistic analysis was used. The IL-8 release values for the stimulated controls were 78,202 ± 9,940 pg·mL^{-1} for non-CF cells and 19,009 ± 943 pg·mL^{-1} for CF cells and were used to express the results as % of the controls.


Reduced form of GSH (nmol.mg protein-1)
25
20
15
10
5
0
a,b
a
a
b
a
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH


Total GSH (nmol.mg protein-1)
30
25
20
15
10
5
0
a,b
a
a
b
a
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH


GSSG (nmol.mg protein-1)
6
5
4
3
2
1
0
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH

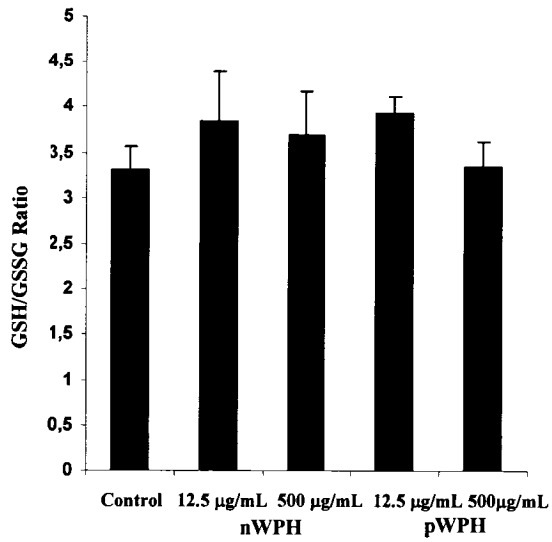
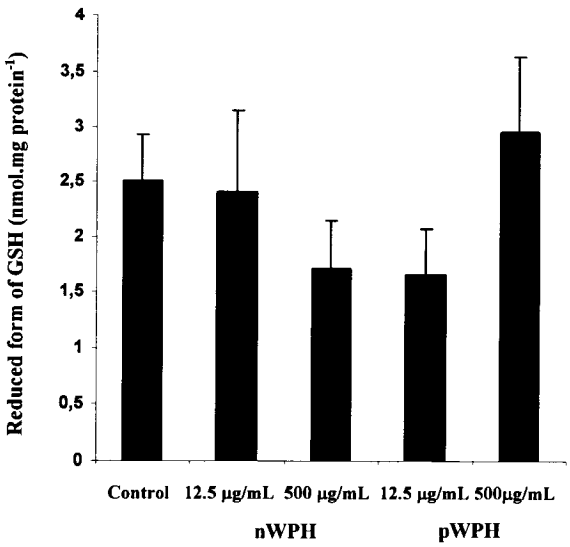
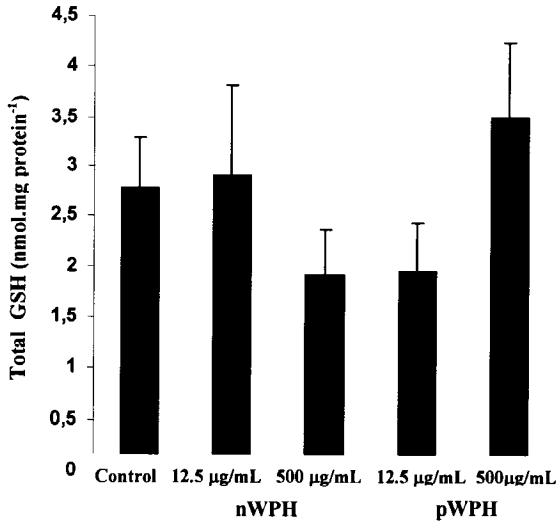
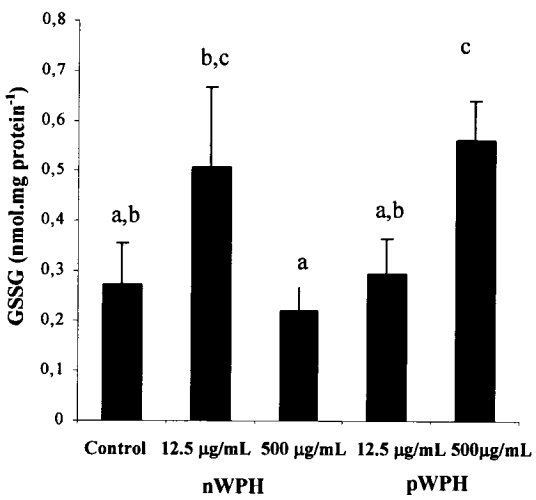

GSH/GSSG Ratio
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH

Figure 11. Intracellular levels of total GSH, the reduced form of GSH, GSSG and the GSH/GSSG ratio in CFTE29o- cells in response to WPH. Nearly confluent CFTR deficient human epithelial cells (CFTE29o-) were incubated for 48 h in MEM containing 2% FBS with two different concentrations (12.5 and 500 μg/mL) of containing peptides smaller than 1 kDa obtained from either native whey protein isolate hydrolysates (nWPH) or hydrolysates from whey protein isolates exposed to 1 cycle (550 MPa) pressurization (pWPH). Wells containing MEM with 2% FBS were used as controls. After 48 h, the cells were collected and the intracellular GSH and GSSG were determined as described in the Methods section. Intracellular GSH was divided by intracellular GSSG to establish the GSH/GSSG ratio. Columns not sharing common letters represent means that differed significantly ($P < 0.05$, ANOVA) by LSD *post hoc* comparison.


Reduced form of GSH (nmol.mg protein-1)
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH


Total GSH (nmol.mg protein-1)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH


GSSG (nmol.mg protein-1)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a,b
b,c
a
a,b
c
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH

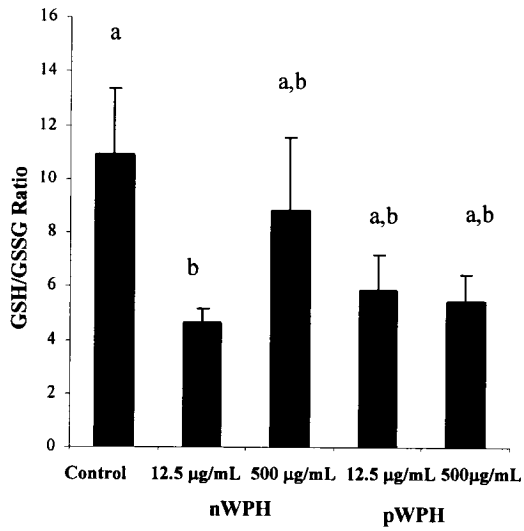

GSH/GSSG Ratio
16
14
12
10
8
6
4
2
0
a
b
a,b
a,b
a,b
Control
12.5 µg/mL
500 µg/mL
12.5 µg/mL
500µg/mL
nWPH
pWPH

Figure 12. Intracellular levels of total GSH, the reduced form of GSH, GSSG and the GSH/GSSG ratio in 9HTEo- cells in response to WPH. Nearly confluent CFTR deficient human epithelial cells (CFTE29o-) were incubated for 48 h in MEM containing 2% FBS with two different concentrations (12.5 and 500 μg/mL) of peptides smaller than 1 kDa obtained from either native whey protein isolate hydrolysates (nWPH) or hydrolysates from whey protein isolates exposed to 1 cycle (550 MPa) pressurization (pWPH). Wells containing MEM with 2% FBS were used as controls. After 48 h, the cells were collected and the intracellular GSH and GSSG were determined as described in the Methods section. Intracellular GSH was divided by intracellular GSSG to establish the GSH/GSSG ratio. Columns not sharing common letters represent means that differed significantly ($P < 0.05$, ANOVA) by LSD *post hoc* comparison.

CONNECTING STATEMENT

The results obtained in Chapter 4 demonstrated that hyperbaric treatment at 1 cycle of 550 and 3 cycles at 400 MPa increased the efficiency of whey protein digestibility by 1.6 and 2.2-fold, respectively, after pepsin treatment for 30 min as compared to the digestibility of native whey protein. As expected, the enhanced digestibility of pressurized whey proteins produced < 1 kDa peptide fragments that differed between the nWPH and pWPH treatments following pepsin and pancreatin digestion. Cell culture studies carried out with wild-type and CFTR deficient tracheal epithelial cells generally showed that CF cells were more sensitive to WPH in terms of GSH and IL-8 modulation and low doses of pWPH appeared to attenuate a pro-inflammatory condition in CF cells without affecting non-CF cells.

Considering the results obtained in Chapter 3 showing that low doses of fenretinide treatment inhibited TNF-α-induced IL-8 production of CF cells without affecting the IL-8 release in non-CF cells, and the results of Chapter 4 showing a similar effect when the cells were treated with pWPH, an additional series of experiments were conducted in CF and non-CF tracheal epithelial cells to assess the combined effect of low dose fenretinide (1.25 μM) and low doses (12.5 μg/mL) of native or pressurized WPH on IL-8 release, apoptosis and cell viability as described in the next chapter.

CHAPTER 5

Inhibition of the Inflammatory Response of CFTR-Deficient Lung Epithelial Cells Following Pre-Treatment with Whey Peptides and Fenretinide

Regina Maria Vilela[1], Larry C. Lands[2], Brian Meehan[2] and Stan Kubow[1,3]

[1]School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
[2]Montreal Children's Hospital
Division of Respiratory Medicine, Room D-380
2300 Tupper Street, Montreal, Quebec H3H 1P3

[3]Corresponding Author: Dr. Stan Kubow
School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore, Ste. Anne de Bellevue
Quebec, Canada H9X 3V9
E-mail: stan.kubow@mcgill.ca

Abstract

A variety of therapies have attempted to treat the infection and the chronic inflammatory state in cystic fibrosis (CF). Nutrient-based therapies such as whey proteins and whey protein hydrolysates (WPH) have been hypothesized to protect the lungs from the deleterious effects of oxidative stress and inflammation in CF via enhancement of the GSH/GSSG redox status. Recently, we have demonstrated that low molecular weight peptides isolated from WPH obtained from hydrostatic pressure treated whey protein isolates (WPI) showed a tendency to enhance GSH status and decrease the pro-inflammatory state in lung epithelial CF cells without affecting non-CF cells. Similarly, our recent work has demonstrated that fenretinide treatment attenuated IL-8 release in response to TNF-α in CFTR deficient lung epithelial cells but not in non-CF cells. In the present work, wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) tracheal cells were treated with a combination of low dose fenretinide (1.25 μM) and low doses (12.5 μg/mL) of peptides isolated from digests of native (nWPH) or pressurized (pWPH) whey proteins to assess the impact of this combined treatment on IL-8 release, apoptosis and cell viability. Following pre-treatment with fenretinide, CFTR deficient cells showed a significant ($P < 0.05$) decrease in TNF-α-induced IL-8 release with no pro-apoptotic effects. A trend for decreased TNF-α-induced IL-8 release in CF cells was also observed in association with the nWPH and pWPH treatments but no effect on apoptosis was observed from these treatments under stimulated conditions. The combination of fenretinide and WPH treatments exerted no enhancement of the reduction in TNF-α stimulated IL-8 release in CF cells seen with fenretinide treatment. Wild-type cells were sensitive to apoptosis induced by fenretinide that was ameliorated in combination with WPH treatment. Hence, the combination of fenretinide and whey peptides provides a potential approach to decrease lung inflammation in CF cells and concurrently limit pro-apoptotic effects of fenretinide in non-CF cells.

5.1 Introduction

Cystic Fibrosis Transmembrane Regulator (CFTR) is a cAMP- and protein kinase A (PKA)-regulated Cl^- channel as well as a regulator of other ion channels and plays a predominant role in both cAMP- and Ca^{2+}- activated secretion of electrolytes (Kunzelman and Mall, 2001). A defect in this transmembrane protein results in failure to secrete Cl^- through the CFTR channel and accelerated Na^+ transport through the epithelial Na^+ channel (ENaC) in epithelial cells leading to a clinical syndrome that affects mainly pancreatic and pulmonary function and contributes to the chronic inflammatory state in cystic fibrosis (CF) (Bals *et al.*, 1999; Knowles and Boucher, 2002). The organ dysfunctions that characterize the CF phenotype are not completely understood and it has been proposed that inflammation may precede infection in the progression of CF (Khan *et al.,* 1995). The decline in lung function in patients with CF may be the result of an exaggerated inflammatory response with overproduction of IL-8, which results in excessive neutrophil infiltration in the lungs and persistent inflammation combined with bacterial infection and reduced mucous clearance (Koehler, 2004).

There is no definitive treatment for CFTR deficiency; however, available therapies that treat the lung dysfunction that characterizes the CF phenoype include antibiotics, physiotherapy, oxygen therapy and lung transplantation (Nelson, 2005; Koehler *et al.*, 2004). Due to the high rates of morbidity and mortality related to lung deterioration, the main focus of the CF treatment is to treat infection and decrease inflammation of the respiratory tract (Nelson, 2005). Although many efforts have been made seeking to improve clinical care for patients with CF, the optimal effective approach to manage airway inflammation has not been achieved to date and pulmonary complications still account for the respiratory failure and mortality in CF.

Nutrient-based therapies such as whey proteins and whey protein hydrolysates (WPH) have been proposed to have immunomodulatory effects on the basis of *in vitro* cell culture (Cross and Gill, 1999; Bounous and Gold, 1991) and *ex vivo* animal studies (Low *et al.*, 2003; Ford *et al.*, 2001; Pentilla *et al.*, 2001). Whey proteins and/or WPH have

been shown to increase lymphocyte proliferation (Mercier *et al.*, 2004) and the immune response to antigens such as red blood cells (Bounous and Gold, 1991), specific vaccine antigens (Low *et al.*, 2003), sporulated oocysts of *Eimeria vermiformis* (Ford *et al.*, 2001), and as well as downregulating immune response to lipopolysaccharide (LPS) (Cross and Gill, 1999), concavalin A (Cross and Gill, 1999) and ovoalbumin oral tolerance (Penttila *et al.*, 2001). In a clinical trial, CF patients receiving whey protein isolates (WPI) demonstrated increases in lymphocyte GSH levels as compared to casein-fed controls (Grey *et al.*, 2003). The induction of tissue GSH is hypothesized to protect CF lungs from the deleterious effects of oxidative stress in response to the inflammation. Based on the above *ex-vivo* animal feeding trials of whey proteins (Low *et al.*, 2003; Ford *et al.*, 2001; Pentilla *et al.*, 2001) and *in vitro* cell culture studies (Cross and Gill, 1999; Kent *et al.*, 2003) involving WPH, it is reasonable to suggest that specific peptides or a combination of peptides present in whey protein could act as an auxiliary treatment for inflammatory diseases such as CF. The mechanism by which such peptides might affect the immune response is unknown and has not been investigated.

We have recently demonstrated that tracheal epithelial cells with a CFTR gene mutation (mutant ΔF508 CFTR) had more than three-fold lower GSH/GSSG ratio as compared to wild-type tracheal epithelial cells (Vilela *et al.*, 2005a). Moreover, when the mutant CFTR epithelial cells were exposed to low doses of low molecular weight (< 1kDa) peptides isolated from WPH that had undergone hydrostatic pressure processing (pWPH), they showed a strong tendency to increase their intracellular GSH content and the redox GSH/GSSG ratio. In addition, when CF cells were treated with peptides isolated from either pWPH or nWPH under the TNF-α stimulated condition, a strong downward trend in IL-8 release was observed. Differences between nWPH and pWPH were also observed as non-CF cells responded to nWPH with a strong downward trend in IL-8 release whereas pWPH treatment did not affect the wild-type lung epithelial cells. These results suggest that pWPH might attenuate the inflammatory response in CF cells without affecting normal epithelial cells in this regard.

In previous work, we have demonstrated that fenretinide, a synthetic derivative of retinoic acid, inhibited TNF-α-induced IL-8 production in CFTE29o- tracheal cells thereby presenting the possibility that the lung inflammation could be attenuated via low dose fenretinide treatment (Vilela *et al.*, 2005a). Fenretinide has been suggested to inhibit nuclear factor (NF)-κB (Shimada *et al.*, 2002), which could be one mechanism of its anti-inflammatory action. In view of our previous observations that both fenretinide and WPH treatments were associated with decreased IL-8 release in CF cells under TNF-α stimulated conditions, the present work examined the effect of the combined use of either nWPH or pWPH with fenretinide on IL-8 release from wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells under both basal and stimulated conditions. The effects of these combined treatments on apoptosis and cell viability was also examined in these two cell lines.

5.2 Materials and Methods

Materials

Wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) immortalized human tracheal epithelial cells were gifts obtained from Dr. D. Gruenert (University of California). The supplies for the maintenance of cell culture such as minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Burlington, Ontario, Canada). Trypsin-EDTA solution (0.25%) was obtained from Sigma-Aldrich Co (Oakville, Ontario, Canada). The solution used to coat the T-75 flasks and 24-well plates was prepared with collagen type I bovine, and human fibronectin obtained from BD Biosciences (Oakville, Ontario, Canada); bovine serum albumin (BSA), and Lechner & LaVeck (LHC) basal medium obtained from Biosource-Biofluids Division (Camarillo California, USA). Human recombinant TNF-α was obtained from BD Pharmigen (Oakville, Ontario Canada) and prepared with 0.1% BSA. To determine IL-8 release ELISA kits (Pharmigen, OptEIA Human IL-8 Set, catalog # 550999) were

obtained from BD Bioscience (Oakville, Ontario, Canada). Cell viability was determining by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma-Aldrich Co. The synthetic retinoid, fenretinide [*N*-(4-hydroxyphenyl) retinamide], was obtained from Sigma-Aldrich Co. InPro90® native whey proteins were obtained from Volac Nutrition, Inc. (England). The pressure treatment involved treatment of 15% (w/v) WPI solutions at 1 cycle of 550 MPa. Briefly, for the pressure treatment, a 15% solution (w/v) of WPI was prepared in tap water and placed into sealed plastic bags. Pressurization was performed using an ABB Cold Isostatic Press Model CIP42260 (Autoclave Engineers, Subsidiary of ABB Autoclave System, Columbus, OH). The temperature was kept at 26-27°C and after reaching 550 MPa (within 3-4 min) the pressure was released within 1 minute (1 cycle pressure treatment). Both native WPI (15% (w/v) solution) and pressurized WPI samples were lyophilized and subjected to dual enzyme pepsin-pancreatin digestion and ultrafiltration to generate peptides with a molecular weight cut-off < 1 kDa as described previously (Vilela *et al.*, 2005b).

Cells Culture

General Procedures

Cells were grown in pre-coated T-75 flasks in Eagle's minimum essential medium (MEM) containing 10% FBS and re-fed every 2-3 days until confluent. The confluent, adherent monolayers were then released from the plastic surface after treatment with polyvinyl-pirrolidone (PVP)-trypsin-EDTA and were seeded to 24-well plates or 60 mm dishes for 24 h before receiving the treatments.

Treatment with Fenretinide, Whey Peptides and TNF-α

Cells were treated with fenretinide at a low dose (1.25 μM) previously tested by our group to effectively decrease IL-8 release without affecting cell viability. This dose mimics the peak plasma levels reached in neuroblastoma patients receiving fenretinide

therapy for 28 days (Garaventa *et al.*, 2003). The whey peptide dose was chosen based on effective peptide doses (12.5 μg/mL), which demonstrated immune-modulating effects in response to relatively small doses of low molecular weight (< 1k Da) whey peptides in previous studies (Li *et al.* 1994; Mercier *et al.*, 2004). Wild-type and mutant ΔF508 CFTR cells seeded at 0.4 and 0.6 x 10^6 cells/mL in 24-well plates, respectively, were grown in MEM containing 10% FBS for 24 h until nearly confluence. The MEM was replaced with fresh medium containing 2% FBS and 1.25 μM of fenretinide (reconstituted in ethanol) alone or with 12.5 μg/mL of pWPH or nWPH reconstituted in nanopure water. The cells were allowed to grow for 24 h at 37°C in 5% CO_2 and after 24 h the medium was replaced with fresh MEM 2% FBS containing the same initial concentration of fenretinide and WPH in order to characterize the impact of fenretinide and/or WPH on IL-8 release in an unstimulated basal condition after an additional 24 h. To assess the effect of fenretinide and/or WPH on IL-8 production in a stimulated state, after the initial 24 h incubation with fenretinide and/or WPH, cells were treated with MEM 2% FBS containing 1.25 μM of fenretinide alone or with 12.5 μg/mL of pWPH or nWPH concurrently stimulated with human recombinant TNF-α (10 $ng \cdot mL^{-1}$) for additional 24 h. All experiments included unstimulated negative control wells and had vehicle ethanol controls.

IL-8 Release and Cell Viability Assays

After the treatment described above, the supernatant was collected to determine IL-8 released using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates were coated with capture antibody (anti-IL-8) overnight, washed with 0.05% Tween-20 in PBS and coated with phosphate buffer (PBS) 10% FBS in order to block non-specific binding. Known concentrations of IL-8 (standard) and the samples containing the IL-8 released by the cells after treatment (supernatant) were added as aliquots into appropriate wells, incubated for 2 h and decanted from the wells. Anti-IL-8 plus enzyme reagent (biotinylated detection antibody conjugated to Streptavidin-Horseradish) were added and incubated for one hour. After washing the plate, a solution was added which contained a substrate for the enzyme (TMB-peroxide

chromogen) present in the anti-IL-8 + enzyme reagent mixture and the plate was incubated for 30 min. The reaction was stopped using a 2 N H_2SO_4 solution and the absorbance was read at 450 nm using a Titertek II Multiscan MCCB40 (Labsystems, Finland). The optical densities were then used to calculate the IL-8 concentration from the standard curve and adjusted by their dilution factor.

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). Briefly, after collecting the supernatant to determine IL-8 release, the cells were gently washed with PBS, MTT solution (MTT, 0,5mg/mL culture medium free of phenol red) was added and the cells were incubated for 3 h at 37°C. After incubation, the supernatant was aspirated, HCl-isopropanol solution (0.04 N HCl in isopropanol) was added and after five min the optical densities were measured at 540 nm using a series 750 microplate reader from Cambridge Technology, Inc. (Cambridge, MA, USA). The O.D. values were converted into cell numbers by using a cell proliferation standard curve with a cell seeding concentration ranging from 0.4 to 1 x 10^6/mL cells (Loodsdrech *et al.*, 1994).

In Situ end Labelling of DNA Strand Breaks

Adherent epithelial cell lines grown on coated coverslips within 24 well plates for 24 h were treated with fenretinide and/or pWPH with the same concentrations used to determine IL-8 release as described above. At 24 h after the incubation with fenretinide and/or pWPH and 24 h before the experiment, the cells were stimulated with TNF-α (experimental group) or with cisplatin at 25 μM or 12.5 μM (as a positive control). After a 48 h period of cell growth exposed to fenretinide (1.25 and 5 μM) and/or WPH (12.5 μg/mL) (24 h after stimulation with TNF-α), cells were fixed using 4% paraformaldehyde in PBS at pH 7.4 for 1 h at room temperature, washed, and then made permeable using 0.1% Triton X-100 in 0.1% citrate buffer for 2 min on ice. After washing in PBS, the coverslips were individually removed from the 24 well plates and then cells were incubated with Terminal deoxynucleotidyl transferase (Tdt) enzyme conjugated to fluorescein (catalog #11684795910, Roche Molecular Biochemicals,

Indianopolis, IN) for 1 h at 37°C. A negative control slide was produced by incubating a slide of both cell lines with buffer minus the Tdt enzyme. The Tdt enzyme binds to the 3-OH free end of DNA strand breaks. Following this procedure, cells were washed twice with PBS. Coverslips were placed on slides using the VectaShield Anti Fade media (catalog # H-1000, Vector Laboratories Canada Inc., Burlington, Ontario). Slides were labelled individually with numbers to keep the observer blinded to the outcome until after the slides were reviewed. The slides were observed at 400 X (total magnification) using a fluorescent capable microscope (Axiophot Microscope, Zeiss, Germany) and photographs were taken using a camera RT slides and Spot RT Image Analysis System software (Diagnostic Instruments, Inc., MI, USA). Nuclei that appeared brightly fluorescent were considered positive for apoptosis.

Statistics

Data were expressed as mean ± standard error of the mean. The minimum number of replicate experiments for all measurements was at least three. Differences among groups were examined by analysis of variance (ANOVA). *Post hoc* LSD comparisons were used for comparing the effect of the treatments on cytokine release and Tukey's test was applied for comparing the effect of the treatments on cell numbers. SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois) was used in all statistical analyses. Group means were considered to be significant when $P < 0.05$.

5.3 Results and Discussion

IL-8 Production in Response to Fenretinide and Whey Protein Hydrolysates

Similarly to our previous studies (Vilela *et al.*, 2005a), unstimulated CF and non-CF and cells produced low basal levels of IL-8 release (Table 1). An analogous situation was observed in previous cell culture studies that indicate small or no differences in basal levels of inflammation in CF and non-CF epithelial cells (Schwiebert *et al.*, 1999; Becker *et al.*, 2004; Venkatakrishnan *et al.*, 2000). There was no impact of sham (ethanol)

vehicle on IL-8 release in either type of culture as also observed in our previous studies (data not shown).

Non-CF cells were more sensitive to TNF-α (10 ng·mL^{-1}) induction of IL-8 release than CF cells as compared to the unstimulated MEM controls (Table 1). These results are also in accordance to our previous studies (Vilela *et al.*, 2005a) and Massengale *et al.* (1999), who have demonstrated that non-CF cells are more sensitive to mediators of inflammation such as LPS. However, a consistent pattern of differences in the secretion of IL-8 between CF and non-CF epithelial under TNF-α is not always present and can differ depending on the cell lines used (Bedard *et al.*, 1993; Schwiebert *et al.*, 1999).

Under basal conditions, fenretinide, nWPH and pWPH and the combination of fenretinide and either nWPH or pWPH increased significantly the IL-8 release ($P < 0.05$) in CF cells as compared to untreated MEM control (Table 1, Fig. 1). In comparison to the significant ($P < 0.05$) 114-fold increase in IL-8 production (19,009 ± 94 pg·mL^{-1}) following TNF-α stimulation, fenretinide (258 ± 31 pg·mL^{-1}) alone and the combination of fenretinide with either nWPH (266 ± 20 pg·mL^{-1}) or pWPH (272 ± 34 pg·mL^{-1}) were associated with only a minimal increase in IL-8 release ranging from 1.55 to 1.76-fold as compared to basal unstimulated control (166.66± 21.5 pg·mL^{-1}). A similar situation was observed for the non-CF cells that showed a significant increase in IL-8 release as compared to the control when treated with either fenretinide or the combination of fenretinide with nWPH or pWPH under basal conditions (Table 1, Fig. 1); however, as also shown in our previous work (Vilela *et al.*, 2005b), neither nWPH nor pWPH treatments alone affected IL-8 release of non-CF cells under basal conditions (Table 1). As seen in the CF cells, the wild-type cells also showed a minimal increase in IL-8 release with fenretinide (415 ± 18 pg·mL^{-1}) (Table 1) and the combination of fenretinide plus native or pressurized WPH (i.e., 1.4 to 1.8-fold) (Fig. 1) when compared with the 271-fold increase in IL-8 production following TNF-α stimulation (78,202 ± 9,940 pg·mL^{-1}) (Table 1).

As observed in our previous work (Vilela *et al.*, 2005a), fenretinide treatment was associated with a significant ($P < 0.05$) decrease in IL-8 release in CF cells under the TNF-α stimulated condition (Table 1). Interestingly, the treatment of CF cells with nWPH, pWPH as well as the combination of the whey peptide treatments with fenretinide all showed a similar downward trend in IL-8 release under TNF-α stimulation (Table 1, Fig 2). Strong trends of lower IL-8 release in the mutant CFTR cells were observed in association with the nWPH ($P = 0.08$) and pWPH ($P = 0.13$) treatments.

Cell Viability and Apoptosis

The cell viability of both CFTR mutant and wild-type cell lines was unaffected either in basal or in TNF-α stimulated conditions by fenretinide (1.25 μM) when assessed using MTT assay (data not shown). As observed in our previous study, however, the higher fenretinide dose of 2.5 μM was shown to affect the viability of non-CF cells under both basal and TNF-α stimulated conditions, which was also associated with the high levels of apoptosis (Fig. 3).

As shown by negative fluorescent *in situ* end labeling DNA (Fig. 4), CF cells were resistant to the apoptosis that was seen in the wild-type cells exposed to fenretinide treatment (1.25 and 2.5 μM) under both basal and TNF-α stimulated conditions (Fig. 3). The resistance to apoptosis of the mutant CFTR epithelial cells was also unaltered by treatment of the peptides isolated from either nWPH or pWPH or the combination of fenretinide and the WPH treatments (Fig. 4). These results are consistent with our previous study whereby CF cells were shown to be resistant to apoptosis when treated with fenretinide at concentrations of 1.25 and 2.5 μM (Vilela *et al.*, 2005a). Other studies have shown that not all cell types are sensitive to the apoptotic effects of fenretinide (Chen *et al.*, 1999). A metabolic characteristic associated with cells with the CFTR mutation that could be related to the resistance to apoptosis could involve an overexpression of the antiapoptotic protein Bcl-2 (Harris *et al.*, 2005). In addition, a high constitutive content of intracellular GSH as demonstrated in lung epithelial mutant CFTR cells in our previous work (Vilela *et al.*, 2005a) and in HeLa cells with CFTR ΔF508

mutation by Jungas *et al.* (2002) could play an anti-apoptotic role. Jungas *et al.* (2002) demonstrated that HeLa cells with the CFTR ΔF508 mutation were resistant to apoptosis induced by hydroperoxide and depended on chemical-induced GSH depletion to allow for the proapoptotic effects of Bax protein activation. Likewise, the LY-ar mouse lymphoma cell line that expresses higher amounts of Bcl-2 protein, showed an increased sensitivity to apoptosis induced by radiation following depletion of the intracellular thiol pool (Mirkovic *et al.*, 1997).

Fenretinide has been demonstrated to promote apoptosis via hydroperoxide production followed by mitochondrial permeability transition, caspase activity, and DNA fragmentation in human cutaneous squamous carcinoma cells (Hail and Lotan, 2001). Based on the above, it is plausible that the relatively high intracellular GSH content of CF cells could have mediated their apoptotic resistance to fenretinide. We previously postulated that high levels of GSH in CFTR-deficient cells could contribute to the CF cells resistance to apoptosis through inhibition of sphingomyelinase (SMase) and that fenretinide would by-pass this resistance through increased *de novo* synthesis of ceramide, which is pro-apoptotic (Vilela *et al.*, 2005a). Erdreich-Epstein *et al.* (2002) have demonstrated that fenretinide can promote an increase of *de novo* synthesis of ceramide in human brain microvascular endothelial cells. In our previous work, however, despite fenretinide-mediated increase in intracellular ceramide content at the higher dose of 2.5 μM, the resistance of CF cells to fenretinide-induced apoptosis persisted suggesting that ceramide generation is ineffective in stimulating apoptosis in CF cells (Vilela *et al.*, 2005a).

Non-CF cells underwent apoptosis in response to fenretinide under both basal and TNF-α stimulated conditions as shown by positive fluorescent *in situ* end labeling DNA revealing strand breaks with classic peripheral nuclear condensation of chromatin (Fig. 3). The proapoptotic effect of fenretinide has been demonstrated in cell culture studies involving cancer cells and has been related to ceramide synthesis (Ferrari *et al.*, 2003; Wu *et al.*, 2001). In our previous study, however, the dose of 1.25 μM of fenretinide, which stimulated apoptosis, was not associated with an increase in intracellular levels of

ceramide, suggesting that other mechanisms are also involved in pro-apoptotic effects induced by fenretinide in wild-type cells. Recently, Lovat *et al.* (2004) have shown in neuroblastoma cells that both *de novo* synthesis and SMase pathways are activated in response to the pro-apoptotic effects of fenretinide and that both these pathways are related to different cell death-signaling mechanisms.

In the case of the *de novo* ceramide synthesis, serine palmitoyltransferase and ceramide synthase are key elements to synthesize ceramide without the involvement of SMase and that there is also the formation of ceramide-derived gangliosides. Gangliosides induce the generation of reactive oxygens species (ROS) via activation of 12-lipoxygenase and thus promote apoptosis (Lovat *et al.*, 2004). Moroever, under extracellular stimulus of fenretinide, SMase generates sphingomyelin (SM), which is subsequently cleaved by SM hydrolase into ceramide and phosphocholine (Lovat *et al.*, 2004). The subsequent increase in ceramide content in cell membranes is crucial for the formation of lipid rafts and efficient activation of the apoptosis initiator caspase-8 through the death-inducing signalling complex (DISC) (Miyaji, 2005). Hence, the high constitutive levels of GSH of CF cells could limit the pro-apoptotic effects of fenretinide via effects that occur both upstream and downstream of ceramide synthesis. Specifically, high concentrations of GSH are known to: (a) limit SMase activity (Liu and Hannun, 1997; Liu *et al.*, 1998) that is upstream of ceramide synthesis; and (b), via antioxidant effects to limit ROS-mediated apoptosis (Tsyupko *et al.*, 2001) that occurs downstream of ceramide synthesis.

Interestingly, under basal conditions, pWPH treatment stimulated apoptosis in non-CF cells as opposed to nWPH that did not differ from the control (MEM 2% FBS) (Fig. 3). This result is likely related to the different profiles of low molecular weight peptides isolated from pWPH as compared to nWPH (Vilela *et al.*, 2005b) and demonstrates that the peptides generated from the hydrolysates of pressurized and native whey proteins differ in their effects on cellular functions. Although TNF-α treatment was associated with a slight increase in apoptosis in non-CF cells, no signs of apoptosis were presented in these cells under TNF-α stimulated conditions after treatment with whey peptides isolated from either nWPH or pWPH (Fig. 3). Similarly, the nWPH and pWPH

treatments protected the cells from apoptosis induced by fenretinide at the two doses tested (1.25 and 2.5 μM) under both stimulated and non-stimulated conditions. Chemical enhancement of cellular GSH content has been shown to protect against TNF-α stimulated apoptosis in MCF-7 cells (Liu *et al.*, 1998); however, this mechanism is unlikely since the nWPH and pWPH treatments did not affect the intracellular GSH content of wild-type cells under basal conditions in our previous work (Vilela *et al.*, 2005b). The possibility, however, that the WPH treatments under inflammatory conditions could stimulate an increase in intracellular GSH content cannot be completely ruled out.

It is possible that mechanisms other than GSH modulation that could play a role in the whey peptide inhibition of fenretinide mediated apoptosis in normal lung epithelial cells. For example, peptides could act as pseudosubstrates for executioner caspases thereby interfering with the apoptosis that occurs downstream of ceramide synthesis. The cleavage sites of the caspases are usually at an Asp-X bond and, based on the recognition sites of substrates, various peptide sequences have been developed as tools for studies of caspases family members and apoptosis (Degterev *et al.*, 2003; Kidd, 1998). In this regard, it is possible that some peptides used in our study could have a sequence that mimics cleavage sites of the caspases. Further studies are needed to assess the possibility that peptides isolated from WPH could act as caspase inhibitors and/or activators as possible explanations for their observed modulation of apoptosis.

As CF cells did not undergo apoptosis or any changes in cell viability following fenretinide treatment, no relationship was observed between apoptosis and decreased in IL-8 release indicating that cell death was not a mechanism of the cytokine inhibition. Also, although ceramide has also been demonstrated to inhibit NF-κB activation (Signorelli *et al.*, 2001), no relationship was observed in our previous studies between cellular ceramide content and decreased IL-8 release (Vilela *et al.*, 2005a). In support of these observations, fenretinide has been shown to directly increase expression of the IκBα-NF-κB inhibitor in human prostate cancer cell lines (Shimada *et al.*, 2002), which

suggests that fenretinide can decrease IL-8 secretion without involving ceramide synthesis and apoptosis.

In our previous work, we postulated that a trend for increased GSH/GSSG ratios associated with the WPH treatments could have played a role in the tendency of IL-8 release to be decreased with WPH treatment (Vilela *et al.*, 2005b). In that regard, the use of the GSH precursor drug, N-acetylcysteine (NAC), has been demonstrated to improve lung inflammatory indices including decreased sputum IL-8 (van Overveldi *et al.*, 2005). Alternatively, or in addition, the inhibition of IL-8 release associated with the WPH treatments could be related to mechanisms involving peptides acting as caspase inhibitors, specifically via inhibition of caspase-8 activation. In studies with human astroglioma cells, IL-8 expression was shown to be dependent on caspase-8 activation of NF-κB transcriptional activity under TNF-α stimulation (Choi *et al.*, 2002).

Despite favourable effects on IL-8 release via treatment with either fenretinide or WPH, the combined treatment of CFTR-deficient cells with either nWPH or pWPH together with fenretinide showed no additional benefit in terms of suppression of IL-8 release. Thus, no enhancement in the fenretinide-mediated suppression of IL-8 secretion was observed when this treatment was combined with either nWPH ($P = 0.05$) or with pWPH ($P = 0.12$). It is conceivable, however, that WPH might provide antioxidant protection in the extracellular environment mediated by induction of GSH. This latter action could thus provide protection to the lung epithelial cells within the oxidant environment associated with inflammation. Hence, further studies are necessary to establish whether WPH could thus provide additional benefit to the effects of fenretinide on IL-8 release and neutrophil influx in the airway surface under stimulated conditions in CF.

In conclusion, the present study suggests a possible benefit for CF via the combined treatment of fenretinide and whey peptides as normal epithelial cells are protected from the apoptosis induced by fenretinide and the fenretinide-mediated decrease in IL-8 release from CF cells stimulated with TNF-α is not adversely affected. Hence, the combination of fenretinide and whey peptides is an interesting candidate as a potential

primary treatment to ameliorate acute lung inflammation in CF, which is a primary cause of mortality and morbidity in CF. Fenretinide has been shown low toxicity in a variety of clinical studies (Ulukaya and Wood, 1999) and no side effects have been reported with the use of WPI.

The present studies represent an *in vitro* environment and conclusions about CF pathogenesis should be made with caution as results were obtained using only wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells. More studies are needed to explore the effects of fenretinide and whey peptides on CF using different doses of both compounds and diverse experimental models such as different cells lines from the entire spectrum of cells in the human respiratory tract and in vivo studies with animal models of CF. Further work is now required to determine whether the observations found in this study can be translated into a clinically meaningful effect in CF patients.

Acknowledgments

The authors thank Jiun-Ni Liu for her technical assistance and help. The first author was sponsored scholarships by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Process n. 200026/93-3 (RN), Brasília, D.F.-Brazil; Federal University of Paraná, and Tuiuti University of Paraná, Curitiba, Paraná, Brazil. The research was funded via grants to SK and LL by the National Sciences and Engineering Council of Canada (NSERC) and the Canadian CF Foundation.

References

Bals R., Weiner D. J., Wilson J. M. 1999. The innate immune system in cystic fibrosis lung disease. *The Journal of Clinical Investigation*. 103: 303-7.

Becker M. N., Sauer M. S., Muhlebach M. S., Hirsh A. J., Wu Q, Verghese M. W., Randell S. H. 2004. Cytokine secretion by cystic fibrosis airway epithelial cells. *American Journal Respiratory and Critical Care Medicine.* 169: 645-53.

Bedard M., McClure C. D., Schiller N. L., Francoeur C., Cantin A., Denis M. 1993. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology.* 9: 455-462.

Boucher R. C. 2004. Relationship of airway epithelial ion transport to chronic bronchitis. *Proceedings of the American Thoracic Society.* 1: 66-70.

Boujaoude L. C., Bradshaw-Wilder C., Mao C., Cohn J., Ogretmen B., Hannun Y. A., Obeid L. M. 2001. Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid. Modulation of cellular activity of sphingosine 1-phosphate. *Journal of Biological Chemistry.* 276: 35258-35264.

Bounous G., Gold F. 1991. The biological activity of undernatured dietary whey proteins: role of glutathione. *Clinical and Investigatigative Medicine.*14: 296-309.

Bush A. 2005. How has research in the last five years changed my clinical practice? *Archives of Disease in Childhood.* 90: 832-836.

Chen Y., Zhou G., Tan Tse-Hua. 1999. c-Jun N-Terminal Kinase Mediates Apoptotic Signaling Induced by *N*-(4-Hydroxyphenyl) retinamide. *Molecular Pharmacology.* 56: 1271-1279.

Choi C., Kutsch O., Park J., Zhou T., Seol D. W, Benveniste E. N. 2002. Tumor necrosis factor-related apoptosis-inducing ligant induces caspase-dependent interleukin-8 expression and apoptosis in human astroglioma cells. *Molecular and Cell Biology.* 22: 724-736.

Conese M., Copreni E., Di Gioia S., De Rinaldis P., Fumarulo R. 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *Journal of Cystic Fibrosis.* 2: 129-135.

Conway S. P., Brownlee K. G., Denton M., Peckham D. G. 2003. Antibiotic treatment of multiresistant organisms in cystic fibrosis. *American Journal of Respiratory Medicine.* 2: 321-332.

Cross M. L., Gill H. S. 1999. Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology.* 77: 345-350.

Cuello M., Coats A. O., Darko I., Ettenberg S. A., Gardner G. J., Nau M. M., Liu J. R., Birrer M. J., Lipkowitz S. 2004. *N*-(4-hydroxyphenyl)retinamide (4HPR) enhances TRAIL-mediated apoptosis through enhancement of a mitochondrial-dependent amplification loop in ovarian cancer cell lines. *Cell Death and Differentiation.* 11: 527-541.

Degterev A., Boyce M., Yuan J. 2003. A decade of caspases. *Oncogene.* 22: 8543-8567.

Erdreich-Epstein A., Tran L. B., Bowman N. N., Wang H., Cabot M. C., Durden D. L., Vlckova J., Reynolds C. P., Stins, M. F., Groshen S., Millard M. 2002. Ceramide in fenretinide-induced endothelial cell apoptosis. *The Journal of Biological Chemistry.* 277: 49531-49537.

Ferrari N., Morini M., Pfeffer U., Minghelli S., Noonan D. M., Albini A. 2003. Inhibition of Kaposi's sarcoma in vivo by fenretinide. *Clinical Cancer Research.* 9: 6020-6029.

Ford J. T., Wong C. W., Colditz I. G. 2001. Effects of dietary protein types on immune reponses and levels of onfection with *Eimeria vermiformis* in mice. *Immunology and Cell Biology.* 79: 23-28.

Garaventa A., Luksch R., Lo Piccolo M. S., Cavadini E., Montaldo P. G., Pizzitola M. R., Boni L., Ponzoni M., Decensi A., De Bernardi B., Bellani F. F., Formelli F. 2003. Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. *Clinical Cancer Research.* 9: 2032-2039.

Grey V., Mohammed S. R., Smountas A. A., Bahlool R., Lands L. C. 2003. Improved glutathione status in young adult patients with cystic fibrosis supplemented with whey proteins. *Journal of Cystic Fibrosis*. 2: 195-198.

Hail N. Jr., Lotan R. 2001. Mitochondrial respiration is uniquely associated with prooxidant apoptotic effects of N-(4-hydroxyphenyl) retinamide. *The Journal of Biological Chemistry*. 276: 4514-45621.

Han E. E., Beringer P. M., Louie S. G., Gill M. A., Shapiro B. J. 2004. Pharmacokinetics of ibuprofen in children with cystic fibrosis. *Clinical Pharmacokinetics*. 43: 145-156.

Harris J. F., Fisher M. J., Hotchkiss, J. R., Monia B. P., Randell, S. H., Harkema J. R., Tesfaigzi Y. 2005. Bcl-2 sustains increased mucuous and epithelial cell numbers in metaplasic airway epithelium. *American Journal Respiratory and Critical Care Medi*cine. 171: 764-772.

Hudson V. M. 2001. Rethinking cystic fibrosis pathology: the critical role of abnormal reduced glutathione (GSH) transport caused by CFTR mutation. *Free Radical Biology and Medicine*. 30: 1440-61.

Jungas T., Motta I., Duffieux F., Fanen P., Stoven V., Ojcius D.M. 2002. Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry Journal of Biological Chemistry*. 277: 27912-27918.

Kennedy R. S., Konok G. P., Bounous G., Baruchel S., Lee T. D. G. 1995. The use of whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I-II clinical study. *Anticancer Research*. 15: 2643-2650.

Khan T. Z., Wagener J. S., Bost T., Martinez J., Accurso F. J., Riches D. W. H. 1995. Early pulmonary inflammation in infants with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*. 151: 1075-1082.

Kidd V. J. Proteolytic activities that mediate apoptosis. 1998. *Annual Reviews in Physiology*. 60: 533-573.

Koehler D. R., Downey G. P., Sweezey N. B., Tanswell A. K., Hu J. 2004. Lung inflammation as a therapeutic target in cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology*. 31: 377-81.

Kolesnic R. 2002. The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *The Journal of Clinical Investigation*. 110: 3-8.

Knowles M. R., Boucher R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways ("Perspective"). *The Journal of Clinical Investigation*. 109: 571-577.

Konstas A. A., Koch J. P., Korbmacher C. 2003. cAMP-dependent activation of CFTR inhibits the epithelial sodium channel (ENaC) without affecting surface expression. *European Journal of Physiology*. 445: 513-521.

Kreda S. M., Mall M., Mengos A., Rochelle L., Yankaskas J., Riordan J. R., Boucher R. C. 2005. Characterization of wild-type and ΔF508 cystic fibrosis transmembrane regulator in human respiratory epithelia. *Molecular Biology of the Cell*. 16: 2154-2167.

Kunzelmann K., Mall M. 2001. Pharmacotherapy of the ion transport defect in cystic fibrosis. *Clinical and Experimental Pharmacology and Physiology.* 28: 857-867.

Lavrentiadou S. N., Chan C., Kawcak T., Ravid T., Tsaba A. van der Vliet A., Rasooly R., Goldkorn T. 2001. Ceramide-mediated apoptosis in lung epithelial cells is regulated by glutathione. *American Journal of Respiratory Cell and Molecular Biology.* 25: 676-684.

Lee T. W. R., Mattews D. A., Blair E. 2005. Novel molecular approaches to cystic fibrosis gene therapy. *The Biochemical Journal.* 387: 1-15.

Li L., Clark K., Lau B. H. S. 1994. Thymic peptides increase glutathione level and glutathione disulfide reductase activity in vascular endothelial cells. *Biotechnology Therapeutics.* 6: 87- 97.

Liu B., Andrieu-Abadie N., Levade T., Zhang P., Obeid L. M., Hannun Y. A. 1998. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. *Journal of Biological Chemistry.* 273: 11313-11320.

Loosdrecht van de A. A., Beelen, R. H. J., Ossenkoppele G. J., Broekhoven M. G., Langenhuijsen, M. M. A. C. 1994. A tetrazolium-based mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of Immunological Methods.* 174: 311-320.

Lovat P. E., Di Sano F., Corazzari M., Fazi B., Donnorso R. P., Pearson A. D. J., Hall A. G., Redfern C. P. F., Piacentini M. 2004. Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. *Journal of the National Cancer Institute.* 96: 1288-1299.

Low P. P. L., Rutherfurd K. J., Gill H. S., Cross M. L. 2003. Effect of dietary whey protein concentrate on primary and secondary antibody responses in immunized BALB/c mice. *International Immunopharmacology*. 3: 393-401.

Massengale A. R., Quinn F. Jr, Yankaskas J., Weissman D., McClellan W. T., Cuff C., Aronoff S. C. 1999. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *American Journal of Respiratory Cella nd Molecular Biology.* 20: 1073-1080.

Mercier A., Sylvie F. G., Fliss, I. 2004. Immunomodulating effects of whey proteins and their enzymatic digests. *International Dairy Journal.* 14: 175-183

Micke P., Beeh K. M., Buhl R. 2002. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. *European Journal of Nutrition*. 41: 12-18.

Micke P., Beeh K. M., Schlaak J. F., Buhl R. 2001. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *European Journal of Clinical Investigation*. 31: 171-178.

Mirkovic N., Voehringer D. W., Story M. D., McConkey D. J., McDonnell T. J., Meyn R. E. 1997. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene.* 15: 1461-1470.

Miyaji M., Jin Z. X., Yamaoka S., Amakawa R., Fukuhara S., Sato S. B., Kobayashi T., Domae T. M., Bloom E. T., Okazaki T., Umehara H. 2005. Role of membrane sphingomyelin and ceramide in platform formation for Fas-mediated apoptosis. *Journal of Internal Medicine*. 202: 249-259.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods.* 65: 55-63.

Nelson T. 2005. Cystic fibrosis: an overview. *Journal of Clinical Gastroenterology.* 39: 307-317.

Parnham M. J. 2005. Immunomodulatory effects of antimicrobials in the therapy of respiratory tract infections. *Current Opinion in Infectious Disease.* 18: 125-131.

Penttila I. A., Zhang M. F., Bates E., Regester G., Read L. C., Zola H. 2001. Immune modulation in suckling rat pups by a growth factor extract derived from milk whey. *Journal of Dairy Research.* 68: 587- 599.

Poot M., Hosier S., Swisshelm K. 2002. Distinct patterns of mitochondrial changes precede induction of apoptosis by all-trans-retinoic acid and *N*-(4-hydroxyphenyl) retinamide in MCF7 breast cancer cells. *Experimental Cell Research.* 279: 128-140.

Riordan R. J., Rommens J. M., Kerem B. S., Alon N., Rozmahel R., Grzelcsak Z., Zielenski J., Lok S., Plavsic N., Chou J. L., Drumm M. L., Iannuzzi M. C., Collins F. S., Tsui L. C.1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 245: 1066-1073.

Rodgers H. C., Knox A. J. 2001. Pharmacological treatment of the biochemical defect in cystic fibrosis airways. *European Respiratory Journal.* 17: 1314-1321.

Schwiebert L. M., Estell K., Propst S. M. 1999. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *American Journal of Physiology.* 276: C700–C710.

Shimada K., Nakamura M., Ishida E., Kishi M., Yonehara S., Konishi N. 2002. Contributions of mitogen-activated protein kinase and nuclear factor kappa B to *N*-(4-hydroxypheniyl)retinamide-induced apoptosis in prostate cancer cells. *Molecular Carcinogenesis.* 35: 127-137.

Stecenko A. A., King G., Torii K., Breyer R. M., Dworski R., Blackwell T. S., Christman J. W., Brigham K. L. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation.* 25: 145-155.

Tsyupko A. N., Dudnik L. B., Evstigneeva R. P., Alessenko A. V. 2001. Effects of reduced and oxidized glutathione on sphingomyelinase activity and contents of sphingomyelin and lipid peroxidation products in murine liver. *Biochemistry (Moscow).* 66: 1028-1034.

Ulukaya E., Wood E. J. 1999. Fenretinide and its relation to cancer. *Cancer Treatment Reviews.* 25: 229-35.

van Overveldi F. J. Demkow U., Góreck D., de Backer W. A., Zielinski J. 2005. New developments in the treatment of COPD: comparing the effects of inhaled corticosteroids and n-acetylcysteine. *Journal of Physiology and Pharmacology.* 56(Supp 4): 135-142.

Vilela R. M., Lands C. L., Meehan B., Kubow S. 2005a. Inhibition of the inflammatory response of CFTR-deficient lung epithelial cells following pre-treatment with fenretinide. Submitted.

Vilela R. M., Lands C. L., Azadi B., Kubow S. 2005b. The impact of high hydrostatic pressure on the digestibility and production of bioactive peptides from whey protein isolates. In preparation.

Venkatakrishman A., Stecenko A. A., King G., Blackwell T. R., Brigham K. L., Christman J. W., Blackwell T. S. 2000. Exaggerated activation of nuclear factor-κB and altered IκB-β processing in cystic fibrosis bronchial epithelial cells. *Respiratory Cell and Molecular Biology.* 23: 1-16.

van der Vliet A., Eiserich J. P., Marelich G. P., Halliwell B., Cross C. E. Oxidative stress in Cystic Fibrosis: Does it occur and does it matter? In: Antioxidants in Disease Mechanisms and Therapy. 1997. *Advances in Pharmacology.* 38: 491-513.

Wang F., Zeltwanger S., Yang I. C. H. Nairin A. C. 1998. Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. *The Journal of General Physiology.* 111: 477-490.

Webber M. M., Bello-Deocampo D., Quader S., Deocampo N. D., Metcalfe W. S., Sharp, R. M. 1999. Modulation of the malignant phenotype of human prostate cancer cells by *N*-(4-hydroxyphenyl)retinamide (4HPR). *Clinical and Experimental Metastasis.* 17: 255-63.

Wu J. M., DiPietrantonio A. M., Hsieh T.-C. 2001. Mechanisms of fenretinide (4-HPR)-induced cell death. *Apoptosis.* 6: 377-388.

Zhang L., Parente J., Harris S. M., Woods D. E., Hancock R. E., Falla T. J. 2005. Antimicrobial peptide therapeutics for cystic fibrosis. 49: 2921-2927.

Zhu S., Yue G., Shoemaker R. L., Matalon S. 1996. Adult alveolar type II cells lack cAMP and Ca^{2+}- activated Cl^- channels. *Biochemical and Biophysical Research Communications.* 218: 302-308.

Table 1. Effect of WPH and fenretinide on IL-8 release from wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells under basal and TNF-α stimulated condition

	IL-8 release ($pg \cdot mL^{-1}$)	
Treatment	**Non-CF cells**	**CF cells**
Basal unstimulated condition (MEM 2% FBS)	287 ± 43^{a}	166 ± 21^{a}
nWPH treated under unstimulated condition	268 ± 16^{a}	266 ± 20^{b}
pWPH treated under unstimulated condition	$329 \pm 7^{a,b}$	272 ± 34^{b}
Fenretinide treated under unstimulated condition	415 ± 18^{b}	258 ± 31^{b}
TNF-α stimulated condition (10 $ng \cdot mL^{-1}$)	$78,202 \pm 9,940^{a,b}$	$19,009 \pm 943^{a}$
nWPH treated under stimulated condition	$62,026 \pm 4,122^{a}$	$15,152 \pm 1,451^{a}$
pWPH treated under stimulated condition	$70,992 \pm 3,357^{a,b}$	$15,643.9 \pm 1,045^{a}$
Fenretinide treated under stimulated condition	$83,799 \pm 7,986^{b}$	$14,432.6 \pm 1,189^{b}$

Results are means ± S.E. of 3 independent experiments. Basal and stimulated conditons were analyzed separately whitin each cell line and numbers not sharing common letters represent means that differed significantly ($P < 0.05$; ANOVA) by LSD's *post hoc* comparison.

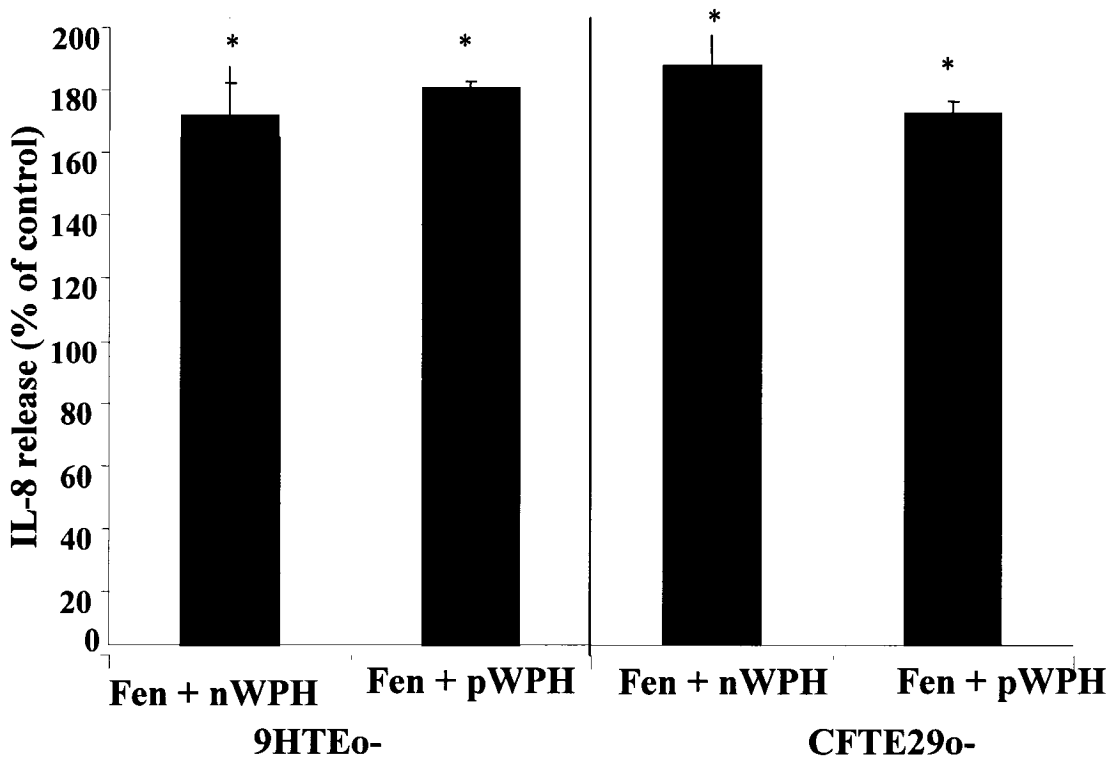


Figure 1. Effect of concomitant fenretinide and WPH treatment on IL-8 release from CF and non-CF cells under unstimulated condition. CFTE29o- and 9HTEo- were incubated in a MEM containing 10% FBS for 24 h. After 24 h the medium was replaced by MEM containing 2% FBS and 1.25 μM of fenretinide and/or 12.5 μg/mL of pWPH or nWPH for additional 24 h. After 24 h, the medium was replaced by MEM 2% FBS and the cells were incubated with the same concentrations of fenretinide and whey peptides and stimulated with human recombinant TNF-α for another 24 h. The supernatant was collected to determine IL-8 release.Vehicle contained ethanol (EtOH) at concentrations equivalent to those used with fenretinide treatments (data not shown). Results are means ± S.E. of 3 independent experiments. For 9HTEo- cells 100% corresponds to IL-8 released by untreated control cells (MEM 2% FBS); 287 ± 43 $pg \cdot mL^{-1}$. For CFTE29o- cells 100% corresponds to IL-8 released by untreated control cells (MEM 2% FBS); 166 ± 21 $pg \cdot mL^{-1}$. Asterisks (*) indicate significant differences ($P < 0.05$) as compared to untreated controls by ANOVA.

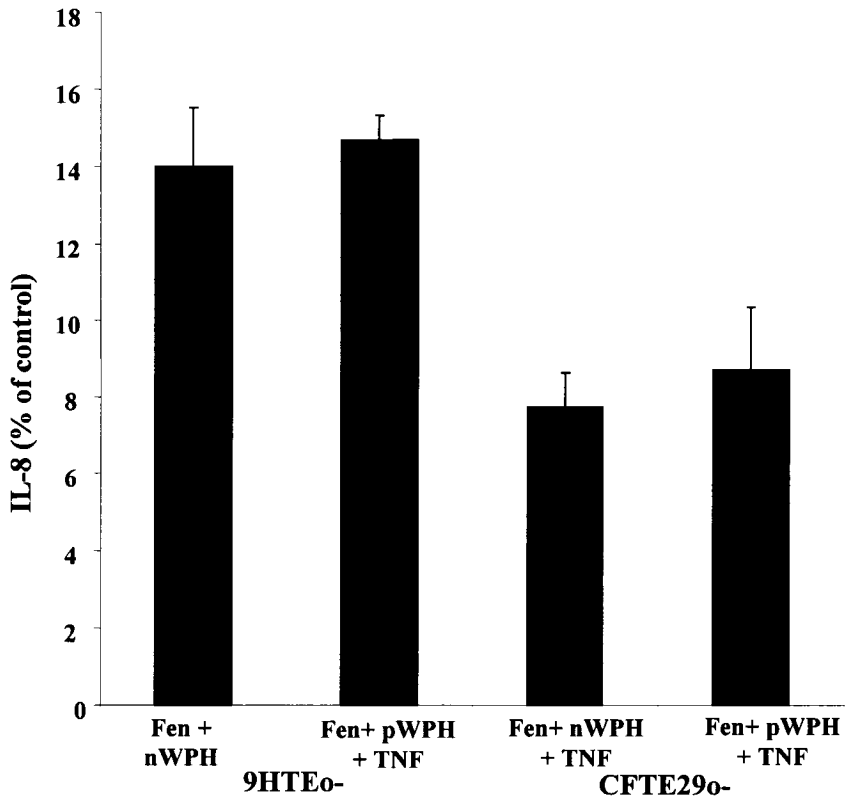


Figure 2. Effect of concomitant fenretinide and WPH treatments on IL-8 release from CF and non-CF cells under TNF stimulated condition. CFTE29o- and 9HTEo- were incubated in MEM containing 10% FBS for 24 h. After 24 h the medium was replaced by MEM containing 2% FBS and 1.25 μM of fenretinide and/or 12.5 μg/mL of pWPH or nWPH for additional 24 h (pre-incubation). After 24 h, the medium was replaced by MEM 2% FBS and the cells were incubated with the same concentrations of fenretinide and whey peptides and stimulated with human recombinant TNF-α (10 $ng.mL^{-1}$) for another 24 h. The supernatant was collected to determine IL-8 release. Vehicle contained ethanol (EtOH) at concentrations equivalent to those used with fenretinide treatments (data not shown). Results are means ± S.E. of 3 independent experiments. For 9HTEo- cells 100% corresponds to IL-8 released by untreated control cells (MEM 2% FBS + TNF-α); 78,202 ± 9,940 $pg{\cdot}mL^{-1}$. For CFTE29o- cells 100% corresponds to IL-8 released by untreated control cells (MEM 2% FBS + TNF-α); 19,009 ± 943 $pg{\cdot}mL^{-1}$.

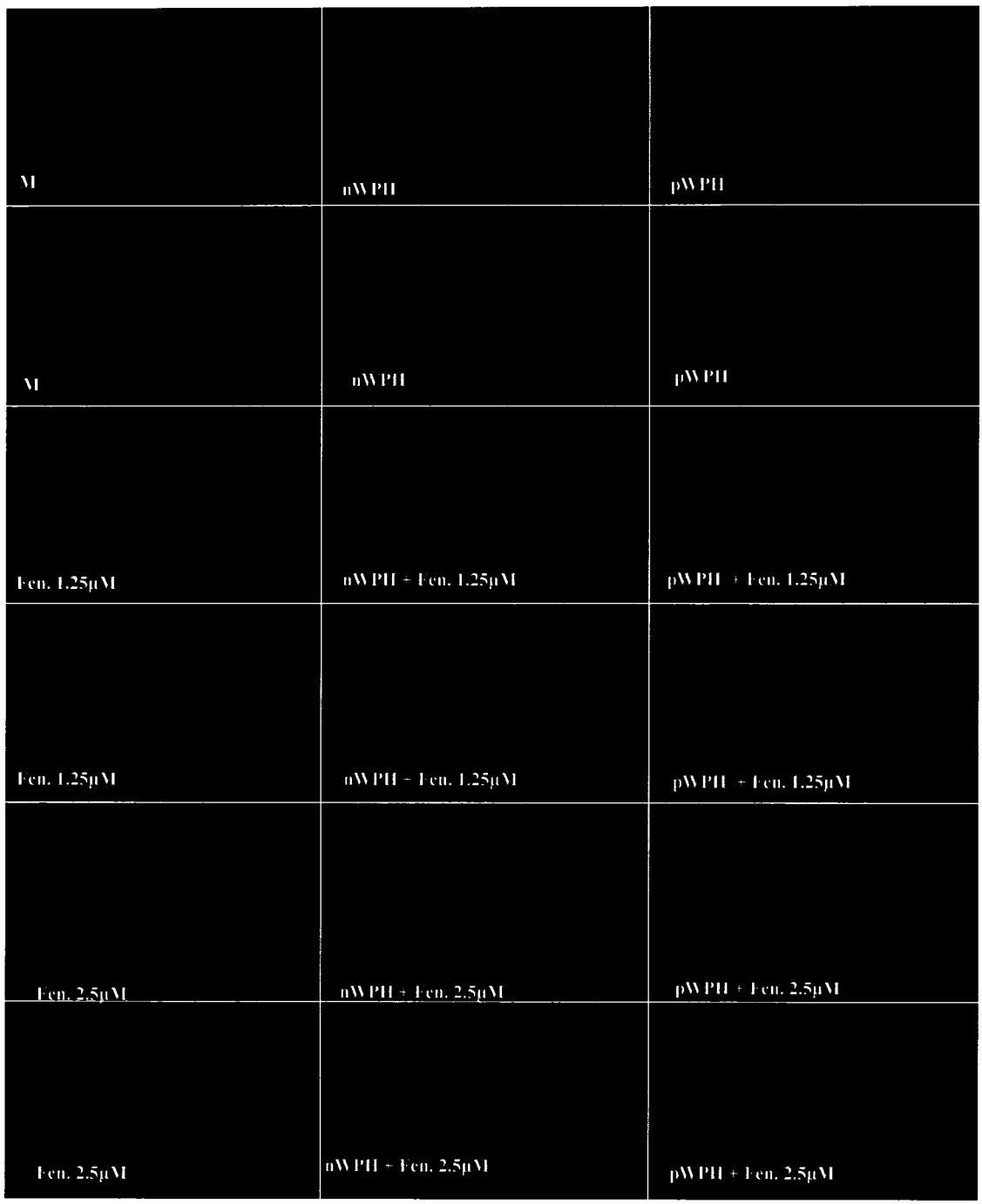

M
nWPH
pWPH
M
nWPH
pWPH
Fen. 1.25μM
nWPH + Fen. 1.25μM
pWPH + Fen. 1.25μM
Fen. 1.25μM
nWPH + Fen. 1.25μM
pWPH + Fen. 1.25μM
Fen. 2.5μM
nWPH + Fen. 2.5μM
pWPH + Fen. 2.5μM
Fen. 2.5μM
nWPH + Fen. 2.5μM
pWPH + Fen. 2.5μM

Figure 3. Images of 9HTEo- *in situ* end labelling of DNA strand breaks. Adherent normal human epithelial cell lines (9HTEo-) grown on coated coverslips within 24 well plates for 24 h were treated with fenretinide and/or WPH with the concentrations indicated in the pictures. At 24 h after the incubation the cells were stimulated with TNF-α (10 $ng.mL^{-1}$), experimental group, or with cisplatin at 25 μM or 12.5 μM as a positive control (data not shown). 24 h after stimulation, cells were fixed, washed, and then made permeable. The coverslips were individually removed from 24 well plates and then cells were incubated with Terminal deoxynucleotidyl transferase (Tdt) enzyme conjugated to fluorescein for 1 h at 37°C. A negative control slide was produced by incubating a slide with buffer minus the Tdt enzyme (data not shown). The coverslips were placed on slides, and the slides were labelled individually with numbers to keep the observer blinded to the outcome until after the slides were reviewed. Cells were observed by using a fluorescent capable microscope and the ones that appeared brightly fluorescent were considered positive for apoptosis. The total magnification used was 400 X. Pictures were taken and the images were edited by using Adobe Photoshop Album 2.0 (Microsoft). The labels in white indicate basal condition and the labels in yellow indicate TNF-α stimulated condition. nWPH and pWPH indicate native and pressurized whey protein hydrolysates (< 1 kDa) treatment respectively. Fen. = fenretinide treatment.

M	nWPH	pWPH
M	nWPH	pWPH
Fen. 1.25μM	nWPH + Fen. 1.25μM	pWPH + Fen. 1.25μM
Fen. 1.25μM	nWPH + Fen. 1.25μM	pWPH + Fen. 1.25μM
Fen. 2.5μM	nWPH + Fen. 2.5μM	pWPH + Fen. 2.5μM
Fen. 2.5μM	nWPH + Fen. 2.5μM	pWPH + Fen. 2.5μM

Figure 4. Images of CFTE29o- *in situ* end labelling of DNA strand breaks. Adherent mutant ΔF508 CFTR epithelial cells (CFTE29o-) grown on coated coverslips within 24 well plates for 24 h were treated with fenretinide and/or WPH with the concentrations indicated in the pictures. At 24 h after the incubation the cells were stimulated with TNF-α (10 $ng.mL^{-1}$), experimental group, or with cisplatin at 25 μM or 12.5 μM as a positive control (data not shown). 24 h after stimulation, cells were fixed, washed, and then made permeable. The coverslips were individually removed from 24 well plates and then cells were incubated with Terminal deoxynucleotidyl transferase (Tdt) enzyme conjugated to fluorescein for one h at 37°C. A negative control slide was produced by incubating a slide with buffer minus the Tdt enzyme (data not shown). The coverslips were placed on slides, and the slides were labelled individually with numbers to keep the observer blinded to the outcome until after the slides were reviewed. Cells were observed by using a fluorescent capable microscope and the ones that appeared brightly fluorescent were considered positive for apoptosis. The total magnification used was 400 X. Pictures were taken and the images were edited by using Adobe Photoshop Album 2.0 (Microsoft). The labels in white indicate basal condition and the labels in yellow indicate TNF-α stimulated condition. nWPH and pWPH indicate native and pressurized whey protein hydrolisates (< 1 kDa) treatment, respectively. Fen. = fenretinide treatment.

FINAL CONCLUSION AND SUMMARY

The elevated pulmonary morbidity and mortality due to the progressive damage of the lungs in response to a chronic inflammatory-immune state is the main concern of CF care. Although mutations of the CFTR channel have been identified as the cause of CF (Dinwiddie, 2000), the pathogenic role of the CFTR channel is still under investigation. Advances in pharmacotherapy have been achieved; however, the discovery of new aspects of the CFTR channel such as permeability to GSH (Lindsdell and Hanrahan, 1998; Kogan *et al.*, 2003) and S-1P (Boujaoude *et al.*, 2001) increases the complexity of the CF pathogenesis and the challenge of determining therapeutically narrowed drug targets. The drugs routinely used to manage CF airway inflammation such as antibiotics and anti-inflammatory agents are still lacking long term efficacy and effective CF treatment depends on advances in the knowledge of metabolic pathways that elicit the inflammatory response and tissue destruction during the course of this chronic fatal disease.

There are numerous unanswered questions regarding the metabolic pathways that might be correlated to the CFTR deficiency and chronic inflammation. Among the various metabolic defects ascribed to CF cells associated with pathogenesis include IL-8 release hyperresponsiveness to TNF-α (Stecenko *et al.*, 2001, Venkatakrishnan *et al.*, 2000), *P. aeruginosa* (Scheid *et al.*, 2001) and LPS (Kammouni *et al.*, 1997); increased activation of NF-κB (Venkatakrishnan *et al.*, 2000); and overexpression of the antiapoptotic protein Bcl-2 (Harris *et al.*, 2005). In addition, the airway environment has been shown to present thick mucus layers (Puchelle *et al.*, 2002), high influx of neutophils (Bals *et al.*, 1999) and ineffective antimicrobial peptide activity (Boucher, 2004). One of the objectives of the present thesis was to study GSH status, ceramide production and IL-8 release in relation to TNF-α stimulation in CFTR deficient cells. As described in Figure 1, inadequate CFTR GSH export (Gao *et al.*, 1999; Day *et al.*, 2004) could lead to low GSH content (Gao *et al.*, 1999) in the airway environment as well as impaired release of S-1P (Boujaoude *et al.*, 2001). These latter events have been suggested to play a pro-inflammatory role in CF lung disease. We have hypothesized in this thesis that

inadequate export of GSH via the mutant CFTR could lead to high basal intracellular concentrations of GSH and resistance to apoptosis. This latter suggestion was demonstrated previously by Jungas *et al.* (2002) in HeLa cells with the CFTR mutation. The studies described herein have demonstrated several-fold higher GSH content relative to wild-type cells also occurs in mutant CFTR lung epithelial cell lines although such high GSH content in mutant CFTR lung epithelial cell lines has not previously been observed (Gao *et al.*, 1999).

The system used to grow the cells in our study is similar to the cell culture system used by Jungas *et al.* (2002) regarding to the use of fetal bovine serum (FBS) to supplement the cell culture medium. The addition of FBS to the medium was considered as essential for our cell culture studies since some components, absent in serum free media, such as albumin, are important to avoid starvation of the cells. As albumin works as carrier of amino acids including disulfide-bonded cysteine and GSH (Groff and Gropper, 2000; Peters, 1996), it is conceivable that lack of albumin in the cell culture medium, as occurred in the Gao *et al.* (1999) study, could impair amino acid uptake and alter metabolism to a catabolic state to induce depressed intracellular levels of GSH in both wild-type and CF cells. In support of this contention, serum starvation in cell culture is typically associated with depressed intracellular GSH concentrations (Shaw and Chou, 1986; Kang and Enger, 1991). Thus, possible differences in intracellular GSH concentrations might be obviated under serum-free conditions.

Interestingly, in our study, despite the relatively high intracellular GSH content, the redox GSH/GSSG ratio that is sensitive marker of oxidative stress, was several-fold lower in CF cells, which is consistent with studies showing that the GSSG content of epithelial lining fluid (ELF) obtained from CF patients is a large proportion of the total GSH pool (Rahman *et al.*, 2005). The lowered redox ratio could be the result of oxidative stress or an outcome of oxidant-dependent effects on signal transduction that affects protein function (Rahman *et al.*, 2005). Hence, the present work is consistent with previous observations of impaired antioxidant protection and elevated oxidant products in CF (Lagrange-Puget *et al.*, 2004). Moreover, an adverse reduction-oxidation (redox)

state is associated with a pro-inflammatory response as the redox ratio reflects the balance between thiols and reactive oxygen species. Intracellular changes to the GSH/GSSG ratio can affect the DNA-binding affinities of pro-inflammatory transcription factors, such as NF-kB (Toledano and Leonard, 1991). Hence, the low GSH/GSSG ratio in CF could represent a condition of oxidative stress that can trigger NF-κB activation and thereby lead to a pro-inflammatory state. The small sample size used to measure GSH and GSSG was a limitation of the present work. In addition, more studies are needed to confirm that CFTR deficiency is responsible for increased intracellular GSH and low GSH/GSSG ratio observed in this study. For example, redox-status assays could be carried out using CFTE cells transfected with the normal CFTR gene.

A major direction of recent research in CF has focused on the theory that the chronic inflammation observed in cells expressing CFTR deficiency might be due to excessive cytokine release, exacerbating the inflammatory response in CF that precedes infection (Bonfield *et al.*, 1999). The present thesis work focused on pathways related to GSH status, ceramide production and IL-8 release in relation to CF mediated inflammation. Based on studies that have shown that GSH inhibits SMase (Liu and Hannun, 1997, Lavrentiadou *et al.*, 2001), we hypothesized that high intracellular GSH would inhibit SMase in TNF-α- stimulated ΔF508 CFTR, and impair ceramide synthesis through the SMase pathway (Levade and Jaffrezou, 1999), and make CF cells more resistant to apoptosis. In addition, considering that ceramide has been shown to downregulate NF-κB activation (Signorelli *et al.*, 2001), we hypothesized that a decrease in ceramide in CF cells leads to increased production of IL-8 in response to TNF-α (Fig. 2).

In view of the above, the present thesis work focused on two possible agents that could decrease IL-8 release from cultured mutant ΔF508 CFTR tracheal epithelial cells, fenretinide through *de novo* synthesis of ceramide, and WPH based on its immune properties and it potential to improve intracellular GSH status, particularly the GSH/GSSG redox ratio. The present thesis has provided new insights regarding these possible mechanisms of inflammation and apoptosis in CF cells via the cell culture treatments using fenretinide and whey peptides in the TNF-α stimulated state.

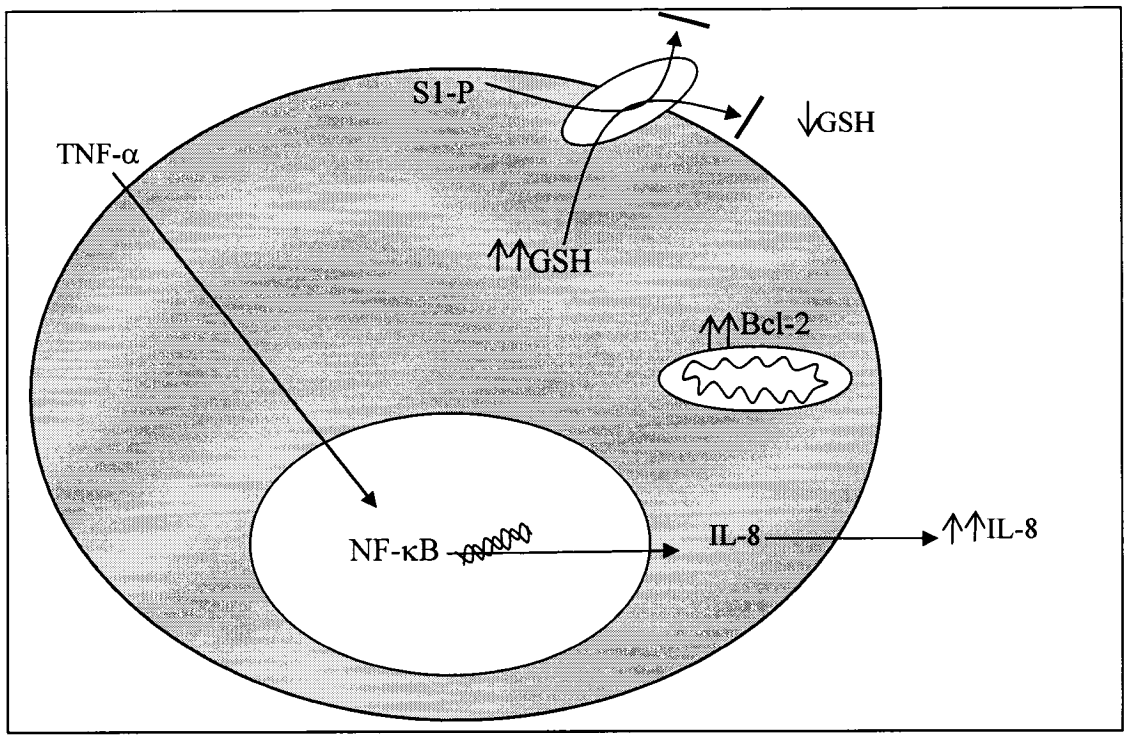


Figure 1. Possible mechanisms of metabolic disturbances in CF cells. CFTR cells have been described as having: IL-8 (interleukin-8) hyperesponsivenes to TNF-α (tumor necrosis factor-α) (Stecenko *et al.*, 2001, Venkatakrishnan *et al.*, 2000); increased activation of NF-κB (nuclear factor kappa B) (Venkatakrishnan *et al.*, 2000); overexpression of the antiapoptotic protein Bcl-2 (Harris *et al.*, 2005); inadequate CFTR glutathione (GSH) export (Gao *et al.*, 1999; Day *et al.*, 2004); low GSH content (Gao *et al.*, 1999); impaired release of S-1P (sphingosine 1-phosphate) (Boujaoude *et al.*, 2001).

The major finding of this thesis work was that IL-8 release in CF cells was downregulated by low dose fenretinide treatment in a dose-dependent manner under TNF-α stimulation.The decrease in IL-8, however, was not correlated to ceramide response to fenretinide at the lowest dose used (1.25 μM). Since the basal ceramide concentration did not change when the cells were treated with 1.25 μM of fenretinide, we could not confirm that the decrease in IL-8 release could be modulated by ceramide *de novo* synthesis, suggesting that other mechanisms are involved in the anti-inflammatory effect of fenretinide.

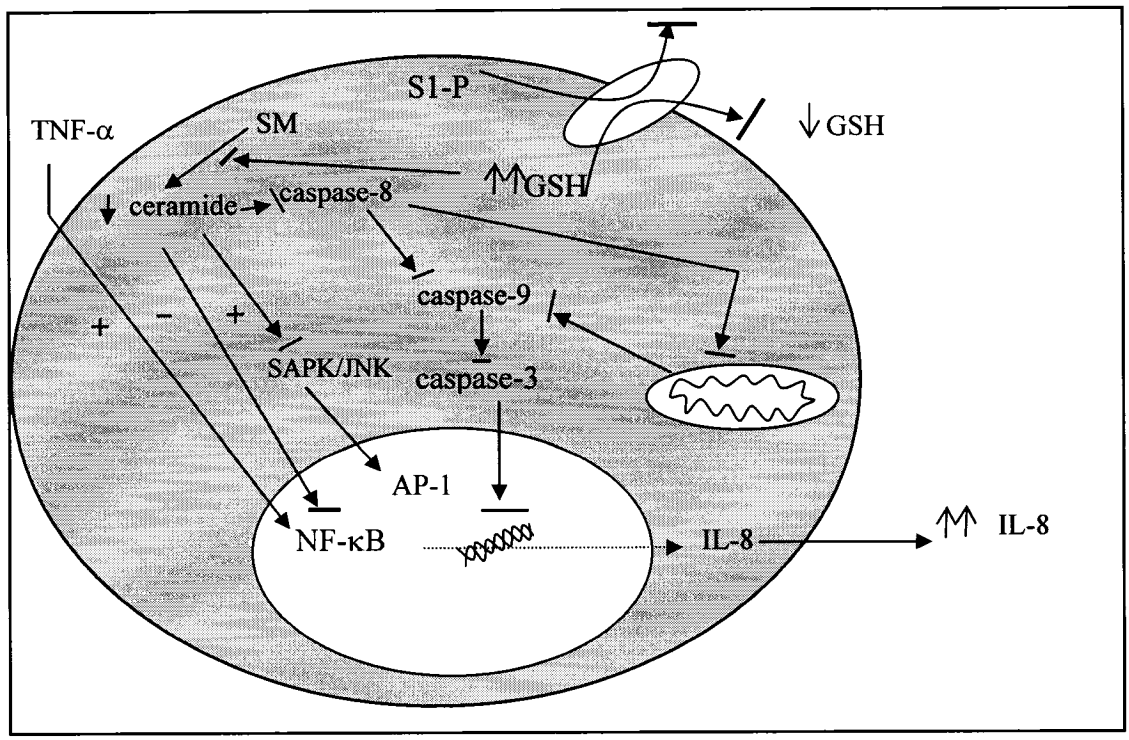


Figure 2. Proposed effects of high intracellular GSH content on ceramide synthesis, the inflammatory response and apoptosis in CF cells. Considering research findings showing that CFTR deficient cells have high intracellular GSH levels (Jungas *et al*,. 2002) and that GSH has been demonstrated to inhibit SMase (sphingomyelinase) (Liu and Hannun, 1997; Lavrentiadou *et al.*, 2001), we hypothesized that low ceramide levels generated through SM (sphingomyelin) pathway would limit apoptosis and contribute to NF-κB (nuclear factor kappa B) activation and overexpression of IL-8 (interleukin 8). SAPK/JNK: stress-activated protein kinase/c-Jun NH_2-terminal kinase.

Based on the potent inhibition of GSH on SMase activity and that fenretinide can generate ceramide via both *de novo* synthesis and via stimulation of SMase (Lovat *et al.*, 2004), we postulated that higher intracellular GSH in CF cells would be associated with greater resistance to ceramide generation via fenretinide treatment; however, a converse result was observed in this regard. The importance of the relatively high ceramide content in CF cells induced by high dose fenretinide treatment is unclear although this

could contribute to the anti-inflammatory action of fenretinide observed at the higher doses. Future studies are required to determine the biochemical significance of this finding. Alternatively, or in addition, fenretinide could attenuate TNF-α-mediated inflammation in CF cells via direct inhibition of IκBα degradation (Shimada *et al.*, 2002), which can lead to inhibition of NFκB activation.

The comparable ceramide content in CF cells to non-CF cells indicates that the resistance of CF cells to apoptosis occurs downstream of ceramide synthesis such as overexpression of Bcl-2, which could contribute to the apoptotic resistance of CF cells (Harris *et al.*, 2005). *De novo* ceramide synthesis occurs in the endoplasmic reticulum, and in addition to its function as a proapoptotic second messenger, this sphingolipid can subsequently be metabolized to gangliosides in the Golgi complex or regenerate sphingomyelin (SM). Gangliosides induce the generation of ROS via activation of 12-lipoxygenase and could promote apoptosis (Lovat *et al.*, 2004). Depite the more pronounced increase in ceramide levels in CF cells relative to non-CF cells following fenretinide treatment, CF cells showed resistance to apoptosis whereas non-CF cells became apoptotic. It is possible that that high intracellular GSH levels in CF cells could neutralize ROS that could be involved in apoptotic events following fenretinide stimulus. Ceramide can also be converted into sphingosine and subsequently to S-1P through sphingosine phosphate kinase (SphK) activity (Pyne and Pyne, 2000), which is activated by TNF-α. Considering that the ability of CFTR cells to export S-1P is impaired in CF (Boujaoude *et al.*, 2001), future studies would be worthwhile to examine whether CF cells would have more intracellular S-1P. High intracellular content of S-1P would limit the conversion of ceramide into S-1P, which could be the reason for the higher ceramide concentrations in CF cells observed following fenretinide treatment.

Another major finding of this thesis was the observation that pressure processing of whey proteins led to their enhanced *in vitro* digestibility as well as different peptide and amino acid profiles as examined by HPLC, capillary electrophoresis and GC-MS. This observation demonstrates for the first time that the partial unfolding of whey protein observed previously with pressure processing (Alvarez, 2004) exerts a long-lasting effect

on protein digestibility, particularly since the pressurized proteins showed consistent enhanced digestibility following long-term storage. The isolated low molecular weight peptides from hydrolysates of pressure processed whey proteins were observed to have significantly different effects relative to peptide obtained from native whey hydrolysates in terms of GSH status in both CFTR deficient and wild type cell culture studies. This latter finding indicates that pressure processing could lead to the generation of bioactive peptides and amino acids from enzymatic hydrolyses that differ in their biofunctional effects as compared to peptides obtained from whey protein hydrolysates.

An increase in pepsin-mediated *in vitro* digestion of whey proteins along with enhanced breakage of disulphide bonds via 1-cycle and 3-cycle pressurization was demonstrated in this thesis. This latter finding provides further supporting evidence that pressure cycling of whey proteins could potentially alleviate the allergic response to these proteins. Hosseini-nia (2000) showed lower IgG- and IgE-mediated antigenic reactions to β-LG, BSA and α-LA via Western blot analysis of the serum obtained from rats fed 3-cycle pressure-treated whey protein isolates. Previous work has demonstrated that thioredoxin treatment of whey proteins, which acts as a reductant of intramolecular disulphide bonds, enhances pepsin digestion and eliminates the allergenic response to β-LG in a dog model of milk protein allergenicity (del Val *et al.*, 1999). It was suggested by del Val *et al.* (1999) that an increased sensitivity to pepsin-mediated digestion and breakdown of the exposed disulphides induced by thioredoxin destroys the protein conformation responsible for allergenicity and the resistance to digestion associated with disulphide-rich proteins such as β-LG. Similar *in vivo* feeding trials would be worthwhile to study whether pressure-cycling of whey proteins could exert such inhibitory effects on whey protein allergenicity.

Although fenretinide and WPH treatments showed similar trends for downregulation on IL-8 in CF cells, no enhancement was observed in this regard from the combined treatment of fenretinide and WPH. In the light of these results we speculated that the inhibition of IL-8 release associated with the WPH treatments could be related to mechanisms involving peptides acting as caspase inhibitors, specifically via inhibition of

caspase-8 activation since IL-8 expression was shown to be dependent on caspase-8 activation of NF-κB transcriptional activity under TNF-α stimulation (Choi *et al.*, 2002). In non-CF cells, fenretinide induced apoptosis and nWPH and pWPH treatments protected the cells from apoptosis induced by fenretinide at the two doses tested (1.25 and 2.5 μM) under both stimulated and non-stimulated conditions. Since WPH did not enhance intracellular GSH concentrations in non-CF cells, mechanisms other than GSH modulation was likely involved in the decreased the susceptibility of fenretinide-induced apoptosis in normal lung epithelial cells exposed to whey peptides. One possibility is that peptides could act as pseudosubstrates for executioner caspases such as caspase-3 thereby interfering with the apoptosis that occurs downstream of ceramide synthesis (Degterev *et al.*, 2003).

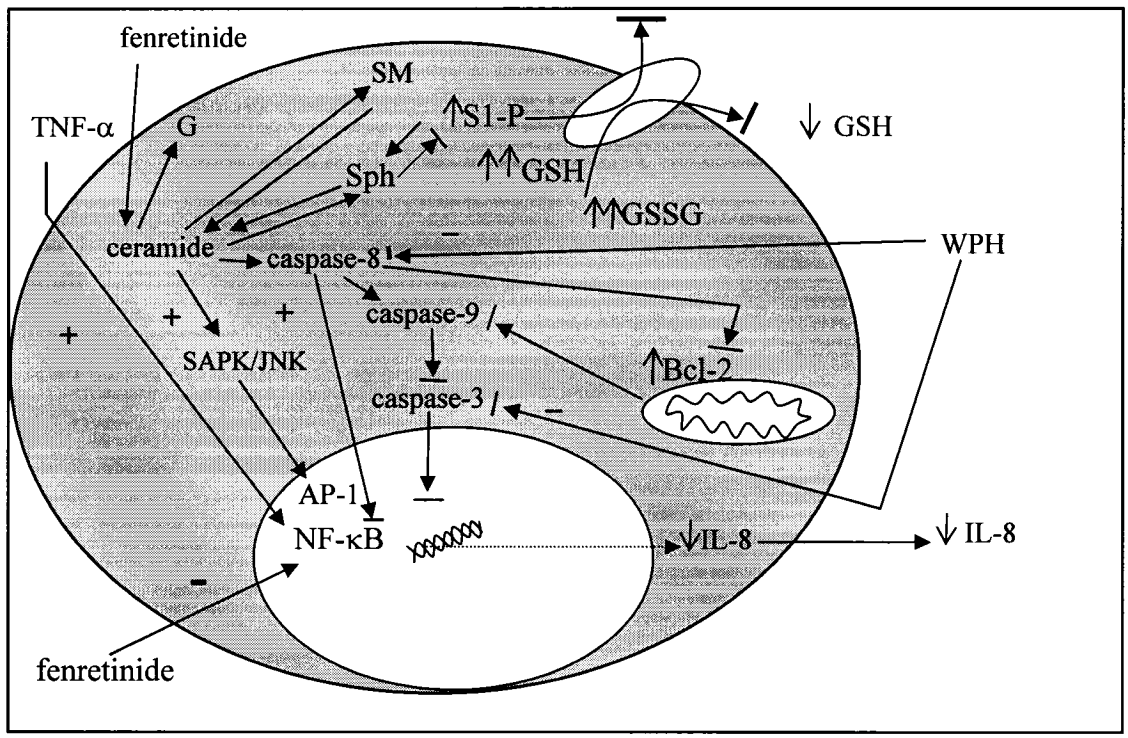


Figure 3. Proposed effects of fenretinide and WPH on CF cells apoptosis and IL-8 release. Considering our findings, we speculated that: the IL-8 (interleukin-8) release inhibition is not only explained by increase of ceramide; the higher levels of ceramide in fenretinide stimulated cells may be due to the impairment of S1-P (sphingosine 1-phosphate) export; whey peptides may act as caspase inhibitors thus contributing to the

antiapoptotic effect of GSH and the antiapoptotic Bcl-2 as well as to the inhibition of NF-κB (nuclear factor kappa B). WPH: whey protein hydrolsates. TNF-α: tumor necrosis factor alpha. SAPK/JNK: stress-activated protein kinase/c-Jun NH_2-terminal kinase.

Our findings are limited to the measurements of IL-8 release, apoptosis, intracellular levels of GSH/GSSG and ceramide. A limitation of the thesis work is that intracellular GSH levels were not measured to assess the redox state in TNF-α stimulated cells as well as following fenretinide treatment or in the combination of fenretinide and peptides treatment under basal and TNF-α stimulated condition. More studies are necessary to clarify the mechanisms by which fenretinide and whey peptides interfere with IL-8 release and apoptosis. For example, studies could assess the possibility that peptides isolated from pWPH and nWPH could act as caspase inhibitors as a possible explanation for their observed modulation of apoptosis and IL-8 release. It would also be important to verify whether fenretinide or whey peptides could inhibit NF-κB activation in CF cells as a mechanism of their anti-inflammatory action. Measurement of intracellular S-1P and other ceramide derivatives could help to explain the strong induction of ceramide levels following fenretinide treatment in CF cells. Also, *in vitro* studies do not necessarily reflect the *in vivo* context and further animal and human studies are required to assess the therapeutic potential of fenretinide and whey peptides in CF. In that regard, however, recent collaborative work in which we participated showed that fenretinide treatment was associated with a major decrease in colony forming units (CFUs) in the lungs of CFTR-knockout mice infected with *Pseudomonas aeruginosa* (personal communication). Hence, these initial *in vivo* studies show promise that fenretinide could play a therapeutic role in CF pathogenesis.

To our knowledge, this is the first demonstration that food-derived peptides can induce functional effects in either normal or CFTR-deficient pulmonary epithelial cells. The peptides chosen were released from enzymatic hydrolysis followed by ultrafiltration to allow the cells to get in contact exclusively with peptides smaller than 1 kDa, mimicking the physiologic state that limits absorption of higher molecular weight peptides. It is also the first time the impact of peptides and fenretinide on IL-8 release was tested in such

cell types opening new avenues for future studies regarding to the effect of these compounds on the immune response in the CF condition.

In conclusion, the results of our work present the possibility that the lung inflammation in CF could be attenuated via low doses of fenretinide and/or whey protein hydrolysates and open new possibilities to explore the therapeutic impact of fenretinide and whey peptides on cellular metabolism in CF.

References

Alvarez P. 2004. Effect of applied hydrostatic pressure on the structure and rheological properties of whey proteins. M.Sc. Thesis, McGill University, Montreal, QC., Canada.

Bals R., Weiner D. J., Wilson J. M. 1999. The innate immune system in cystic fibrosis lung disease. *The Journal of Clinical Investigation.* 103: 303-7.

Bonfield T., Konstan M. W., Berger M. 1999. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *Journal of Allergy and Clinical Immunology.* 104: 72-78.

Boucher R. C. 2004. Relationship of airway epithelial ion transport to chronic bronchitis. *Proceedings of the American Thoracic Society.* 1: 66-70.

Boujaoude L. C., Bradshaw-Wilder C., Mao C., Cohn J., Ogretmen B., Hannun Y. A., Obeid L. M. 2001. Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid. Modulation of cellular activity of sphingosine 1-phosphate. *Journal of Biological Chemistry.* 276: 35258-35264.

Choi C., Kutsch O., Park J., Zhou T., Seol D. W, Benveniste E. N. 2002. Tumor necrosis factor-related apoptosis-inducing ligant induces caspase-dependent interleukin-8 expression and apoptosis in human astroglioma cells. *Molecular and Cell Biology.* 22: 724-736.

Day B. J., van Heeckeren A. M., Min E., Velsor L. W. 2004. Role for cystic fibrosis transmembane conductance regulator protein in a glutathione response to bronchopulmonary *Pseudomonas* infection. *Infection and Immunity.* 72: 2045-2051.

Degterev A., Boyce M., Yuan J. 2003. A decade of caspases. *Oncogene.* 22: 8543-8567.

del Val G., Yee B. C., Lozano R. M., Buchanan B. B., Ermel R.W., Lee Y. M., Frick O. L. 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *Journal of Allergy and Clinical Immunology.* 103: 690-697.

Dinwiddie R. 2000. Pathogenesis of lung disease in cystic fibrosis. *Respiration.* 67: 3-8.

Gao L, Kim K. J., Yankaskas J. R., Forman H. J. 1999. Abnormal glutathione transport in cystic fibrosis airway epithelia. *American Journal of Physiology.* 277: L113-L118.

Hosseini-nia T. 2000. Structural and nutritional properties of whey proteins as affected by hyperbaric treatment. 2000. PhD. Thesis. McGill University, Montreal, QC, Canada.

Jungas T., Motta I., Duffieux F., Fanen P., Stoven V., Ojcius D. M. 2002. Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry.* 277: 27912-27918.

Harris J. F., Fisher M. J., Hotchkiss J. R., Monia B. P., Randell S. H., Harkema J. R., Tesfaigzi Y. 2005. Bcl-2 sustains increased mucuous and epithelial cell numbers in metaplasic airway epithelium. *American Journal of Respiratory and Critical Care Medicine.* 171: 764-772.

Kammouni W., Figarella C., Marchand S., Merten M. 1997. Altered cytokine production by cystic fibrosis tracheal gland serous cells. *Infection and Immunity.* 65: 5176-5183.

Kogan I., Ramjeesingh M., Li C., Kid J. F., Wang Y., Leslie E. M., Cole S. P. C., Bear C. E. 2003. CFTR directly mediates nucleotide-regulated glutathione efflux. *European Molecular Biology Organization.* 22: 1981-1989.

Lagrange-Puget M., Durieu I., Ecochard R., Abas-Chorfa F., Drai J., Steghens J. P., Pacheco Y., Vtal-Durand D., Bellon G. 2004. Longitudinal study of oxidative status in 312 cystic fibrosis patients in stable state and during bronchial exacerbation. *Pediatric Pulmonology.* 38: 43- 49.

Lavrentiadou S. N., Chan C., Kawcak T., Ravid T., Tsaba A. van der Vliet A., Rasooly R., Goldkorn T. 2001. Ceramide-mediated apoptosis in lung epithelial cells is regulated by glutathione. *American Journal of Respiratory Cell and Molecular Biology.* 25: 676-684.

Levade T., Jaffrezou J. P. 1999. Signalling sphingomyelinases: which, where, how and why? *Biochimica et Biophysica Acta.* 1438: 1-17.

Lindsdell P., Hanrahan J. W. 1998. Glutathione permeability of CFTR. *The American Physiological Society.* 275: C323-326.

Liu B., Hannun Y. A. 1997. Inhibition of the neutral magnesium-dependent sphimgomyelinase by glutathione. *The Journal of Biological Chemistry.* 272: 16281-16287.

Lovat P. E., Di Sano F., Corazzari M., Fazi B., Donnorso R. P., Pearson A. D. J., Hall A. G., Redfern C. P. F., Piacentini M. 2004. Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. *Journal of the National Cancer Institute.* 96: 1288-1299.

Peters T. Jr. 1996. *All About Albumin: Biochemistry, Genetics, and Medical Applications*. San Diego: Academic Press. pp. 51-124.

Puchelle E., Bajolet O., Abély M. 2002. Airway mucus in cystic fibrosis. *Paediatric Respiratory Reviews*. 3: 115-119.

Pyne S., Pyne N. J. 2000. Sphingosine 1-phosphate signalling in mammalian cells. *Biochemical Journal*. 349: 385-402.

Rahman I., Biswas S. K., Jimenez L. A., Torres M., Forman H. J. 2005. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxidants and Redox Signaling*. 7: 42-59.

Scheid O., Kempster L., Griesenbach U., Davies J. C., Dewar A., Weber P. P., Colledge W. H., Evans M. J., Geddes D. M., Alton E. W. 2001. Inflammation in cystic fibrosis airways: relationship to increased bacterial adherence. *European Respiratory Journal*. 17: 27-35.

Shimada K., Nakamura M., Ishida E., Kishi M., Yonehara S., Konishi N. 2002. Contributing of mitogen-activated protein kinase and nuclear factor kappa B to *N*-(4-hydroxyphenyl) retinamide-induced apoptosis in prostate cancer cells. *Molecular Carcinogenesis*. 35: 127-137.

Signorelli P., Luberto C., Hannun Y.A. 2001. Ceramide inhibition of NF-kappaB activation involves reverse translocation of classical protein kinase C (PKC) isoenzymes: requirement for kinase activity and carboxyl-terminal phosphorylation of PKC for the ceramide response. *The FASEB Journal*. 15: 2401-2414.

Stecenko A. A., King G., Torii K., Breyer R. M., Dworski R., Blackwell T. S., Christman J. W., Brigham K. L. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation*. 25: 145-155.

Toledano M. B., Leonard W. J. 1991. Modulation of transcription factor NF-κB binding activity by oxidation-reduction *in vitro*. *Proceedings of the National Academy of Sciences USA*. 88: 4328-4332.

Venkatakrishnan A., Stecenko A. A., King G., Blackwell T. R., Brigham K. L., Christman J. W., Blackwell T. S. 2000. Exaggerated activation of nuclear factor-κB and altered IκB-β processing in cystic fibrosis bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*. 23: 396-403.

APPENDICES