EFFECT OF NATIVE AND PRESSURIZED WHEY PROTEIN ISOLATES ON INFLAMMATION IN RESPIRATORY EPITHELIAL CELLS EXPRESSING EITHER WILDTYPE OR MUTANT CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR)

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This thesis is dedicated to my grandparents, Juliette, Romaine, Edmond, and particularly Georges, whose foresight made this possible.

"Science is a wonderful thing if one does not have to earn one's living at it."

Albert Einstein

TABLE OF CONTENTS

ABSTRACT

Cystic fibrosis (CF) is a fatal inherited disease characterized by chronic, persistent and exaggerated inflammation. The airways of CF patients exhibit a sustained influx of neutrophils and interleukin (IL)-8, the major neutrophil chemoattractant. Anti-inflammatory therapy is a focus of CF treatment, with a clear need for novel, safe and effective therapies. Whey proteins have been attributed a number of biological activities, including antioxidant and anti-inflammatory effects. Previous research has suggested a suppression of the inflammatory response to tumor necrosis factor (TNF)α by whey protein hydrolysates (WPH) in respiratory epithelial cell lines. Furthermore, it is suggested that hyperbaric pressure (HP) treatment of whey proteins can potentiate their biological activities. The effect of HP treatment on the digestibility, anti-inflammatory and antioxidant activity of whey protein isolates (WPI) was explored in a series of mechanistic *in vitro* studies and in a clinical trial. Hydrolysates of pressurized (pWPH) and native (nWPH) whey protein isolates were generated using two differing *in vitro* digestion protocols that mimic human gastrointestinal digestion. Hyperbaric pressure pre-treatment resulted in enhanced *in vitro* digestibility and antioxidant activity of WPI regardless of major differences in enzymatic conditions and the resulting peptide profiles, signifying that pressure processing generates an enhanced release of bioactive peptides regardless of the proteolytic environment. The peptide profiles obtained from the hydrolysates of pWPH exhibited quantitative and qualitative differences from those of nWPH. To explore the possible mechanisms by which WPH may exert their anti-inflammatory effects, wild-type and cystic fibrosis (CF) conductance regulator-deficient cells were treated with either nWPH or pWPH and stimulated with TNFα, interleukin (IL)-1β or lipopolysaccharide (LPS). Both nWPH and pWPH suppressed LPS-induced IL-8 secretion, although pWPH were more potent requiring lower doses to exert significant inhibition. pWPH increased the ferric-reducing antioxidant activity of cell culture supernatant in both cell lines. Neither type of hydrolysate suppressed TNFα- or IL-1β-induced IL-8 secretion. Since LPS and IL-1β, beyond the activation of their respective receptors, share a common intracellular pathway, further experiments were conducted on signalling events that occur prior to the convergence of the two pathways,

at the level of Toll-like receptor (TLR)4. There was no effect of either nWPH or pWPH on the cell membrane expression of TLR4. Neither type of WPH exhibited direct binding and neutralization of LPS, but both significantly reduced the binding of LPS to cell surface receptors. It is therefore likely that the WPHs exerted the observed LPS-induced IL-8 suppression by suppressing the binding of LPS to its receptor with the consequent inhibited activation of TLR4. To explore the possible *in vivo* clinical effects of pressurized WPI, a one-month, open-label pilot study of supplementation with pressurized WPI in children and adults with CF was conducted. Nutritional status was enhanced in both children and adults. An improvement in lung function was observed in children, and C-reactive protein decreased in the majority of patients with initially high values. Overall, the thesis results provide a novel mechanism by which whey proteins and pressurized whey can exert anti-inflammatory effects and suggest new potential avenues for the use of pressurized whey protein isolate as a nutrition-based anti-inflammatory therapeutic or preventative agent.

RESUME

La fibrose kystique (FK) est une maladie héréditaire fatale caractérisée par une inflammation chronique et exagérée. Les voies respiratoires des patients atteints de FK présentent un afflux soutenu de neutrophiles et d'interleukine (IL)-8, l'agent chimiotactique majeur des neutrophiles. La thérapie anti-inflammatoire est un focus du traitement de la FK et il existe un besoin évident de nouvelles thérapies sûres et efficaces. Des effets biologiques, incluant des effets antioxydants et anti-inflammatoires ont été attribués aux protéines de lactosérum. Des recherches antérieures ont suggéré une suppression de la réponse inflammatoire induite par le facteur de nécrose tumorale (TNF)α par des hydrolysats de protéines de lactosérum (WPH). De plus, il a été suggéré que le traitement par pression hyperbare (PH) des isolats de protéines de lactosérum (WPI) peut potentialiser leurs activités biologiques. Les effets d'un traitement PH sur la digestibilité et les activités anti-inflammatoires et antioxydants des WPI ont été explorés dans une série d'études mécanistes in vitro et dans un essai clinique. Des hydrolysats de protéines de lactosérum pressurisées (pWPH) et non pressurisées (nWPH) ont été générés par le moyen de deux différents protocoles de digestion in vitro imitant le processus de digestion gastro-intestinal humain. Le prétraitement HP a entrainé une augmentation de la digestibilité in vitro et de l'activité antioxydante des WPI, malgré les différences entre les deux protocoles de digestion et entre les profiles de peptides résultant des digestions, signifiant que la pression génère une augmentation des peptides bioactifs générés quel que soit l'environnement protéolytique. Les profiles des peptides des pWPH ont exhibé des différences qualitatives et quantitatives comparés aux nWPH. Pour explorer les mécanismes possibles par lesquels WPH pourraient exercer leurs effets anti-inflammatoires, des lignées cellulaires normales et déficientes du Régulateur de la Conductance Transmembranaire Mucoviscidose (CFTR) ont été traitées avec nWPH ou pWPH et stimulées avec TNFα, IL-1β, ou lipopolysaccharide (LPS). Les nWPH et pWPH ont diminué la sécrétion d'IL-8 induite par LPS, bien que les pWPH aient été plus puissants, requérant de moindres doses pour exercer une inhibition significative. pWPH a augmenté l'activité antioxydante des surnageants des cultures des deux lignées cellulaires. Aucun type de WPH n'a inhibé la sécrétion d'IL-8 induite par TNFα ou IL-

1β. Puisque LPS et IL-1β partagent une voie commune de signalisation suivant l'activation de leurs récepteurs respectifs, des expériences ont été menées sur des événements prenant place en amont de la convergence des deux voies de signalisation, au niveau du récepteur Toll-like receptor (TLR)4. Les nWPH ni les pWPH n'ont eu d'effet sur l'expression du TLR4, mais la liaison entre LPS et les récepteurs de surface a été réduite par les deux traitements. Aucun WPH n'a exhibé de liaison directe ou de neutralisation du LPS. Il est donc probable que les WPHs aient exercé la suppression d'IL-8 induite par LPS en inhibant la liaison du LPS avec son récepteur et par conséquent l'activation du TLR4. Pour explorer les effets cliniques in vivo des WPI pressurisés, un essai clinique pilote d'une durée d'un mois a été mené, où des enfants et adultes souffrant de FK ont été supplémentés avec des protéines pressurisées. Le statut nutritionnel des enfants et adultes a été amélioré. Une amélioration des fonctions pulmonaires chez les enfants a été observée et le taux sanguin de protéine C réactive a diminué chez la majorité des patients chez qui les taux étaient élevés au début de l'étude. Globalement, les résultats de cette dissertation fournissent un nouveau mécanisme par lequel les WPI pressurisés peuvent exercer des effets anti-inflammatoires et suggèrent de nouvelles avenues potentielles pour l'usage des WPI pressurisés en tant qu'agent nutritionnel thérapeutique ou préventif.

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ADVANCE OF SCHOLARLY KNOWLEDGE

1. Claims of Original Research

In this thesis, it is demonstrated for the first time that:

1. Hydrolysates from the *in vitro* digestion of whey protein isolates act upon wild-type and cystic fibrosis (CF) respiratory epithelial cells to decrease lipopolysaccharide (LPS)-induced interleukin (IL)-8 secretion.

2. The mechanism by which whey protein hydrolysates interfere with LPS-induced IL-8 is through decreasing the binding of LPS to cell surface receptors.

3. Treatment of whey protein isolates with hyperbaric pressure prior to *in vitro* digestion enhances the LPS-mediated interleukin-8 suppressing effects of the resulting hydrolysates.

4. Treatment of whey protein isolates with hyperbaric pressure prior to *in vitro* digestion enhances the *in vitro* antioxidant capacity of the resulting hydrolysates in both aqueous cell-free and cell culture systems.

5. Short-term supplementation with pressurized whey protein improves BMI in adults, BMI z-score and lung function in children, and decreases serum C-reactive protein levels in patients with initially high values.

This thesis presents improvements over previous research:

6. A number of modifications were brought to an *in vitro* digestion procedure in order to further approximate physiological human gastro-intestinal digestion and absorption.

7. Using the modified digestion procedure, different peptide profiles and a larger population of peptides can be obtained via *in vitro* hydrolysis of whey protein isolates than previously shown. The effects of hyperbaric pressure pre-treatment are such that differences in peptide profiles between pressurized and unpressurized whey protein isolates are detected regardless of the digestion procedure used.

8. Hydrolysates of pressurized whey protein isolates produced via different digestion procedures yield varying biological effects in some respects: hydrolysates generated using the newer modified digestion procedure do not upregulate IL-8 secretion by CF cells under basal conditions nor show a tendency to suppress tumor necrosis factor α-induced IL-8 secretion, as opposed to hydrolysates from previous digestion conditions. However, these different hydrolysates behave similarly in other respects (antioxidant activity, suppression of LPS-induced IL-8 secretion).

1. Research Manuscripts or Abstracts Published or in Preparation for Publication:

1. Iskandar M., Kubow S., Skinner C.D., Meehan B., Mawji N., Sabally K., and Lands L.C. Effect of hyperbaric pressure on the digestibility and antioxidant potential of whey protein isolates. In preparation.

2. Iskandar M., Kubow S., Dauletbaev N., Mawji N., and Lands L.C. Inhibition of interleukin-8 release by pressurized whey protein hydrolysates in respiratory epithelial cells: mechanism of action. In preparation.

3. Lands L.C., Iskandar M., Beaudoin N., Meehan B., Dauletbaev N., and Berthiaume Y. 2010. Dietary supplementation with pressurized whey in patients with cystic fibrosis. *J Med Food.* 13:1-6

4. Iskandar M.M., Skinner C., Kubow S., and Lands L.C. 2008. Anti-inflammatory effects of pressurized whey digestates on wild-type and mutant cystic fibrosis respiratory epithelial cell lines. Abstract, American Thoracic Society (2008)

5. Lands L.C., Beaudoin N., Iskandar M., Meehan B., and Berthiaume Y. Pressurized whey supplementation in CF patients: short-term clinical results. Abstract, North American Cystic Fibrosis Conference (2008)

6. Lands L.C., Beaudoin N. Meehan B., Iskandar M., and Berthiaume Y. Immune modulating effects of pressurized whey protein supplementation in CF patients. Abstract, North American Cystic Fibrosis Conference (2007).

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS

The present thesis involved the collaboration of Dr. Stan Kubow, School of Dietetics and Human Nutrition, MacDonald Campus of McGill University; Dr. Larry C. Lands, Paediatric Respiratory Medicine, The Montreal Children's Hospital, Montreal, Quebec, and Dr. Cameron D. Skinner, Department of Chemistry and Biochemistry, Concordia University.

Drs. Lands and Kubow, the candidate's primary supervisors, were the originators of the research project and were closely involved in the study design, providing ongoing guidance and feedback in all aspects of the thesis. Both supervisors provided extensive feedback and thorough critiques of all manuscripts and sections of the thesis.

Dr. Skinner provided the facilities, instrumentation and training to perform the capillary zone electrophoresis experiments, training to carry out analyses of the results, as well as helpful comments and suggestions on the thesis project.

The candidate and author of the thesis was responsible for carrying out all of the experiments described in this project, including *in vitro* digestions and lyophilisation, protein and peptide assays, antioxidant assays, capillary zone electrophoresis measurements and analyses, basal cell culture maintenance, cell viability determination, cell culture studies involving cell stimulation and treatment with whey hydrolysates, including ELISA analyses of cell culture supernatants. The candidate performed all statistical analyses and produced all tables and figures, with the exception of those in chapter 5. The candidate was responsible for writing all sections of the thesis with the exception of the manuscript represented in chapter 5, which was written by Dr. Lands. In said chapter, the candidate contributed a portion of written text, proofed and organized the data collected at the end of the study, performed analyses of cytokine concentrations in *ex vivo* stimulated blood samples, collected diet history information from subjects and instructed them on the proper use of the protein supplement.

Dr. Dauletbaev performed the glutathione measurements and flow cytometric measurements on samples produced by the candidate in cell culture. He also provided ongoing guidance and training in cell culture procedures and general laboratory techniques, as well as scientific insight and assistance.

Brian Meehan participated in the development of the amended digestion procedure, provided initial training in general laboratory procedures, and performed the *ex vivo* whole blood cultures and stimulations. Nadir Mawji helped to perform replicates of digestions and a number of ELISA measurements. Dr. Kebba Sabally ran the HPLC separations of hydrolysates. Nadia Beaudoin coordinated the clinical study and performed skinfold measurements.

LIST OF TABLES

CHAPTER 2. LITERATURE REVIEW

CHAPTER 3.

CHAPTER 5.

LIST OF FIGURES

CHAPTER 2. LITERATURE REVIEW

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

LIST OF ABBREVIATIONS

ACE	Angiotensin I converting enzyme
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
BALF	Bronchoalveolar lavage fluid
BCAA	Branched chain amino acid
BMI	Body mass index
BSA	Bovine serum albumin
BSO	L-buthionine-(S,R)-sulfoximine
C/EBP	CAAT/enhancer-binding protein
CD14	Cluster of differentiation 14
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl^-	Chloride ion
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase 2
CRP	C-reactive protein
Cys	Cysteine
CZE	Capillary zone electrophoresis
Da	Dalton
DEXA	Dual x-ray absorptiometry
DNA	Deoxyribonucleic acid
DS	Dextran sulphate
dsRNA	Double-stranded RNA
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid
EDTA	Ethylenediamine tetra-acetic acid
ELF	Epithelial lining fluid
ENA-78	Epithelial neutrophil activating peptide

ENaC	Epithelium Na^+ channel
ERK	Extracellular-regulated kinase
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in one second
FFM	Fat-free mass
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methyl-leucyl-phenylalanine
FRAP	Ferric-reducing antioxidant power
FVC	Forced vital capacity
GCS	Gamma-glutamylcysteine synthetase
GEE	Glutathione ethyl ester
GGT	γ-glutamyltranspeptidase
Glu	Glutamine
Gly	Glycine
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Glycomacropeptide
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HP	High pressure
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitory kappa B
IKK	IκB Kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist

iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase(s)
IRF-3	Interferon regulatory factor 3
JNK	C-jun N-terminal kinase
L. paracasei	*Lactobacillus paracasei*
LAL	Limulus amebocyte lysate
LBP	LPS-binding protein
Lf	Lactoferrin
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB4	Leukotriene B4
mAb	Monoclonal antibody
Mal	MyD88 adaptor-like
MALP-2	Diacylated lipopeptide
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein
MD-2	Myeloid differentiation protein 2
mDC	Myeloid dendritic cell
MEK	MAP kinase/Erk kinase kinase
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MPa	MegaPascal
MPM	Malleable protein matrix
MPO	Myeloperoxidase
MTT	3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MWCO	Molecular Weight Cut-Off
MyD88	Myeloid differentiation factor 88
NAC	N-acetyl L-cysteine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

NE	Neutrophil elastase
NEM	*N*-ethylmeleimide
NF-κB	Nuclear factor-kappa B
NG	Nase-gastric
NK	Natural killer
NO	Nitric oxide
nWPH	Native whey protein hydrolysates
O_2^-	Superoxide anion
OPA	*o*-Phthaldialdehyde
OTC	2-oxothiazolidine-4-carboxylate
P. aeruginosa	*Pseudomonas aeruginosa*
Pam3	Triacylated lipopeptide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PHA	Phytohemagglutinin
PVP	Polyvinyl-pyrrolidone
pWPH	Pressurized whey protein hydrolysates
pWPI	Pressurized WPI
RANTES	Normal T cell expressed and secreted
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high performance liquid chromatography
RSV	Respiratory syncytial virus
RV	Residual volume
S. aureus	*Staphylococcus aureus*
SAM	*S*-adenosyl-L-methionine
sCD14	Soluble CD14
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
-SH	Sulfhydryl
S-S	Disulfide
β-LG	Beta-lactoglobulin
ssRNA	Single-stranded RNA
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-beta
TIR	Toll/IL-1R
TLC	Total lung capacity
TLR	Toll-like receptor
TNBS	Trinitrobenzene-sulfonic acid
TNFR	TNF receptor
TNF-α	Tumor necrosis factor alpha
TPTZ	Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll-IL-1R domain-containing adaptor inducing IFN-β
Tyr	Tyrosine
WPC	Whey protein concentrate
WPE	Whey protein extract
WPH	Whey protein hydrolysate
WPI	Whey protein isolate
α-LA	Alpha-lactalbumin
γ-Glu-L-Cys	Gamma-glutamylcystine

CHAPTER 1

INTRODUCTION

1.1 Rationale and Statement of Purpose

Cystic fibrosis (CF) is the most common lethal hereditary disease in the Caucasian population, affecting approximately one in 3,600 live births in North America (WHO/ECFTN, 2002; Gibson *et al.*, 2003). It is a multisystem condition caused by mutations in the gene encoding for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a cyclic AMP regulated chloride channel in epithelial cell membranes (Davis, 2006). The absence of a functional CFTR in cells impairs the flux of water and electrolytes across the cell membrane (Zemanick *et al.*, 2010). These imbalances lead to clinical manifestations in the form of severe pulmonary disease, pancreatic insufficiency, elevated sweat chloride concentrations and infertility in males (Knowles and Durie, 2002). Progressive chronic obstructive lung disease is the major cause of morbidity and mortality in CF (Davis, 2006; Gao *et al*., 1999), and is characterized by chronic bacterial infection (most commonly by *Pseudomonas aeruginosa*) and persistent and vigorous inflammation. The airways of CF patients are dominated by neutrophil infiltration and are subject to high concentrations of pro-inflammatory mediators such as interleukin (IL)-8, the major neutrophil chemoattractant (Terheggen-Lagro, 2005; Koehler, 2004). Another major feature of CF is a state of oxidative stress and decreased anti-oxidant defences including low levels of glutathione, an important tripeptide thiol reducing agent, systemically and in the lung epithelial lining fluid (Roum *et al.*, 1993). The presence of neutrophils, which release oxidants and proteinases, leads to epithelial damage and exacerbates the vicious cycle of infection-inflammation (Koehler, 2004). Current treatment for CF includes antibiotic therapy, enzyme replacement to treat malabsorption due to pancreatic insufficiency, and anti-inflammatory therapies (Jones and Helm, 2009; Munck, 2010). At this time, although several therapeutic modalities have shown promise in the treatment of inflammation, concerns over long-term effectiveness and adverse effects highlight the ongoing need for new, safe and effective therapeutic agents that

address both inflammation and oxidative stress (Auerbach *et al.*, 1985; Florescu *et al.*, 2009).

Whey proteins are widely used as nutritional supplements and have been shown to increase glutathione (GSH) levels *in vitro*, in animal models and in clinical studies (Kent *et al.*, 2003; Hamad *et al.*, 2011; Grey *et al.*, 2003). In addition, several immune modulating and anti-inflammatory effects of whey proteins have been suggested in a number of experimental settings and disease conditions (Sprong *et al.*, 2010; Beaulieu *et al.*, 2007; Shin *et al.*, 2005). The beneficial antioxidant effects of whey proteins have been largely attributed to their high cysteine content, thereby acting via the provision of substrate for GSH synthesis. In addition to its role in regulating redox equilibrium, GSH is involved in many critical cellular functions including the regulation of redox-sensitive transcription factors. Thus, GSH deficiency can trigger inflammation via increased transcription of nuclear factor κB, which results in the secretion of IL-8 (Dröge *et al.*, 1994; Haddad *et al.*, 2000). Specifically in the case of CF, previous *in vitro* work has suggested that hydrolysates from whey protein isolates (WPI) can increase cellular GSH levels and tend to suppress IL-8 secretion by respiratory epithelial cells (Vilela *et al.*, 2006). These studies have also suggested that the prior application of hyperbaric pressure to WPI potentiates their GSH-enhancing and IL-8 suppressing effects. Hyperbaric pressure induces conformational changes that can render whey proteins more susceptible to gastro-intestinal digestive enzymes (López-Fandiño, 2006), thereby altering the profile of peptides obtained through enzymatic hydrolysis. It has been postulated that these alterations in peptide profiles represent an increase in the availability of bioactive peptides, thereby enhancing the anti-inflammatory and GSH-promoting effects of WPI. The aforementioned work did not investigate the possible molecular mechanism(s) by which whey-derived peptides could exert their bioactive effects. Furthermore, the enzymatic digestion of WPI and peptide recovery methodologies employed in these studies present with some limitations which, if addressed, could better approximate *in vivo* gastro-intestinal digestive processes. Finally, the effects of WPI hydrolysates on IL-8 suppression were examined only in response to Tumor Necrosis Factor (TNF)-α. Chronic bacterial infection being one of the hallmarks of CF, it would be fitting to investigate the

possible anti-inflammatory effects of WPI hydrolysates in situations of cell exposure to bacterial gene products, such as lipopolysaccharide (LPS).

1.2 Project Objectives

1. Building upon previous work into the *in vitro* digestibility of whey protein isolates (WPI), to evaluate the effect of hyperbaric pressure pre-treatment on the digestibility of WPI with the use of a modified digestion and peptide isolation protocol that better approximates *in vivo* gastro-intestinal digestive processes.

2. To evaluate the effect of hyperbaric pressure pre-treatment of WPI on the peptide profiles of hydrolysates obtained from the digestion of WPI, and how these profiles reflect the antioxidant capacity of the hydrolysates.

3. To study the potential anti-inflammatory effects of WPI hydrolysates, specifically their effects on IL-8 release by wild-type and CFTR-deficient human respiratory epithelial cells, either under basal conditions or stimulated with endogenous (TNFα; IL-1β) or exogenous (LPS) proinflammatory stimuli.

4. To explore and identify the molecular mechanism(s) by which WPI hydrolysates exert their effects on IL-8 secretion by immortalized wild-type and CFTR-deficient human respiratory epithelial cells.

5. To explore the effects of pressurized whey protein supplementation on nutritional status, markers of inflammation and lung function in patients with cystic fibrosis.

1.3 Hypotheses

1) Hydrolysates of native and pressurized WPI obtained via an amended digestion and peptide isolation protocol will contain a larger peptide population upon analysis of peptide profiles. Furthermore, such hydrolysates will exhibit biological activities

(antioxidant capacity, IL-8 suppression) that are either equivalent or enhanced when compared to hydrolysates obtained via the digestion protocol of Vilela *et al.* (2006).

2) Hyperbaric treatment will enhance the digestibility of WPI when subjected to the amended digestion protocol, and will enhance the antioxidant capacity of the resulting hydrolysates in comparison to native WPI hydrolysates.

3) WPI hydrolysates will suppress the secretion of IL-8 by immortalized wild-type and CFTR-deficient respiratory epithelial cell lines either under basal or stimulated conditions.

4) Hyperbaric treatment will potentiate the IL-8 suppressing effects of WPI hydrolysates.

5) Pressurized whey supplementation will result in improvements in nutritional status, markers of inflammation, and lung function in patients with cystic fibrosis.

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CHAPTER 2

LITERATURE REVIEW

2.1 Cystic Fibrosis: Epidemiology and Pathophysiology

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, and affects an estimated one in 3,600 live births in North America (WHO/ECFTN, 2002). The frequency is highest among Caucasians, where one in 25 are heterozygote carriers (Collins, 1992). The mutation affects a 250 kb gene on chromosome 7, which encodes a protein 1,480 amino acids long, CFTR, which functions as a cyclic AMP regulated chloride channel (Davis, 2006; Gao *et al.*, 1999) in epithelial cell membranes. More than 1,500 different mutations have been found so far (CCFF, 2008), the most common mutation being DelF508, which is caused by deletion of phenylalanine at position 508 and represents 70% of CF alleles in North America (Davis, 2006).

The median predicted survival age of CF patients has increased from 22.8 years in 1977 to 37.4 years in the United States and 46.6 years in Canada in 2008; about 58.8% of Canadians with CF are adults (CCFF, 2008; CFF, 2008). Pulmonary disease constitutes the major cause of morbidity and mortality (Davis, 2006; Gao *et al*., 1999); in fact, impaired lung function has been found to be the best predictor of mortality (Kerem *et al.*, 1993). However, lung disease is not the only clinical feature of CF. Other clinical characteristics include obstructive azoospermia resulting in infertility in 98% of males, and elevated chloride levels in sweat (Knowles and Durie, 2002).

Another important clinical feature of CF involves pancreatic insufficiency. This is due to obstruction of the pancreatic ducts, followed by tissue damage and subsequent loss of exocrine function. Although supplementation with fat-soluble vitamins and administration of pancreatic enzymes are standard practice in the care of CF patients with pancreatic insufficiency, malabsorption of protein, fat and fat-soluble vitamins is seldom

entirely resolved and leads to a failure to meet the increased energy demands (Amin and Ratjen, 2008). These demands are increased due to endobronchial infection, which results in a hypermetabolic state. In turn, lung infections can lead to reduced food intake, further aggravating malnutrition (Ratjen and Doring, 2003).

Malnutrition plays an important prognostic role in CF, as pancreatic insufficiency and subsequent intestinal malabsorption adversely affect the patient's ability to fight infection and repair lung damage. In turn, increased energy demands and appetite suppression due to pulmonary disease lead to poor growth and nutritional status (Amin and Ratjen, 2008). The close relationship between lung function, nutritional status and clinical course in CF has been demonstrated in cross-sectional and longitudinal studies, where a strong correlation was found between a decline in nutritional status and a severe decrease in lung function, irrespective of bacterial status (Davis, 2006; Steinkamp and Wiedemann, 2002; Schoni and Casaulta-Aebischer, 2000; Konstan *et al.*, 2003). At an age as young as 3 years, CF patients exhibit considerable growth retardation as shown by mean weight-for-age and height-for-age values that are 0.5 standard deviations below the 50^{th} percentile. These growth indexes were shown to be strong predictors of lung function at the age of 6 years, and independently of clinical indexes of pulmonary disease (Konstan *et al.*, 2003).

Pulmonary symptoms include mucus accumulation, infection by several opportunistic pathogens particularly *Pseudomonas aeruginosa* (PsA), and chronic inflammation (Gao *et al.*, 1999). One explanation for bacterial colonization involves the reduced chloride secretion by airway epithelial cells and increased sodium reabsorption, which result in reduced water content of the apical surface fluid, causing inhaled bacteria to be trapped in the thick mucus and slowing mucociliary clearance (Ratjen and Doring, 2003; Saiman, 2004). Inflammation in CF is characterized by activation of immune and inflammatory cells, specifically neutrophils, and their recruitment to the lungs, where they secrete cytokines, oxidants and other pro-inflammatory mediators (Rahman and MacNee, 1998). In addition, it has been shown that airway epithelial cells also secrete cytokines, chemokines, and oxidants (Thompson *et al.*, 1995). This leads to a chronic state of inflammation, which results in tissue injury (Rahman and MacNee, 1998). The ensuing

damage to the epithelium, which is the first line of defence against infectious agents, sets the stage for a vicious cycle of infection-inflammation.

2.1.2 Cystic Fibrosis: Inflammation and Anti-Inflammatory Therapy

CF pulmonary disease is mainly characterized by an exaggerated and persistent inflammatory response, and a sustained influx of neutrophils into the airways. The bronchoalveolar lavage fluid (BALF) of CF patients contains high concentrations of neutrophils and pro-inflammatory mediators including interleukin (IL)-8, tumor necrosis factor (TNF)-α, IL-6, leukotriene (LT)B4, and low levels of nitric oxide (NO) and the anti-inflammatory cytokine IL-10 (Terheggen-Lagro, 2005; Koehler, 2004). In addition to proinflammatory cytokines, neutrophils secrete oxidants and proteinases such as elastase, which cause further damage to the respiratory epithelium. DNA release from the nuclei of degenerated neutrophils also contributes to increased viscosity of mucus/sputum. The above events lead to structural damage to the airway epithelium, perpetuating susceptibility to bacterial infection and resulting in decreased lung function, characterized by decreased % predicted Forced Expiratory Volume in 1 second (FEV_1) (Downey *et al.*, 2009), mucopurulent plugging, bronchiectasis and intermittent bronchopneumonia (Koehler, 2004).

The involvement of the absent or decreased function of the chloride channel CFTR in the process of inflammation is unclear. Reduced chloride secretion and increased sodium reabsorption by airway epithelial cells result in reduced water content of the apical surface fluid, causing inhaled bacteria to be trapped in the thickened mucus and slowing mucociliary clearance (Ratjen and Doring, 2003; Saiman, 2004). In some infants and young children with CF, however, airway inflammation has been observed as an early event that is identified in the absence of positive bacterial or viral cultures. The BALF of some infants with CF has been found to have high concentrations of neutrophils and IL-8 without evidence of infection (Balough *et al.*, 1995; Armstrong *et al.*, 1995; Khan *et al.*, 1995). Several studies have shown a constitutively elevated expression of IL-8, IL-6 and constitutive activation of nuclear factor (NF)-κB in CF human glandular cells (Kammouni

et al., 1997; Tabary *et al.*, 1999). Moreover, Tabary *et al.* (1999) have shown that reducing extracellular chloride concentrations could correct the increased IL-8 secretion by CF human glandular cells. The CF airway therefore seems to be characterized by a markedly dysregulated cytokine profile, although it is unclear whether this is directly related to the absence of a functional CFTR (e.g. arising from a dysregulated ion transport in the airway epithelium regardless of the presence of infection). Weber *et al.* (2001) have shown that absent Cl^- channel function contributes to endogenous NF-κB activation. In addition, the ΔF508 mutation leads to the mistrafficking of CFTR and its accumulation in the endoplasmic reticulum, causing cell stress leading to NF-κB activation (Weber *et al.*, 2001). The inherent pro-inflammatory nature of the CF pulmonary epithelium is also suggested by models of CF fetal tracheal xenografts in mice, which show that CF grafts, before the onset of infection, have increases in the localization of subepithelial leukocytes as well as augmented airway lumen IL-8 secretion (Tirouvanziam *et al.*, 2000; Puchelle and Peault, 2000; Puchelle *et al.*, 2001). Airway epithelial cells are sentinel players in the innate immune system, as they respond to neutrophilic stimuli such as TNFα, neutrophil elastase, or to bacterial gene products via Toll-like receptors (TLRs), by secreting IL-8. The modulation of the epithelial inflammatory response is therefore an important therapeutic target in the treatment of CF.

The secretion of IL-8, which is now known as CXCL8 according to the new nomenclature system for chemokines, results primarily in the chemotactic recruitment of neutrophils to a site of inflammation, and angiogenesis. It is part of the CXC family of chemokines, characterized by the presence of one non-conserved amino acid separating the first two cysteine residues at the NH_2-terminal, and is considered the prototypical chemokine (Remick, 2005; Mukaida, 2003; Strieter, 2002). It is not constitutively produced, but its secretion is induced by a number of stimuli, including the early proinflammatory cytokines IL-1 and TNFα (Standiford *et al.*, 1990), bacteria and their gene products (DiMango *et al.*, 1995), viruses and their products (Garofalo *et al.*, 1996; Mahé *et al.*, 1991), oxidants and oxidative stress (DeForge *et al.*, 1993; Shono *et al.*, 1996), and other cellular stress such as hypoxic conditions (Xu *et al.*, 1999) or osmotic stress (Shapiro and Dinarello, 1995; Hoffman *et al.*, 2002). The IL-8 gene is encoded on

human chromosome 4, and contains promoter sequences for the transcription factors NF-κB, activator protein (AP)-1 and CAAT/enhancer-binding protein (C/EBP). In order to activate IL-8 mRNA transcription, NF-κB interacts with either C/EBP or AP-1, although only NF-κB is indispensable in some cell types. These processes are regulated upstream by mitogen-activated protein (MAP) kinases (C-jun N-terminal kinase, extracellular-regulated kinase), by NF-κB dependent or independent processes. Additionally, p38 MAPK affects IL-8 production post-transcriptionally by stabilizing IL-8 mRNA (Li *et al.*, 2002; Strieter, 2002; Hoffman *et al.*, 2002, Roebuck, 1999). IL-8 is secreted by leukocytic as well as nonleukocytic somatic cells, including epithelial cells, and in addition to neutrophil, basophil and T lymphocyte chemotaxis, it affects neutrophil function in a number of ways, including but not limited to the induction of L-selectin shedding, degranulation, and the respiratory burst (Remick, 2005; Mukaida, 2003).

Pulmonary epithelial cells can contribute to the inflammatory response by secreting a cocktail of inflammatory mediators, including IL-6, IL-8, granulocyte/macrophage colony-stimulating-factor (GM-CSF), regulated upon activation, normal T cell expressed and secreted (RANTES), epithelial neutrophil activating peptide (ENA)-78, and monocyte chemotactic protein (MCP)-1 (Stellato *et al.*, 1995; Bédard *et al.*, 1993; Crestani *et al.*, 1994; van Wetering *et al.*, 2002). Among these, IL-8 has received much attention as a major player in the process of inflammation in CF via its role as a powerful neutrophil chemoattractant. A substantial contribution of IL-8 to neutrophil chemotactic activity in CF sputa has been reported (Richman-Eisenstat *et al.*, 1993). IL-8 concentrations are characteristically elevated in the BALF of CF patients, often as early as infancy and even after controlling for bacterial burden (Muhlebach *et al.*, 1999; Muhlebach and Noah, 2002; Dean *et al.*, 1993; Khan *et al.*, 1995). Clinical findings involving BALF or bronchial tissue from CF patients show more consistently or more markedly increased IL-8 concentrations than, for example, IL-6 or IL-1β (Noah *et al.*, 1997; Tabary *et al.*, 1998).

There is considerable debate regarding the relationship between infection and inflammation in CF. For a given burden of infection, the BALF from CF patients

typically exhibits quantitatively higher concentrations of IL-8 and neutrophils than non-CF patients (Noah *et al.*, 1997; Muhlebach *et al.*, 1999; Chmiel and Davis, 2003), suggesting an excessive inflammatory response relative to bacterial load. Neutrophilic inflammation has been detected in the BALF of CF infants in the absence of detectable infection or colonization by bacterial pathogens (Khan *et al.*, 1995; Muhlebach 1999). Much research has been directed at determining whether the inflammatory response is inherent to the CF lung and independent of infection, or is exaggerated, dysregulated and persistent following infection. Tissue and cell culture studies have yielded conflicting evidence in this regard. Several reports show a higher constitutive IL-8 secretion by bronchial epithelial or gland cells expressing mutant CFTR when compared to cells expressing wild-type CFTR (Muhlebach *et al.*, 2004; Tabary *et al.*, 1998; Tabary *et al.,* 2000; Kammouni *et al.*, 1997; Weber *et al.*, 2001). In contrast, a number of other studies have found no difference in basal IL-8 secretion between CF and non-CF cells (Schwiebert *et al.*, 1999; Pizurki *et al.*, 2000; Scheid *et al.*, 2001; Black *et al.*, 1998), and some report a decreased constitutive secretion of IL-8 by CF cells (Massengale *et al.*, 1999). In response to *P. aeruginosa*, bacterial gene products or other stimuli such as IL-1β or TNFα, there seems to be no consensus either regarding IL-8 secretion by CF cells in comparison to wild-type cells, despite the abundance of available data. Studies either show an augmented stimulated IL-8 secretion or NF-κB activation in CF cells (Aldallal *et al.*, 2002; Scheid *et al.*, 2001), a lower magnitude of response (Massengale *et al.*, 1999; Carrabino *et al.*, 2006), no consistent differences (Schwiebert *et al.*, 1999; Bédard *et al.*, 1993; Pizurki *et al.*, 2000; Black *et al.*, 1998), or initially similar responses but with differences becoming more apparent after long-term incubation with *P. aeruginosa* or bacterial supernatants, suggesting an elevated and more sustained response by CF cells when assessed over time (Joseph *et al.*, 2005; Becker *et al.*, 2004). Such discrepancies could be explained in part by differing experimental designs, the use of immortalized cell lines or primary cells, the comparison of nonisogenic cell lines, cell culture conditions (e.g. submerged cells on plastic culture plates or in an air-liquid interface), the bacterial strains used or the origin of bacterial products. Nevertheless, although it is not yet fully clear whether the apparent hyperinflammatory response is intrinsic to CF epithelia, or is a consequence of early infection and/or the accumulation of large amounts of bacteria, data

from clinical studies are in agreement that the airways of patients with CF show high concentrations of proinflammatory mediators, and that this inflammatory response is sustained and exaggerated compared to healthy subjects, after controlling for bacterial burden (Noah *et al.*, 1997; Muhlebach *et al.*, 1999; Konstan and Berger, 1997; Khan *et al.*, 1995).

The presence of high concentrations of IL-8 and infiltrating neutrophils in the CF airway points to the suppression or reduction of IL-8 secretion as an appealing therapeutic target (Konstan and Davis, 2002). Fully human monoclonal antibodies (mAbs) recognizing IL-8 have been developed and shown to interfere with IL-8 binding to neutrophils as well as with chemotaxis and a number of IL-8-dependent neutrophil functions *in vitro* (Yang *et al.*, 1999; Kurdowska *et al.*, 1995). A randomized, double-blind, placebo-controlled pilot clinical trial involving COPD patients has shown that intravenous administration of an anti-IL-8 mAb was well tolerated and resulted in an improvement in the transitional dyspnea index score. However, no improvements in lung function were observed (Mahler *et al.*, 2004). The safety and efficacy of another anti-IL-8 mAb has recently been shown in patients with palmoplantular pustulosis, an inflammatory condition heavily involving neutrophils (Skov *et al.*, 2008). As of yet, no such mAbs have been evaluated in CF. Other approaches such as the development of antagonists to the IL-8 receptor CXCR2 have been considered (Widdowson *et al.*, 2004; Quan *et al.*, 1996). Some corticosteroids and macrolide antibiotics, employed as anti-inflammatory therapeutics in CF, have been shown to exert their effects in part by down-regulating the production of IL-8 (Suzuki *et al.*, 1997; Noah *et al.*, 1998; Hashimoto *et al.*, 2000; Mukaida *et al.*, 1994; Dauletbaev *et al.*, 2009). Other targeted approaches to the control of inflammation via IL-8 down-regulation, such as the modulation of NF-κB signalling, or blocking of TLR4, have been proposed (Koehler, 2004; Konstan and Davis, 2002). Strategies such as interferon-γ therapy (Moss *et al.*, 2005), or the administration of recombinant IL-10 (Chmiel *et al.*, 1999), two cytokines whose effects include IL-8 down-regulation (de Waal Malefyt *et al.*, 1991; Wang *et al.*, 1995; Schnyder-Candrian *et al.*, 1995), are under investigation.

2.1.3 Pathways to IL-8 Secretion

Airway epithelial cells respond to a variety of stimuli, activating pathways that converge to upregulate IL-8 secretion. Notably, TLRs that are located either internally or on epithelial cell surfaces, recognize highly diverse pathogen-associated molecular patterns (PAMPs). In humans, at least 10 different receptors populate the TLR family, each recognizing a specific set of PAMPs. Functional TLRs 1-6 and TLR9 have been found to be expressed in airway epithelial cells (Greene *et al.*, 2005; Mayer *et al.*, 2007), and mRNA for TLRs 1-10 were expressed by such cells in one study (Muir *et al.*, 2004). Table 1 lists the different TLRs and their respective ligands.

Table 1. Toll-like receptors and their corresponding ligands

Receptor	Ligand	Location
TLR1	Triacylated lipopeptide (with TLR2)	Monocytes, plasmacytoid dendritic cells (pDCs), B, T, NK cells
TLR2	Triacylated lipopeptide (lipoteichoic acid, LTA from Gram-positive bacteria), diacylated lipopeptide (e.g. MALP-2 from mycoplasma), peptidoglycans, zymosan, heat shock proteins	NK cells, neutrophils, mast cells, myeloid dendritic cells (mDCs), T cells
TLR3	dsRNA, Poly(I:C)	Fibroblasts, mDCs, NK cells
TLR4	LPS (Gram-negative bacteria), F protein (murine retroviruses), heat shock proteins, fibrinogen fragments, respiratory syncytial virus (RSV) fusion protein. Neutrophil elastase, hyaluronan (endogenous)	Epithelial, endothelial cells; mast cells, monocytes, neutrophils; regulatory/gamma-delta T cells
TLR5	Flagellin (Gram-negative bacteria)	Epithelial, monocytes, NK cells, mDCs
TLR6	Diacylated lipopeptide (with TLR2), zymosan	Mast, B cells mDCs, pDCs, monocytes

TLR7	ssRNA, imidazoquinoline Principal ssRNA receptor in mice.	B cells, eosinophils, pDCs, neutrophils
TLR8	ssRNA, imidazoquinoline Principal ssRNA receptor in humans.	NK, T cells, mDCs, monocytes
TLR9	Bacterial DNA (unmethylated CpG)	pDCs, NK, B cells; tonsillar cells
TLR10	Unidentified	pDCs, B cells

Sources: Greene and McElvaney, 2005; Greene *et al.*, 2008; Asea *et al.*, 2002; Gribar *et al.*, 2008; Akira, 2003; Iwasaki and Medzhitov, 2004; Hopkins and Sriskandan, 2005; Hornung *et al.*, 2002.

TLR4, the best known cell surface TLR, recognizes lipopolysaccharide (LPS), originating from the cell wall of Gram-negative bacteria. The activation of TLR4 by LPS necessitates the presence of other proteins such as cluster of differentiation (CD)14, myeloid differentiation protein (MD)-2 and LPS binding protein (LBP), and results in the recruitment of the adaptor proteins myeloid differentiation factor 88 (MyD88) and MyD88 adaptor-like (Mal) (Greene and McElvaney, 2005). In turn, MyD88 associates with the IL-1 receptor-associated kinases (IRAKs) and TNF receptor associated factor-6 (TRAF6) and dissociates from the TLR4 complex. This results in activation of the Inhibitory (I)-κB Kinase (IKK) complex, which catalyses IκBα phosphorylation and subsequent degradation by the proteasome, thereby allowing NF-κB to translocate into the nucleus, where it activates the gene transcription of IL-8 and other pro-inflammatory cytokines (Akira, 2003). An alternative, MyD88 independent pathway, is used by TLRs 2 and 4 and involves the recruitment of the adaptors Toll-IL-1R domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), inducing Interferon regulatory factor (IRF)-3 activation and the subsequent production of type I IFN (Greene and McElvaney, 2005). Figure 1 depicts the signalling cascades originating from the activation of TLR4, IL-1 receptor (IL-1R) and TNF receptor (TNFR) and leading to the transcription of proinflammatory mediators, namely IL-8. As shown in the figure, stimulation of cells with IL-1β through the IL-1 receptor (IL-1R) activates the same MyD88-dependent pathway as associated with TLRs. Indeed, members of the IL-1R

family and TLRs share homology in the intracellular domain responsible for signalling, which is termed Toll/IL-1R (TIR) (Verstrepen *et al.*, 2008). In addition, exposure to TNFα can also lead to NF-κB activation via the TNF receptor (TNFR), which recruits the adaptor protein TNF receptor-associated death domain (TRADD). TRADD then associates with additional adaptor proteins, TNF-R-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), initiating a signalling cascade that leads to NF-κB activation (Hsu *et al.*, 1995 and 1996; Chen and Goeddel, 2002).

The possibility that the CFTR mutation affects the expression of TLRs and thereby influences airway hyperresponsiveness and the inflammatory process has been investigated. Muir *et al.* (2004) found that both CF and non-CF respiratory epithelial cell lines expressed mRNA for all 10 known TLRs, although some TLRs such as TLR7 or TLR8 have not been detected on the surfaces of either cell type (Greene *et al.*, 2005; Mayer *et al.*, 2007). Some studies report no major differences in the basal expression of most TLRs between CF and non-CF cell lines (Muir *et al.*, 2004; Firoved *et al.*, 2004; Greene *et al.*, 2005), while others have detected differences, such as decreased TLR4 expression (John *et al.*, 2010), or increased TLR5 expression (Blohmke *et al.*, 2008) in CF cells in comparison to wild-type cells. In response to bacterial exposure or to stimulus by a TLR2/6 agonist, diacylated lipopeptide (MALP-2), some report a slight increase in surface TLR2 in CF cells but not in normal cells (Muir *et al.*, 2004), or increased TLR2-dependent signalling in CF cells relative to non-CF cells (Firoved *et al.*, 2004; Greene *et al.*, 2005). However, some of these works (Greene *et al.*, 2005) compared nonisogenic cell lines and confirmatory studies are needed to rule out specific differences that may be inherent to the cell lines used. Table 2 summarizes some of the findings regarding the behaviour of different TLRs in CF and normal cells.

Table 2. Toll-like receptors in CF and non-CF cells

Cell Type	Stimulus	Findings	Authors
Wild-type, CF and CFTR-corrected respiratory	*Staphylococcus aureus* and *P. aeruginosa*	No effect of CFTR mutation on expression of TLRs. Small consistent increase in surface TLR2 in CF cells after	Muir *et al.*, 2004

epithelial cell lines		bacterial exposure. Magnitude of increase in IL-8 expression in response to stimulus: 40-fold in CF cells vs. 20-fold in non-CF cells. No differences in TLR distribution in lung sections of *cftr* -/- vs. wild-type mice.	
Wild type, CF and CFTR-corrected respiratory epithelial cell lines; primary normal bronchial epithelial cells	Lipopeptides from *P. aeruginosa*	No difference in unstimulated TLR2 expression between CF and non-CF cells. Augmented TLR2-dependent activation of NF-κB in response to lipopeptides in CF vs. non-CF cells.	Firoved *et al.*, 2004
Wild-type and CF respiratory epithelial cell lines	MALP-2, triacylated lipopeptide (Pam3), LPS from *P.aeruginosa*, uCpG	Cell surface expression of TLRs1–5 and TLR9 detected in CF and non-CF cells. Higher basal levels of IL-8 secretion by CF cells, but no difference in relative amounts of IL-8 induced by stimuli between CF and non-CF cells. Only CF cells respond to MALP-2.	Greene *et al.*, 2005
Wild-type, CF and CFTR-corrected respiratory epithelial cell lines	Living and heat-inactivated *P.aeruginosa*; LPS from *Salmonella enterica sv* Arizona	CFTR-corrected cells secrete more IL-8 than CF cells basally and in response to bacteria or LPS. Higher basal surface expression of TLR4 in CFTR-corrected cells, and slightly greater increase in response to LPS stimulus vs. CF cells.	John *et al.*, 2010
CF and CFTR-corrected respiratory epithelial cell lines; PBMCs from CF and healthy patients	Heat-killed, wild-type and flagellin-mutant strains of *P. aeruginosa*; *Burkholderia multivorans; Burkholderia cenocepacia*; pure TLR ligands	CF respiratory epithelial cells and PBMCs produce markedly more elevated levels of IL-6 in response to flagellin or bacterial exposure. Minimal response to TLR ligands other than flagellin. Augmented response by CF cells attributed to higher TLR5 expression and responsiveness.	Blohmke *et al.*, 2008

Much research is needed to more fully characterize the behaviour of different TLRs in CF cells compared to normal cells, and how these differences may influence the inflammatory response by airway epithelial cells.

2.1.4 Oxidative Stress in CF

Oxidative stress plays an important role in the pathophysiology of CF. In addition to cytokines/chemokines, neutrophils release reactive oxygen species (ROS). The transcription factors NF-κB and AP-1 can be activated in response to oxidative/nitrosative stress (Rahman and MacNee, 1998). Oxidative stress can thereby promote the transcription and secretion of more inflammatory mediators by airway epithelial cells such as TNF-α, IL-1 and IL-8 which, in turn, induce further neutrophil recruitment, thus perpetuating the inflammatory response and tissue injury. This leads to an imbalance in the redox status towards a state of oxidative stress, and a concomitant depletion of glutathione, the most abundant thiol reducing agent in mammalian tissue (Rahman and MacNee, 2000). In its reduced form, glutathione (GSH) is a tripeptide thiol (L-γ-glutamyl-L-cysteinyl-glycine) ubiquitously found in mammalian cells. The reactive sulfhydryl group present in the cysteinyl moiety is the key functional group that confers GSH its ability to play a number of crucial roles in cellular metabolism. Critical cellular functions attributed to GSH include regulating the activation and expression of redox-sensitive transcription factors (Dröge *et al.*, 1994; Haddad *et al.*, 2000), regulation of redox equilibrium and sulfhydryl status, and antioxidant activity via the peroxidase-coupled reaction (Hayes and McLellan, 1999). In the lung, GSH is present in the epithelial lining fluid (ELF) at high concentrations (200 – 300 μmol/L, as opposed to less than 5 μmol/L in plasma) (Hudson, 2001; Kelly, 1999; Cantin *et al.*, 1987), where it confers powerful antioxidant protection in a milieu highly exposed to reactive oxygen and nitrogen species. GSH concentrations in the ELF are altered in several respiratory diseases associated with oxidative stress; they are upregulated in patients suffering from primary pulmonary hypertension (Kaneko *et al.*, 1998), asthma (Smith *et al.*, 1993) and chronic smokers (Cantin *et al.*, 1987; Morrison *et al.*, 1999), and decreased in patients with idiopathic pulmonary fibrosis (Cantin *et al.*, 1989) and cystic fibrosis (Roum *et al.*,

1993). In CF patients, reduced GSH concentrations are not limited to the ELF as they have also been observed in the plasma, denoting to a systemic deficiency (Roum *et al.*, 1993; Hudson, 2001; Lands *et al.*, 1999). The CFTR mutation has been linked to the chronic and progressive decrease in the reduced extracellular GSH:GSSG ratio as it is also permeant to larger organic anions (Lindsell and Hanrahan, 1998; Hudson, 2001; Gao *et al.*, 1999). A decreased efflux of GSH into the extracellular environment is thought to be a direct consequence of the lack of functional CFTR (Hudson, 2001). The consequences of reduced GSH concentrations in the lung are several fold. The decreased antioxidant protection places the pulmonary epithelium at greater risk of oxidant induced damage, contributing to fibrosis and the impairment of lung function (Rahman *et al.*, 1999; Rahman and MacNee, 1999; Kelly, 1999; Lands *et al.*, 1999). Furthermore, exposure to oxidants inactivates the anti-protease system (Vogelmeier *et al.*, 1997; Gillissen *et al.*, 1993; Buhl *et al.*, 1996), which leads to increased levels of neutrophil elastase (NE). The ramifications of the latter phenomenon are many, including the stimulation of mucus secretion by goblet cells (Nadel, 2000), facilitation of adherence of *P. Aeruginosa* (Plotkowski *et al.*, 1989), and inhibition of ciliary beating (Amitani *et al.*, 1991). Due to its free sulphydryl group, GSH has the ability to cleave disulfide bonds in proteins via thiol-disulfide exchange, a reaction catalyzed by thiol transferase:

Protein – SSG + GSH ↔ Protein – SH + GSSG (DeLeve and Kaplowitz, 1991)

Because the cleavage of disulfide bonds between mucus glycoproteins promotes mucolysis, a deficiency in GSH therefore leads to increased viscoelasticity of mucus, which inhibits ciliary beating and further facilitates bacterial colonization (Houtmeyers *et al.*, 1999; Fuloria and Rubin, 2000; Smith *et al.*, 1996). Another effect of cellular GSH deficiency is the triggering of inflammation, as it is directly related to increased transcription of AP-1 and NF-κB (Cho *et al.*, 1998; Haddad *et al.*, 2000; Sen *et al.*, 1997). Conversely, improving GSH status or the intracellular GSH:GSSG ratio via supplementation or treatment with glutathione ethyl ester (GEE), or cysteine-donating compounds such as *N*-acetylcysteine (NAC), has been shown to attenuate the inflammatory response *in vivo* (Tirouvanziam *et al.*, 2006; Koike *et al.*, 2007) and *in vitro* (Haddad *et al.*, 2001). In addition, GSH deficiency is also related to a decreased oxidative burst by neutrophils (Atalay *et al.*, 1996), decreased phagocytosis, and causes premature

apoptosis (Watson *et al.*, 1996; O'Neill *et al.*, 2000). The increase in the inflammatory response is therefore accompanied by a reduction of the bactericidal ability of neutrophils and macrophages.

Glutathione is synthesized in a two-step process, each catalyzed by a cytosolic enzyme. Gamma-glutamylcysteine synthetase (GCS) catalyzes γ-glutamylcysteine formation from cysteine (L-cys) and glutamate (L-glu), and glutathione synthetase (GS) catalyzes GSH formation from glycine and γ-glutamylcysteine. Both reactions are ATP-dependent, with the first being the rate-limiting step and subject to feedback inhibition by GSH (Seelig and Meister, 1985; Wu *et al.*, 2004; Lu, 2009):

$$\text{L-Glutamate + L-cys + ATP} \leftrightarrow \text{L-}\gamma\text{-glutamyl-L-cysteine + ADP + } P_i$$

$$\text{L-}\gamma\text{-Glutamyl-L-cysteine + glycine + ATP} \leftrightarrow \text{glutathione + ADP + } P_i$$

In addition to feedback inhibition by GSH, the first step in GSH synthesis by GCS is also regulated by the availability of precursor amino acids, most notably cysteine (De Leve and Kaplowitz, 1991; Lu, 2009).

Epithelial cells can also obtain glutathione from the extracellular milieu through the membrane-bound enzyme γ-glutamyltranspeptidase (GGT). GGT hydrolyzes extracellular GSH into cysteinyl-glycine and a γ-glutamyl moiety, which is then coupled to cystine, resulting in the formation of γ-glutamylcystine. The latter is then imported into the cell and reduced to γ-glutamylcysteine and cysteine. Finally, the action of GS results in the resynthesis of GSH by the addition of glycine (Berggren *et al.*, 1984; Hayes and McLellan, 1999).

The presence of GSH as a substrate is instrumental in the detoxification of lipid peroxides and hydrogen peroxide (H_2O_2). A selenium-dependent enzyme, GSH peroxidase, catalyzes the reduction of peroxides by GSH, resulting in the formation of glutathione disulfide (GSSG). GSH-*S*-transferase can also catalyze the detoxification of organic peroxides by GSH. GSH can then be restored from GSSG through the action of glutathione reductase, in the presence of nicotinamide adenine dinucleotide phosphate

(NADPH), thereby maintaining GSH:GSSG homeostasis (Rahman *et al.*, 1999; Lu, 2009; De Leve and Kaplowitz, 1991; Rahman and MacNee, 2000).

In view of the role played by GSH in the regulation of redox status, the protection against oxidative injury, and the modulation of immune responses, reducing oxidative stress and increasing tissue GSH concentrations have been the targets of antioxidant therapies in CF (Cantin *et al.*, 2007; Rahman and MacNee, 2000). Attempts have been made at improving GSH status in a number of pulmonary diseases via the administration of GSH intravenously, orally, or by aerosol inhalation (Roum *et al.*, 1999; Buhl *et al*., 1990; Borok *et al.*, 1991). Intravenous administration of GSH does not lead to an augmentation of GSH levels in the ELF due to its short plasma half-life (Buhl *et al.*, 1990; Wendel and Cikryt, 1980). The use of amino acid or peptide precursors of GSH such as NAC, 2-oxothiazolidine-4-carboxylate (OTC) or *S*-adenosyl-L-methionine (SAM) or other thiol derivatives have also been investigated (Haddad *et al.*, 2001; Geudens *et al.*, 2008; Tirouvanziam *et al.*, 2006; Meyer *et al.*, 1994), with variable success. A recent Cochrane review into the effects of oral and nebulized thiol derivatives in CF has found no significant clinical benefit of such therapies (Nash *et al.*, 2009).

2.1.5 Therapies for CF

The search for safe, effective anti-inflammatory therapies in CF is ongoing. Several studies have investigated the use of oral corticosteroids, showing improvements in lung function as assessed by FEV_1 and forced vital capacity (FVC), decreased inflammation (erythrocyte sedimentation rate (ESR) & Immunoglobulin (Ig)G, Auerbach *et al.*, 1985) and a decreased frequency of pulmonary exacerbations. However, oral corticosteroids have exhibited unacceptable adverse effects, including glucose intolerance, growth impairment, cataract formation, and bone fractures (Auerbach *et al.*, 1985; Eigen *et al.*, 1995; Rosenstein and Eigen, 1991; Dinwiddie, 2005; Koehler, 2004). Delivery of corticosteroids through the inhalation route can decrease the occurrence of systemic side effects, but there is a paucity of studies convincingly demonstrating long-term efficacy (Schiotz *et al.*, 1983; Van Haren *et al.*, 1995; Koehler, 2004). The non-steroidal anti-

inflammatory drug ibuprofen has been shown to slow the decline of lung function and weight loss in CF patients; however, the frequency of hospitalizations has been unaffected. Other limitations include a small risk of gastrointestinal haemorrhage, as well as the need for regular monitoring of plasma ibuprofen levels, which have prevented its more widespread use as a long-term therapeutic agent (Cardario and McKinnon, 1991; Dinwiddie, 2005). Macrolide antibiotics present a promising prospect for the treatment of CF, as they have been shown to significantly improve FEV_1 and FVC. However, much research is still needed to determine their effectiveness in patient subgroups such as young children, the appropriate dosage, and long-term efficacy. Other concerns are possible gastrointestinal side effects such as diarrhea and nausea, and the potential development of resistant organisms (Florescu *et al.*, 2009; Yousef and Jaffe, 2010; Equi *et al.*, 2002; Wolter *et al.*, 2002; Saiman *et al.*, 2003 and 2010).

CF is a multisystem disease. Effective treatment needs to be multifaceted, and must address the many pathophysiologic aspects of the disease, including nutritional support, airway clearance techniques, antimicrobial, antioxidant and anti-inflammatory therapies. Although tremendous progress has been achieved in extending current understanding of CF pathogenesis and in improving treatment, resulting in a markedly improved prognosis over the past four decades, there remains a clear need for new therapies with established long-term safety and efficacy.

2.2 Whey Proteins: an Overview

Whey proteins, a by-product of the cheese making industry (Walzem *et al.*, 2002), are the proteins that remain soluble in milk after precipitation of caseins either at low pH (4.6) and 20^{o}C or by rennet (Farrell *et al.*, 2004). They constitute 20% of total proteins in cow's milk (Walzem *et al.*, 2002). Depending on the type of cheese produced and the method of manufacture, either sweet whey (derived from ricotta, cottage or cream cheese) or acid whey (derived from mozzarella, cheddar or Swiss cheese) can be produced. Technological progress has allowed for an expansion of whey-based products such as demineralized whey, reduced lactose whey, specific or single whey protein fractions, as

well as whey protein concentrates (WPC), with a protein content ranging from 34 to 80 percent) and whey protein isolates (WPI), with a protein content of no less than 90 percent (USDEC, 2005). Whey proteins consist of five major proteins; ß-lactoglobulin (ß-LG), α-lactalbulmin (α-LAC), bovine serum albumin (BSA), glycomacropeptide (GMP) and immunoglobulins (Ig), including IgA, IgM, and IgG (Farrell *et al.* 2004), each having unique functional, nutritional or nutraceutical properties. In addition, whey contains a number of minor proteins occurring in low abundance, the most notable being lactoferrin (Lf) and lactoperoxidase (LPO), as well as a number of growth factors (de Wit, 1998). The complete composition of bovine whey proteins is shown in Table 2.

Table 3. Composition of Whey Proteins

Protein	Concentration in Milk (g/L)	% of Whey Proteins	MW (g/mol)
Major Proteins			
β-lactoglobulin	2.0 – 4.0	55 – 65	18,400 – 36,800
α-lactalbumin	1.0 – 1.5	15 – 25	14,200
Immunoglobulins	0.6 – 1.0	10 – 15	160,000
Bovine serum albumin	0.1 – 0.4	5 – 10	66,000
Glycomacropeptide	–	10 – 15	700
Minor Proteins			
Lactoferrin	0.2	1 – 2	80,000
Lactoperroxidase	0.03	0.5	77,500
Proteose-peptones	0.6 – 1.8	<1	4,000 – 80,000

Adapted from Beaulieu *et al.*, 2006; de Wit, 1998; Marshall, 2004; Dairy Export Council, 2005.

Compared to caseins, the secondary and tertiary structures of whey proteins are generally more organized; they are globular molecules with molecular weights ranging from 0.7 to 900 KDa, and containing disulfide bonds which confer stability to their structure (de Wit, 1998; Creamer *et al.* 1983). These conformational features have been implicated in the

observed resistance of most whey proteins to enzymatic digestion (Reddy, 1988; Singh and Creamer, 1993). Furthermore, during digestion, casein micelles are hydrolyzed by pepsin to a much greater extent than whey proteins, as they form clots and exit the stomach slowly, as opposed to whey proteins, which, under acidic conditions do not coagulate and have a much shorter gastric half-emptying time (Mahé *et al.*, 1995; Boirie *et al.*, 1997). Indeed, intact whey proteins (namely ß-LG, α-LAC, Ig, and lactoferrin) have been found in the intestinal lumen in animal studies (Mahé *et al.*, 1996; Walzem *et al.* 2002).

As part of breast milk, the main function of whey proteins is their high biological value and high protein content in milk, which thus provides nourishment for the newborn for the first six months of life (Krissansen, 2007). Whey proteins are rich in essential amino acids, and have a high protein digestibility corrected amino acid score of 100, comparable to egg protein (Hoffman and Falvo, 2004). They are also rich (about 26%) in branched chain amino acids (BCAA) (Walzem *et al.*, 2002), which are primal for muscle protein synthesis (Ha and Zemel, 2003). A number of biological effects have been attributed to whey protein intake and to the peptides derived from them, including antioxidant activity, antimicrobial, antiviral, anti-carcinogenic and anti-inflammatory and ACE-inhibitory activities (Kirssansen, 2007; Brody, 2000; Clare and Swaisgood, 2000; Oner *et al.*, 2006). The immunomodulating effects of whey are of particular interest (Cross and Gill, 1999; Lin *et al.*, 2008; Low *et al.*, 2003; Mercier *et al.*, 2004; Loimaranta *et al.*, 1999). Indeed, the abundance of research into the potential nutritional and therapeutic applications of whey is such that it is no longer considered a by-product, but a major product of cheese manufacture (Walzem *et al.*, 2002).

2.2.1 Antioxidant Potential of Whey Proteins and Peptides

The antioxidant properties of whey have been linked to their high content of sulphur-containing amino acids (namely cysteine, a precursor for GSH synthesis) as well as γ-glutamyl-cysteine residues, which are more desirable for the synthesis of GSH, as they by-pass the rate-limiting step in GSH biosynthesis (Bounous *et al.*, 1991; Rahman and

MacNee, 2000). Indeed, increased lymphocyte GSH levels in healthy and CF patients have been achieved through dietary whey protein supplementation. In a randomized placebo controlled study, 18 healthy young adults were given either two 10 g-daily doses of whey protein or a casein control for 3 months. A significant 35.5% increase in lymphocyte GSH levels (a marker of tissue GSH) was observed in the whey-supplemented group, with no change in the placebo group. The whey supplemented group also exhibited improved skeletal muscle performance, as assessed by a 30-second isokinetic cycling sprint, and a decrease in percent body fat with no change in weight, suggesting an increase in fat-free mass (FFM) (Lands, Grey, and Smountas, 1999). The latter findings could have implications for CF patients beyond the increase in glutathione stores, as the loss of FFM is a common occurrence in CF, and has been associated with reduced inspiratory muscle function (Ionescu *et al.*, 1998; 2002). A similar whey protein supplementation regimen of 21 young adult patients with CF resulted in a significant 46.6% increase in lymphocyte glutathione after 3 months. Although there were no significant changes in lung function or body composition, the limited study sample size may have prevented the detection of such changes (Grey *et al.*, 2003). Furthermore, it is possible that increasing GSH levels may not reverse, but slow the progression of lung damage, an outcome that requires assessment over a longer period of time. Whey protein supplementation has been shown to improve GSH levels along with clinical or biochemical outcomes in a number of disease states, including hepatitis B (Watanabe *et al.*, 2000), HIV infection (Micke *et al.*, 2001), atopic asthma (Lothian *et al.*, 2006), acute ischemic stroke (de Aguilar-Nascimento *et al.*, 2011), and non-alcoholic steatohepatitis (Chitapanarux *et al.*, 2009).

Several animal studies have demonstrated the GSH-enhancing effects of whey protein feeding in a range of disease models. Animals placed on whey protein diets as the main source of protein exhibited an increase in liver GSH levels following resistance exercise (Haraguchi *et al.*, 2010), carbon tetrachloride intoxication (Balbis *et al.*, 2009), high carbohydrate diet-induced fatty liver (Hamad *et al.*, 2011), and in a guinea pig asthma model (Kloek *et al.*, 2011). Whey feeding also increased GSH levels in immune cells after acute ethanol ingestion (Tseng *et al.*, 2010), and in heart tissue after iron overload

(Bartfay *et al.*, 2003). Concomitant to increases in GSH concentrations, whey proteins protected corresponding tissues against oxidative stress (Bartfay *et al.*, 2003; Elia *et al.*, 2006; Blouet *et al.*, 2007) and tissue damage (Rosaneli *et al.*, 2002), and resulted in an increased humoral immune response (Bounous *et al.*, 1989; Bounous and Gold, 1991). Interestingly, Blouet *et al.* (2007) found that diets enriched with cysteine either in the form of NAC or as part of α-LAC ameliorated GSH status and protected against oxidative stress and glucose intolerance induced by high sucrose intake. However, in another study, the effect of NAC, but not α-LAC, on postprandial glucose levels was abrogated by prior injection with the GSH synthesis inhibitor buthionine sulfoximine (BSO), suggesting that α-LAC may exert its effects by other mechanisms, in addition to GSH synthesis (Blouet *et al.*, 2007b).

In vitro studies have shown an increase in intracellular GSH levels in response to whey protein isolate (WPI) by different cell types. Pre-treatment of PBMCs with WPI protected against subsequent alcohol-induced GSH reduction (Tseng *et al.*, 2008). Similarly, administration of WPI to a pheochromocytoma cell line prior to treatment with ethanol augmented intracellular GSH and had a weak non-significant protective effect against ethanol-induced cytotoxicity, but did not improve cell viability (Tseng *et al.*, 2006). Treatment with WPI has been shown to differentially modulate intracellular GSH levels in normal cells as opposed to cancer cells, exerting an enhancing effect in the former and a suppressing effect in the latter. These effects are associated with decreased proliferation of cancer cells (Baruchel and Viau, 1996), and enhanced cytotoxicity of an anticancer agent (Tsai *et al.*, 2000). These *in vitro* studies are limited by the use of intact undigested proteins to which cells or tissue other than the gut epithelium would not normally be exposed, as will be discussed below. Taking this into consideration, Kent *et al.* (2003) treated human prostate epithelial cells with WPI hydrolyzed with trypsin, chymotrypsin and peptidase, at doses up to 500 μg/mL. Treatment for 12 – 48 h significantly increased intracellular GSH concentrations while similar treatment with hydrolyzed casein had no effect. WPI also protected cells against oxidant induced cell death, and the addition of BSO abrogated the GSH enhancing effects of WPI. Although this study used enzymatically hydrolyzed WPI as opposed to intact protein, the hydrolysates were

unfiltered and may have contained digestive enzyme remnants including trypsin, and large peptide fragments, which may exhibit weak gut absorption or diminished biological activity (Roberts *et al.*, 1999). There is a dearth of *in vitro* data addressing the GSH-enhancing or antioxidant effects of peptides derived from the gastro-intestinal digestion of whey proteins. Nonetheless, the above set of studies indicating potentially potent biofunctional properties associated with whey protein intake has sparked increasing research investigating the biological properties of whey as functional foods.

In addition to their glutathione enhancing effects, amino acids and peptides derived from the hydrolysis of whey proteins have been shown to provide antioxidant activity in their own right. The antioxidant potential of whey derived peptides and amino acids has been investigated in a number of studies employing several different methodologies. Peptides from β-LG and α-LA exhibit free-radical scavenging activity *in vitro* (Hernández-Ledesma *et al.*, 2005), and inhibit iron-catalyzed lipid oxidation in liposomes (Colbert and Decker, 1991; Peña-Ramos *et al.*, 2004). Single amino acids with antioxidant capacity, such as cysteine, tyrosine or tryptophan (Udenigwe *et al.*, 2011) can also contribute to the overall antioxidant activity of whey hydrolysates. However, although many of the *in vitro* methods employed provide useful evidence of the antioxidant capacity of whey peptides in food systems, their results are difficult to extrapolate to biological systems. Methods commonly used to assess antioxidant capacity of foods include the oxygen radical absorbance capacity (ORAC) and the total radical trapping antioxidant parameter (TRAP) assays, which are based on the hydrogen-donating ability of an antioxidant and the subsequent scavenging of peroxyl radicals. Although convenient, these methods do not involve a chain propagation step following radical production, rendering questionable their relevance to the measurement of radical chain-breaking antioxidants (Antunes *et al.*, 1999). Furthermore, reliable modified versions of these assays capable of measuring lipophilic antioxidants have yet to be developed (Huang *et al.*, 2005). Other methods assessing the antioxidant capacity of food components include assays based on the reduction of an oxidant probe via single electron transfers, such as the ferric ion reducing antioxidant power (FRAP), the Trolox equivalent antioxidant capacity (TEAC), the 2,2-diphenyl-1-picrylhydrazyl radical scavenging

(DPPH), the Cu(II) reduction capacity assays, and the total phenols assay by Folin-Ciocalteu reagent (FCR). These assays involve the assumption that the reducing capacity of a sample is equivalent to its antioxidant capacity, since they do not employ oxygen radicals. These assays also present with other limitations. The TEAC values obtained from the measurement of pure antioxidants do not clearly reflect the number of electrons that can be donated, the reaction time for FRAP must be adjusted for certain compounds that react more slowly, and DPPH, a stable nitrogen radical, lacks resemblance with peroxyl radicals which are greatly reactive. Finally, the relevance of results obtained from such assays to *in vivo* situations has yet to be established, as they do not take into consideration the bioavailability of antioxidants when consumed and transformations they may undergo through the digestion and absorption process. The conditions in which the assays are conducted do not reflect physiological conditions, such as pH and temperature. Nonetheless, information gleaned from such assays can provide useful insight into the relative antioxidant activities of food molecules (Huang *et al.*, 2005).

2.2.2 Immunomodulatory and Anti-Inflammatory Potential of Whey Proteins and Peptides

The effects of whey, individual whey proteins or peptides derived from them on a variety of immune functions have been extensively investigated. A number of immune modulating effects of whey proteins have been reported, including but not limited to *in vitro* stimulation of peripheral blood lymphocyte proliferation (Kayser and Meisel, 1996), stimulation of neutrophil phagocytic activity (Miyauchi *et al.*, 1998); *in vivo* enhancement of the humoral immune response (Low *et al.*, 2003), and increased white blood cell count in mice with a parasitic infection (Ford *et al.*, 2001). An exaggerated and sustained inflammatory response being a hallmark of CF pathophysiology, evidence attributing anti-inflammatory effects to whey proteins is of particular interest. Such effects have been reported in a number of *in vitro*, animal and clinical studies, most notable of which are summarized in Table 3.

Table 3. Anti-inflammatory effects of whey proteins and peptides

Study objective	Study design	Results	Authors
Studies involving undigested whey proteins			
Effect of whey protein isolate, α-LA and β-LG on IL-6 release during ischemia/reperfusion.	Male Sprague-Dawley rats received duodenal injection of 300 mg/kg protein 1 h prior to ischemia.	WPI, α-LA and β-LG suppressed IL-6 release; more potent effect of α-LA, which may be mediated by NO.	Yamaguchi and Uchida, 2007
Effect of Lf in a rat model of adjuvant induced arthritis.	Daily oral Lf administered to male Wistar rats before and after adjuvant injection.	Suppression of TNF-α and increased IL-10 production in LPS-stimulated adjuvant-injected rats.	Hayashida *et al.*, 2004
Effects of Lf and LPO in a mouse model of influenza infection.	BALB/c mice orally administered Lf or LPO in solution before and after infection.	Lower numbers of infiltrated cells in the lungs of both Lf and LPO fed mice. No difference in BALF IL-6 levels between groups, but decreased serum IL-6 in LPO fed mice.	Shin *et al.*, 2005
Effect of GMP in a rat model of trinitrobenzene-sulfonic acid (TNBS)-induced colitis	GMP orally administered to female Wistar rats 2 d prior or 3 h post colitis induction.	Dose-dependent decrease in body weight loss, anorexia, colonic damage, colonic alkaline phosphatase activity and IL-1β.	Daddaoua *et al.*, 2005
Effect of a malleable protein matrix (MPM) of lactic acid bacteria-fermented whey protein in a mouse model of atopic dermatitis induced by oxazolone.	MPM orally administered to female CD-1 mice 7 d prior or post dermatitis induction.	Decreased ear inflammation and neutrophil extravasation. Effects comparable to the hydrocortisone positive control.	Beaulieu *et al.*, 2007
Effect of a MPM of lactic acid bacteria-fermented whey protein in a mouse air pouch model of inflammation.	MPM orally administered to female C57BL/6J mice 2 wks prior to creation of air pouch and LPS injection.	50% inhibition of infiltrating leukocytes, decreased TNFα, IL-1β, and IL-6. No effect on neutrophil phagocytosis.	Beaulieu *et al.*, 2009
Effect of dietary cheese whey	Male Wistar rats fed diets	Cheese whey and positive	Sprong *et al.*,

feeding in a rat model of dextran sulphate sodium (DSS)-induced colitis	containing casein (control), cheese whey, or casein + threonine/cysteine (positive control) for 14 d prior to DSS consumption for 7 d.	control diets decreased colonic expression of IL-1β, calprotectin and iNOS; softened stools, decreased diarrhea, and increased mucin secretion.	2010
Effect of bovine and human Lf and lactoferricin B on a monocytic cell line.	LPS-stimulated THP-1 cells incubated with Lf or lactoferricin B.	Both Lf and lactoferricin B decreased IL-6 production.	Mattsby-Baltzer *et al.*, 1996
Effect of bovine GMP in a rat model of TNBS-induced ileitis.	GMP (500 mg/kg/d) orally administered to female Wistar rats 2 d prior to induction of ileitis.	Significant reduction of intestinal damage and necrosis, normalization of MPO and AP activities, reduction of COX-2 and iNOS expression. Decreased levels of TNF-α, IL-1β, IL-1ra and IL-17 mRNA.	Requena *et al.*, 2008
Effect of a whey protein extract (65 – 75% LPO) on the immune response of rat pups to oral ovalbumin.	Whey protein extract (200 µg/pup) orally administered to suckling Hooded Wistar rat pups on days 4 – 9 after ovalbumin gavage.	Decreased proliferation of spleen cells cultured with ovalbumin and Concavalin A. Increased (5-fold) TGF-β secretion after culture with ovalbumin. Decreased MHC I expression on ileal epithelial cells.	Penttila *et al.*, 2001
Effect of early naso-gastric (NG) whey protein feeding in elderly patients with acute ischemic stroke.	Patients randomized to be given early NG feeding containing either hydrolyzed casein or hydrolyzed whey for a minimum of 5 days.	No difference in C-reactive protein between groups. Lower serum IL-6, glutathione peroxidase and glutathione in the whey protein vs. casein group.	De Aguilar-Nascimento *et al.,* 2011
Studies involving digested whey proteins or whey-derived peptides			
Effect of whey protein hydrolysate (WPH) in a mouse model of mite-induced	Female NG/Nga mice fed diet containing WPH for 2 wks prior to induction of	Lower skin scores (erythema, excoriation, edema) in mice fed WPH-	Shimizu *et al.*, 2006

dermatitis.	dermatitis.	diet; lower soluble E-selectin levels; decreased tissue neutrophil infiltration. No difference in total IgE levels between groups.	
Effect of acidic and basic peptide fractions from tryptic-chymotryptic digestion of β-LG hydrolyzed with *Lactobacillus paracasei* on spleen cells.	Spleenocytes from female BALB/c mice incubated with peptide fractions (proliferation); peptides and PHA (cytokines).	Peptide fractions stimulated lymphocyte proliferation. The acidic *L. paracasei* hydrolysed fraction upregulated IL-10 production, while the non-hydrolysed fraction stimulated IFN-γ production.	Prioult *et al.*, 2004
Immunomodulatory, antioxidative and anti-inflammatory effects of Enprocal (41% WPC) in gut cell proliferation	Caco-2 cells treated with digested and undigested Enprocal	Downregulation of TNFα and IL-1β, upregulation of IL-2, IL-10 and IFNγ secretion. Decreased adhesion of Jurkat E6-1 and THP-1 cells to Caco-2 monolayer	Kanwar and Kanwar, 2009

Despite the panoply of publications demonstrating various anti-inflammatory effects of whey proteins and their derived peptides in a wide range of disease states, surprisingly few have investigated possible molecular mechanisms by which these effects are exerted. In a recent study by Rusu *et al.* (2009), the effects of a bovine whey protein extract (WPE) on neutrophil function were investigated. The WPE was obtained by acid precipitating a commercial whey solution and subjecting the recovered precipitate to microfiltration, and contained mostly β-LG, α-LA, GMP, IgG, BSA and small amounts of TGF-β2. Treatment of neutrophils from healthy volunteers with WPE in doses up to 100 mg/L was shown to reduce apoptosis and improve viability. Furthermore, pre-treatment with WPE for 24 h primed the production of superoxide anion (O_2^-), increased chemotaxis and the release of myeloperoxidase (MPO) in neutrophils subsequently stimulated with a bacterial peptide, N-formyl-methyl-leucyl-phenylalanine (fMLP). Phagocytosis of zymosan particles was also enhanced by WPE pre-treatment. In a

subsequent study, a similar whey protein extract at doses up to 1000 mg/L stimulated the production by neutrophils of IL-1Ra (Rusu *et al.*, 2010), which regulates the biological activity of IL-1β by blocking its binding to IL-1 receptors (Seckinger *et al.*, 1987; Arend *et al.*, 1990). Neutrophil production of IL-1β was also increased, however the ratio of IL-1Ra:IL-1β increased in a dose-dependent fashion, resulting in a partial inhibition of the effects of IL-1β. Interestingly, the effects of WPE on the production of IL-1Ra by neutrophils were significantly reduced by pre-treatment with inhibitors of p38 MAPK, MEK/ERK1/2 and IκBα phosphorylation, suggesting that the observed effects implicated the MAPK and NF-κB pathways. In both studies, further analysis revealed that β-LG and α-LA were the components primarily responsible for the observed effects of the whey protein extract, although the addition of GMP enhanced the effects of the former two proteins on O_2^- production. Interestingly, a separate study investigated the inhibitory effects of GMP on LPS-induced proliferation of mouse spleen cells (Otani and Monnai, 1995). It was determined that the inhibitory effect was due to the induction by plastic adherent cells of a soluble inhibitory factor that reacted strongly with an anti-IL-1Ra antibody.

Other possible mechanisms are suggested by work involving Lf or Lf-derived peptides. Lee *et al.* (1998) induced lethal endotoxin shock by an intravenous injection of LPS following gastric tube feeding of either bovine Lf or BSA to germ-free neonatal Minnesota piglets. The piglets fed Lf exhibited a significantly lower mortality rate (16.7%) than those fed BSA (73.7%) after 48 h, as well as a decreased incidence of endotoxin shock related symptoms, including hypothermia. Porcine peripheral blood mononuclear cells (PBMCs) were then pre-incubated with Lf at different concentrations (up to 1000 μg/mL) followed by incubation with fluorescein isothiocyanate (FITC)-labelled LPS. Flow cytometric analysis of PBMCs showed that Lf inhibited the binding of FITC-LPS to monocytes in a dose-dependent manner. Other studies have shown that Lf is able to bind with LPS (Shahriar *et al.*, 2006; Appelmelk *et al.*, 1994; Caccavo *et al.*, 1999). Lf can also interact with soluble (s)CD14, thereby significantly decreasing the sCD14-LPS-induced expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin by human umbilical vein endothelial cells (HUVEC) (Baveye *et al.*, 2000).

Affecting the expression of these two adhesion molecules implies that Lf may down-regulate the recruitment and adhesion of leukocytes to sites of inflammation. These findings are extended in a subsequent study by the same group, showing that a similar treatment of HUVEC with Lf reduces LPS-induced IL-8 expression and secretion (Elass *et al.*, 2002). The peptide lactoferricin B, found in the N-terminal region and obtained from the pepsin cleavage of bovine Lf, has been found to exert potent bactericidal ability (Bellamy *et al.*, 1992), and has also been determined as the sequence primarily responsible for the LPS-binding ability of Lf (Elass-Rochard *et al.*, 1995). However, the anti-inflammatory activity of Lf could also be mediated by mechanisms other than LPS-binding. Mattsby-Baltzer *et al.* (1996) have shown that both Lf and lactoferricin can inhibit IL-6 secretion of human monocytic cells even when added 30 min following LPS, or 15 min following TNF-α stimulation. This suggests that Lf may exert a direct effect on the cells themselves.

The above described work offers many insights and suggests numerous possibilities for future investigations into the mechanisms by which whey proteins and peptides exert their anti-inflammatory effects. At this time, a number of cellular functions have been shown to be affected by whey treatment, and several, often seemingly unrelated mechanisms have been proposed. Whey itself is composed of a number of different proteins, and each of these individual proteins can yield a diverse array of peptide combinations upon hydrolysis, depending on the choice of enzyme(s) used and hydrolysis conditions. In view of the above, it is likely that the ensemble of whey proteins and their derived peptides exert their effects via a concert of different mechanisms. Continued research into the subject holds the promise of expanding the current list of identified peptides with their respective effects and modes of action. In turn, this may lead to the future development of various whey-based nutritional supplements with general anti-inflammatory activity, or whey-derived components with targeted or specific biological activities.

These and other studies into the *in vitro* effects of whey proteins on cellular functions share one major limitation. Although it is rightly argued that certain whey proteins resist gastric digestion and can be absorbed intact (Caillard and Tomé, 1994; Gardner, 1988),

this resistance is only partial and a certain degree of enzymatic hydrolysis does take place, resulting in the production of whey-derived peptides that would reach the systemic circulation. Whey proteins and their derived products are studied mostly for their potential value as a dietary supplement. Therefore, a better approximation of physiological processes *in vivo* would involve the hydrolysis of test proteins, preferably with a combination of pepsin and pancreatic enzymes and under conditions that closely mimic human digestion, followed by investigations into the effects of the resulting hydrolysates *in vitro*.

In that regard, a number of studies have evaluated the effects of peptides derived from whey proteins. Small peptides are postulated to be released from the ingested parent protein through gastro-intestinal digestion and to reach the systemic circulation in physiologically significant quantities (Pihlanto-Leppala, 2001; Meisel, 2005). For example, a synthetic form of the α-LA-derived tripeptide, Tyr-Gly-Gly, stimulated the proliferation of peripheral blood lymphocytes from healthy subjects *in vitro* (Kayser and Meisel, 1996). Kanwar and Kanwar (2009) investigated the *in vitro* effects of a commercially available whey-based supplement (Enprocal) on gut immune function. Enprocal was digested *in vitro* using a commercial enzyme mixture containing pancreatin, papain, bromelain, trypsin, α-chymotrypsin, lipase, amylase and rutin. Administration of intact or digested Enprocal showed no cytotoxicity and enhanced the proliferation of normal intestinal epithelial cells at doses up to 8000 μg/mL. In contrast, the treatment was cytotoxic to the Caco-2 adenocarcinoma cell line at doses above 2000 μg/mL, suggesting an anti-cancer effect. In addition, digested Enprocal increased transepithelial resistance, upregulated the expression of tight junction protein, and inhibited the adhesion of lymphocytes and monocytes to Caco-2 cells. The treatment also downregulated the LPS-induced expression of TNFα, IL-1β and IL-6 by THP-1 and Jurkat E6-1 cells in coculture with Caco-2 cells, but had no effect on IL-8 expression. IFN-γ and IL-10 expression by Jurkat E6-1 cells was increased. However, the effects of digested as opposed to undigested Enprocal were only compared in cell viability and cytotoxicity assays. Furthermore, a limitation of this study is that, in an attempt to mimic *in vivo* exposure of

the human digestive tract to the supplement, a digested sample was prepared using an enzyme mixture that is not representative of the human gut enzyme population.

Prioult *et al.* (2004) reported that acidic and basic fractions of tryptic and chymotryptic hydrolysates of β-LG stimulated mouse lymphocyte proliferation, while intact β-LG did not. Further hydrolysis of these fractions by a *Lactobacillus paracasei* extract significantly reduced these stimulating effects. In addition, while the acidic fraction of the β-LG hydrolysate enhanced the production of IFN-γ by mouse spleenocytes, the same fraction further hydrolyzed by *L. paracasei* had an opposite effect. Although Kanwar and Kanwar (2009) did not detect differences in the effects of digested versus undigested protein, the findings of Prioult *et al.* illustrate the fact that the effects of proteins can differ from those of their derived peptides, and that varying degrees or conditions of hydrolysis can result in peptides with dramatically differing effects.

Only a handful of studies have explored the *in vitro* anti-inflammatory effects of hydrolyzed whey proteins, and only one in the context of CF (Vilela *et al.*, 2006). Whey protein hydrolysates were generated using a two step digestion protocol mimicking human gastro-intestinal digestion and were used to treat cultured human respiratory epithelial cells. Under unstimulated conditions, wild-type tracheal epithelial cells did not respond to treatment with whey hydrolysates, but CFTR deficient cells displayed a significant increase in IL-8 secretion. In contrast, the hydrolysates showed a tendency to decrease TNFα-induced IL-8 secretion in both cell lines. However, in light of the suggested *in vitro* anti-inflammatory effect of WPI hydrolysates under stimulated conditions, the stimulation of IL-8 secretion under basal conditions warrants further inquiry. The two-step digestion procedure used to produce the hydrolysates involved pepsin (step 1) and pancreatin (step 2), which are enzyme combinations that closely resemble those found in the human GI tract. However, some of the conditions under which hydrolysis was performed, such as incubation temperature, were chosen for optimal enzyme activity rather than strictly physiological. Also, in order to separate small peptides (less than 1 kDa) from enzymes and undigested protein, ultrafiltration was performed using a membrane with a Molecular Weight Cut-Off (MWCO) of 1 kDa. The

MWCO of ultrafiltration membranes being defined by their ability to reject molecules of a particular size, a membrane with a larger pore size would be more appropriate to recover the desired peptides in the permeate.

2.2.3 Protein Modification by High Hydrostatic Pressure Treatment

Pressure treatment of food or food components is increasingly used by the food industry in order to sterilize food products (Mozhaev *et al.*, 1994), inhibit enzymatic activity, extend shelf-life whilst preserving flavours and other quality attributes, minimize oxidative browning, and can also be used to enhance the functionality of certain food ingredients (Rastogi *et al.*, 2007). High pressure (HP) disrupts protein structure and leads to alterations in protein conformation, or to denaturation. In its native state, a protein is kept stable by covalent and hydrogen bonds, and by electrostatic and hydrophobic interactions (Huppertz *et al.*, 2004). Covalent bonds, which stabilize the primary structure of a protein, are generally unaffected by HP treatment between 0 and 40^{o}C (Mozhaev *et al.*, 1994). The quaternary structure of a protein, which is stabilized by non-covalent bonds, is disrupted at low pressures (up to 150 MPa) (Huppertz *et al.*, 2002). Increased pressure (>200 MPa) is required to disrupt the hydrophobic and ionic interactions that define the protein's tertiary structure (Hendrickx *et al.*, 1998). The secondary structure, stabilized by hydrogen bonds, can be ruptured only at very high pressures (>500 MPa), resulting in irreversible protein denaturation. Some of the effects of HP on the molecular interactions within proteins remain to be fully elucidated, but include dissociation of ion pairs, hydrogen bond disruption and reformation (López-Fandiño, 2006), shortening the length of these bonds, and the promotion of intermolecular hydrogen bond formation while reducing intramolecular bonds. The latter is partly mediated by a process of hydration, whereby water forms intermolecular hydrogen bonds upon moving into the protein interior (Boonyaratanakornkit *et al.*, 2002). The penetration of water into the protein can lead to an alteration of conformation towards that of a molten globule, a partially folded compact state with no specific tertiary structure (Oliveira *et al.*, 1994; Creighton, 1990). At very high pressures, augmented protein exposure to the solvent via hydration can lead to aggregation (Webb *et al.*, 2001). Hydration also mediates secondary

structure perturbation leading to irreversible protein unfolding at very high pressures (>500 MPa) (Payne *et al.*, 1997). Depending on the level of pressure, these processes can alter protein conformation in multiple ways and give rise to a number of intermediate denatured states (López-Fandiño, 2006). In contrast, chemical or thermal protein denaturation methods lead to more dramatic structure alterations and afford much less control over the induced structural changes (Jonas, 2002).

2.2.3 Effect of High Pressure on Whey Proteins

β-LG, the dominant whey protein in bovine milk, is a 162 amino acid long globular protein with two disulfide (S-S) bridges and one free cysteine (Farrell *et al.*, 2004). HP has been shown to induce reversible and irreversible changes to the structure of β-LG (Hosseini-nia, 1999; Hayakawa *et al.*, 1996; Ikeuchi *et al.*, 2001). Tanaka *et al.* (1996b) found that the reactivity of the sulfhydryl (SH) group of ß-LG B, which is normally buried in the protein, increased as a function of pressure due to exposure of the sulfhydryl group to the surface. This facilitates intermolecular reactions between SH groups and the formation of intermolecular S-S bonds, and is believed to contribute to the irreversible denaturation and aggregation induced by HP (Funtenberger *et al.*, 1997).

α-LA is a compact globular protein, containing 123 amino acid residues and four S-S bonds, and is more baroresistant than β-LG (Farrell *et al.*, 2004). Pressure treatment in excess of 400 MPa is required for irreversible unfolding of α-LA (Tanaka *et al.*, 1996; López-Fandiño, 2006). The presence of four S-S bonds (as opposed to two S-S bonds in β-LG), and its binding with calcium partly confer α-LA its relatively higher resistance to HP treatment (Hosseini-nia *et al.*, 2002). BSA, also a globular protein with 583 amino acid residues, contains 17 S-S bonds and one free SH group (Farrell *et al.*, 2004). Its secondary structure can be disrupted at higher pressures (>300 MPa), although these changes are largely reversible, probably due to the large number of S-S bonds stabilizing the protein (Hosseini-nia *et al.*, 2002). However, aggregation can occur at these high pressures via the protein's free SH group (Galazka *et al.*, 1997).

Such pressure-induced conformational changes have implications in terms of proteins' susceptibility to enzymatic reactions. Most whey proteins are resistant to enzymatic digestion. β-LG is especially resistant to hydrolysis by pepsin, trypsin and chymotrypsin (Reddy *et al.*, 1988; Singh and Creamer, 1993). HP treatment can help expose peptide sequences normally embedded in the proteins' hydrophobic core, rendering them more accessible for cleavage by proteolytic enzymes. Recent work by Vilela *et al.* (2006) has demonstrated that pressurization of whey protein isolates (WPI) resulted in improved *in vitro* digestibility and potentiated the GSH enhancing capability of WPI in CFTR deficient respiratory epithelial cells.

In vivo studies have also demonstrated enhancements of the biological effects of whey proteins due to pressurization. Rats fed a pressurized WPI-based diet exhibited a significantly higher elevation in tissue GSH levels than rats fed a native WPI-based diet (Hosseini-nia, 2000). More recently, in a mouse model of *P. aeruginosa* lung infection, it was suggested that mice fed a pressurized WPI-based diet for one month showed a lower post-infection lung bacterial burden and decreased weight loss as opposed to mice fed native WPI (Kishta *et al.*, 2009). A study by Zavorsky *et al*. (2007) in healthy men and women showed a dose-response increase in lymphocyte GSH levels (by up to 24%) after supplementation with pressurized WPI for a relatively short period of two weeks. This augmentation in lymphocyte GSH levels with 45 g/day of pressurized WPI is comparable to that observed in a previous study involving native WPI supplementation for three months (Lands *et al.*, 1999). Based on these observations, the authors suggested that pressurized whey supplementation can achieve beneficial GSH-enhancing effects in a significantly shorter period of time, and using less protein overall. Another, recent study in children and adults with CF showed that one-month supplementation with pressurized WPI improved lung function in children, enhanced nutritional status in both children and adults, and decreased serum C-Reactive Protein (CRP) in patients with elevated CRP at baseline (Lands *et al.,* 2010). A randomized, double-blind placebo-controlled study investigated the effects of pressurized WPI supplementation (20 g/day) alone or in combination with an exercise training program in patients with COPD (Laviolette *et al.*, 2011). Whey supplementation resulted in a significant increase in cycling endurance test

time, as well as significant improvements in self-reported levels of fatigue and emotional control as assessed by the Chronic Respiratory Questionnaire, while supplementation with the casein placebo did not. These studies, in addition to the body of work showing the beneficial effects of whey proteins, suggest a number of applications of pressurized whey as a modified and improved form of the proteins. Further research is needed in order to identify the specific areas with a demonstrable benefit of pressurized over native whey, as well as to shed light on the manner in which the physical/chemical changes induced in whey proteins by pressurization correlate with changes in functional biological outcomes.

The present thesis work builds on and extends preceding research into the effects of pressurization on whey protein digestibility and antioxidant capacity. Using immortalized respiratory epithelial cells and varying stimulation conditions, previous findings suggesting an anti-inflammatory effect of whey proteins are strengthened, and possible mechanism(s) of action are investigated.

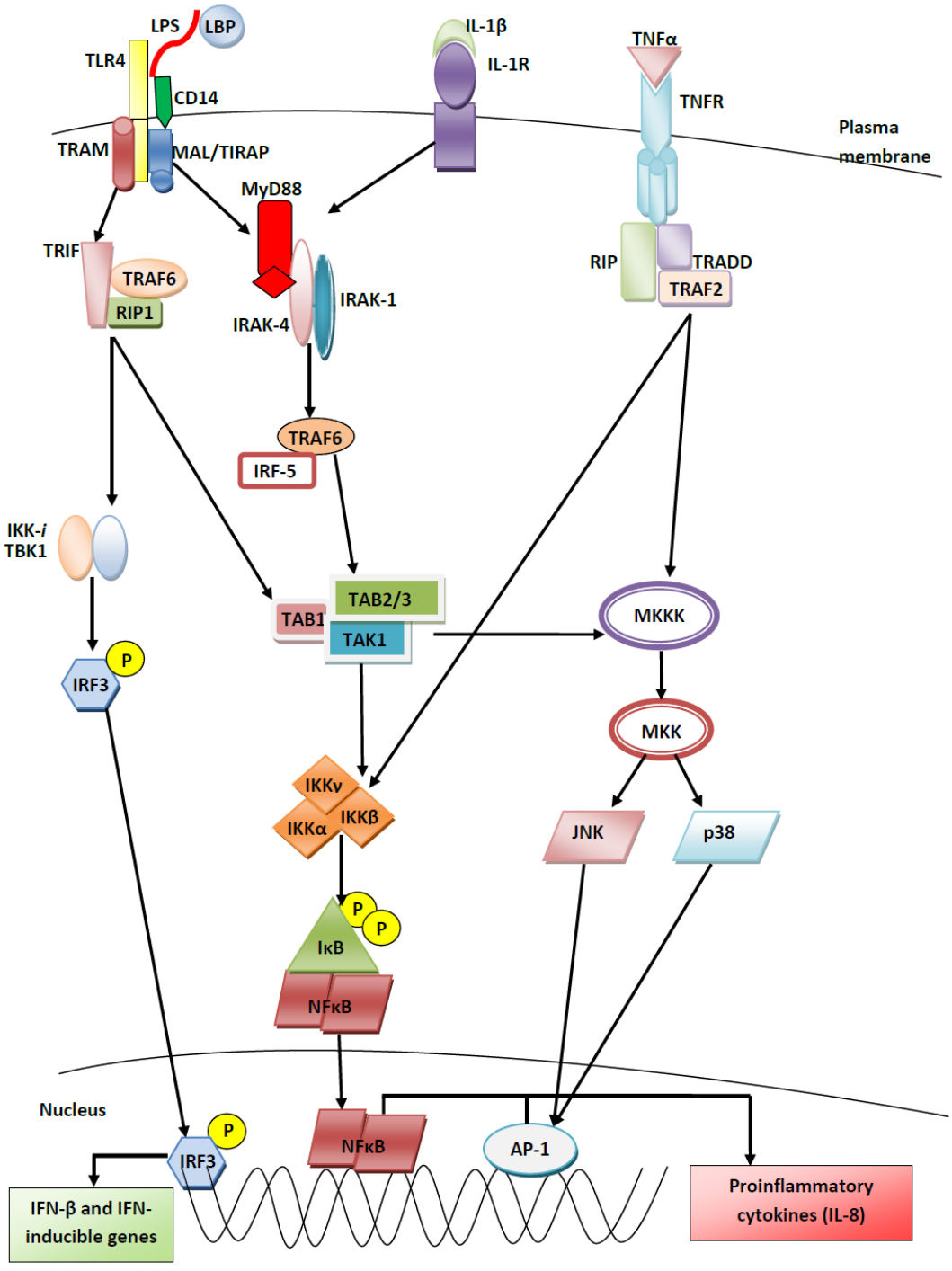

Figure 1. Molecular pathways leading to IL-8 expression. Adapted from: Greene and McElvaney 2005; Verstrepen *et al.*, 2008.

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CHAPTER 3

Effect of hyperbaric pressure on the digestibility and antioxidant potential of whey protein isolates

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Abstract

Whey proteins have been the subject of increasing interest as a functional food due to their well-established antioxidant activities *in vitro* and *in vivo* as well as a number of other biological activities. Hyperbaric pressure treatment of proteins induces conformational changes, which have been shown to increase the *in vitro* digestibility of whey proteins, as well as alter the peptide profiles of the corresponding hydrolysates. An objective of this study was to implement a series of modifications to a previously adapted *in vitro* digestion procedure in order to better mimic human gastrointestinal digestion and absorption, as well as to investigate the effects of hyperbaric pressure pre-treatment on the digestibility, antioxidant capacity and the peptide profiles of hydrolysates from the digestion of whey protein isolates (WPI). Pressurized (pWPI) and native (nWPI) were hydrolyzed using pepsin and pancreatic enzymes in a protocol based on a previous methodology, but with alterations in temperature and pH conditions, incubation times, the enzymes used, the enzyme-to-substrate ratio, and the choice of ultrafiltration membrane molecular weight cut-off. High pressure pre-treatment significantly enhanced the digestibility of WPI as assessed by the rate of decrease of protein content and the rate of appearance of primary α-amino groups. The peptide profiles of nWPI and pWPI hydrolysates, analyzed by HPLC and CZE, were markedly different, showing quantitative differences in the relative abundances of a number of eluting peptides, as well as qualitative differences in terms of the appearance of new peaks in pWPI that were absent from nWPI hydrolysates. Pressurization also enhanced the ferric-reducing antioxidant power of WPI hydrolysates. These results suggest potential applications of hyperbaric pressure treatment as a means to enhance the bioactivities of food proteins.

3.1 Introduction

Whey proteins are extensively used in the food industry for their gelling, foaming, and emulsifying properties (de Wit, 1998; López-Fandiño, 2006). They are also widely utilized as nutritional supplements for athletes due in part to their high branched-chain amino acid (BCAA) content, which is thought to reduce protein degradation and muscle loss during heavy exercise (Blomstrand and Newsholme, 1992; Schena *et al.*, 1992). In recent years, a number of biological activities beyond their nutritional value have been attributed to whey proteins, making them the subject of increasing interest as a functional food. Research has demonstrated that whey proteins can have antimicrobial, antiviral, anti-carcinogenic, opioid and ACE-inhibitory activities (Krissansen, 2007; Brody, 2000; Clare and Swaisgood, 2000), immunomodulatory effects (Cross and Gill, 1999; Lin *et al.*, 2008; Low *et al.*, 2003; Mercier *et al.*, 2004) and can decrease blood pressure and serum lipid levels (Kawase *et al.*, 2000).

Many of the beneficial effects of whey proteins are attributed to their ability to afford protection against oxidative stress. In addition to being rich in BCAAs, they are notable for their high content of sulphur-containing amino acids (cysteine, methionine) (Walzem *et al.*, 2002). Cysteine is a rate-limiting precursor for the biosynthesis of glutathione (L-γ-glutamyl-L-cysteinyl-glycine), a ubiquitous tripeptide thiol reducing agent. In addition to their glutathione-enhancing effects, amino acids and peptides derived from the hydrolysis of whey proteins have been shown to provide anti-oxidant activity in their own right. Peptides from β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) exhibit free-radical scavenging activity *in vitro* (Hernández-Ledesma *et al.*, 2005). Whey peptides or hydrolysates also inhibit iron-catalyzed lipid oxidation in liposomes (Colbert and Decker, 1991; Peña-Ramos and Xiong, 2001; Peña-Ramos *et al.*, 2004). The antioxidant effects of these peptides could be mediated by particular peptide structures or via specific side-chain groups in amino acid residues (Chan *et al.*, 1994; Chen *et al.*, 1996). Single amino acids with antioxidant capacity, such as cysteine, tyrosine or tryptophan (Udenigwe *et al.*, 2011) can also contribute to the overall antioxidant activity of whey hydrolysates. In animals, whey protein feeding can decrease tissue free radical concentrations (Elia *et al.*,

2006). In addition to the direct antioxidant effects of whey peptides and amino acids, animal and *in vitro* studies have suggested an effect of whey protein feeding via upregulation of antioxidative enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Zommara *et al.*, 1996; Xu *et al.*, 2011; Abdel-Aziem *et al.*, 2011; Takayanagi *et al.*, 2011). How exposure to whey proteins leads to the upregulation of antioxidant defense enzymes is still unclear. The induction of NF-E2-related factor 2 (Nrf2), a transcription factor largely responsible for the transcription of genes encoding antioxidant enzymes (Johnson *et al.*, 2008) has been suggested (Xu *et al.*, 2011).

Enzymatic hydrolysis of proteins is often used to produce hydrolysates with functional or bioactive properties, or to investigate the digestibility and bioavailability of particular proteins. Whey proteins are globular molecules with organized secondary and tertiary structures stabilized by disulfide (S-S) bonds. β-LG, α-LA and bovine serum albumin (BSA) contain two, four and seventeen S-S bonds, respectively (Farrell *et al.*, 2004). These features help render most whey proteins relatively resistant to proteolytic hydrolysis by enzymes such as pepsin, trypsin or chymotrypsin (Reddy *et al.*, 1998). The primary sequence of β-LG, for example, contains peptide bonds that can be cleaved by trypsin or pepsin, but are located within the protein's hydrophobic core and therefore inaccessible to the enzyme (Chobert *et al.*, 1996; Knudsen *et al.*, 2002). In addition, being soluble at acidic pH, whey proteins exhibit a relatively shorter gastric emptying time and exit the stomach after a shorter exposure to peptic hydrolysis relative to other dietary proteins. In view of the above and of the significant functional effects of whey proteins observed in animal and clinical studies, enhancing the susceptibility of these proteins to gastro-intestinal digestion could potentially increase the bioavailability of bioactive peptides present in their primary sequence.

High hydrostatic pressure treatment can disrupt protein structure and alter their conformation, thereby exposing otherwise hidden peptide sequences to proteolytic cleavage (López-Fandiño, 2006). This can enhance their digestibility and the bioavailability of peptides derived from their enzymatic hydrolysis, and may potentiate the bioactive properties of such hydrolysates. Much research involving the enzymatic

digestion of proteins under hyperbaric pressure has shown accelerated reaction rates that were attributed to the conformational changes undergone by the substrate rather than pressure-induced increase in enzymatic activity (Dufour *et al.*, 1995; Stapelfeldt *et al.*, 1996; Bonomi *et al.*, 2003). However, in the context of whey proteins as functional foods, the effect of pressure-induced conformation changes on subsequent digestibility by human gastro-intestinal enzymes is more relevant. A few studies have shown that hyperbaric pressure pre-treatment increases the hydrolysis rate and alters the resulting peptide profiles of β-LG (Knudsen *et al.*, 2002) and whey protein isolates (WPI) (Vilela *et al.*, 2006). In the study by Vilela *et al.*, a two-stage *in vitro* digestion protocol was adapted to mimic human gastrointestinal digestion and absorption. WPI was first subjected to hydrolysis by pepsin at acidic pH, followed by pancreatin at near-neutral pH and a final ultrafiltration step through a 1 kDalton (Da) membrane to isolate low molecular weight peptides. It has been shown that peptides of sizes up to 1 kDa can be absorbed through the intestinal lumen via di- and tri-peptide transporters or paracellular pathways (Arbit *et al.*, 2006; Nellans, 1991; Silk *et al.*, 1985) in quantitatively significant amounts to exert their bioactive effects. It is possible that the peptide mixture obtained via the digestion procedure of Vilela *et al.* may have quantitatively and qualitatively underestimated the peptide population under 1 kDa, as the Molecular Weight Cut-Off (MWCO) of ultrafiltration membranes are based on their ability to retain molecules of a given size. Several peptide species may have therefore been eliminated from the final permeate.

In addition to peptide isolation methods, variations in hydrolysis methods such as the choice of enzymes, the enzyme to substrate (E:S) ratio, temperature and pH can result in hydrolysates with different peptide profiles, and their biological activities can markedly differ in nature or in magnitude (Pacheco and Sgarbieri, 2005; Prioult *et al.*, 2004). One objective of this study was to apply modifications to the *in vitro* digestion protocol originally adapted by Vilela *et al.* such as incubation time, temperature and pH, enzymes and E:S ratios, and peptide isolation, so as to approximate more closely the physiological gastrointestinal digestive process. Vilela *et al.* showed that hyperbaric pressure pre-treatment of WPI improved its *in vitro* digestibility and altered the peptide profiles of the

resulting hydrolysates. A second objective of this study was to determine whether these effects of pressurization on WPI digestibility and hydrolysate composition are still observed when using a modified, more physiological *in vitro* digestion system. Finally, the effect of high pressure pre-treatment on the *in vitro* antioxidant capacity of WPI hydrolysates was tested.

3.2 Materials and Methods

Materials

Inpro 90 Whey Protein Isolate (WPI) was purchased from Vitalus (Abbotsford, BC), with the following composition: protein (dry basis) $\geq$ 92%; β-Lg 43-48%; GMP 24-28%; α-La 14-18%; BSA 1-2%; Immunoglobulins 1-3%; Lf <1%. Pepsin from porcine stomach mucosa, porcine pancreatic trypsin, bovine pancreatic chymotrypsin, porcine intestinal peptidase, pancreatin from porcine pancreas, and *o*-phthalaldehyde (OPA), were purchased from Sigma-Aldrich. Amicon ultrafiltration membranes (Molecular Weight Cut-Off 1 and 10 kDa) and ultrafiltration stirred units were purchased from Millipore. Bradford reagent was purchased from BD Biosciences. Ferric Chloride was purchased from ACP chemicals Inc., L-Ascorbic Acid from Fisher scientific. Sodium acetate trihydrate, glacial acetic acid and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich and were of highest analytical grade.

Hyperbaric Treatment of WPI

The WPI was dissolved (15% solution) in double-distilled water (ddH_2O) and pressurized with an Avure High Pressure Processing System model QFP 215L-600 (Avure Technologies, Columbus, OH). As pressures above 500 MPa are required to denature most whey proteins (Huppertz *et al.*, 2002), one cycle of pressurization at 550 MPa with 1 min holding time was carried out. Control native WPI (nWPI) underwent the same treatment with omission of the pressurization step. The solutions were then frozen

overnight at – 80°C and immediately freeze-dried and stored at – 20 °C under nitrogen gas until further use.

In Vitro Enzymatic Digestion and Peptide Isolation

Pressurized (pWPI) and native (nWPI) whey protein isolates were dissolved in ddH_2O at a concentration of 3 mg/mL and at 37°C. The pH was adjusted to 1.9 with addition of 1 N or 10 N HCl. First-stage digestion was performed with pepsin (prepared in 0.01 M HCl; E:S ratio 1:200) for 15 min, after which the pH was adjusted to 7.4 with addition of 10 N NaOH. Second-stage digestion was performed with trypsin, chymotrypsin and peptidase (prepared in phosphate buffer pH 7.0, E:S ratios 1:200, 1:87, and 1:120 respectively) for 60 min, after which the enzymes were inactivated with the addition of 10 N NaOH (pH 10.5).

Immediately upon inactivation of the proteolytic enzymes, the entire mixture was chilled on ice and subjected to ultrafiltration. Briefly, to remove high molecular weight peptides, a membrane filter with a molecular weight cut-off (MWCO) of 10 kDa (Millipore, Nepean, ON) was used in a stirred ultrafiltration membrane reactor (Amicon Ultrafiltration Cell, model 8050) at 4°C and under nitrogen gas pressure of 40 psi. The filtrates were freeze-dried and stored at – 80°C under nitrogen gas until further use.

The modifications applied to the previous digestion protocol of Vilela *et al.* (2006) are as follows:

Temperature. Incubation temperatures were previously chosen for optimal activities of the digestive enzymes (37°C and 40°C for pepsin and pancreatin, respectively). In the present study, temperature was maintained at 37°C throughout the entire digestion procedure.

pH. The pH of the solution was maintained at 1.9 during pepsin digestion and at 7.4 during digestion with pancreatic enzymes, based on the work of Gauthier *et al.* (1986), Agudelo *et al.* (2004) and Qiao *et al.*, 2004. Furthermore, since the digestion was carried

out in a non-buffered solution, the drop in pH during pancreatic digestion due to protein hydrolysis was off-set by the continuous addition of 1N NaOH to maintain a constant pH of 7.4.

Incubation time. The initial length of peptic digestion was set at 30 min, based on the gastric half-emptying time observed *in vivo* (Mahé *et al.*, 1994). However, it has been observed that whey proteins, which do not coagulate under acidic conditions, remain soluble and exit the stomach more rapidly than caseins (Mahé *et al.*, 1996, Boirie *et al.*, 1997). Therefore the length of peptic digestion was shortened to 15 min.

Enzymes. Pancreatin is a concentrated and lyophilized mixture of several enzymes including amylase, trypsin, ribonuclease, and lipase. However, the relative concentrations of the different enzymes within the mixture are undefined. Although it is possible to adjust the E:S for pancreatin per se, the actual amounts of individual proteolytic enzymes may vary. Batch to batch variability could also hinder the standardisation of the digestion procedure. In order to control the concentrations of proteolytic enzymes used during the digestion, the proteases trypsin, chymotrypsin and aminopeptidase were obtained separately and added simultaneously during the second stage of digestion. Furthermore, aminopeptidase, a brush-border enzyme, was included as it is not found in pancreatin.

Enzyme to substrate ratio (E:S). For pepsin, the E:S was decreased to 1:200. Rat feeding experiments have suggested that, prior to reaching the intestine for further hydrolysis, about 30% of ingested protein is hydrolyzed in the stomach (Sheffner *et al.*, 1956). In an in-depth study of different enzymatic conditions for *in vitro* protein digestion, Gauthier *et al.* (1986) have shown that a lower E:S ratio for peptic hydrolysis allows for the production of approximately 30% of trichloroacetic acid (TCA)-soluble nitrogen after 15 – 30 min. In addition, the lower E:S ratio reduces the amount of amino acid contamination from pepsin hydrolyzed by subsequent digestive enzymes (Gauthier *et al.*, 1986). Similar ratios for pepsin hydrolysis (1:200 to 1:250) have been used in most studies involving *in vitro* protein digestion (Kitabatake and Kinekawa, 1998; Pihlanto-Leppala *et al.*, 2000; Agudelo *et al.*, 2004). The E:S ratios for the pancreatic enzymes were calculated based on the methodologies of Kent *et al.* (2003), Wong and Cheung (2001), and Hsu *et al.* (1977).

Ultrafiltration membrane molecular weight cut-off (MWCO). The MWCO of an ultrafiltration membrane is a nominal value expressed in Daltons (Da) and is defined by the ability of the membrane to retain at least 90% of a globular molecule of said molecular weight (Millipore Technical Note, 1999). Therefore, in order to obtain a filtrate containing a maximal recovery of peptides of approximately 1000 Da in size, it is recommended by the manufacturer that a filtration membrane with a significantly greater MWCO than the size of the desired solute should be used. In that regard, ultrafiltration membranes with a MWCO of 10,000 Da were used in the current study.

Digestibility Assays

Digestibility was assessed as follows: samples were collected during the digestion procedure at 0, 5, 10 and 15 minutes of stage 1 (pepsin), and at 15, 30, 45 and 60 minutes of stage 2 (pancreatic enzymes). Protein content was assessed using the Bradford method (Gotham *et al.*, 1988). Samples were incubated for 5 min with Bradford reagent at a 1:50 ratio and absorbances were read on a spectrophotometer at 590 nm. A standard curve was constructed using BSA as a standard. The α-amino group content was determined using the *o*-phthalaldehyde (OPA) reagent, according to the method of Church *et al.* (1985), which measures primary amine groups in amino acids, peptides and proteins. The OPA reagent was prepared by adding 25 mL of 100 mM sodium tetraborate in water, 2.5 mL of 20% (wt/wt) SDS, 40 mg of OPA dissolved in 1 mL of methanol, 100 µL of β-mercaptoethanol and ddH_2O to a final volume of 50 mL. A 50 µL sample of digestion mixture was incubated for 2 min with 950 µL OPA reagent solution. Absorbances were read at 340 nm on a spectrophotometer. A standard curve was constructed using leucine-glycine as a standard peptide.

Characterization of Hydrolysates Peptide Profiles

The peptide profiles of lyophilized hydrolysates were analyzed by capillary zone electrophoresis (CZE) and Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). CZE was carried out using a Beckman Coulter P/ACE MDQ capillary

electrophoresis system (Fullerton, CA, USA). A fused-silica capillary (60 cm in length, window at 50 cm, 75 μm i.d., 360 μm o.d.) from Polymicro Technologies (Phoenix, AZ, USA) was conditioned in between runs by flushing for 1 min at 20 psi, followed by a 0.25-min wait at 0 bar with 5 mM SDS and then 100 mM NaOH. The capillary was filled with 50 mM phosphate buffer (pH 2.3; 2 min at 20 psi) and conditioned under 15 kV for 1 min (0 psi). Samples were injected hydrodynamically (0.5 psi, 5 s) and separated using 50 mM phosphate buffer (pH 2.3) with 15 kV at 28°C. Electropherograms were generated at 190, 254, and 275 nm (5 nm bandwidth, 4 Hz) from the photodiode array. Peak integration of the resulting electropherograms was done using OriginPro v8.0. For HPLC, samples were analyzed using a Varian HPLC system with a tertiary gradient pump, a variable wavelength UV/VIS detector, and an autosampler with refrigerated sample compartment (Varian Canada Inc, Mississauga, ON). Samples were eluted using a Onyx reverse-phase HPLC column (100 x 4.5 mm) (Phenomenex, CA), using a solvent flow rate of 1 mL/min and detection was at 215 nm. Gradient elution was carried out with a mixture of two solvents. Solvent A: 0.05% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile (ACN) and solvent B: 0.05%TFA in 60% aqueous ACN, (v/v) starting with 100% solvent A and reaching 40% solvent A and 60% solvent B in 30min.

Anti-oxidant Capacity of Hydrolysates

The antioxidant capacity of the freeze-dried digestates was assessed using the Ferric Reducing Antioxidant Power (FRAP), based on the reduction of the Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex to the ferrous (Fe^{2+}) form, and performed according to the method of Benzie and Strain (1996): FRAP reagent was prepared with sodium acetate buffer (300 mMol), 2.5 mL TPTZ solution (10 mMol in 40 mMol HCl), and 2.5 mL ferric chloride solution (20 mM in ddH_2O) in a 10:1:1 ratio, respectively. Lyophilized whey protein digestates were reconstituted to a concentration of 10 mg/mL and incubated with FRAP reagent for 90 min, at a 1:30 ratio. A standard curve was constructed with BSA and absorbances were read at 593 nm.

Statistical Analysis

Data are presented as mean ± SEM. All experiments were performed in triplicate. For digestibility assays, results were compared by two-way analysis of variance with Tukey's post-hoc test for multiple comparisons. Differences in antioxidant capacities between native and pressurized WPI hydrolysates and differences in CZE profiles percent peak area were analyzed using t-tests. A *p*-value less or equal to 0.05 was considered significant. Statistical analyses were performed using Sigma Stat v2.03 (Systat Software Inc., Chicago, IL).

3.3 Results

Effect of Hyperbaric Treatment on *in vitro* Digestibility of WPI

Using a modified Bradford method for protein quantitation (Bradford, 1976; Gotham *et al.*, 1988), the protein content of the hydrolysate mixture was analyzed at different time points throughout the digestion procedure. Figure 1 shows the *in vitro* digestibility of nWPI and pWPI as assessed by protein disappearance via the Bradford method. During the first 5 min of peptic digestion, the protein concentration decreased by 28.8% for pWPI, whereas the decrease was by 12.1% only for nWPI. At both the 5 and 10 min time points, the difference in degree of hydrolysis (DH) was significantly different between nWPI and pWPI (p= 0.0047 and 0.009, respectively), indicating that pWPI was hydrolyzed at a higher rate than nWPI. By the end of the 15 min digestion with pepsin, nWPI and pWPI showed a comparable degree of hydrolysis (DH) (36.8 and 37.2%, respectively). During stage 2 or pancreatic digestion, most of the protein disappearance occurred during the first 15 minutes for both pWPI and nWPI. However, the DH had reached 95.8% for pWPI, whereas it had only reached 83.3% for nWPI after 15 min. After 30 min of digestion, the DH was 99.7% and 95.31% for pWPI and nWPI, respectively. The difference in DH between pWPI and nWPI was statistically significant at both the 15 and 30 min times points (p=0.02 and 0.04, respectively).

Analysis of primary α-amino group content using the OPA reagent also showed an enhanced release of primary amines in pWPI compared to nWPI (Figure 2). During the first 5 min of pepsin hydrolysis, the rate of peptide formation was similar between pWPI and nWPI, respectively, producing a 116.9 and 110.14% (p=0.1) increase in peptide content relative to baseline (time 0 min). However, peptide production rate in nWPI reached a plateau at 5 min, a slowing down which was only observed at 10 min for pWPI. The percent increase in peptide content was significantly different between pWPI and nWPI at 10 and 15 min (p=0.01 and 0.005, respectively). During pancreatic digestion, peptide content was significantly higher in pWPI relative to nWPI at incubation times 15, 30, and 45 min (p=0.04, 0.01 and 0.005, respectively). At 60 min, pWPI peptide content was still higher than nWPI, although the difference did not reach statistical significance.

Effect of Hyperbaric Treatment on the Peptide Profiles of WPI Hydrolysates

Preliminary analyzes of the hydrolysates by electrospray ionization – mass spectrometry showed that the great majority of peptides were of sizes ≤ 1 kDa (data not shown). Figure 3 shows the peptide profiles of hydrolysates of native (nWPH) and pressurized (pWPH) whey protein obtained by CZE. Lyophilized hydrolysates were reconstituted at a concentration of 2 mg/mL and separated over a 50 min elution time. The electropherograms in figure 3 show that the peptide profiles obtained from pWPH differed from those of nWPH in terms of relative abundance. The percent area of each peak relative to the entire area under the curve was compared between pWPH and nWPH. A number of peaks were significantly increased in pWPH relative to nWPH, while others were significantly lower. In addition, two novel peaks appeared in the pWPH that were absent from nWPH, while one peak from nWPH was absent from the pWPH peptide profiles. Table 1 lists the percent areas of each peak in the nWPH and pWPH profiles. Figure 4 shows the peptide profiles of nWPH and pWPH obtained by HPLC. Here as well, differences in relative abundance were observed, and two peaks appeared in pWPH that were absent from nWPH.

Effect of Hyperbaric Treatment on the Ferric-Reducing Antioxidant Power of WPI Hydrolysates

Figure 5 shows the FRAP values for pWPH and nWPH. The ability of pWPH to reduce the Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex was higher than that of nWPH by 21.1% ($p<0.05$).

3.4 Discussion

This study describes a series of modifications applied to a previously adapted *in vitro* digestion protocol that was intended to imitate *in vivo* gastrointestinal digestion. Since this process and its resulting hydrolysates are intended to investigate protein digestibility as well as the potential bioactive effects of the peptides produced, mimicking physiological processes as much as possible in a laboratory setting is highly desirable. The changes introduced herein, including temperature, pH, enzymatic conditions and ultrafiltration method, constitute noticeable improvements in terms of further approximating human gastrointestinal digestion and absorption. The effects of such modifications on the resulting hydrolysates are illustrated by the HPLC and CZE peptide profiles from the current study, which differ markedly from those of Vilela *et al.* The modified digestion protocol employed in this study increased the peptide population by more than 3-fold in comparison to previous work. Although a number of adjustments were introduced to the current digestion procedure, the major factor responsible for the increased number of peptides is likely the use of a 10 kDa ultrafiltration membrane to isolate the peptides, as opposed to a 1 kDa membrane. Preliminary analyses by liquid chromatography-mass spectrometry confirmed that these peptides were mostly of 1 kDa in size or smaller (data not shown).

It has been shown that hydrolysates produced via different *in vitro* digestion procedures can exhibit dissimilar peptide profiles as well as markedly different biological activities. Pacheco and Sgarbieri (2005) produced hydrolysates of whey protein concentrate (WPC) using either pancreatin, alcalase, or protamex^{TM}, under temperature and pH conditions

optimal for the enzymes used. The pancreatin and protamex hydrolysates yielded peptide profiles that were distinct from that of the alcalase hydrolysate. In addition, when mice were fed diets based on these hydrolysates, liver glutathione content was increased only in response to pancreatin and protamex hydrolysates. Prioult *et al.* (2004) showed that, while an acidic fraction of tryptic and chymotryptic hydrolysates of β-LG could stimulate interferon-γ production by mouse spleenocytes, the same fraction further hydrolyzed by a *Lactobacillus paracasei* extract resulted in an opposite effect. Since results from the Vilela *et al.* study suggest an anti-inflammatory effect of WPI hydrolysates in TNFα-stimulated respiratory epithelial cells, it would be of interest to verify whether the modified hydrolysis protocol used in this study also yields hydrolysates with similar effects.

Results from this study show that hyperbaric treatment enhances the *in vitro* digestibility of WPI. Vilela *et al.* (2006) previously showed that WPI digestibility by pepsin was enhanced by pressure pre-treatment, a finding confirmed herein. In addition, the present results extend previous work, showing that subsequent digestion by pancreatic enzymes is also enhanced. The process of pressurization likely helped to expose cleavage sites within the proteins that would be otherwise unavailable to the action of pepsin. The resulting improved digestibility by pepsin can lead to the formation of intermediate polypeptides that are, in turn, more readily hydrolyzed by pancreatic enzymes.

Analyses of the peptide profiles of nWPH and pWPH hydrolysates by CZE and HPLC show distinct quantitative differences in the relative abundance of peaks, as well as qualitative differences illustrated by the appearance of novel peaks in pWPH that are absent in nWPH. This extends previous findings by Vilela *et al.*, further demonstrating that the effects of pressurization are such that differences in digestibility and peptide profiles are still observed even when major modifications are brought to the digestion procedure.

Our results also show that hydrolysates from pWPH had a significantly higher ferric-reducing antioxidant power than nWPH. The α-amino group content of pWPH

hydrolysates was not significantly different from that of nWPH at the end of the digestion. Therefore the increased antioxidant power was not attributable to an increase in peptide concentration, but to a relative increase in the abundance of antioxidant peptides and amino acids in pWPH. It has been recently determined that acidic amino acids (glutamic acid and aspartic acid) are strong contributors to the ferric reducing antioxidant power of food protein hydrolysates, due to their hydrogen-donating ability. Sulphur-containing amino acids (cysteine and methionine) are the most potent at reducing the Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex owing to their sulfhydryl group (Udenigwe and Aluko, 2011). It is likely that the process of pressurization exposed particular cleavage sites within the whey proteins, affording the proteolytic enzymes better access to more readily produce specific peptides sequences containing these amino acids. Hence, it is conceivable that the observed quantitative and qualitative differences in peptide profiles induced by pressure treatment reflect an enrichment of the final mixture with specific peptides possessing antioxidant activity. Limitations of the FRAP assay include the fact that it does not detect thiol-type antioxidants, and that the reaction occurs under conditions that are not physiological, such as a highly acidic pH (Apak *et al.*, 2004). Therefore, results of the FRAP assay may not reflect the antioxidant capacities of the tested compounds *in vivo*. However, one purpose of this study was to investigate whether hyperbaric pressure pre-treatment of WPI leads to hydrolysates with altered functionality; the FRAP assay is a simple method suited for the direct comparison between hydrolysates from native and pressure-treated WPI.

At the end of stage 2 digestion, both pWPH and nWPH solutions had undetectable protein levels as assessed by the Bradford assay. The limit of detection of this assay are polypeptides of size below 3 kDa, indicating that the final hydrolysate mixture consisted chiefly of small polypeptides and amino acids. It is noteworthy that, during the digestion procedure, the protein concentration of pWPH hydrolysates reached zero after 60 min, noticeably faster than nWPH. At times 30 and 45 min, there remained respectively 4 times and 14 times more protein in the nWPH solution as compared to pWPH. This indicates that proteins and peptides larger than 3 kDa disappear faster during pWPI digestion. It is likely that the increased rate of appearance of small peptides is

accompanied by an increase in their rate of absorption. The use of an open digestion system whereby the reaction mixture is maintained within a dialysis membrane, allowing a continuous separation of small molecules, would be better suited to identify the peptides produced at different times. Another advantage of the use of an open digestion system and the continuous removal of small peptides is the prevention of end-product inhibition of the proteolytic enzymes. A limitation of a closed system such as the one employed herein, is the possibility of enzyme inhibition due to end-product accumulation, possibly leading to an underestimation of the rate of digestion, particularly for pWPI where small molecules are produced at a faster rate.

It has been shown that the rate of absorption of amino acids from dietary proteins influences whole body protein deposition by affecting the breakdown, synthesis and oxidation of proteins (Boirie *et al.*, 1997). As leucine balance can be used as an index of whole body protein deposition (El-Khoury *et al.*, 1995), the postprandial rise in plasma amino acids was examined in healthy subjects using L-[1-^{13}C]leucine-labeled casein or whey protein (Boirie *et al.*, 1997). It was found that the ingestion of whey protein resulted in a higher, more rapid and transient increase in plasma amino acid levels than casein. These differences were also related to an augmented rate of protein synthesis and oxidation as compared to casein. However, protein breakdown was inhibited by casein whereas it was unaffected by whey protein ingestion, although the addition of energy in the form of carbohydrate did induce inhibition of proteolysis. In contrast, elderly subjects given a whey protein meal exhibited a higher postprandial protein utilization efficiency than after a meal with casein, as well as a higher postprandial leucine balance (Dangin *et al.*, 2003; Pennings *et al.*, 2011). Although a slow release of amino acids into the circulation is desirable in some conditions such as hepatic encephalopathy or renal insufficiency (Fruhbeck, 1998), strong hyperaminoacedemia could be beneficial for the elderly, as it can reverse the impaired muscle protein synthesis following feeding (Welle *et al.*, 1994; Volpi *et al.* 1998). The resulting improvement in protein synthesis could be advantageous in limiting muscle protein loss in the elderly. Other implications of such differences in postprandial aminoacidemia are illustrated by findings in patients with Type 2 Diabetes Mellitus, where a mixed meal containing whey protein resulted in a

greater beta-cell response than a casein meal, as assessed by levels of insulin, pro-insulin and C-peptide. The differences in beta-cell responses were attributable to the proteins' digestion and absorption pattern rather than their amino acid composition, as the administration of a meal containing casein-like free amino acids elicited similar responses to those of whey protein (Tessari *et al.*, 2007). An increased rise in plasma amino acids was also observed in conjunction with increased satiety after whey protein ingestion in comparison to casein, suggesting usefulness in weight management and the treatment of obesity (Hall *et al.*, 2003). All of the above postprandial effects of whey proteins were attributed to their more rapid gastric emptying leading to a more pronounced appearance of amino acids in the circulation as opposed to casein, a "slower" protein. These findings, in conjunction with the increased *in vitro* digestibility of pWPI observed herein, suggest that the pressurization of whey proteins could potentiate the observed beneficial effects described above.

We have shown that pressurization of whey proteins improves their *in vitro* digestibility and modifies the resulting peptide profiles even when different digestion procedures are used. In addition, pressurization enhances the *in vitro* antioxidant capacity of whey proteins. These *in vitro* effects suggest that hyperbaric pressure treatment offers promising prospects for the modification of food proteins leading to enhancements of their functional and bioactive properties *in vivo.*

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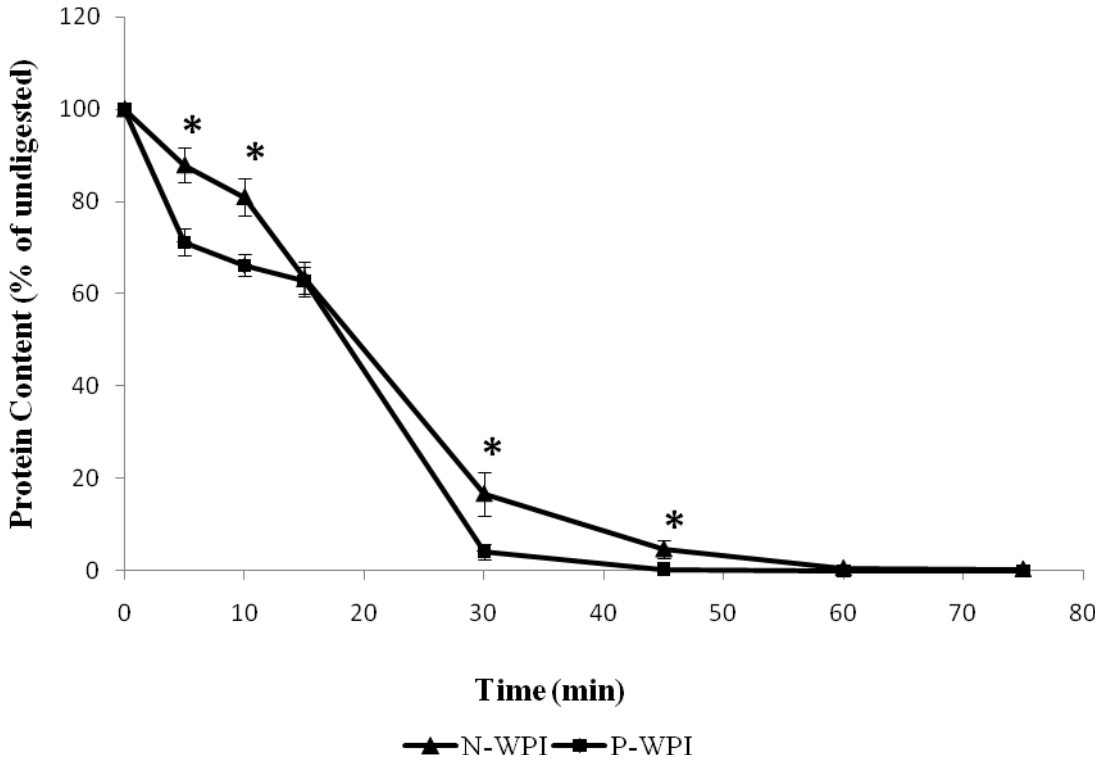

Figure 1. Effect of high hydrostatic pressure on the *in vitro* proteolysis of WPI. Solutions of 3 mg/mL pWPI or nWPI were subjected to *in vitro* digestion with pepsin (15 min) followed by trypsin, chymotrypsin and peptidase (60 min). Samples were taken every 5 min during pepsin digestion, and every 15 min during digestion with pancreatic enzymes. The protein content was determined using the Bradford method. Data are expressed as a percentage of baseline values ± SEM. Statistically significant differences in protein content between nWPI and pWPI (Tukey's *post hoc* comparison) are designated by * $p<0.05$.

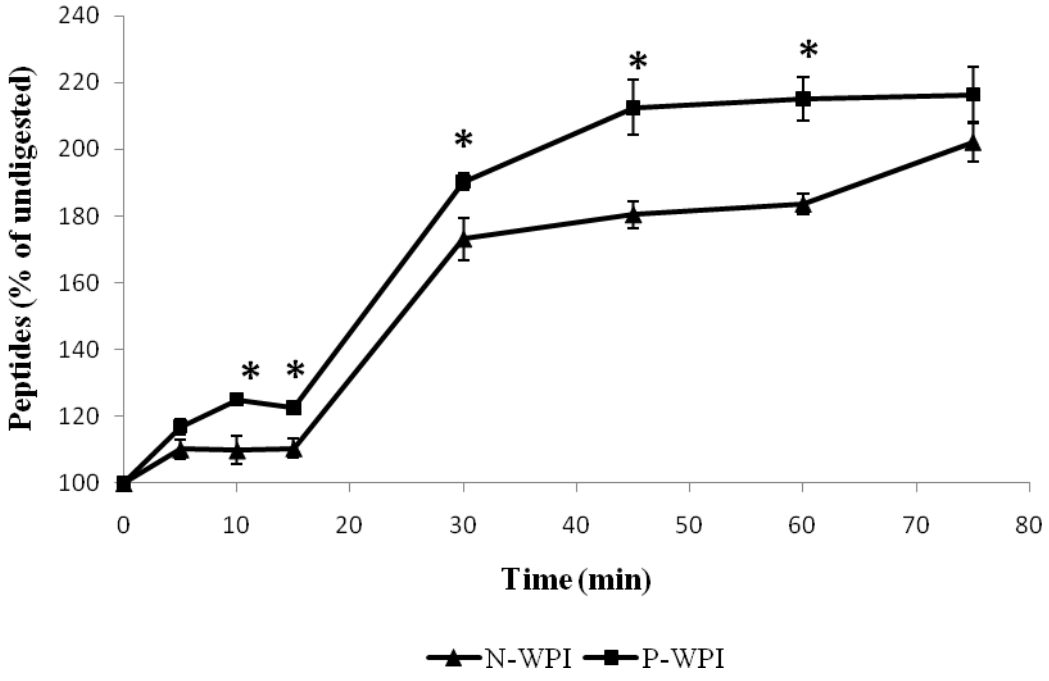

Figure 2. Effect of high hydrostatic pressure on *in vitro* enzymatically-driven peptide release from WPI. Solutions of 3 mg/mL pWPI or nWPI were subjected to *in vitro* digestion with pepsin (15 min) followed by trypsin, chymotrypsin and peptidase (60 min). Samples were taken every 5 min during pepsin digestion, and every 15 min during digestion with pancreatic enzymes. The α-amino group content was determined using the OPA reagent based on the method of Church *et al.* (1985). Data are expressed as a percentage of baseline values ± SEM. Statistically significant differences in α-amino group content between nWPI and pWPI (Tukey's *post hoc* comparison) are designated by * $p<0.05$.

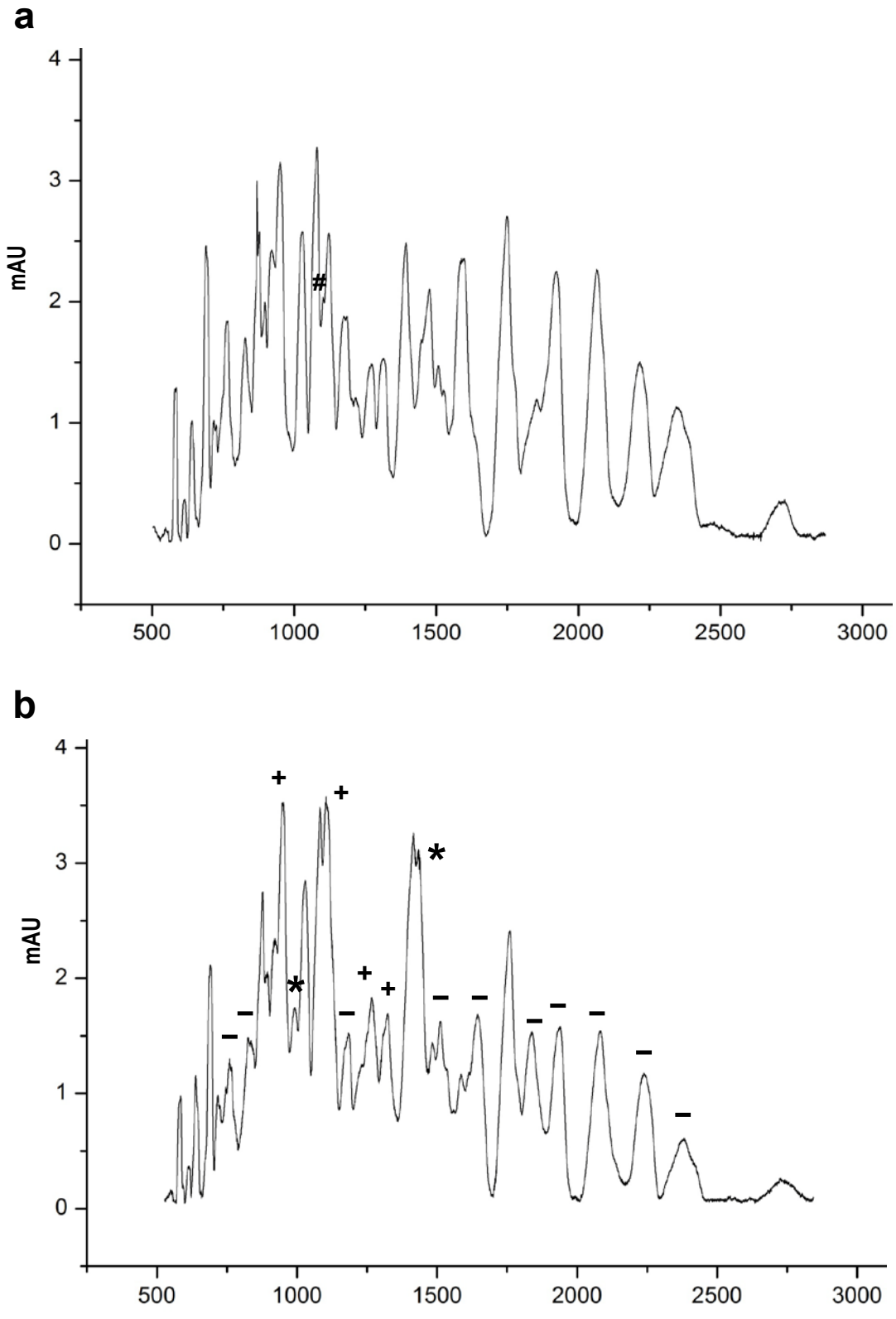
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Figure 3. CZE profiles of peptide extracts with MWCO ≤ 1 kDa derived from pepsin, trypsin, chymotrypsin and peptidase hydrolysis of (a) nWPH and (b) pWPH. The symbols identify extra peaks found in pWPH hydrolysates (*) and indicate differences in the relative abundances of peptides as a result of pressure treatment (+, higher abundance, -, lower abundance in pWPH relative to nWPH; #, peak found in nWPH but not in pWPH hydrolysates).

Table 1. Differences in percent peak areas between nWPI and pWPI hydrolysates, relative to the total area under the curve, as assessed by CZE.

Peak Number	% of Area Under the Curve		*p*-value
	N-WPI	P-WPI	
1	0.9100	0.7336	NS
2	0.2508	0.2149	NS
3	0.8530	0.6995	NS
4	0.0215	0.1164	NS
5	2.2934	1.8091	NS
6	0.7993	0.2026	0.0154
7	0.3922	1.1154	NS
8	2.6619	1.5572	0.027
9	2.7604	2.4542	NS
10	2.4608	2.9801	NS
11	1.4659	1.4913	NS
12	2.7565	2.4610	NS
13	4.5988	4.8079	0.046
14	0	2.0727	Only in pWPI hydrolysate
15	3.7926	4.1746	NS
16	4.2629	4.2212	NS
17	0.9752	0	Only in nWPI hydrolysate
18	3.3845	6.1931	<0.0001
19	3.7332	2.5659	0.002
20	1.3635	1.0267	NS
21	2.5261	4.2416	0.0476
22	2.6743	3.5632	0.0005
23	5.0190	5.4883	NS
24	0	4.2459	Only in pWPI hydrolysate
25	1.5836	1.5758	NS
26	3.2332	2.1505	0.0038

27	1.4856	1.1582	NS
28	1.2950	1.4485	NS
29	5.9445	3.699	0.007
30	6.8146	6.0910	NS
31	2.8035	4.1246	0.0005
32	6.0780	3.8966	0.002
33	6.3993	5.0982	0.024
34	4.7252	3.6685	0.006
35	5.3344	2.7964	0.002

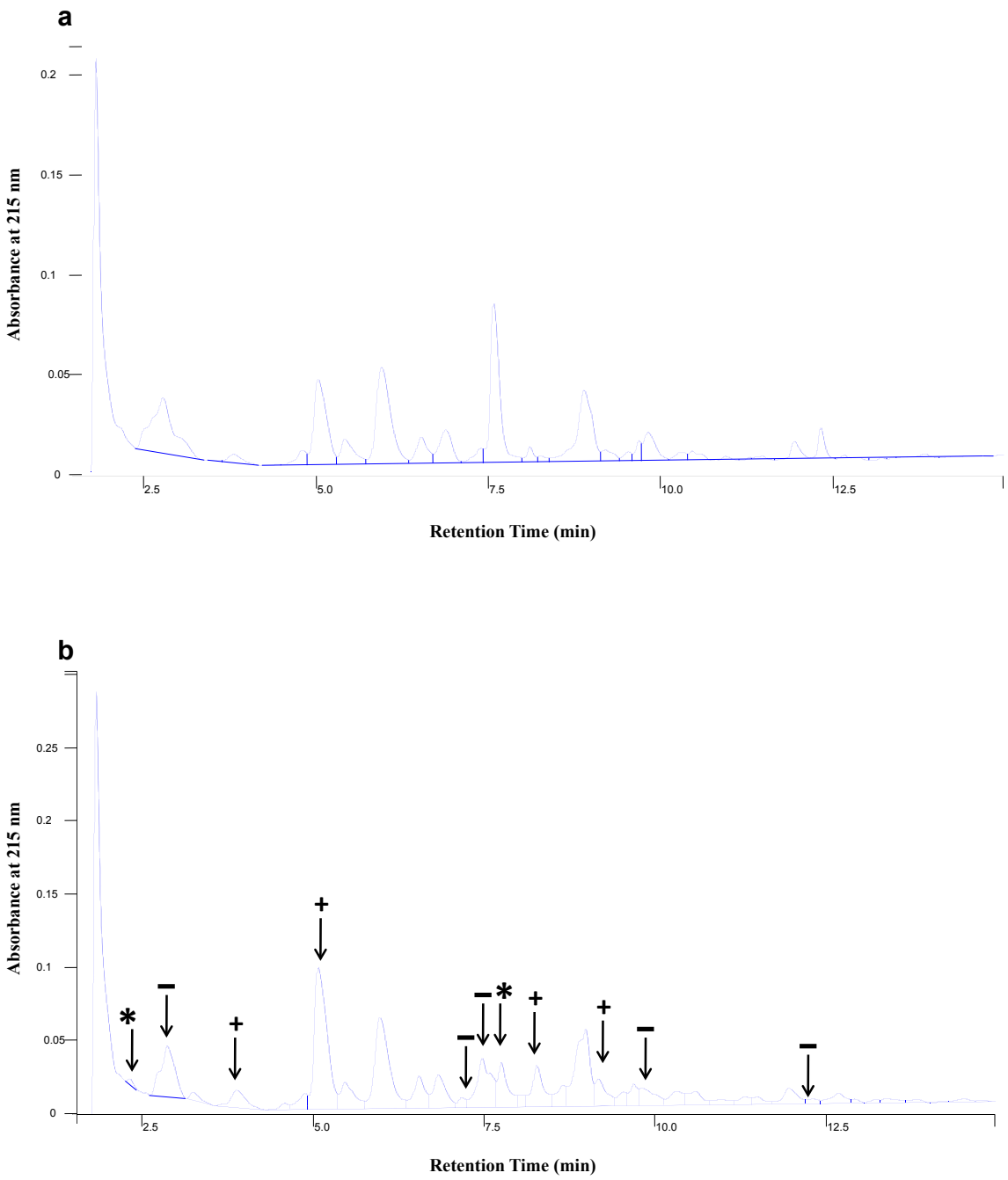

Figure 4. HPLC profiles of peptide extracts with MWCO ≤ 1 kDa derived from pepsin, trypsin, chymotrypsin and peptidase hydrolysis of (a) nWPH and (b) pWPH. The arrows identify extra peaks found in pWPH hydrolysates (*) and indicate differences in the relative abundances of peptides as a result of pressure treatment (+, higher abundance, -, lower abundance in pWPH relative to nWPH).

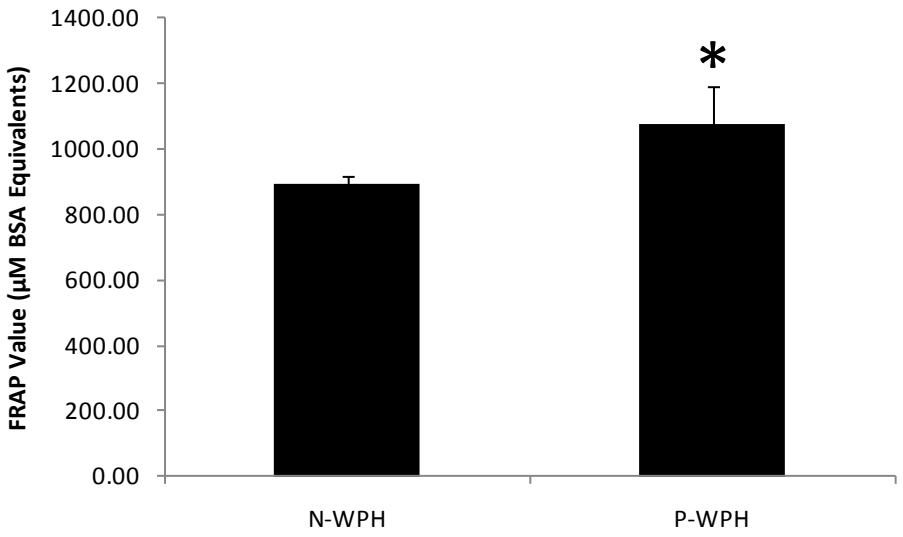

Figure 5. Effect of high hydrostatic pressure on the ferric-reducing antioxidant power (FRAP) of WPH hydrolysates. Freeze-dried hydrolysates resulting from the *in vitro* digestion and subsequent ultrafiltration were reconstituted in ddH_2O at a concentration of 10 mg/mL and incubated with the FRAP reagent for 90 min at a 1:30 ratio. BSA was used to construct a standard curve and absorbances were read at 593 nm. Results are expressed in µM BSA equivalents ± SEM.

CONNECTING STATEMENT

The results obtained in Chapter 3 showed that the effects of hyperbaric pressure pre-treatment resulted in improved *in vitro* digestibility of whey protein isolates and altered the peptide profiles of the resulting hydrolysates regardless of the digestion system utilized. These hydrostatic pressure mediated changes were accompanied by an enhancement in the ferric-reducing anti-oxidant power of the lyophilized hydrolysates. The preceding chapter examined the effects of hyperbaric pressure on the physicochemical characteristics of whey protein hydrolysates. The following chapter investigates whether these effects also result in enhancements in the biological activities of whey protein hydrolysates, namely their anti-inflammatory and antioxidant activities, in a cell culture system. Hence, a wild-type cell line as well as a cystic fibrosis transmembrane conductance regulator (CFTR) mutant cell line were used to examine the effects of whey protein hydrolysates on the secretion of IL-8, under basal or stimulated conditions. The effect of the hydrolysates on the cell supernatant antioxidant activity was also tested. Possible mechanisms of action by which the hydrolysates may exert their IL-8 suppressing activity were investigated. Finally, since the hydrolysates produced in Chapter 3 exhibited different peptide profiles than those in previous studies (Vilela *et al.*, 2006), the effects of hydrolysates produced via these two digestion procedures on IL-8 secretion by wild-type cells were compared.

CHAPTER 4

Inhibition of Interleukin-8 Release by Pressurized Whey Protein Hydrolysates in Respiratory Epithelial Cells: Mechanism of Action

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Abstract

Whey proteins exert immunomodulatory and anti-inflammatory effects in various experimental settings, apart from their value as a dietary source of essential amino acids. Hyperbaric pressure treatment of whey proteins increases their digestibility, changes the spectrum of peptides released by gastrointestinal digestive enzymes, and improves nutritional status and systemic inflammation in patients with Cystic Fibrosis (CF). To examine possible mechanisms by which native and pressurized whey proteins can modulate the inflammatory response in CF, we conducted a series of *in vitro* studies. Hydrolysates of pressurized whey suppressed lipopolysaccharide (LPS)-stimulated interleukin (IL)-8 production in respiratory epithelial cells expressing either wild-type or mutant ΔF508 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Native whey hydrolysates had no effect on wild-type cells and required higher concentrations for an effect on ΔF508 cells. Neither had an effect on tumor necrosis factor-alpha (TNFα)- or IL-1β-induced IL-8 secretion. There was no effect of either hydrolysate on Toll like receptor (TLR)4 expression. The hydrolysates did not exhibit direct neutralization of LPS as assessed by the limulus amebocyte lysate (LAL) test, but LPS binding to surface TLR4 was reduced by both native and pressurized whey hydrolysates. In addition, pressurized whey hydrolysates increased cell culture supernatant antioxidant capacity. The beneficial immune modulating effects observed in CF patients receiving pressurized whey protein could be due to the enhanced suppression of IL-8, which could be partly explained by inhibition of the binding of LPS to TLR4.

4.1 Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, and affects an estimated one in 3,600 live births in the USA. Pulmonary disease, which constitutes the major cause of CF morbidity and mortality (Davis, 2006; Gao *et al.*, 1999), is characterized by pulmonary recruitment of inflammatory cells, primarily neutrophils, which secrete cytokines, oxidants and other pro-inflammatory mediators (Rahman and MacNee, 1998). The bronchoalveolar lavage fluid of CF patients contains particularly elevated levels of IL-8 (Noah *et al.*, 1997), which results in further neutrophil recruitment, leading to a state of chronic inflammation and tissue injury (Davis, 2006; Chmiel *et al.*, 2002).

Airway epithelial cells are sentinel players in the innate immune system. IL-8 is secreted by epithelial cells in response to neutrophilic stimuli such as Tumor Necrosis Factor α (TNFα), neutrophil elastase (Nakamura *et al.*, 1992) or to bacterial gene products via Toll-like receptors (TLRs) (DiMango *et al.*, 1995; Greene *et al.*, 2005). There is also evidence that the CF pulmonary epithelium itself is pro-inflammatory, as demonstrated by models of CF fetal tracheal xenografts in mice, where increases in subepithelial leukocytes and airway lumen IL-8 were observed in CF grafts before the onset of infection (Tirouvanziam *et al.*, 2000; Puchelle and Peault, 2000; Puchelle *et al.*, 2001). Modulation of the epithelial inflammatory response therefore is an important therapeutic target in the treatment of CF.

Malnutrition also plays an important prognostic role in CF, as pancreatic insufficiency and subsequent intestinal malabsorption adversely affect the patient's ability to fight infection and repair lung damage; in turn, increased energy demands and appetite suppression due to pulmonary disease lead to poor growth and nutritional status (Amin and Ratjen, 2008). The close relationship between lung function, nutritional status and clinical course in CF has been demonstrated in cross-sectional and longitudinal studies, where a strong association was found between a decline in nutritional status and a severe

decrease in lung function, irrespective of bacterial status (Davis, 2006; Steinkamp *et al.*, 2002; Schoni and Casaulta-Aebischer, 2000; Konstan *et al.*, 2003).

Whey proteins, a by-product of the cheese making industry, possess nutritional benefits as a source of protein of high biological value (de Wit, 1998; Krissansen, 2007), and a number of anti-inflammatory effects (Brody, 2000; Clare and Swaisgood, 2000; Oner *et al.*, 2006). These effects of whey proteins include decreased cytokine release in rodent models of ischemia reperfusion (Yamaguchi and Uchida, 2007) and lipopolysaccharide (LPS) injection (Beaulieu *et al.*, 2009). In addition, individual whey proteins such as lactoferrin (Mattsby-Baltzer *et al.*, 2009; Lee *et al.*, 1998; Hayashida *et al.*, 2004), or glycomacropeptide (GMP), a casein-derived peptide commonly found in whey protein isolates (WPI) (Otani *et al.*, 1996), and peptides generated from their pepsin-pancreatin digests (Bruck *et al.*, 2006) have exhibited immunomodulatory effects including anti-inflammatory *in vivo* effects or *in vitro* inhibition of inflammatory cytokine release.

High-pressure treatment induces conformational changes in whey proteins (Hendrickx *et al.*, 1998). These conformational changes can expose peptide sequences normally embedded in the hydrophobic core, rendering them more accessible to enzymatic digestion. For example, Tanaka *et al.* (1996) found that the chemical reactivity of the sulfhydryl group of the whey protein β-lactoglobulin B, which is normally buried in the protein, increased as a function of pressure due to exposure of the sulfhydryl group to the surface. Recent work by Vilela *et al.* (2006) demonstrated that pressurization of WPI resulted in improved *in vitro* digestibility, promoted the release of novel peptides by gastrointestinal digestive enzymes, and showed a trend to decrease IL-8 secretion by TNFα-stimulated CF respiratory epithelial cells. Significant *in vivo* functional outcomes have also been attributed to the pressurization of WPI. Zavorsky *et al.* (2007) found that a short, two-week supplementation with pressurized WPI increased lymphocyte glutathione (GSH) levels (by up to 24%) in healthy men and women. A recent study of pressurized WPI supplementation (1 month) in CF patients improved nutritional status and markers of systemic inflammation (Lands *et al.*, 2010).

The aim of this study was to investigate the *in vitro* immunomodulatory effects of enzymatically digested native WPI (nWPI) and pressurized (pWPI) on wild-type and CF respiratory cell lines. We hypothesized that peptides present in the hydrolysates of WPI act on epithelial cells to decrease IL-8 secretion and that this inhibition is enhanced by the pressurization of WPI. Specific objectives of the work were to investigate and compare the IL-8 suppressing capabilities of the hydrolysates of nWPI and pWPI in response to endogenous (TNFα, IL-1β) and exogenous (LPS) immunostimulants, as well as to investigate possible mechanisms by which the hydrolysates might exert their effects.

4.2 Materials and Methods

Materials

Inpro 90 Whey Protein Isolate (WPI) was purchased from Vitalus (Abbotsford, BC), with the following composition: protein (dry basis) ≥93%; β-Lactoglobulin 43-48%; GMP 24-28%; α-Lactalbumin 14-18%; Bovine Serum Albumin 1-2%; Immunoglobulins 1-3%; Lf<1%. Pepsin from porcine stomach mucosa, porcine pancreatic trypsin, bovine pancreatic chymotrypsin, porcine intestinal peptidase, LPS and FITC-LPS from *E.coli* (O55:B5), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma-Aldrich. Amicon ultrafiltration membranes (Molecular Weight Cut-Off 10 kDa) and ultrafiltration stirred units and 0.22 μm filters were purchased from Millipore. Human recombinant TNFα and IL-1β were purchased from BD Biosciences. Dulbecco's phosphate-buffered saline (PBS) was obtained from Invitrogen. Antibodies (rabbit polyclonal anti-TLR4 IgG and Alexa Fluor 488 anti-rabbit goat antibody) were purchased from Santa Cruz. Wild type (non-CF; 1HAEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human respiratory epithelial cells were gifts from Dr. D. Gruenert (University of California, SF). Cells were routinely maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Wisent), L-glutamine and penicillin/streptomycin (Invitrogen). All other chemicals were purchased from Sigma-Aldrich and were of highest analytical grade.

Hyperbaric Treatment of WPI

The WPI was dissolved (15% solution) in double-distilled water (ddH_2O) and pressurized with an Avure High Pressure Processing System model QFP 215L-600 (Avure Technologies, Columbus, OH). As pressures above 500 MPa are required to denature most whey proteins (Huppertz *et al.*, 2002), one cycle of pressurization at 550 MPa with 1 min holding time was carried out. Control native WPI (nWPI) underwent the same treatment with omission of the pressurization step. The solutions were then frozen overnight at – 80°C and immediately freeze-dried and stored at – 20 °C under nitrogen gas until further use.

***In Vitro* Enzymatic Digestion**

Pressurized (pWPI) and native (nWPI) whey protein isolates were dissolved in ddH_2O at a concentration of 3 mg/mL and at 37°C. The pH was adjusted to 1.9 with addition of 1 N or 10 N HCl. First-stage digestion was performed with pepsin (prepared in 0.01 M HCl; E:S ratio 1:200) for 15 min, after which the pH was adjusted to 7.4 with addition of 10 N NaOH. Second-stage digestion was performed with trypsin, chymotrypsin and peptidase (prepared in phosphate buffer pH 7.0, E:S ratios 1:200, 1:87, and 1:120 respectively) for 60 min, after which the enzymes were inactivated with the addition of 10 N NaOH (pH 10.5). Pressurized WPI hydrolysates (pWPB) were also prepared following a protocol from previous work (Vilela *et al.*, 2006). Briefly, pWPI was dissolved in ddH2O at a concentration of 3 mg/mL, following which the pH was adjusted to 1.5 with 10 N HCl. First-stage digestion was performed with pepsin (prepared in 0.01 M HCl; E:S ratio 1:100) for 30 min. The pH was then raised to 7.8 using 1 N NaOH. Second-stage digestion was performed with pancreatin (40°C; E:S ratio 1:30) for 1 hour, and the reaction was stopped by raising the pH of the solution to 10.5.

Isolation of Peptides

Following the digestion described above, the hydrolysates from native (nWPH) and pressurized (pWPH) whey protein isolates were subjected to ultrafiltration. Briefly, to remove high molecular weight peptides, a membrane filter with a molecular weight cut-off (MWCO) of 10 kDa (Millipore, Nepean, ON) was used in a stirred ultrafiltration membrane reactor (Amicon Ultrafiltration Cell, model 8050) at 4°C and under nitrogen gas pressure of 40 psi. The hydrolysates pWPB were filtered according to Vilela *et al.* (2006), using a MWCO of 1 kDa. The filtrates were freeze-dried and stored at – 80°C under nitrogen gas until further use.

Cell Culture

Two immortalized respiratory epithelial cell lines (CFTE29o-, expressing mutant ΔF508 CFTR; and 1HAEo-, expressing wild type CFTR) were used to determine the effects of whey protein hydrolysates on IL-8 secretion. Cells were seeded at a density of 5 x 10^5 cells/mL in 24-well cell culture plates, in Minimal Essential Medium (MEM) supplemented with L-glutamine, antibiotics (penicillin/streptomycin) and 10% heat-inactivated Fetal Bovine Serum (FBS), and incubated at 37°C and 5% CO_2 for 24 hours or until confluent. The cells were then pre-incubated with hydrolysates at several doses (0 – 1000 μg/mL) for 1 h with fresh antibiotic-free MEM, in low-serum conditions (2%). Cells were then stimulated with LPS (2.5 μg/mL), TNFα (1 ng/mL) or IL-1β (0.05 ng/mL), along with a fresh dose of hydrolysates. After incubation for 24 h, cell viability was assessed (see below) and the cell-free supernatant was collected and frozen at – 20°C for subsequent IL-8 determination. For analysis of total glutathione (GSH), cells were seeded in 35-mm culture dishes at a density of 5 x 10^5 cells/mL and incubated under the same conditions until confluent, then pre-treated with 1000 μg/mL of pWPI or nWPI hydrolysates for 1 h. The cells were then stimulated with LPS along with a fresh dose of hydrolysates. After incubation for 24 h, cells were collected for GSH analyses with a slight modification of our previous protocol (Vogel *et al.*, 2005). Briefly, cells were washed twice with PBS, scraped into 200 μL of fresh PBS, and transferred into a

microtube with 200 μL of ice cold 5% trichloroacetic acid. Samples were mixed vigorously and incubated on ice for 30 min with intermittent vortexing. Samples were then centrifuged at high speed (microcentrifuge, 16,000 g, 10 min, 4°C) to pellet acid-precipitated proteins. Supernatants were stored at – 80°C for GSH analysis and the pellets were saved for protein determination.

Cell Viability

Cell viability was assessed using the MTT assay (Mosmann, 1983; Dauletbaev *et al.*, 2005), based on the reduction of MTT reagent into purple formazan crystals by viable metabolically active cells. Briefly, cells were washed with PBS and incubated with MTT solution (0.5 mg/mL) for 3 h. The formazan crystals were then dissolved in 0.4 N HCl in isopropanol and dual absorbances were measured at 540 – 600 nm. Cell viability was expressed as % of control.

Interleukin-8 Analysis

IL-8 secretion in cell-free supernatant was assessed with a commercial human IL-8 ELISA kit (BD Biosciences) according to the manufacturer's instructions.

Analysis of Total Intracellular GSH (Total Gluathione)

To quantify glutathione, we utilized the enzymatic kinetic assay, adopted for Cobas Mira S chemistry analyzer (Lands *et al.*, 1999b), and further modified for the current studies. Protein-free supernatants were neutralized with 100 mM sodium phosphate buffer (pH 7.4; 5 mM EDTA) and automatically pipetted into a thermostated (37°C) cuvette. The assay reagents (dithiobis-nitrobenzoic acid, NADPH and glutathione reductase) were thereafter added sequentially. Optical density was monitored at 412 nm. Glutathione concentration in the sample was calculated using a serially diluted (0.25 – 8 μM) glutathione standard curve. Reagent concentrations were as described previously

(Dauletbaev *et al.*, 2001). Protein content was measured using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Analysis of Antioxidant Capacity of Cell-Free Supernatants

1HAEo- and CFTE29o- cells were incubated with pWPH or pWPB hydrolysates for 6 h, following which cell-free supernatant was collected. The antioxidant capacity of the supernatant was assessed using the Ferric Reducing Antioxidant Power (FRAP), based on the reduction of the Fe^{3+}-2,4,6-tripyridyl-*S*-triazine complex to the ferrous (Fe^{2+}) form, and was performed according to the method of Benzie and Strain (1996): FRAP reagent was prepared with sodium acetate buffer (300 mMol), 2.5 mL TPTZ solution (10 mMol in 40 mMol HCl), and 2.5 mL ferric chloride solution (20 mM in ddH_2O) in a 10:1:1 ratio, respectively. Cell-free supernatants were incubated with FRAP reagent for 60 min, at a 1:20 ratio. A standard curve was constructed with BSA and absorbances were read at 593 nm.

Analysis of TLR4 Surface Expression

Cells were seeded in 50 mm culture dishes at a density of 5 x 10^5 cells/mL, incubated at 37°C and 5% CO_2 until confluent, then treated with pWPH (1000 μg/mL) and LPS as described above. After 24 h stimulation, cells were detached using 50 mM EDTA, washed with PBS and incubated with blocking solution (PBS[1%BSA]) for 30 min. Cells were then washed and incubated with anti-TLR4 rabbit antibody for 1 h, washed again and incubated with Alexa Fluor 488 conjugated goat anti-rabbit antibody for 45 min. A FACScalibur flow cytometer coupled with CELLQuest software (BD Biosciences) was used to analyze 10,000 events. Cells were initially gated based on forward and side scatter characteristics and results were expressed as percent of cells with TLR4 surface expression.

Analysis of LPS Binding

LPS binding to surface TLR4 was assessed using a protocol adapted from Abate *et al.* (2010). Cells were seeded in 50 mm culture dishes and pretreated with 1000 µg/mL of pWPH. Cells were then detached using 50 mM EDTA, washed and incubated with 1000 µg/mL hydrolysates and 2.5 µg/mL FITC-LPS for 30 min at 4°C, in PBS(10% FBS) as a source of LPS-binding protein. After washing, cells were resuspended in PBS and 10,000 events were analyzed with a FACScalibur flow cytometer. Cells were initially gated based on forward and side scatter characteristics and results were expressed as percent of cells bound to FITC-LPS.

Limulus Amebocyte Lysate (LAL) Assay

The possibility of direct LPS binding and neutralization by the hydrolysates was assessed by the chromogenic Limulus Amebocyte Lysate (LAL) assay using a commercial kit (Endochrome). Briefly, pWPH at concentrations 12.5 – 1000 µg/mL in endotoxin-free water were incubated with LPS (2.5 µg/mL) for 30 min. Thereafter, 50 µL of the LPS-hyrolysate solution was placed into 96-well microtiter plates with an equal volume of LAL reagent and incubated at 37°C for 7 min. Addition of 100 µL of chromogenic substrate solution and further incubation for 5 min led to the development of a yellow color. The reaction was stopped with addition of 20% acetic acid and absorbances were read on a microplate reader at 405 nm.

Statistical Analysis

Data are presented as mean ± SEM. All experiments were performed in triplicate. For cell culture assays, results were compared by one or two-way analysis of variance for each cell line, with Tukey's *post hoc* test to determine statistically significant differences between treatment and control groups. A *p*-value less or equal to 0.05 was considered significant. Statistical analyses were performed using Sigma Stat v2.03 (Systat Software Inc., Chicago, IL).

4.3 Results

Effect of WPI Hydrolysates on Basal IL-8 Secretion

After a 24 h incubation, nWPH up to 1000 μg/mL had no effect on unstimulated IL-8 secretion by either cell line. However, in response to pWPH, IL-8 secretion decreased to 75.29% ± 12.73 of baseline in CF cells, and to 73.61% ± 5.93 of baseline in wild-type cells at a dose of 1000 μg/mL; although these decreases were not statistically significant (Figure 1).

Effect of WPI Hydrolysates on LPS-Induced IL-8 Secretion

Stimulation of CFTE29o- and 1HAEo- cells with LPS resulted in a significant increase (3.8 and 5.7-fold, respectively) in IL-8 secretion relative to baseline ($p<0.05$; data not shown). Figures 2a and 2b show the inhibitory effects of WPI on LPS-induced IL-8 secretion in both cell lines. In CF cells, both native and pressurized WPI hydrolysates induced a dose-dependent decrease in IL-8 secretion (Figure 2a). nWPH induced a maximum decrease of IL-8 release by 34% (i.e., 66.2% ± 0.11 relative to cells receiving LPS alone, $p<0.05$) at a dose of 1000 μg/mL. pWPH significantly suppressed IL-8 by 40% at a lower dose, of 500 μg/mL ($p<0.05$) and by 48% at 1000 μg/mL ($p<0.05$).

In wild type cells, there was a slight but non-significant decrease in IL-8 secretion at doses of nWPH up to 1000 μg/mL (Figure 2b). A more pronounced response was observed with pWPH, which significantly suppressed IL-8 by 38% ($p<0.05$) at 1000 μg/mL.

Effect of WPI Hydrolysates on TNFα-Induced IL-8 Secretion

Upon 24 h stimulation with 1 ng/mL of TNFα, CFTE29o- and 1HAEo- cells exhibited a greater increase in IL-8 secretion than under LPS stimulation (21.2-fold and 10.0-fold compared to controls, respectively; $p<0.05$; data not shown). To assess the possible

inhibitory effect of WPH on TNFα-induced IL-8 secretion, cells were pre-treated for 1 h with nWPH or pWPH (0 – 1000 μg/mL), then medium was replaced with new medium containing a fresh dose of hydrolysates and 1 ng/mL of TNFα for 24 h. No effect of either type of WPH was observed on IL-8 secretion by CFTE29o- cells (Figure 3a). As the IL-8 responses of both cell lines to 24 h stimulation with TNFα was significantly ($p<0.05$) more pronounced than with LPS, the exposure to TNFα was shortened to 1 h to rule out that the lack of effect was not due to an overwhelming IL-8 response. Cell-free supernatants were collected 23 h later. The resulting increase in IL-8 secretion was more moderate, 6.3-fold for CFTE29o- cells and 2.1-fold for HAEo- cells ($p<0.05$; data not shown). To test the effect of WPH under these stimulation conditions, cells were pre-treated with hydrolysates (500 and 1000 μg/mL) for 1 h, then washed and exposed to TNFα along with a fresh dose of hydrolysates for 1 h, and finally washed and given fresh medium with hydrolysates for an additional 23 h. Again, no effect with either type of WPI hydrolysate was seen on IL-8 secretion after exposure of either cell line to TNFα for 1 h (Figure 3b and 3c).

Effect of WPI Hydrolysates on IL-1β-Induced IL-8 Secretion

In view of the fact that WPH inhibited LPS- but not TNFα-induced IL-8 secretion, we tested whether an inhibition of IL-1β-induced IL-8 secretion would take place. Since LPS and IL-1β share a common intracellular pathway leading to IL-8 secretion after activation of their respective receptors (Verstrepen *et al.*, 2008), the presence or absence of an inhibitory effect against IL-1β-induced IL-8 production would provide information as to whether the inhibitory effect occurs intra- or extracellularly.

In preliminary experiments, cells were stimulated with 1 ng/mL of IL-1β for a duration of 1 h or 24 h, and cell-free supernatants collected at 24 h. These exposures resulted in large increases in IL-8 secretion by CFTE29o- cells (71.8-fold and 13-fold for the 24 and 1 h exposure, respectively) and in 1HAEo- cells (51.5-fold and 12.3-fold, respectively). Subsequently, the dose of IL-1β was decreased to 50 pg/mL. Exposure of CFTE29o- and 1HAEo- cells to this dose for 24 h resulted in a respective 7.9-fold and 21.1-fold increase

in IL-8 production ($p<0.05$; data not shown). Treatment with WPI hydrolysates as described above did not affect IL-1β-induced IL-8 secretion (Figure 4a and 4b). A shorter, 1 h exposure of the cells to this low dose of IL-1β was also tested, resulting in a more moderate IL-8 increase, 2.4-fold and 4.2-fold in CFTE29o- and 1HAEo- cells, respectively ($p<0.05$). Again, there was no effect of either WPI hydrolysates on IL-8 secretion under these conditions (Figure 4c and 4d).

Effect of WPI Hydrolysates on Cell Viability

Cytotoxicity of increasing doses of native and pressurized WPH was assessed by the MTT test, which assays mitochondrial activity as an indicator of cytotoxicity leading to mitochondrial dysfunction (Mosman, 1983). Neither nWPH nor pWPH affected cell viability at doses up to 1000 μg/mL (data not shown).

Effect of LPS and/or WPI Hydrolysates on Total Intracellular Glutathione

In order to assess the impact of treatments with LPS and WPH on cellular GSH status, total intracellular GSH content of cells was analyzed following 24 h stimulation with LPS (2.5 μg/mL), nWPH or pWPH (1000 μg/mL), or both. None of the treatment combinations significantly affected GSH levels (data not shown).

LPS-Neutralizing Activity

The ability of pressurized or native WPI hydrolysates to neutralize LPS from *E. coli* O55:B5 was determined by a quantitative chromogenic LAL assay. Neither of the hydrolysates inhibited the LPS procoagulant activity up to the highest concentration used in cell-culture experiments (1000 μg/mL of LPS). The use of two-fold higher concentrations of hydrolysates did not inhibit LPS-induced activation of the LAL (data not shown).

Effect of WPI Hydrolysates on Surface Expression of TLR4

Flow cytometry was used to assess the surface expression of TLR4 in response to 24 h treatment with LPS alone or in combination with pWPH (1000 μg/mL). There was no effect of either treatment on TLR4 surface expression by CFTE29o- or 1HAEo- cells in comparison to untreated controls (Figure 5a and 5b).

Effect of WPI Hydrolysates on LPS Binding to Surface TLR4

Figure 6 shows the effect of 1 h pre-treatment with pressurized and native WPH (1000 μg/mL) on the ability of LPS to bind to cell-surface receptors. Treatment with pWPH (Figure 6a) resulted in a significant reduction in cell-surface bound FITC-LPS in CFTE29o- and 1HAEo- cells (27.3 and 31.5%, respectively; $p<0.05$). On the other hand, treatment with nWPH resulted in a similar magnitude of decrease (31.1% in CFTE29o- and 31.7% in 1HAEo- cells) but did not reach statistical significance (Figure 6b).

Effect of pWPH and pWPB Hydrolysates on LPS-Induced IL-8 Secretion

Hydrolysates of pressurized WPI were also prepared according to a different digestion protocol (pWPB) previously adapted by Vilela *et al.* (2006). pWPB were compared to pWPH in terms of suppression of LPS-induced IL-8 secretion in 1HAEo- cells. pWPB hydrolysates inhibited IL-8 secretion by 16.6 and 26.2% at doses of 500 and 1000 μg/mL, respectively. pWPH inhibited IL-8 secretion by 25.3 and 28.1% at doses of 500 and 1000 μg/mL, respectively. For both types of hydrolysates, statistical significance was reached only at the higher dose of 1000 μg/mL. There was no significant difference between the two types of hydrolysates in terms of IL-8 suppression.

Effect of pWPI and pWPIB Hydrolysates on the Antioxidant Capacity of Cell-Free Supernatants

The ferric-reducing antioxidant power (FRAP) of cell-free supernatants was assessed after exposure of CFTE29o- and 1HAEo- cells to either pWPH and pWPB hydrolysates (1000 μg/mL) for 6 h. In CFTE29o- cells, both pWPH and pWPB induced an increase in FRAP, by 38.8 and 33.2%, respectively ($p<0.05$). Similar results were observed in 1HAEo- cells, where pWPH and pWPB induced a respective 35.2 and 30.2% increase in FRAP ($p<0.05$). Although in both cell lines, the increase in FRAP following pWPH treatment was slightly higher than following pWPB treatment, the differences were not statistically significant (Figure 8).

4.4 Discussion

The major original finding from the present work is that pre-treatment of both CF and non-CF respiratory epithelial cells with low molecular weight peptide and amino acid products from WPI digestion causes a decrease in LPS-induced IL-8 secretion. The present findings are a first demonstration that whey-derived peptides can downregulate the LPS-induced inflammatory response, which extends previous studies showing that administration of whey proteins demonstrates immunomodulatory effects in animal models (Beaulieu *et al.*, 2009; Hayashida *et al.*, 2004; Shimizu *et al.*, 2006; Shin *et al.*, 2005) and *in vitro* (Mattsby-Baltzer *et al.*, 2009; Otani *et al.*, 1996; Otani and Monnai, 1995).

A key observation in the present work was that IL-8 secretion was suppressed by the WPI hydrolysates following stimulation with LPS but not with TNFα or IL-1β, regardless of the duration of the exposure and the levels of IL-8 release. Ligand-binding to Toll-Like Receptor 4 (TLR4) by LPS or TNF-Receptor (TNFR) via TNFα results in two distinct signalling cascades which lead to transcription factor Nuclear Factor(NF)-κB activation and IL-8 release (Cario *et al.*, 2000; Yamin and Miller, 1997). Activation of the TLR4 by the LPS-LPS Binding Protein-sCD14 complex involves an adaptor protein, MyD88, and

the recruitment of the serine-threonine kinase IRAK (Medzhitov *et al.*, 1998; Wu *et al.*, 2005), which is not involved in TNFR signalling (Yamin and Miller, 1997). In view of the above, the present findings suggested that WPI hydrolysates may exert an inhibitory effect in the LPS pathway upstream of the convergence with the TNFα pathway leading to NFκB activation (Wu *et al.*, 2005). As part of the investigation into the mechanism of action of IL-8 suppression by WPH, we further stimulated the cells with IL-1β. Activation of the IL-1β receptor (IL-1R) leads to an intracellular signalling cascade that is shared by both the IL-1R and TLR4 (Verstrepen *et al.*, 2008). Since there was no effect of WPH on IL-1β-induced IL-8 secretion, this indicates that the WPH exert their effect on an event either prior to the recruitment of MyD88, at the receptor level, or on the adaptors recruited through the MyD88 independent pathway. In light of this information and of previous findings involving down-regulation of TLR4 expression by the mucolytic agent carbocysteine (Tokuda *et al.*, 2009), we tested the effect of LPS with or without WPH on cell-surface TLR4 expression but found no effect of either treatment. Previous studies have shown contradictory findings in terms of the possible induction of TLR4 surface expression by LPS, where some find an up-regulation (John *et al.*, 2010) and others find no effect (Muir *et al.*, 2004). These differential responses could be due to tissue specificity and differences in cell culture conditions (e.g. cells in submerged culture or at an air-liquid interface) employed in these and our studies.

As some food derived peptides may interact with cell-surface receptors (Foltz *et al.*, 2008; Kitazawa *et al.*, 2007; Tani *et al.*, 1990), inhibition of LPS activity by whey peptides could involve inhibition of the activation of TLR4. Our results show that the binding of LPS to cell-surface receptors was inhibited by WPH. Lee *et al.* (1998) proposed that a direct inhibition of the binding of LPS to monocytes by lactoferrin was the mechanism by which lactoferrin feeding of neonatal piglets exerted anti-inflammatory effects following intravenous exposure to LPS. A variety of anti-bacterial peptides including whey-derived peptides have been shown to neutralize LPS via direct binding to the lipid A portion of LPS (Zhang *et al.*, 1999). Since the lipid A portion of LPS is responsible for both TLR and LAL activation (Iwanaga *et al.*, 1992; Rietschel *et al.*, 1993), inhibition of LPS activation of the LAL reagent in the presence of WPI peptides

would indicate the neutralization of the biological effects of LPS. In the present study, however, no interaction between the whey protein-derived peptides and LPS was indicated by the LAL assay. Hence, the whey peptide mediated inhibition of LPS-induced IL-8 release was not a result of a consequent neutralization of the endotoxin on the lipid A portion of the molecule. Taken together, the data suggest that the inhibitory effect was likely due to a direct interaction of the WPH with the receptor, thereby preventing LPS recognition, or to interference with the recognition of LPS by CD14 or LPS-binding protein, events which are necessary for TLR4 activation.

Another possible mechanism by which WPI hydrolysates may have modulated the inflammatory response is via improved antioxidant status either by provision of hydrogen donating peptides or by contributing amino acids with antioxidant capacity, such as cysteine, tyrosine or tryptophan (Pihlanto, 2006). Redox status is a known modulator of the inflammatory response (Rahman *et al.*, 2005), as oxidative stress up-regulates the production of inflammatory cytokines *in vitro* (Gomez-Quiroz *et al.*, 2003; Gutierrez-Ruiz *et al.*, 2001). Whey proteins have antioxidative *in vivo* effects (Pihlanto, 2006, Zommara *et al.*, 1996, Elia *et al.*, 2006) and whey-derived peptides have been shown to exert *in vitro* (Hernandez-Ledesma *et al.*, 2005) and *in vivo* free radical-scavenging activities. Our results show that WPH significantly increased the antioxidant capacity of cell culture supernatant as assessed by the FRAP assay. Although cell supernatant antioxidant activity is not an ideal measure of overall antioxidant status, the relative intracellular antioxidant activities of flavonoids has been found to correspond to their antioxidant capacity in cell culture medium (Kim *et al.*, 2006). Alternatively or in addition, WPI hydrolysates may also have influenced intracellular redox status by affecting cellular GSH status. The provision of antioxidants such as N-acetyl-cysteine down-regulates the IL-8 response to LPS (Gosset *et al.*, 1999; Tanaka *et al.*, 1997), either through direct action as an antioxidant or via induction of intracellular GSH. Conversely, GSH depletion results in exacerbation of the LPS-induced inflammatory response (Haddad, 2002). Whey proteins and peptides, a rich source of cysteine, the rate-limiting precursor for GSH synthesis, have been shown to increase GSH content *in vitro* (Vilela *et al.*, 2006; Kent *et al.*, 2003; Baruchel and Viau, 1996) and *in vivo* (Zavorsky *et al.*, 2007;

Bounous *et al.*, 1989; Bounous and gold, 1991; Lands *et al.*, 1999a). Although measurements of total intracellular GSH content indicated that whey peptide treatment had no effect, it is conceivable that whey protein hydrolysates may have improved the reduced to oxidized glutathione ratio (GSH:GSSG) to modulate the inflammatory response. Previous work has shown that total GSH concentrations may be unchanged despite relative changes in the GSH:GSSG (Glosli *et al.*, 2002). Due to time constraints, we were unable to measure the GSH:GSSG ratio in this study.

Our results show that pressurized WPI hydrolysates are more effective than unpressurized WPI at suppressing LPS-induced IL-8 secretion as lower doses are required to obtain a significant decrease as opposed to native WPI hydrolysates. Previous results (Vilela *et al.*, 2006) and the present study findings demonstrate that pressurization results in qualitatively different peptide profiles from those obtained from native whey digestion as opposed to an increased number of available peptides (Iskandar *et al.*, 2011). As pressurization of whey proteins potentiated the inhibitory effect of hydrolysates on the IL-8 response to LPS, this likely reflects a profile of relatively greater amounts of immunomodulatory peptides generated from the hydrolysis of pressurized WPI. In that regard, hyperbaric pressure treatment of whey proteins induces changes in their secondary and tertiary structures, exposing hidden and otherwise unavailable peptide sequences to enzymatic digestion. Indeed, whey proteins are highly resistant to enzymatic digestion (de Wit, 1998; Reddy *et al.*, 1988), and undigested whey proteins (namely β-lactoglobulin, α-lactoglobulin, immunoglobulins and lactoferrin) have been found in the intestinal lumen (Walzem *et al.*, 2002). Increasing the susceptibility of whey proteins to digestion via pressurization may alter the spectrum of absorbable peptides to increase the availability of bioactive peptides for intestinal absorption. The effects of hyperbaric pressure processing may be considered analogous to fermentation, which has been indicated to release bioactive peptides with greater antioxidant and GSH stimulating *in vitro* or *in vivo* effects (Zommara *et al.*, 1996; 1998). Fermentation, however, involves the use of bacterial enzymes to produce bioactive peptides, while pressurization facilitates their enhanced production through digestion involving the host's gastrointestinal enzymes.

Although the inhibition of LPS binding to TLR4 by nWPH did not reach statistical significance, the magnitude of inhibition was comparable with that of pWPH. It was observed, however, that pWPH exhibited a 21.1% higher antioxidant activity (Iskandar *et al.*, 2011) than nWPH. This difference could partly explain the improved efficiency of LPS-induced IL-8 suppression of pWPH over that of nWPH. Thus, it is likely that the mechanism of action of the observed IL-8 inhibition by WPH involves a combination of antioxidant activity in addition to preventing the binding of LPS to surface receptors.

Cell viability was unaffected from the treatment with either LPS (2.5 μg/mL) or WPH ranging at doses up to 1000 μg/mL. Therefore, the decrease in LPS-induced IL-8 secretion in CFTE29o- and 1HAEo- cells in response to WPH was not accountable by a decrease in cell viability. In contrast, Kent *et al.* (2003) found a significant decrease in viability in prostate epithelial cells exposed to whey protein hydrolysates at a dose of 1000 μg/mL. Differences in peptide composition of the unfiltered hydrolysates (Kent *et al.*, 2003), which may contain large peptide fragments as well as enzyme remnants including trypsin, could have accounted for differences in cell viability.

Our results contrast those from previous work using a different hydrolysis protocol that demonstrated a trend of WPI hydrolysates to decrease TNFα-induced IL-8 release in CF cells (Vilela *et al.*, 2006), which was unobserved in the present study. Moreover, in contrast to the present work, under unstimulated conditions, Vilela *et al.* showed an increase in IL-8 release following whey hydrolysate treatment. The contrasting results could be due to differences in the digestion and peptide isolation protocols, as hydrolysates generated via different enzyme mixtures or digestion procedures can yield differing biological effects (Pacheco and Sgarbieri, 2005; Prioult *et al.*, 2004). The use of an ultrafiltration membrane of MWCO 10 kDa in the current study as opposed to the 1 kDa membrane utilized by Vilela *et al.*, would result in a peptide mixture with a higher proportion of peptides relative to free amino acids and a peptide fraction containing higher and medium MW peptides. Preliminary analyses of the pWPH hydrolysates by ESI-MS revealed that the great majority of peptides are of sizes below 1 kDa (data not shown), which is within the molecular weight range typically presumed to be suitable for

gastrointestinal absorption (Arbit *et al.*, 2006). Furthermore, ultrafiltration with a 1 kDa membrane could lead to a higher proportion of salts in the final lyophilized product, which could affect IL-8 production under basal conditions (Tabary *et al.*, 2000). The differences in peptide profiles between the pWPH (Iskandar *et al.*, 2011) and pWPB (Vilela *et al.*, 2006) could correspond to the differences in biological activities observed. However, the hydrolysates do not differ with respect to all of their possible biological activities as both decreased LPS-induced IL-8 secretion and increased cell culture supernatant FRAP values to a similar extent.

To our knowledge, the present findings demonstrate for the first time that whey-derived peptides could impact upon inflammatory responses involving LPS-mediated IL-8 release and extends clinical work showing a trend to IL-8 down-regulation with pressurized whey supplementation in CF patients, in addition to enhanced nutritional status (Lands *et al.*, 2010). There is increasing evidence that gram-negative bacterial infections that lead to LPS-induced IL-8 release can exacerbate the inflammatory responses in chronic lung diseases such as CF (Sethi and Murphy, 2001; Sethi *et al.*, 2006; Watt *et al.*, 2005) and thereby contribute to lung pathophysiology. Although more studies are needed, the present work provides new insight and opens new avenues of research into the potential utilization of whey proteins in proinflammatory conditions involving IL-8 such as CF, particularly in relation to bacterial induced inflammation. Effective suppression of IL-8 hypersecretion is a valid therapeutic target in CF (Konstan and Davis, 2002). Oral corticosteroids have unacceptable adverse side effects, inhaled corticosteroids have yet to prove long-term efficacy, and ibuprofen has not gained popularity due to its varying bioavailability and potential adverse effects (Dinwiddie, 2005). The IL-8 suppressing effects demonstrated herein add an interesting possible therapeutic dimension to whey proteins as a nutrition-based adjuvant to conventional therapy towards restoration of a homeostatic anti-inflammatory balance in CF.

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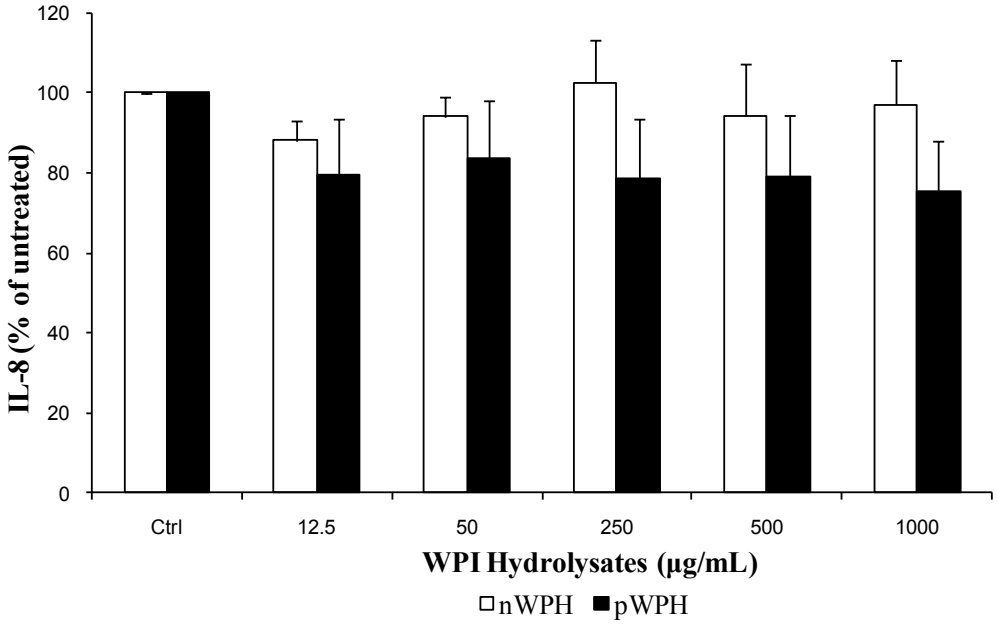
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WPI Hydrolysates (µg/mL)
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b

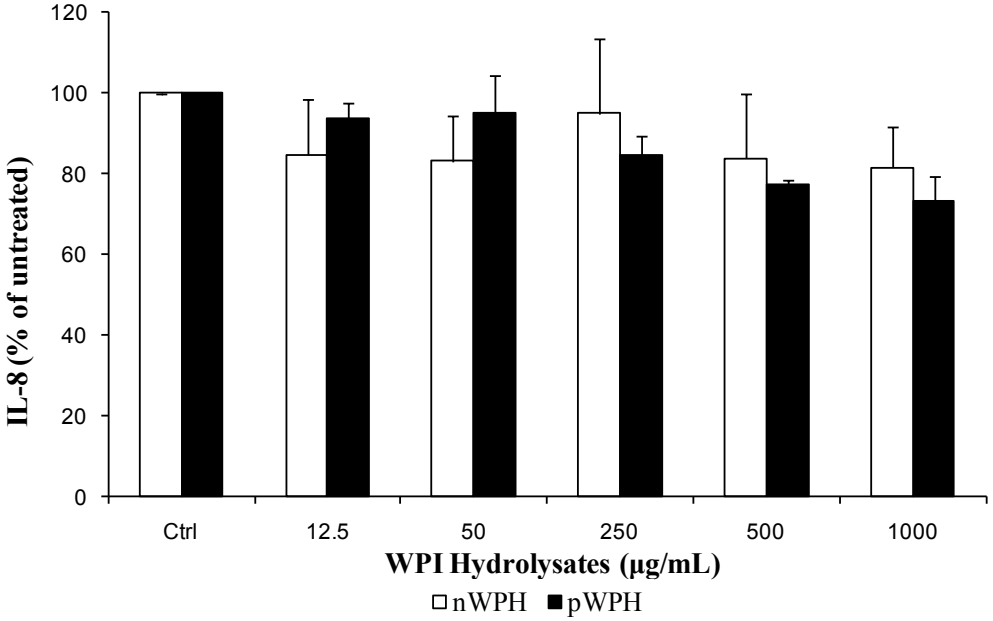
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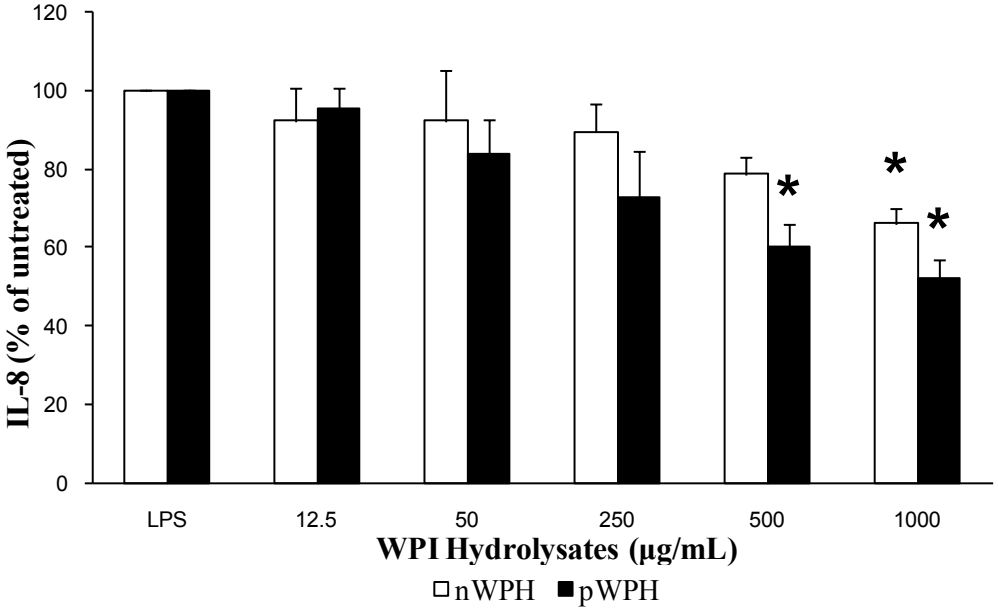
Figure 1. Effect of native and pressurized WPI hydrolysates on IL-8 secretion by (a) CFTE29o- and (b) 1HAEo- cell lines under unstimulated conditions. Cells were treated with nWPH or pWPH at doses 12.5 – 1000 μg/mL for 24 h and supernatant IL-8 concentrations were assessed. Data are represented as mean ± SEM of three to five independent experiments.

a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IL-8 (% of untreated)
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pWPH

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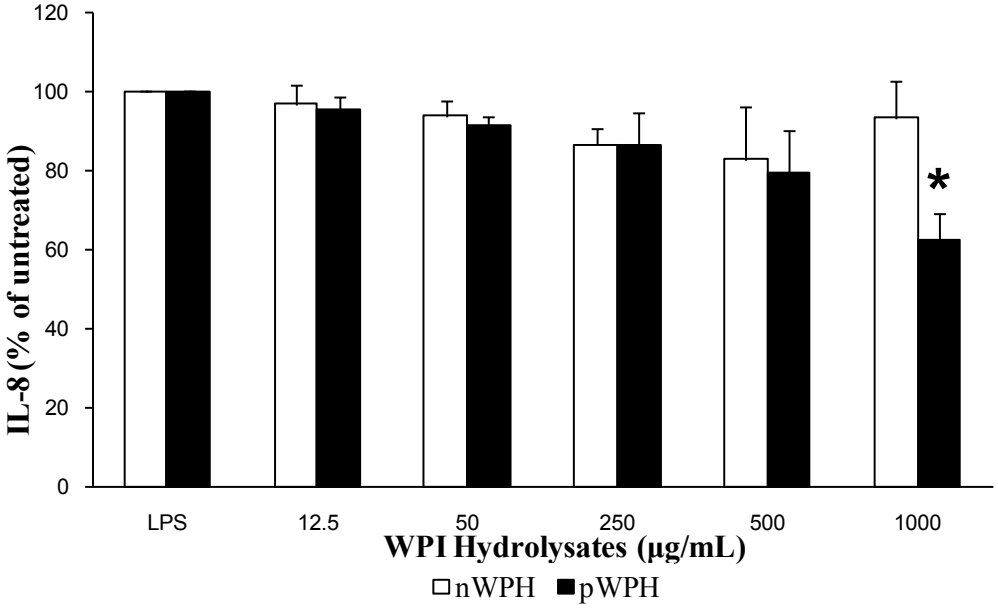
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IL-8 (% of untreated)
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LPS
12.5
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250
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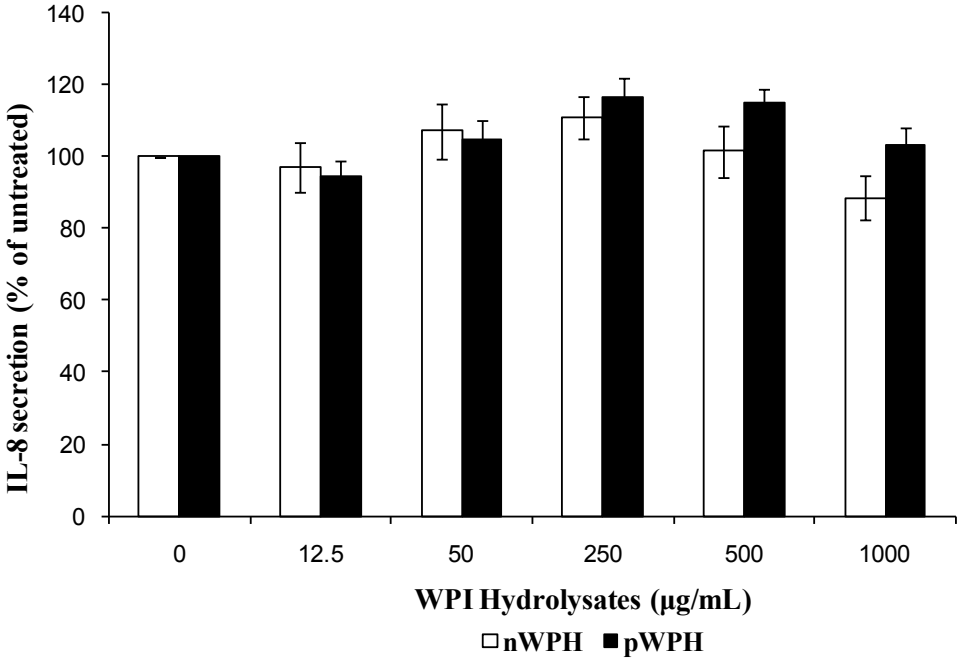
1000
WPI Hydrolysates (µg/mL)
nWPH
pWPH

Figure 2. Effect of native and pressurized WPI hydrolysates on LPS-induced IL-8 secretion by (a) CFTE29o- and (b) 1HAEo- cell lines. Cells were pre-incubated with WPH for 1 h, then stimulated with LPS (2.5 μg/mL) and fresh WPH for 24 h. Data are represented as mean ± SEM of three to five independent experiments. Asterisks (*) indicate significant differences ($p < 0.05$) as compared to untreated controls by ANOVA and Tukey's *post hoc* analysis.

a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IL-8 secretion (% of untreated)
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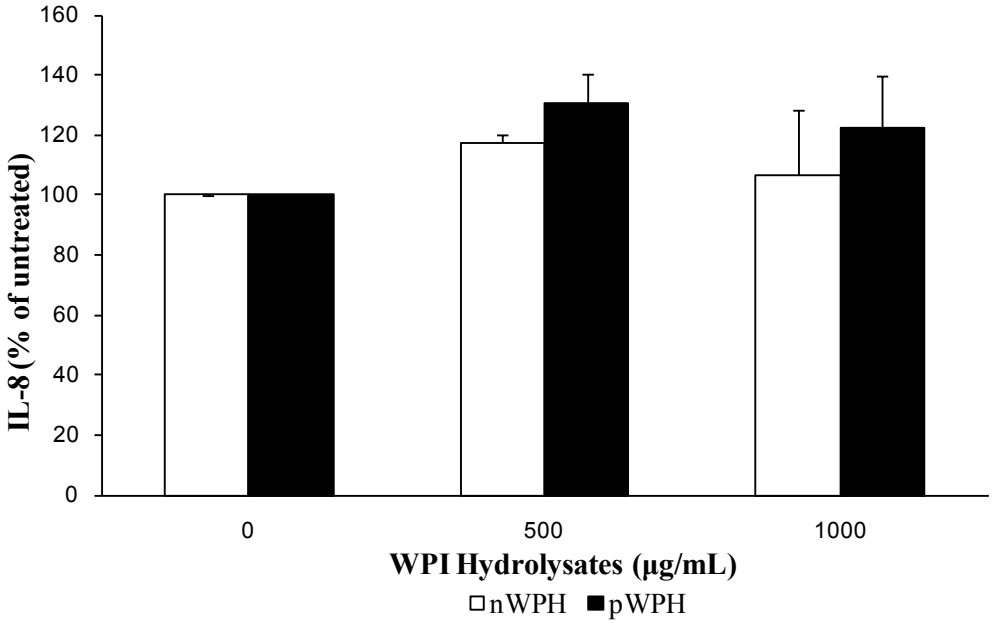
1000
WPI Hydrolysates (µg/mL)
nWPH
pWPH

b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80
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0
IL-8 (% of untreated)
0
500
1000
WPI Hydrolysates (µg/mL)
nWPH
pWPH

c

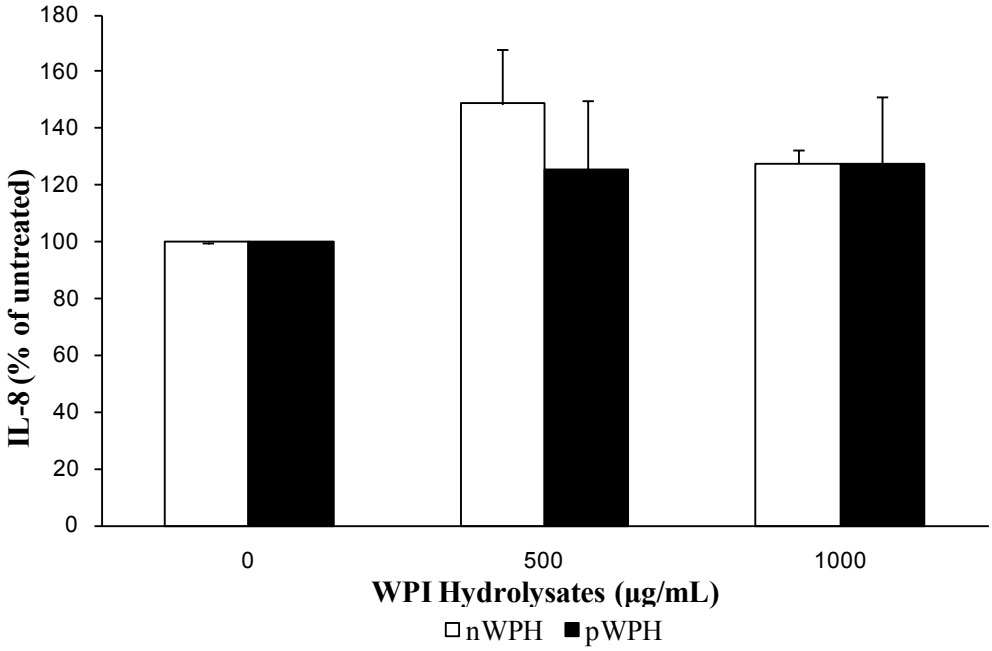
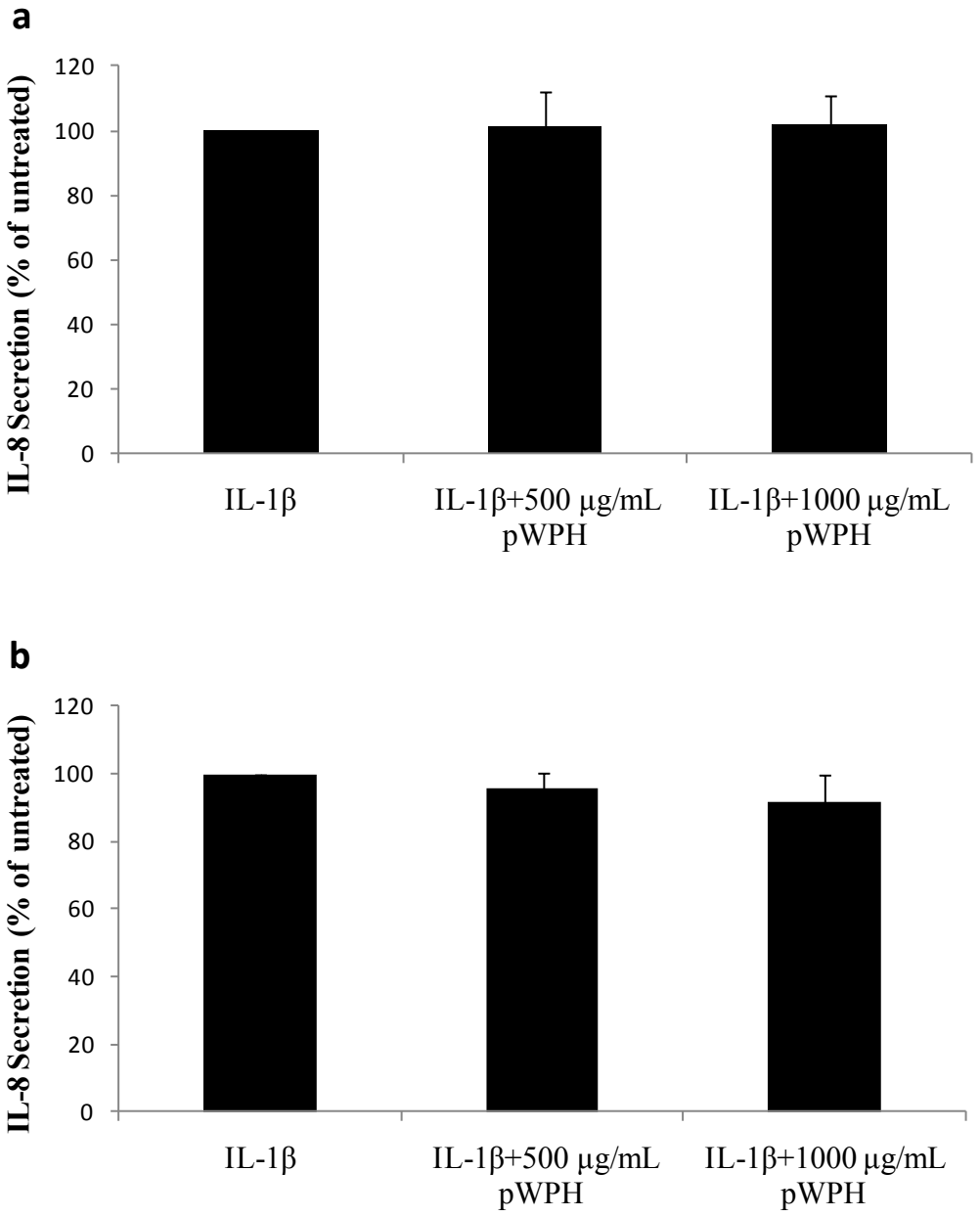


Figure 3. Effect of native and pressurized WPI hydrolysates on TNFα-induced IL-8 secretion by CFTE29o- and 1HAEo- Cell Lines. (a) CFTE29o- cells were pre-treated with WPH for 1 h, then stimulated with 1 ng/mL of TNFα along with fresh WPH for 24 h. (b) CFTE29o- and (c) 1HAEo- cells were pre-treated with WPH for 1 h, stimulated with 1 ng/mL of TNFα along with fresh WPH for 1 h, and supernatant collected for IL-8 analysis after 23 h of further incubation with WPH. Data are represented as mean ± SEM of three to five independent experiments.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IL-8 Secretion (% of untreated)
120
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0
IL-1β
IL-1β+500 µg/mL pWPH
IL-1β+1000 µg/mL pWPH
b
IL-8 Secretion (% of untreated)
120
100
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20
0
IL-1β
IL-1β+500 µg/mL pWPH
IL-1β+1000 µg/mL pWPH

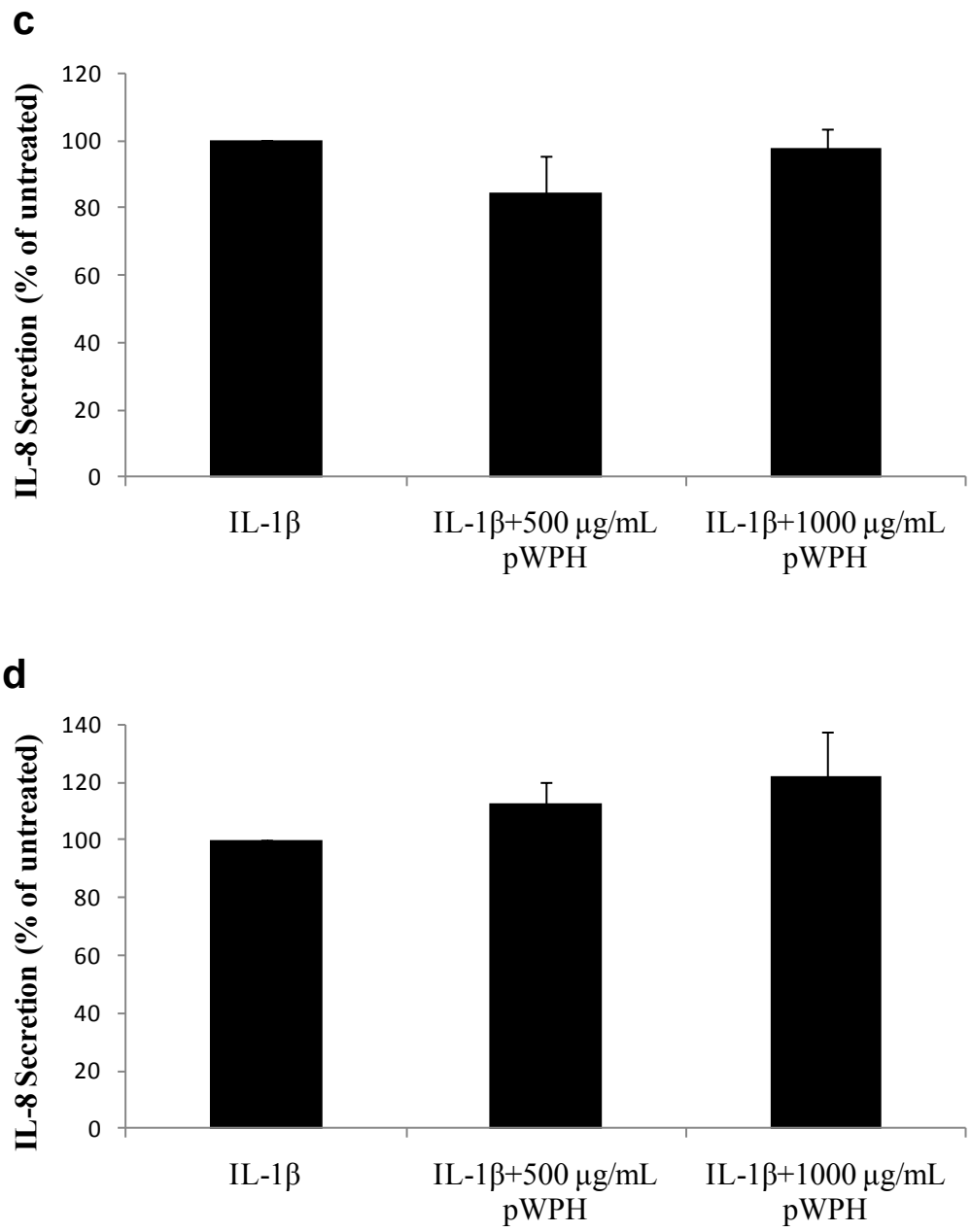
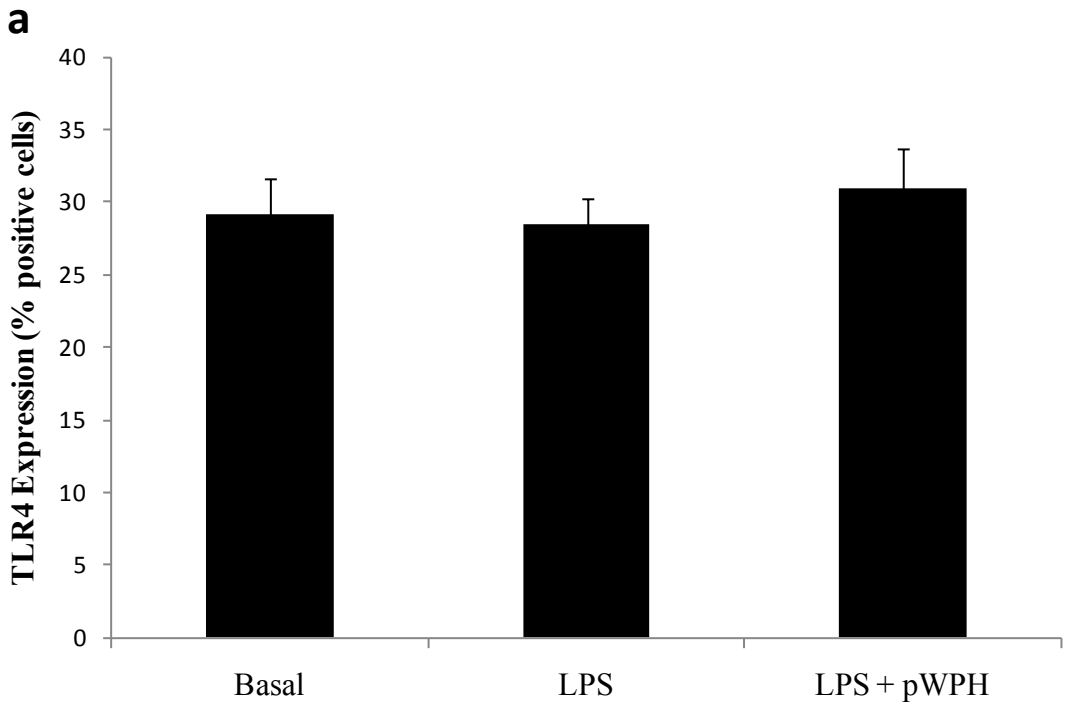


Figure 4. Effect of pressurized WPI hydrolysates on IL-1β-induced IL-8 secretion by CFTE29o- and 1HAEo- cell lines. (a) CFTE29o- and (b) 1HAEo- cells were pre-treated with WPH for 1 h, then stimulated with 50 pg/mL of IL-1β along with fresh WPH for 24 h. (c) CFTE29o- and (d) 1HAEo- cells were pre-treated with WPH for 1 h, stimulated with 50 pg/mL of IL-1β along with fresh WPH for 1 h, and supernatant collected for IL-8 analysis after 23 h of further incubation with WPH. Data are represented as mean ± SEM of three to five independent experiments.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TLR4 Expression (% positive cells)
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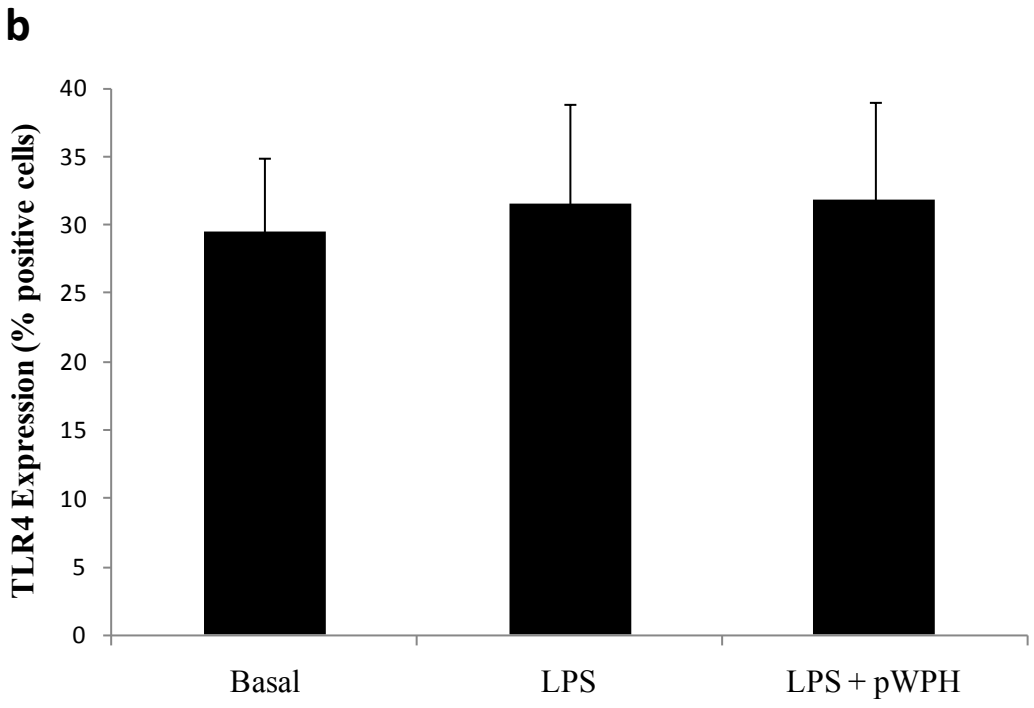
LPS
LPS + pWPH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TLR4 Expression (% positive cells)
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Basal
LPS
LPS + pWPH

Figure 5. Effect of pressurized WPI hydrolysates on surface TLR4 expression in (a) CFTE29o- and (b) 1HAEo- cell lines. Cells were pre-treated with WPH (1000 μg/mL) for 1 h, stimulated with LPS along with fresh WPH for 24 h, and assessed for TLR4 surface expression using an anti-TLR4 rabbit antibody by flow cytometry. Data are represented as mean ± SEM of three independent experiments.

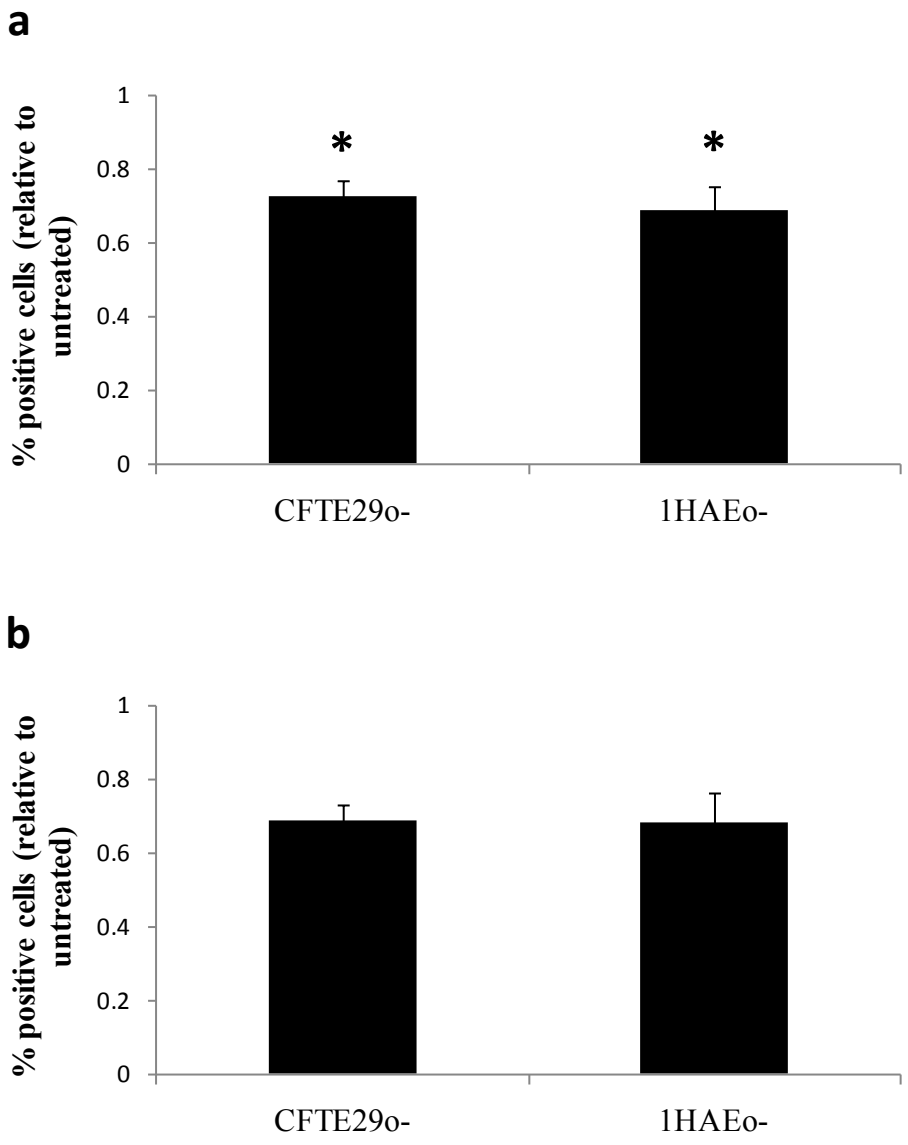


Figure 6. Effect of (a) pressurized and (b) native WPI hydrolysates on LPS binding to surface TLR4 in CFTE29o- and 1HAEo- cell lines. Cells were pre-treated with WPH (1000 µg/mL) for 1 h, detached and incubated with 1000 µg/mL hydrolysates and 2.5 µg/mL FITC-LPS for 30 min at 4°C, washed and resuspended in PBS for flow cytometric analysis. Data are represented as mean ± SEM of four independent experiments. Asterisks (*) indicate significant differences ($p < 0.05$) as compared to untreated controls by paired t-test.

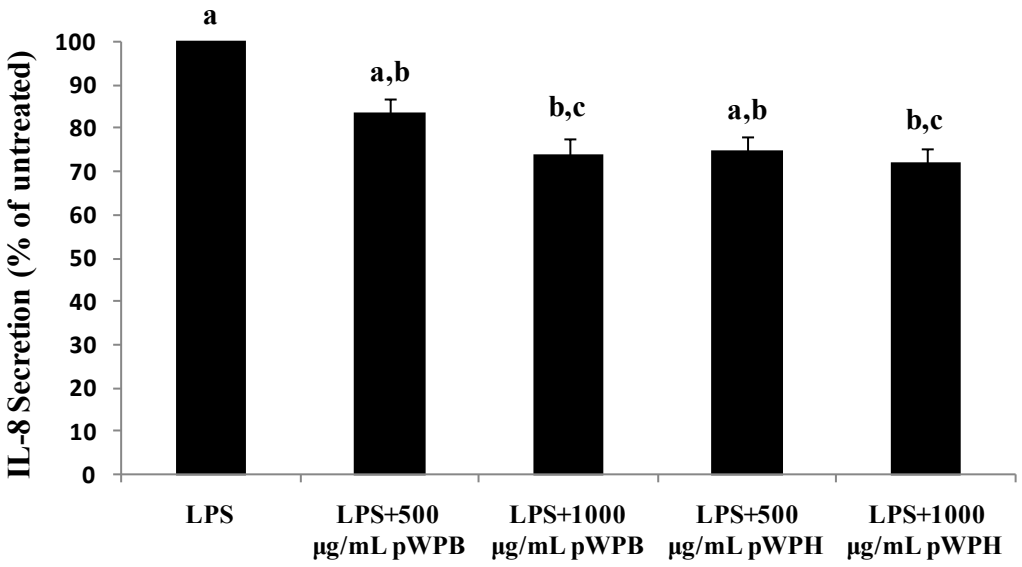
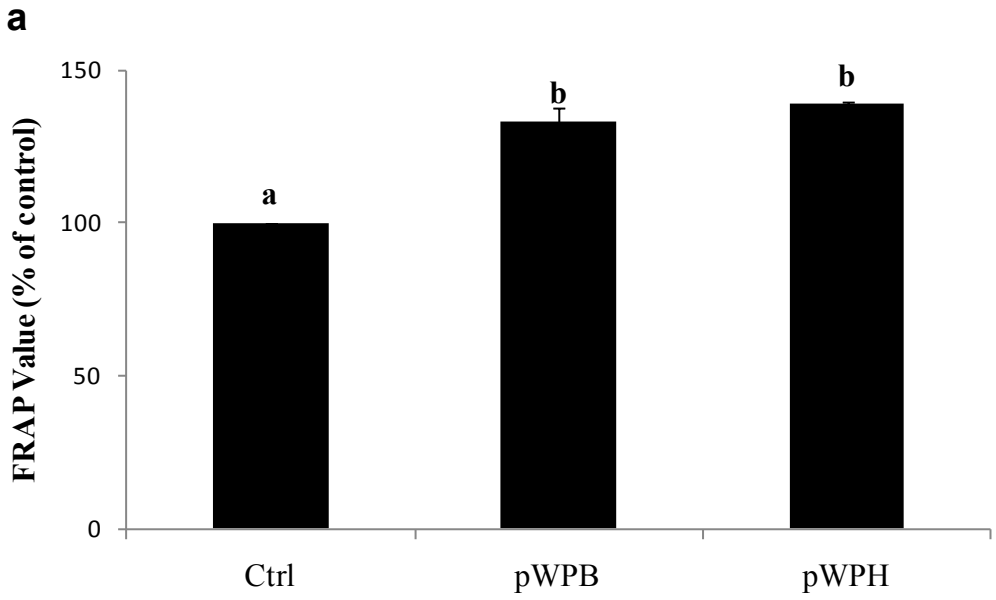


Figure 7. Effect of hydrolysates prepared using two different digestion protocols (pWPH and pWPB) on LPS-induced IL-8 secretion. 1HAEo- cells were pre-treated with pWPH or pWPB for 1 h followed by incubation with LPS and fresh pWPH or pWPB for 24 h. Data are represented as mean ± SEM of five independent experiments. Treatments not sharing common letters are significantly different ($p < 0.05$) by one way ANOVA and Tukey's *post hoc* analysis.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FRAP Value (% of control)
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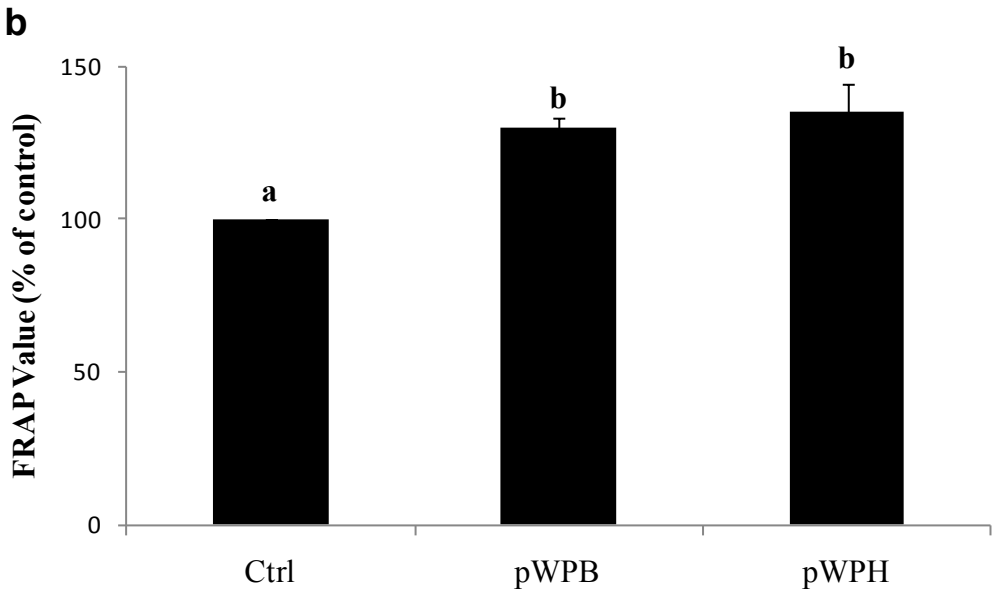
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FRAP Value (% of control)
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Figure 8. Effect of hydrolysates prepared using two different digestion protocols (pWPH and pWPB) on the FRAP of cell-free supernatants. (a) CFTE29o- and (b) HAEo- cells were incubated with either pWPH or pWPB (1000 μg/mL) for 6 h and cell culture supernatant was analyzed using the FRAP assay. Data are represented as mean ± SEM of three to four independent experiments. Treatments not sharing common letters are significantly different ($p < 0.05$) by one way ANOVA and Tukey's *post hoc* analysis.

CONNECTING STATEMENT

Results from Chapter 4 have shown that hydrolysates from native and hyperbaric pressure treated whey protein isolates suppress the LPS-induced IL-8 secretion in CF respiratory epithelial cells. Furthermore, the combined results from Chapter 3 and 4 have shown that hyperbaric pressure pre-treatment enhances the antioxidant and anti-inflammatory activities of whey protein hydrolysates. In view of the *in vitro* data obtained in the previous chapters, the next chapter investigates possible *in vivo* effects from the intake of pressurized whey protein isolate in CF patients. To that end, an open-label pilot clinical trial in children and adults with CF was conducted in order to assess the potential effects of short-term supplementation with pressurized whey protein isolate on nutritional status, lung function and markers of inflammation.

CHAPTER 5

Dietary Supplementation with Pressurized Whey in Patients with Cystic Fibrosis

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Abstract

Cystic Fibrosis (CF) is characterized by malnutrition, chronic pulmonary inflammation, and oxidative stress. Whey protein is rich in sulfhydryl groups and is recognized for its ability to increase glutathione and reduce oxidative stress. Previously, we have shown that supplementation with whey increased intracellular glutathione levels in patients with CF. We have subsequently shown that hyperbaric pressure treatment of whey protein promotes the release of novel peptides for absorption, increases intracellular glutathione in healthy subjects, and reduces *in vitro* production of interleukin (IL)-8. We hypothesized that pressurized whey supplementation in children and adults with CF could have significant nutritional and anti-inflammatory benefits. A pilot open-label study of 1-month dietary supplementation with pressurized whey in CF patients was undertaken to assess the effects. Twenty-seven patients with CF (nine children, 18 adults) were enrolled. The dose of pressurized whey was 20 g/day in patients less than 18 years of age and 40 g/day in older patients. Anthropometric measures, pulmonary function, serum C-reactive protein (CRP), whole blood glutathione, and whole blood IL-8 and IL-6 responses to phytohemagglutinin (PHA) stimulation were measured at baseline and at 1 month. Three adults withdrew (one with gastrointestinal side effects, two with acute infection). Both children and adults showed enhancements in nutritional status, as assessed by body mass index. Children showed improvement in lung function (forced expiratory volume in 1 second). The majority of patients with an initially elevated CRP showed a decrease. PHA-stimulated IL-8 responses tended to decrease in the adults. Whole blood glutathione levels did not change. Thus, oral supplementation with pressurized whey improves nutritional status and can have additional beneficial effects on inflammation in patients with CF.

Keywords: glutathione, inflammation, interleukin-8, nutrition

5.1 Introduction

Cystic fibrosis (CF) is the most common lethal genetic mutation in the Caucasian population, affecting approximately 1:2,900 white and 1:10,000 non-white live births in North America (Anselmo and Lands, 2008). CF is characterized by chronic suppurative lung disease with both pulmonary and systemic inflammation, malnutrition, and intestinal malabsorption. The major therapeutic goals are preserving lung function and improving nutritional status.

Lung disease in CF is characterized by a chronic neutrophilic inflammation (Elizur *et al.*, 2008). This inflammation is associated with oxidative stress and leads to progressive lung damage (Sagel *et al.*, 2002; Smountas *et al.*, 2004) and premature death. Oxidants damage the respiratory epithelium, leading to activation of redox sensitive transcription factors such as nuclear factor κB and production of pro-inflammatory cytokines such as the neutrophil chemoattractant interleukin (IL)-8. Oxidants also reduce antiprotease activity, leading to decreased killing of *Pseudomonas* by neutrophils (Birrer *et al.*, 1994; Buhl *et al.*, 1996; Starosta *et al.*, 2006; Voynow, Fischer and Zheng, 2008; Hartl *et al.*, 2007; Leavy, 2008). Oxidative stress thus propagates the dysregulated neutrophilic lung inflammation characteristic of CF that ultimately results in respiratory failure and untimely death.

Current anti-inflammatory therapies have either unacceptable adverse effects (systemic corticosteroids) (Eigen *et al.*, 1995), or demonstrate effectiveness but have concerns over potential adverse effects (high-dose ibuprofen) (Lands *et al.*, 2007) or effects that may not be long-lasting (azithromycin) (Tramper-Stranders *et al.*, 2007). These therapies have not addressed the oxidative stress in CF.

We, and others, have shown that CF patients are at risk for deficiencies of the central thiol antioxidant, glutathione (Lands *et al.*, 1999). Thiols (molecules with functional sulphydryl groups) are important peroxide scavengers and determinants of cellular redox potential. Redox potential affects the oxidation status of redox sensitive transcription

factors, such nuclear factor κB, that regulate the expression of pro-inflammatory cytokines like IL-8. Therefore provision of thiol donors could have significant antioxidant and anti-inflammatory benefits for CF patients.

Thiol donors have antioxidant properties and can serve as precursors for biosynthesis of the central anti-oxidant, glutathione. A recent observational study of patients using a combination of oral and inhaled glutathione found significant improvement in clinical markers (Visca *et al.*, 2008). *N*-acetylcysteine, a cysteine donor that can be used as a substrate for glutathione synthesis, decreases neutrophils and neutrophil elastase in CF sputum (Tirouvanziam *et al.*, 2006).

Cow's milk whey protein is rich in thiol amino acids, especially cysteine, and is recognized for its ability to increase glutathione and reduce oxidative stress. In our previous work, we have shown that supplementation with whey increased intracellular glutathione levels in patients with CF (Grey *et al.*, 2003). Hyperbaric pressure treatment of whey protein results in protein unfolding (López-Fandiño, 2006). This unfolding increases pressurized whey's *in vitro* digestibility and enhances feed efficiency in animal models (Jing *et al.*, 2004; Vilela *et al.*, 2006). Pressurization also alters the spectrum of peptides released upon *in vitro* digestion, increases intracellular glutathione in healthy subjects (Zavorsky *et al.*, 2007), and reduces *in vitro* production of IL-8 (Vilela *et al.*, 2006).

We hypothesized that supplementation with pressurized whey protein could result in improved nutritional and potentially modulate inflammatory status in CF patients. This pilot study was undertaken to look for short-term effects of pressurized whey protein supplementation in children and adults with CF.

5.2 Subjects and Methods

Subjects

Patients with CF (positive sweat test and/or two CF transport regulator mutations) above the age of 6 years were recruited. Patients had to be able to perform pulmonary function tests, have a baseline FEV_1 >50% predicted (Morris *et al.*, 1971), be willing to take the supplement for 1 month, and return for evaluation at the end of 1 month. Participants needed to have no change in their clinical status for the month preceding entry into the study. Exclusion criteria were patients with significant hepatic or renal impairment as judged by their treating physician, patients already taking a protein supplement, and patients with a documented allergy to cow's milk protein. The study had the approval of the Ethics boards of the McGill University Health Centre – Montreal Children's Hospital and Centre Hospitalier de L'Université de Montréal.

Intervention: In adults, participants were given 40 g of pressurized whey in applesauce (5 g/50 mL applesauce) per day for 28 days. Children under 18 years of age were given 20 g (20 participants). Data suggests that the recommended 1 – 1.5 g/kg/day of protein intake may be too low for CF patients (Geukers *et al.*, 2005). The daily dose of 40 g for an adult and 20 g for a child was projected to represent about 0.5 – 1.0 g/kg/day added protein and other studies have used a total of 2.9 – 3.8 g/kg/day in long term pediatric studies. Participants were instructed to take the supplement apart from a meal and to take pancreatic enzyme supplements equivalent to what they take for a snack. Patients were asked to otherwise continue their habitual dietary habits.

At the beginning and end of the intervention, subjects were weighed on an electronic balance and height measured on a stadiometer. For children, BMI was expressed as a z-score. Lean body mass was assessed by 4-site skinfold measurement (Durnin and Womesley, 1974). The skinfold measurements were done by one individual (NB). Lung function was assessed by spirometry and whole body plethysmography, according to American Thoracic Society criteria (Miller *et al.*, 2005; Wanger *et al.*, 2005). Blood was

drawn at the beginning and end to assess total and differential white blood cell count, high-sensitivity C-Reactive Protein serum concentration, whole blood glutathione concentration, and hepatic (alanine aminotransferase, aspartate aminotransferase, glucose tolerance test) and renal function (blood urea nitrogen, creatinine) safety laboratories. White blood cell count and safety laboratories were measured in the routine hospital laboratories of the participants' base hospital. High-sensitivity CRP was measured in the routine biochemistry laboratory of the Centre Hospitalier de L'Université de Montréal. Whole blood glutathione was assessed using the glutathione reductase recycling method by spectrophotometry as previously described by us (Lands *et al.*, 1999; Anderson, 1985; Dauletbaev *et al.*, 2005).

To assess cytokine production, heparinized whole blood was diluted (1:10) in RPMI 1640 supplemented with 2mM L-glutamine, penicillin/streptomycin (Invitrogen), and seeded in 24-well cell culture plates. 10µL of phytohemmaglutinin (PHA) solution (final concentration 5 µg/mL) or vehicle (PBS) were added to each well and the samples were incubated for 24 h at 37°C, 5% CO_2. Samples were then collected, centrifuged (900 g for 5 min), and the supernatants stored at - 80°C for subsequent analysis of IL-6 and IL-8 by ELISA (BD Biosciences, San Jose, CA, USA) (De Groote *et al.*, 1992).

Data Analysis: Values were expressed as mean ± SD. As both children and adults were included in this study, children and adults were analyzed separately, and comparisons between initial and follow-up values were performed by paired t-tests. A p-value <0.05 was considered as significant.

5.3 Results

Twenty-seven patients were enrolled, 18 of whom were adults of at least 18 years of age. Three adult subjects did not complete the study, one for gastrointestinal side effects, and two because of acute pulmonary infections. The final 24 subjects consisted of 9 children (6 males, 3 females) and 15 adults (5 males, 10 females).

Baseline data are found in Table 1. Overall patients were reasonably nourished, with an average BMI z-score of – 0.7 SD's in children and a BMI of 21 kg/m^2 in adults. Airflow limitation (Forced Expiratory Volume 1 sec, FEV_1) was mild in the children and tended to be more advanced in the adults ($p<0.06$). Airtrapping (RV/TLC, normal <25%) was evident in both groups, but more so in the adults ($p<0.01$). The C-Reactive Protein trended higher in the adult patients ($p<0.09$), suggesting greater inflammation. Genotype was available on 22 of the participants. All but one had at least one deltaF508 allele (9 homozygous) and the remaining participant was homozygous for Class I mutations.

The pressurized whey supplement represented an average protein intake of 0.7 g/kg in both the children (0.7 ± 0.32 g/kg/day) and adults (0.72 ± 0.12 g/kg/day). The applesauce employed for this study contained 20 g carbohydrate/120 mL and no fat or protein, and had a caloric content of 80 kcal/120 mL; each 50 mL package contained 5 g of whey protein. The four packages/day in children added 133 nonprotein kcal and 80 protein kcal in the children and the eight packages/day in adults added 266 nonprotein kcal and 160 protein kcal. This represented an increase of 7 – 8 kcal/kg/day in both the children (7.4 ± 3.42 kcal/kg/day) and adults (7.7 ± 1.31 kcal/kg/day).

In the children, there was an average weight gain of 480 g ($p>0.1$) (Table 2). BMI z-score was improved after 1 month of supplementation (from – 0.69 to – 0.52, $p<0.05$). Lean body mass (322 g increase) and percentage body fat (0.5%) increase did not change significantly.

In the adults, there was a significant weight gain of 840 g ($p<0.02$). BMI increased significantly (from 20.96 to 21.26, $p<0.03$). Lean body mass tended to increase with an average gain of 700 g, $p<0.08$, with no change in percentage body fat (0.1% increase).

Combining the children and adults, there was a significant relation between the change in weight and change in lean body mass ($r = 0.54$, $p<0.01$).

In children, there was a significant increase in the percent predicted FEV_1, increasing from 81% to 91% (Figure 1). There was no change in the adult values. Neither children nor adults had any significant change in the degree of airtrapping (RV/TLC).

Neither group had a significant change in the C-Reactive Protein values. However 10 of the 13 patients (2 children) with initial C-Reactive Protein values greater than 1.0 showed decreased values at follow-up ($p<0.05$).

There were no significant changes in total white count, percentage neutrophils, or absolute neutrophil count for either the children or adults. Whole blood glutathione measures were available at both time points in 19 participants (6 children) and this did not change significantly for either group.

In the adult patients, PHA-stimulated IL-8 tended to decrease following supplementation from 2053 (2469) to 1108 (981) pg/mL ($n=12$, $p<0.08$). This was not seen in the children. Neither group showed a change in the PHA-stimulated IL-6 response.

Of the 24 patients completing the trial, five adults, in addition to the one who withdrew, complained of abdominal pain that did not stop them from completing the study. One adult patient developed a transient facial rash. There were no changes in hepatic or renal function parameters.

5.4 Discussion

In this pilot open-label study, we have found that short-term supplementation of children and adults with CF with pressurized whey resulted in enhanced nutritional status (BMI z-score in children and BMI in adults), improved lung function (% predicted FEV_1) in children, and trends towards decreased inflammation. This is consistent with our previous work with pressurized whey supplementation in healthy individuals (Zavorsky *et al.*, 2007) and *in vitro* studies (Vilela *et al.*, 2006).

This pilot study is limited by the relatively small number of participants, the short time period of supplementation, and the lack of a control group. Another potential limitation is the use of skinfolds to assess lean body mass, rather than dual-x-ray absorptiometry (DEXA) or bioelectrical impedance. However, DEXA and skinfold measurements give similar values in this magnitude of change (Lands *et al.*, 1996). We designed this pilot with a short intervention period to look for any immediate signals, and to allow comparison with other short term interventions with cysteine donors. While it is yet to be demonstrated for CF patients, one year of N-acetylcysteine supplementation in patients with idiopathic fibrosis was helpful (Demedts *et al.*, 2005). It is therefore possible that maximal benefits were not realized in this pilot study in the one-month period and that prolonged beneficial effects may occur.

It is possible that the increased calories of the pressurized whey in applesauce may have been partially responsible for the improvements in nutritional status. However, although the use of supplements has been shown by some studies to increase total energy intake, several studies have not been successful in showing a positive effect of supplement use of comparable caloric contribution on nutritional status (Hanning *et al.*, 1993; Kalnins *et al.*, 2005). Furthermore, the increase in calories is unlikely to explain the improvement in lung function in children and the anti-inflammatory trends, suggesting functional effects of whey beyond its contribution in terms of energy and nutrients. We attempted to record habitual dietary intakes at the beginning and end of the study, but the number of returned records and their quality did not allow us to adequately assess the quantity and quality of the participants' diets. The lack of glutathione response in the current trial may potentially be due to the lack of concordance between whole blood glutathione concentrations and concentration in other tissue spaces, such as inflammatory cells or lung tissue (Zavorsky *et al.*, 2007; Otterbein and Choi, 2002).

The changes in nutritional status are similar to what we have witnessed in rodent studies using semi-purified diets composed of either unpressurized whey or pressurized whey (Jing *et al.*, 2004). Typically we have witnessed that after two weeks of feeding, rodents fed pressurized whey diets grow faster, and demonstrate enhanced feed efficiency.

CF is characterized by an excessive neutrophilic inflammation and accompanying oxidative stress that contribute to worsening lung function (Sagel *et al.*, 2002; Smountas *et al.*, 2004). Decreased lung function is the primary cause of early death in CF, with FEV_1 being the primary prognostic factor for mortality in CF. Nutritional status is also a significant prognostic factor (Sharma *et al.*, 2001). Reduction in neutrophilic recruitment and the consequences of this dysregulated neutrophilic inflammation has shown benefit (Eigen *et al.*, 1995; Tramper-Stranders *et al.*, 2007). However, concerns over safety or lack of long term efficacy suggest that alternative approaches are needed. Thiol donors can have benefits both from their direct peroxide scavenging potential, but also from their ability to alter gene expression through redox-sensitive transcription factors.

There are several potential mechanisms by which thiol donors could act. Interleukin-8 suppression through reduced activation of redox sensitive transcription factors Nuclear Factor κB and Activating Protein-1 would reduce neutrophil recruitment. The protection of intraluminal proteases from oxidant damage would reduce the amount of free elastase (Birrer *et al.*, 1994; Buhl, Meyer and Vogelmeier, 1996; Starosta *et al.*2006; Voynow, Fischer and Zheng, 2008). This would enhance the killing of *Pseudomonas aeruginosa* by neutrophils (Hartl *et al.*, 2007; Leavy, 2008) and diminish elastase-stimulated mucin and Interleukin-8 production by epithelial cells (Voynow, Fischer and Zheng, 2008). An observational study of inhaled plus oral glutathione noted a decrease in the number of positive cultures for *Pseudomonas aeruginosa* (Visca *et al.*, 2008).

Whey, a natural byproduct of cheese manufacturing, is rich in thiols (Nicodemo *et al.*, 2000). Hyperbaric pressure treatment of whey increases the digestibility of whey, by unfolding the whey proteins and making them more susceptible to digestion by pancreatic enzymes (López-Fandiño; 2006; Vilela *et al.*, 2006). We have found pressurized whey to be relatively well-tolerated and result in increased intracellular glutathione levels in healthy individuals (Zavorsky *et al.*, 2007) and decreased IL-8 secretion in respiratory epithelial cells (Vilela *et al.*, 2006).

In a 3-month study of non-pressurized whey supplementation in CF patients (Grey *et al.*, 2003), supplementation increased glutathione levels, but there were no accompanying changes in nutritional status or lung function. No changes in nutritional status were reported in a recent 1-month pilot study of N-acetylcysteine (Otterbein and Choi, 2002). The study of patients using inhaled and oral glutathione also did not show nutritional changes at 1 month, but did show significant improvements at 5.5 months (Visca *et al.*, 2008). We speculate that the beneficial effects of pressurized whey could be due to enhanced feed efficiency, which we have observed in animal studies (unpublished) or anti-inflammatory effects that are not necessarily related to glutathione concentrations.

N-acetylcysteine reduced sputum neutrophil counts and IL-8 concentrations in those CF patients with initially elevated levels (Otterbein and Choi, 2002). Consistent with this finding, we found that whole blood PHA-stimulated IL-8 tended to decrease. The effect of pressurized whey on PHA-stimulated cytokine production may be mediated through suppression of NF-κB (Janeway *et al.*, 1997). NF-κB is redox sensitive (Roebuck *et al.*, 1999), and so the abundance of thiols in pressurized whey may suppress its activation.

The differences in lung function responses between children and adults that we observed are consistent with the results of high-dose ibuprofen in children (Konstan *et al.*, 1995; Lands and Stanojevic, 2007). Lung function reflects both current status and previous health and injury. Further, with time there tends to be more parenchymal damage and scarring, which is unlikely to change with any therapy. Thus it is not surprising that young lungs, especially with well-maintained function, are more plastic and amenable to therapy. The results of pressurized whey, whether it is helping with clearance of secretions or modulating inflammation, are consistent with this previous work.

In conclusion, pressurized whey supplementation represents a natural dietary-based approach to enhancing nutritional status. Pressurized whey supplementation also appears to have potential immune modulating effects that could be beneficial for CF patients over the long term.

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Table 1. Baseline Characteristics of Study Participants

	Mean (SD)	
	Children (n=9)	*Adults (n=15)*
Age (years)	10.8 (3.8)	29.9 (5.9)
Male:female	6:3	5:10
Height (cm)	141.0 (24.69)	164.1 (8.31)
Weight (kg)	34.4 (15.16)	56.68 (9.28)
Lean Body Mass (kg)	28.3 (12.22)	44.0 (8.87)
Body Fat Percentage (%)	17.3 (2.72)	22.4 (7.46)
BMI z-score (SD's)	-0.69 (0.698)	
BMI (kg/m^2)		20.96 (2.46)
FEV_1 (% predicted)	80.1 (16.78)	67.1 (15.43)
RV/TLC (%)	30.7 (7.91)	42.9 (7.06)
CRP (pg/ml)	0.7 (0.74)	7.5 (11.18)
White Blood Cell Count (10^9/l)	8.65 (3.48)	8.22 (1.80)[a]
Relative Neutrophil Count (%)	50.8 (16.48)	66.6 (9.09)[a]

[a]n=11.

Table 2. Response to Pressurized Whey Supplementation in Children and Adults with CF

	Change posttreatment – pretreatment (SD)	
	Children (n=9)	*Adults (n=15)*
Weight (g)	480 (832)	840 (1,309)*
Lean body mass (g)	322 (736)	703 (1,467)**
Body fat percentage (%)	0.5 (2.25)	0.08 (2.07)
BMI z-score (SD)	0.16 (0.206)*	
BMI (kg/m^2)		0.30 (0.487)*
FEV_1 (% predicted)	10.7 (13.77)*	0.7 (6.63)
RV/TLC (%)	0.4 (6.2)	1.6 (4.53)
CRP (pg/ml)	3.4 (10.95)	3.0 (9.6)

Data are absolute changes from baseline (SD).

* $P<0.05$, ** $P<0.01$.

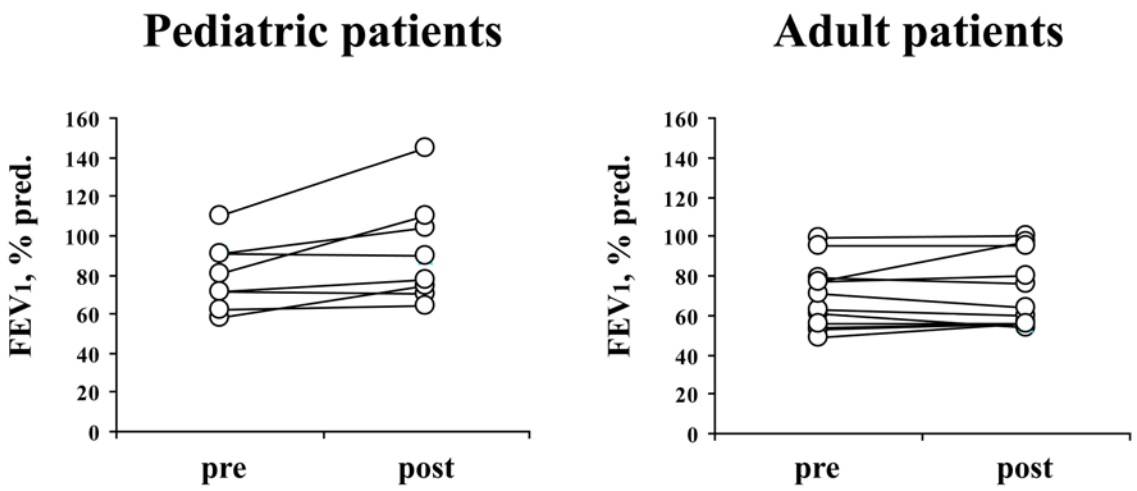

Figure 1. Changes in lung function (FEV_1 percentage predicted) in children and adults with CF supplemented with pressurized whey for 1 month.

CHAPTER 6

FINAL CONCLUSION AND SUMMARY

A number of biological activities have been attributed to whey proteins, ranging from angiotensin-converting-enzyme-inhibitory to antioxidant activities (Kirssansen, 2007; Brody, 2000; Clare and Swaisgood, 2000; Oner *et al.*, 2006). The present work highlights the anti-inflammatory effects of whey protein via the suppression of IL-8 secretion, a potent pro-inflammatory chemokine, as well as the potentiation of these effects by hyperbaric pressure pre-treatment. Following the inhibition of lipopolysaccharide (LPS)-induced IL-8 secretion by whey protein hydrolysates, our investigations into the possible mechanisms of action have indicated that whey peptides prevented the binding of LPS to cell surface receptors. The above findings present implications beyond bacterial-induced inflammation in CF. LPS is a cell wall constituent of gram-negative bacteria (Lüderitz *et al.*, 1982). A key event in the induction of the host's inflammatory response to invading microorganisms is the recognition of bacterial components such as LPS by the innate immune system (Gabay and Kushner, 1999; Ulevitch and Tobias, 1999)

The pulmonary epithelium represents the body's largest surface area exposed to the external environment (Delclaux and Azoulay, 2003). It is constantly exposed to a myriad of airborne microorganisms, irritants and toxins. It is well established that pulmonary epithelial cells actively participate in innate immunity by contributing to the inflammatory response, namely via the production of IL-8 (Strieter, 2002). In CF, a chronic, dysregulated inflammation leads to lung tissue damage, resulting in a gradual worsening of lung function and eventually death (Chmiel *et al.*, 2002). Frequent bacterial infections, particularly with *Pseudomonas aeruginosa,* exacerbate the inflammatory response and lung function deterioration (Gibson *et al.*, 2003). LPS, also termed endotoxin, is a component of the outer membrane of gram-negative bacteria (Rietschel *et al.*, 1994). LPS concentrations in the airway lumen correlate with IL-8 and neutrophil concentrations (Muhlebach and Noah, 2002). Although LPS concentrations in the bronchoalveolar lavage fluid (BALF) of CF patients are not particularly elevated when

compared to non-CF patients (Muhlebach and Noah, 2002), it has been shown that exposure of airway epithelial cells to LPS markedly up-regulates the expression of *MUC2* (Li *et al.*, 1997), the gene coding for mucin, a glycoprotein component of mucus. In normal airways, the mucosal layer lubricates and protects the pulmonary epithelium (Rose, 1992). In CF, however, an overproduction of underhydrated thick mucus takes place, slowing mucociliary clearance and facilitating bacterial adherence (Saiman, 2004). Increased mucin secretion in CF in response to LPS exposure can therefore contribute to increased mucus plugging of the airways (Li *et al.*, 1997). Thus, in addition to the modulation of LPS-induced IL-8 secretion by the airway epithelium, preventing other cellular responses to endotoxin exposure could represent great benefit in attenuating the exaggerated and persistent inflammation that is one of the hallmarks of CF pathology.

Mucus overproduction, to which exposure to LPS can contribute, is also a feature of other chronic lung pathologies, such as asthma, allergic rhinitis and chronic obstructive pulmonary disease (COPD) (Voynow and Rubin, 2009; Wang *et al.*, 2008). In COPD, bacterial pathogens, particularly gram-negative bacteria such as *Haemophilus influenza or Moraxella catarrhalis,* are implicated in a large proportion of acute exacerbations (Sethi and Murphy, 2001; Sethi *et al.*, 2002). In addition, LPS and TLR4 on pulmonary epithelial cell surfaces have been implicated in a number of aspects of the pathology of asthma and allergic rhinitis, such as airway hyper-responsiveness. LPS stimulation of cultured mouse tracheae has been shown to augment the bradykinin-induced contractile response (Bachar *et al.*, 2004). Bronchial reactivity, airway neutrophil-dominated inflammation, and airflow obstruction can be induced by LPS inhalation in patients with mild asthma (Eldridge and Peden, 2000).

LPS can be found in significant amounts in air pollution particulate matter (Mueller-Anneling *et al.*, 2004), cigarette smoke (Hasday *et al.*, 1999), but also in grain dust, textiles, indoor ventilation systems, carpeting and household pets (Reed and Milton, 2001). Its ubiquitous nature coupled with the fact that endotoxin inhalation by healthy subjects can also lead to acute airway inflammation and obstruction, albeit at higher doses

than required for asthmatics (Sandstrom *et al.*, 1992), point to potential benefits of pressurized whey protein isolate (WPI) as a means to control or abate such reactions.

The stimulation of monocytes and macrophages by LPS to secrete TNF-α, IL-1 and IL-6 is thought to be an important cause of septic shock (Wood *et al.*, 2004), a leading cause of death in intensive care patients (Martin *et al.*, 2003). The production of pro-inflammatory cytokines stimulates neutrophils and endothelial cells to release reactive oxygen species, hypochlorous acid, and nitric oxide, among others, in order to destroy the invading pathogens. However, these same mediators can lead to damage to tissues and organs, ultimately resulting in multiple organ dysfunction syndrome (MODS) (Werdan, 2001). Mortality rates from septic shock are estimated at 40 – 60% (Brun-Buisson, 2000). Attempts to abate the systemic inflammatory response by targeting, for example, TNFα, IL-1 or NO using soluble receptors, antibodies, IL-1R antagonist or nitric oxide synthase inhibitors have yielded marginal results at best (Zeni *et al.*, 1997; Freeman and Natanson, 2000). The activation of monocytes/macrophages results in diverse cellular responses; it is therefore unlikely that pharmacological agents targeting downstream inflammatory mediators would be successful at modulating their various effects. Thus a more preferable approach is the targeting of LPS before its initiation of early events (Marshall, 2003; Zeni *et al.,* 1997). Strategies to neutralize LPS such as monoclonal antibodies or endotoxin binding agents have shown inconsistent or disappointing results (Marshall, 2003). Including whey proteins or their hydrolysates as a means to meet the protein requirements of critically ill patients, whether through oral or enteral feeding, could potentially be beneficial in abating the cascade of events initiated by LPS at its root.

Many gastrointestinal tract inflammatory disorders, such as ulcerative colitis and Crohn's disease (CD), involve neutrophil mucosal infiltration (Reaves *et al.*, 2005). Similar to the case of CF, this infiltration is intimately related to epithelial injury and disease severity (Nusrat *et al.*, 1997). The healthy intestinal barrier normally restricts the LPS access from the lumen, via epithelial tight junctions (Nusrat *et al.*, 2000). However, in an inflamed bowel, where intestinal permeability is altered (Wellmann *et al.*, 1986; Wyatt *et al.*, 1993), bacterial gene products including LPS can find their way into the plasma

(Caradonna *et al.*, 2000). Endotoxemia in CD patients has been suggested to be associated with acute exacerbation (Wellmann *et al.*, 1986). Furthermore, it has been suggested that increased levels of interleukin (IL)-8 mRNA, which is released by stimulation with LPS, may contribute to the heavy neutrophilic infiltration characteristic of inflammatory bowel disease (MacDermott, 1999). Recent work has shown that, in a piglet model of dextran sulphate-induced colitis, pressurized whey feeding resulted in lower neutrophil infiltration as reflected by lower myeloperoxidase activity, in comparison to skim milk fed piglets. Moreover, pressurized whey-fed piglets had decreased levels of proinflammatory cytokines such as TNFα and IL-8 (Piccolomini *et al.*, 2011).

Chronic and exaggerated inflammation in response to bacterial pathogens or their gene products is a concern in a number of pathological conditions. Although great strides have been made in terms of anti-inflammatory therapy, there remains a need for effective therapeutic agents with proven long-term safety. A nutrition-based supplementation with bioactive effects such as with pressurized WPIs offers a number of possibilities in that regard.

An interesting observation in the present work is the fact that whey protein hydrolysates generated via two different digestion procedures differed in peptide profiles and in some of their biological activities (such as TNFα-induced IL-8 suppression or basal IL-8 stimulation), but not in others (such as LPS-induced IL-8 suppression). In addition, it is known that modifying the conditions of hyperbaric treatment such as pressure level, pH, and temperature, can result in pressurized whey protein isolates with different properties (López-Fandiño, 2006). This further extends the possibilities for applications of pressurized whey proteins and their hydrolysates. Varying hyperbaric pressure conditions coupled with variations in enzymatic hydrolysis procedures could lead to the production of hydrolysates tailored to exert different functions.

In the food industry, whey proteins are extensively used for their gelling, foaming, emulsifying, or dough-forming properties. The application of hyperbaric pressure is known to affect these attributes, which can be advantageous based on the desired

properties of the final product (López-Fandiño, 2006). The antioxidant properties of whey derived peptides are well documented, particularly in iron-catalyzed liposomal systems (Pena-Ramos and Xiong, 2001; Colbert and Decker, 1991), suggesting their value in preventing or slowing lipid and fatty acid peroxidation in foods. The increased ferric-reducing antioxidant power of pressurized in comparison to native whey protein isolates points to new possibilities for the application of hyperbaric pressure to enhance the value of whey peptides as natural functional ingredients with antioxidant properties in food products.

The glutathione enhancing effects of whey protein *in vitro* and *in vivo* are discussed in chapter 2 of this thesis. In the pilot clinical trial herein, no GSH enhancing effects were observed after supplementation with pressurized whey protein for 1 month. It is possible that whole blood glutathione concentrations do not reflect concentrations in other compartments, such as lung tissue or inflammatory cells. Previous studies showing significant increases in glutathione concentrations following supplementation with whey protein have measured the glutathione content of lymphocytes (Lothian *et al.*, 2006; Lands *et al.*, 1999; Grey *et al.*, 2003; Zavorsky *et al.*, 2007), plasma (Micke *et al.*, 2001; Chitapanarux *et al.*, 2009), or serum (de Aguilar-Nascimento *et al.*, 2011). Several of these studies also involved a significantly longer period of supplementation than the current trial (Lands *et al.*, 1999; Grey *et al.*, 2003; Chitapanarux *et al.*, 2009; Middleton *et al.*, 2004). A study of the effects of energy restriction lasting 4 days, preceded by whey or casein supplementation for 17 days, a period shorter than that of the current trial, showed no effect of dietary protein on the reduced to oxidized glutathione ratio in whole blood (Rankin *et al.*, 2006).

The studies in this dissertation present with a few limitations:

The studies in Chapter 4 are difficult to extrapolate to *in vivo* situations as they were conducted using an *in vitro* cell culture model, a reductionist approach. However, certain mechanistic aspects of molecular interactions, such as ligand-receptor interaction, are better examined using cell or tissue culture models. The use of primary cells is often

preferred to that of artificially immortalized cell lines, but it has been suggested that CF primary cells may display a hyperinflammatory phenotype due to a prior chronic exposure to inflammatory conditions *in vivo* (Ribeiro *et al.*, 2005). In addition, the large variability between donors and the limited amount of cells that can be obtained from them can be a hindrance to study feasibility. Nevertheless, recent advances in tissue culture-related technologies have included the development of air-liquid interface (ALI) methods of cell culture. This allows the maintenance of respiratory epithelial cell lines in the form of polarized and differentiated monolayers that develop tight junctions, thereby providing a closer representation of the *in vivo* airway epithelium (Lin *et al.*, 2007).

Another limitation is that the peptides measured *in vitro* do not necessarily reflect those that are seen systemically since peptides are not all absorbed and many are acted upon by tissue and blood proteases (Vermeirssen *et al.*, 2004), resulting in a short plasma half-life in the order of minutes (Foltz *et al.*, 2010). Hence, not all the bioactive peptides affecting cells *in vitro* survive the process of absorption and proteolysis to affect lung cells *in vivo*. It is therefore possible that the observed anti-inflammatory and antioxidant effects of whey *in vivo* are in part due to their amino acid content. Although data can be found in the literature regarding the relative rates of absorption of amino acids from different food proteins, little is known of the kinetics, plasma concentrations and half-lives of dietary peptides. A comparison of the concentration ranges and exposure times used *in vitro* with physiological conditions is therefore difficult to conduct. On the other hand, food peptides derived enzymatically have been shown to survive the above physiological processes to exert effects such as ACE inhibition and blood pressure lowering (Vermeirssen *et al.*, 2004). The appearance in the systemic circulation following enteral administration of thyrotropin releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), and insulin, three bioactive peptides with increasing respective chain lengths, has been investigated in Sprague-Dawley rats (Roberts *et al.*, 1999). Enteral TRH administration resulted in circulating levels of thyroid-stimulating hormone comparable to those following intravenous TRH administration. Enteral administration of LHRH and insulin also lead to significant release of follicle-stimulating hormone and decreases in blood glucose, respectively, although larger doses of the longer peptides were required to exert

a significant effect. It is therefore possible to obtain a physiological response following the ingestion of μg to mg amounts of dietary peptides (Roberts *et al.*, 1999). Further, there is always the possibilty to by-pass these processes using inhalation nebulizers to directly expose lung cells to the peptides.

Another limitation is the use of a closed or contained digestion system as opposed to an open system with concomitant dialysis. During gastrointestinal digestion, small peptides are absorbed gradually as they are produced in the lumen. In a closed digestion system, the peptides produced from enzymatic hydrolysis remain in the reaction solution and may lead to end-product inhibition of the enzymes. In contrast, an open system allows for the continuous removal of small peptides as they are produced, through a dialysis membrane with a pore size of choice. This offers an added advantage of collecting and identifying the specific peptides that are released at any given time of the digestion. However, the open digestion system also involves the use of large quantities of buffered solutions, greatly affecting the feasibility of recovering lyophilized peptides. Nevertheless, both closed and open digestion systems have been shown to provide different but complementary information with regards to hydrolysis products (Bassompierre *et al.*, 1997).

The findings from the present work suggest a number of future research directions:

It has recently been shown that the addition of glutathione to cell culture medium can inhibit the secretion of IL-8 by CF epithelial cells stimulated with *Pseudomonas aeruginosa* diffusible material. Moreover, the inhibition of NADPH oxidase, a reactive oxygen species (ROS)-generating enzyme, was also shown to significantly reduce the transcription of IL-8 in these cells. These results suggest that the intracellular generation of ROS is an important intermediate signal leading to an inflammatory response following the activation of TLRs (Roussel *et al.*, 2011). In that regard, results from the present work have shown that pressurized whey protein hydrolysates (WPH) were more potent at suppressing the LPS-induced IL-8 secretion by respiratory epithelial cells than native WPH. Furthermore, both native and pressurized WPH inhibited LPS binding to

cell surface receptors with a comparable magnitude, although the effect of native WPH did not reach statistical significance. Combining this finding with the enhanced antioxidant activity of pressurized WPH observed herein, this could explain the enhanced LPS-induced IL-8 suppression by pressurized WPH in comparison to nWPH. This contention is in agreement with the findings of Roussel *et al.*, and can be verified by assessing intracellular epithelial cell ROS generation in response to native and pressurized WPH.

The *in vitro* digestion of WPIs as conducted in this work results in a population of at least 35 peptides. It is likely that these peptides differ in their respective antioxidant or anti-inflammatory activities, some being more potent than others. Interesting information can be obtained from the fractionation of these hydrolysates via a chromatographic method. First, the effects of different fractions can be tested for their bioactive effects, and the most potent fractions can be further fractionated, followed by the use of mass spectrometry to identify the sequences of the most active peptides. Second, this process can help determine whether the peptides act synergistically, as would be evidenced by the loss of bioactivity of individual fractions.

Bioactive peptides with known sequences can be used to further elucidate the mechanism by which WPH suppress LPS-induced IL-8 secretion. For example, peptides with a known sequence can be synthesized (Slivka *et al.*, 2009) and conjugated with a fluorescent probe, and their interaction with immobilized TLR4, myeloid differentiation protein (MD-2), LPS-binding protein (LBP) or CD14 can be assessed (Araña *et al.*, 2003).

Based on the results of the pilot clinical trial described in Chapter 5, a clinical study would allow the assessment of the different potencies of native and pressurized WPIs *in vivo.* Ideally, a double-blind clinical trial would involve randomizing subjects into three groups: one receiving a daily dose of pressurized WPI, one receiving an equivalent dose of native WPI, and one receiving a control protein such as casein. Outcome measures would include C-reactive protein, whole blood and bronchoalveolar lavage fluid

cytokines, differential white blood cell count, and nutritional status. A trial period longer than one month would likely be required to show significant differences in serum glutathione levels and lung function.

In conclusion, the results of this work suggest a potential for the use of pressurized WPI or their derived hydrolysates as a nutrition-based adjunct therapeutic agent for the treatment of inflammation in CF. Possibilities are also presented for the exploitation of the antioxidant potential of pressurized WPI for a benefit to human health as well as in food systems.

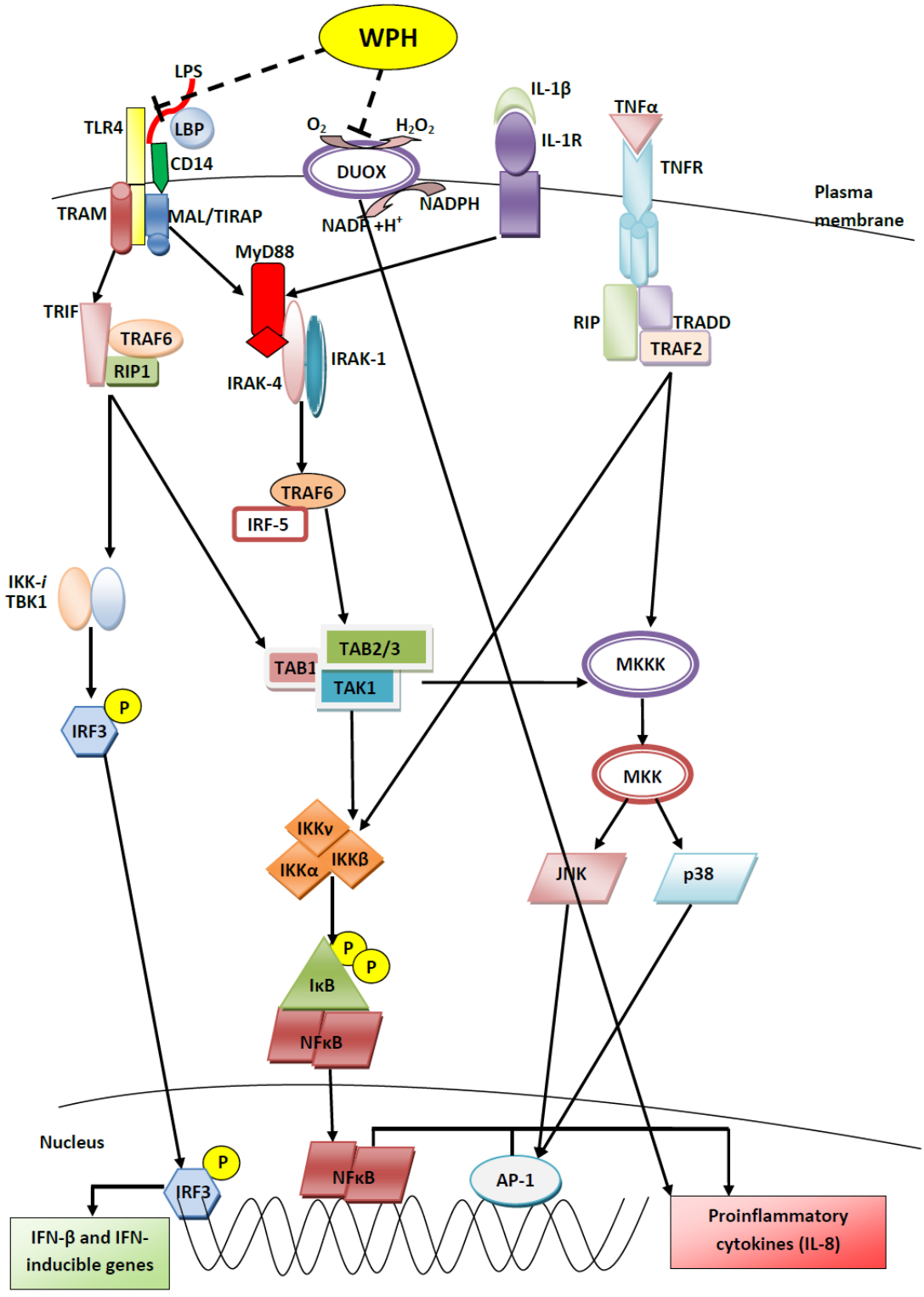

Figure 1. Possible molecular pathways leading to IL-8 expression and affected by whey protein hydrolysates (dashed lines). Adapted from: Greene and McElvaney 2005; Verstrepen *et al.*, 2008.

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