PREVENTION OF TYPE 1 DIABETES WITH PHOSPHODIESTERASE INHIBITORS

BY

EVETTE A.BESHAY

Department of Pathology

McGill University

Montreal

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ABSTRACT

The phosphodiesterase (PDE) inhibitors Pentoxifylline (PTX), a general inhibitor, and Rolipram (ROL), a type 4 inhibitor, have been shown to have anti-inflammatory effects. They elevate intracellular cAMP levels and suppress the production of inflammatory cytokines such as tumor necrosis factor (TNF)α, interferon (IFN)γ, and interleukin-12 (IL-12). These drugs have also been reported to modulate the immune response in favor of Th2 responses and to be therapeutically effective in various models of autoimmune and/or inflammatory disorders. Their effects on nitric oxide (NO) production are not well studied. Inflammatory cytokines and NO are important mediators implicated in islet β-cell destruction.

In the first part of the study, we examined the effect of PTX and ROL in preventing insulitis and diabetes in non-obese diabetes-prone (NOD) mice as a spontaneous model of insulin-dependent diabetes (IDDM). We found that a 4 week treatment with either PTX or ROL had a strong protective effect, that was still apparent 11 weeks after withdrawing the drugs. Both drugs were equally effective at optimal doses in preventing insulitis and diabetes in NOD mice.

In the second part of the study, we examined the effects of PDE inhibitors on NO production by peritoneal macrophages and RAW 246.7 cells. We also correlated these effects with elevated cAMP levels. We found that both PTX and ROL suppress NO production by IFNγ and lipopolysaccharide (LPS)-activated

macrophages. The inhibitory effects correlated with elevated cAMP levels and were mimicked by other agents which elevate cAMP levels such as dibutyryl cAMP, 8-bromo cAMP, and Forskolin. This suppression was found to be at the transcriptional level. *In vivo*, ROL treatment prevented macrophage activation by staphylococcal enterotoxin B (SEB) and suppressed NO production by these macrophages in *ex vivo* culture.

In the third part of the study, we examined the effects of PDE inhibitors on NO production by insulin-producing NIT-1 insulinoma cells and normal islet cells. It has been reported that islet β-cells express PDE3 and PDE4. We found that inhibitors of PDE4 (ROL), PDE3 (Cilostamide; CIL), or a general inhibitor (PTX), suppressed NO production by islet cells. A combination of ROL and CIL appeared to have more than an additive effect, suggesting synergism. Like in macrophages, the suppression was at the transcriptional level and mimicked by other agents which elevate cAMP levels. *In vivo*, ROL treatment suppress iNOS expression in the islets of NOD mice with cyclophosphamide-accelerated disease, as determined by immunohistochemistry.

These studies establish for the first time that PDE inhibitors have a therapeutic potential in IDDM and other NO-and/or cytokine-mediated inflammatory disorders.

RÉSUMÉ

La pentoxifylline (PTX), un inhibiteur générale des phosphodiestérases (PDE) et rolipran (ROL), un inhibiteur spécifique des PDE de type 4 montrent des effets anti-inflammatoire. Ils augmentent l'adénosine-monophosphate (AMP) cyclique intracellulaire et suppriment la production des cytokines telles le facteur nécrotique tumoral (TNF-a), l'interféron-g (IFN-g) et l'interleukine-12 (IL-12) impliquées dans le processus. Ces inhibiteurs montrent également une modulation de la réponse immunitaire vers le type 2 (Th2) et démontrent une efficacité thérapeutique dans plusieurs modèles de maladies auto-immunes et/ou inflammatoires. Leur effet sur la production d'oxyde nitrique (NO) n'a pas bien été étudié. Les cytokines inflammatoires et le NO sont d'importants médiateurs impliqués dans la destruction des cellules bêta des îlots de Langerhans du pancréas. Nous avons examiné l'implication des inhibiteurs de PDE dans le diabète.

Dans la première partie de notre étude, nous avons examiné le rôle de la PTX et du ROL dans la prévention de l'infiltration de cellules inflammatoires des îlots de Langerhans et le diabète chez les souris non-obèse susceptible de devenir diabétique (NOD), un modèle spontané de diabète de type insuline dépendant (IDDM). Nous avons montré que l'administration de la PTX ou le ROL pendant quatre semaines avaient un important effet protecteur qui était encore apparent après onze semaines du traitement. Les deux inhibiteurs étaient également efficaces dans la prévention de l'infiltration de cellules inflammatoires des îlots de Langerhans et du diabète chez les souris NOD.

Dans la seconde partie, nous avons examiné les effets des inhibiteurs de PDE sur la production de NO par les macrophages péritonéaux et des RAW 246.7, une lignée de types macrophages. Nous avons également étudié la corrélation avec les taux d'AMP

cycliques intracellulaires. Nous avons trouvé que PTX et ROL diminuent la production de NO des macrophages induits par l'INF-g et les lipopolysaccharides (LPS). Leur effet inhibiteur corélère avec une augmentation de l'AMP cyclique intracellulaire et mimique par les analogues de l'AMP cyclique, le dibutyryl AMP cyclique et le 8-bromo AMP cyclique, ou par la forskoline. Cet effet est au niveau de la transcription. Les injections de ROL chez des animaux traités à l'entérotoxine B du staphylocoque qui est connu pour activer les macrophages du péritoine, supprime la production de NO ex-vivo de ces macrophages en culture.

Dans la troisième partie, nous avons examiné l'effet directe des inhibiteurs des PDE sur la production d'insuline des insulomes NIT-1 et les cellules des îlots de Langerhans puisqu'il est connu dans la littérature que les PDE de type 3 et 4 sont exprimées par les des îlots de Langerhans. Nous avons trouvé que l'inhibiteur de type 4 (ROL), de type 3 (Cilostamide, CIL) et l'inhibiteur général des PDE (PTX) supprime la production de NO par les cellules des îlots de Langerhans. L'utilisation de ROL avec le CIL semble synergique sur la suppression de NO puisque l'effet est plus qu'additif. Tout comme pour les macrophages, la suppression se trouve au niveau de la transcription et est imitée par les agents agissant sur le tau d'AMP cyclique. In vivo, ROL supprime la NO syntase inducible (iNOS) exprimé par les des îlots de Langerhans de souris NOD dont le diabète a été accéléré par le cyclophosphamide tel que vue par immunohistochimie.

Ces études établissent pour la première fois que les inhibiteurs des PDE ont un potentiel thérapeutique dans la prévention de l'IDDM et autres maladies du trouble du NO et/ou de maladies inflammatoires associées aux cytokines.

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ABBREVIATIONS

AG : aminoguanidine

APC : antigen presenting cell

BB rat : Bio-Breeding rats

CaM : calmodulin

cAMP : cyclic adenosine monophosphate

CBV : coxsackie B virus

cGMP : cyclic guanine monophosphate

CIL : cilostamide

CMV : cytomegalovirus

CTL : cytotoxic T lymphocyte

CTLA-4 : cytotoxic T lymphocyte antigen 4

CYP : cyclophosphamide

DAG : diacylglycerol

db-cAMP : dibutyryl cAMP

DM : diabetes mellitus

DNA : deoxyribonucleic acid

DTH : delayed type hypersensitivity

EAE : experimental allergic/autoimmune encephalomyelitis

ELISA : enzyme-linked immunosobent assay

eNOS : endothelial nitric oxide synthase

GAD : glutamic acid decarboxylase

Gpx : glutathione peroxidase

HLA : human leukocyte antigen

IAA : insulin autoantibody

ICA : islet cell autoantibody

IDDM : insulin dependent diabetes

IFN : interferon

IRE : interferon response element

Ig : immunoglobulin

IL : interleukin

iNOS : inducible nitric oxide synthase

IP : intraperitoneal

JAK : Janus Kinase

LPS : lipopolysaccharide

mAb : monoclonal antibody

MAPK : mitogen-activated protein kinase

MDSD : multiple low-dose streptozotocin-induced diabetes

MHC : major histocompatibility complex

NAD : nicotinamide adenine dinucleotide

NF_KB : nuclear factor kappa B

NIDDM : non-insulin dependent diabetes mellitus

NK : natural killer

nNOS : neuron nitric oxide synthase

NO : nitric oxide

NOD mouse: non-obese diabetic mouse

PBS : phosphate-buffered saline

PDE : phosphodiesterase

PGE : prostaglandin E

PKA : protein kinase A

PKC : protein kinase C

PTX : pentoxifylline

ROL : rolipram

RT-PCR : reverse transcriptase- polymerase chain reaction

SEB : staphylococcal enterotoxin B

SLE : systemic lupus erythematosus

STAT : signal transducer and activator of transcription

STZ : streptozotocin

TCR : T cell receptor

TGF : transforming growth factor

Th : T-helper

TNF : tumor necrosis factor

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'In life the beginning and the end are determined.

It is what occurs in the interim, who we meet and

what we do, that charts our course'

To the memory of my Mother, Mary

To my Father, Anwar

To my husband, Wahib

To my lovely daughter, Mary

Your love makes me live

I dedicate my work to you with love

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

Introduction and literature review

A.1. Diabetes Mellitus

A.1.1. Definition and classification

Diabetes mellitus (DM) is a syndrome with disordered metabolism and inappropriate hyperglycemia resulting from an absolute or relative lack of insulin. Diabetes was recognized as early as 1550 BC, when the Egyptians described the polyuric symptom which characterize it [1]. Generally, diabetes mellitus is classified into two major clinical forms based on dependency for survival on insulin treatment, insulin-dependent diabetes mellitus (IDDM) or type I diabetes, and non insulin-dependent diabetes mellitus (NIDDM) or type II diabetes.

IDDM is a catabolic disorder in which circulating insulin is virtually absent, plasma glucagon is elevated, and the pancreatic β-cells fail to respond to insulinogenic stimuli. It occurs most commonly in juveniles but occasionally in adults. Clinically, IDDM is characterized by polyuria, polydipsia, rapid weight loss and random plasma glucose of more than 200 mg/dL. This is the most severe form of DM which is usually associated with ketosis.

On the other hand, NIDDM occurs predominantly in adults but occasionally in juveniles. More than 90% of all diabetics in the USA are included in this group. In most cases, the cause is unknown. However, deficiency in the response of β-cells to glucose and insensitivity of the tissue to insulin had been noted. This form of the

disease is often associated with hypertension, hyperlipidemia, and atherosclerosis. Two subgroups of patients are currently distinguished by the absence or presence of obesity. In addition, there is a gestational form of diabetes, and these women have a markedly increased risk of developing postpartum DM. Unlike type I diabetes, type II diabetes lacks an autoimmune component. The focus of this thesis is on the immunologic aspects of IDDM.

A.1.2 Epidemiology of IDDM

IDDM occurs most frequently in persons of northern European descent. The disease is less common among other racial groups, such as blacks, Native Americans, and Asians [2]. The incidence ranges from a low of 1 to 2 per 100,000 people per year in Japan to a high of more than 40 per 100,000 in parts of Finland [3]. In a large prospective study in Europe, there was a pronounced north-south gradient in the incidence of diabetes [3]. The difference in the incidences is largely explainable by the prevailing susceptibility genes for IDDM in racially distinct populations, but environmental factors may also be important. In United States, the prevalence of IDDM by the age of 20 years is about 0.26 percent [4]. The incidence is markedly age-dependent, increasing from a near absence during the early months of life to a peak coincident with pubertal development [5]. However, the disease can occur at any age, with a small mid-life second peak in incidence [6]. Seasonal variability has been noted in all parts of the world, with an increased incidence in the late fall or early winter [4].

A.1.3. Pathogenesis

IDDM is characterized by increased plasma glucose. It is an autoimmune disease characterized by progressive immunologic destruction of insulin-secreting pancreatic β-islet cells by autoreactive leukocytes and their mediators [7]. Even though the precise causes of the disease remain unclear, a combination of genetic, immunologic, and environmental factors appear to contribute to the onset and progression of IDDM [7,8]. However, it has been difficult to demonstrate a strong cause-and-effect link between some of these factors and IDDM, which highlights the need for further investigation [9].

A.1.3.1. Genetic factors

The role of genetic factors in diabetes is complex. It is determined by several genes, and does not follow a Mendelian pattern of inheritance [10]. The genes involved in the disease are susceptibility genes rather than disease genes, as the disease is expressed only in a minor proportion of susceptible individuals. About 60% of genetic predisposition has been mapped to the major histocompatibility complex (MHC) class II genes [11, 12], but several other genes contribute importantly. MHC class II molecules are membrane-associated and they are essential for foreign antigen recognition by T helper (Th) cell. Few cell types express these molecules, other than antigen-presenting cells (APCs). APCs present peptides of foreign antigens in context

with these molecules to Th cells. The human MHC is designated histocompatibility locus A (HLA) (HLA-DP, HLA-DQ, HLA-DR) and the disease association is with DR and DQ HLA genes on chromosome 6 [11,12].

Initially, an association was demonstrated with DR3 and DR4, but subsequently it was found that DQ genes are more strongly implicated. Following further characterization of HLA genes, and a new nomenclature, studies have indicated that HLA-DQB1*0302-A1*0301 is more important than any of the 12 or more HLA-DRB1*04 subtypes [13,14]. It is also demonstrated that the HLA-DRB1*04 subtype, DRB1*0401 confers a risk that is additive to that of DQB1*0302-A1*0301 [15, 16]. In other words, it may be argued that the DR*0401 allele confers a risk for diabetes that is independent of DQ. On the other hand, HLA-DQ6 is negatively associated with type I diabetes. It is speculated that HLA-DR or DQ class II molecules associated with type I diabetes provide antigen presentation that generate T-helper (Th) 1 cells that initiate immune response to specific islet cell autoantigens.

Several laboratories have used families with multiple affected members to carry out complete genome scans to identify other type I diabetes genes [17, 18]. Over 15 such candidate loci have been identified. It is possible that a combination of HLA with other genetic loci may either enhance or decelerate the type I diabetes process. The insulin gene on chromosome 11 [19], and cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene on chromosome 2 have been shown to be prominent susceptibility genes [20]. Upstream of the insulin gene are a variable number of tandem repeats. Class I alleles (26-63 repeats) predispose in a recessive way to type I diabetes, while

class III alleles (140-200 repeats) seem dominantly protective. The protective effect of the latter may be explained by the presence of high concentration of proinsulin mRNA in thymus, possibly enhancing the immune tolerance to preproinsulin, which is a key autoantigen [21].

CTLA-4 delivers negative regulatory signals to T cells, and mutation of these genes have been implicated in various autoimmune diseases. The role of CTLA-4 gene in type I diabetes is not fully understood; however, it has been speculated that a gene polymorphism involving an AT repeat at C terminus at the 3' end of the gene may affect the stability of CTLA-4 mRNA. The longer the repeat, the less stable the CTLA-4 mRNA. Since CTLA-4 is critical to T-cell suppression, it is possible that the long AT repeat may lead to greater T-cell activity because the CTLA-4 protein is not formed.

The role of other genetic loci is not clear, and further experiments will be necessary to understand their link to type I diabetes.

A.1.3.2 Environmental factors

Several studies in monozygotic twins are consistent with the hypothesis that environmental factors are important risk factors for type I diabetes. Indeed, genetic predisposition alone is not sufficient to explain IDDM, since there is a 60 -70% discordance rate in monozygotic twins [22]. In addition, epidemiological studies show that the incidence of IDDM correlates with geographic location [23]. Numerous

environmental factors have been proposed and the main candidates among these factors are viral infections, dietary factors, toxins and stress.

A.1.3.2.1 Viruses

Viral infections have been implicated in the etiology of IDDM for a long time, but in humans their role remains unclear. Viruses may act either by a direct cytolytic effect, or by triggering an autoimmune process leading to the destruction of islet β-cells [24]. Studies on the association of recent outbreaks of viral infection, such as Coxsackie B virus (CBV), and the onset of type I diabetes have been published. These studies reported the presence of specific IgM antibodies to CBV in 39% of the children who had recently developed diabetes while 5.5% of the control group was positive [25]. In addition, CBV type 4 was isolated from the pancreas of diabetic patient [26]. CBV can also induce diabetes in experimental animals [27]. Recent evidence suggests that this virus is directly cytopathic to islet-β cell [28].

The most compelling evidence of viral involvement is congenital rubella, which is strongly associated with the appearance of diabetes in the affected childen [29, 30]. In this case, the disease and HLA association are similar to usual IDDM, and the rubella virus appears to predispose to autoimmunity.

Studies from Sweden [31] and Finland [32] have provided evidence that maternal enterovirus infection increases the risk of type I diabetes in the offspring. These studies also reported that the group-specific antibodies to enteroviruses were higher in mothers whose children later developed diabetes as compared with control

mothers. In addition enterovirus RNA was detected in the sera of IDDM patients more often than in controls [33]

Other viruses such as cytomegalovirus (CMV) are also apparently involved in the pathogenesis of diabetes in some cases. For instance, a case report indicated an association between congenital CMV infection and diabetes [34].

Some viruses are clearly diabetogenic in some species. It is thought that there are two diabetogenic groups of viruses; some of which induce the development of diabetes and others which suppress its development [35]. The most frequently used viruses for the induction of diabetes in mice include encephalomyocarditis virus (EMC) (myocardial strain), mengovirus (clone 2T), and Coxsackie virus (B group) [36].

A.1.3.2.2 Nutritional Factors

Studies have shown that removal of bovine milk compounds in dietary chows can prevent diabetes in BB rat [37] or in NOD mice [38]. Based on that, the cow milk protein hypothesis was developed. In human, several reports also showed that lack of or short duration of breast-feeding is associated with increased risk for type I diabetes [39]. In addition, these studies have shown the presence of antibodies to bovine serum albumin (BSA) in up to 100% of newly diagnosed type I diabetes patients [40].

Some investigators have demonstrated that poor nutritional status correlates with a high incidence of type I diabetes. [41]. Poor nutritional status has been shown

to produce an environment in which antioxidant defenses are often low, resulting in higher susceptibility to oxidative damage.

Some substances or their metabolites may be toxic to islet cells. Interestingly, a Swedish case control study demonstrated that a high dietary intake of N-nitroso compounds, nitrite or nitrate is a high risk factor of type I diabetes [42], and a dose response relationship was shown. Nitrosamine compounds reduce the nicotinamide adenine dinucleotide (NAD) content of β-cells, which may represent a possible mechanism of β-cell dysfunction and destruction.

A.1.3.2.3. Other environmental factors.

Some chemicals such as Alloxan, streptozotocin, Zinc chelating compounds and vacor can induce diabetes. Both alloxan and streptozotocin have been shown to induce the production of free radicals and mediate DNA breaks [43].

Other studies have demonstrated that acute psychological stress may also increase the incidence of diabetes [44].

A.1.3.3. Autoimmunity

Type I diabetes has an autoimmune etiology. Both humoral and cell-mediated autoimmunity against islet cells have been identified. Bottazzo and coworkers [45], in 1974, were the first to describe humoral autoimmunity against islet cells. After that, several autoantibodies were described. These autoantibodies included islet-cell

autoantibodies (ICA) [46], and insulin autoantibodies (IAA) [47]. More recently, anti-glutamic acid decarboxylase (GAD) autoantibodies were described. [48]. GAD is a cytoplasmic enzyme found in cells of the brain and in β cells of the pancreas. In addition, autoantibodies to gangliosides [49] and carboxypeptide H [50] were reported. Most of these autoantibodies against islet cells can be detected in the serum months to years before the clinical onset of diabetes. Based on this evidence, investigators have analyzed the titers of various autoantibodies to predict the onset of disease in high-risk persons, such as siblings of children with diabetes [51].

From animal models (see below), cell-mediated autoimmunity in type I diabetes appears to be more important than humoral autoimmunity. Inflammatory cell infiltration of the islets of Langerhans (insulitis) is seen in humans, rats, and mice with early onset diabetes. Many studies have been performed in non-obese diabetic (NOD) mice to characterize these cells. It was shown that these infiltrating cells include CD4+ and CD8+ T cells, B cells, macrophages and dendritic cells [52]. Among these cells, both T cells and macrophages appear to be effector cells mediating β-cell destruction.

Another line of evidence for autoimmunity is that the disease can be adoptively transferred by T cells [53]. In NOD mice, it has been found that diabetes can be transferred from newly diabetic to non-diabetic animals with a combination of CD4+ and CD8+ lymphocytes [54]. Immunosuppressant drugs like cyclosporine prevent diabetes, but have adverse effects [55, 56]. Moreover, patients with IDDM have a high risk for other autoimmune diseases [57].

A.1.4 Animal Models of IDDM:

" The major breakthroughs in the progress toward the cure of diabetes have been made by investigation of animal diabetes " Eleazar Shaffrir 1996 [58].

Much of our present knowledge concerning the etiology, pathogenesis, treatment and prevention of human diabetes has been acquired from animal models [58 - 60, 35]. The main models of IDDM can be divided into two groups: spontaneous models such as Bio-Breeding (BB) rats and NOD mice; and induced models. In the latter, diabetes can be induced by pancreatectomy, chemicals (alloxan, streptozotocin) or viruses.

A.1.4.1 Spontaneous animal models of IDDM

Spontaneous animal models of IDDM have been particularly useful for studying the role of genetic factors and immune responses in the pathogenesis of IDDM. At present, the best models are the BB rat and NOD mouse, as their diabetic syndrome closely resembles human IDDM [43]. Other species with at least occasional spontaneous type I diabetes [35, 43] are: the Long-Evans Tokushima Lean rat; the Chinese hamster; the Keeshond dog; the Macaca nigra (Celebes black ape); some

Colonies of guinea-pigs; and the New Zealand white rabbit. However, these animals have received much less attention than BB rats and NOD mice.

A.1.4.1.1 NOD mouse

The NOD mouse was discovered in 1974 in Osaka, Japan. The diabetic syndrome of this animal is similar to that of human IDDM, including glucosuria, hyperglycemia and hypoinsulinemia. Insulitis is observed in virtually all diabetes-prone NOD as early as 4-5 weeks of age. Diabetes starts at about 12 weeks of age, and by 27 weeks of age over 80% of females and 20% of males are diabetic [61].

A.1.4.1.1.1 Immunologic factors

Humoral autoimmunity occurs in the pre-diabetic state in NOD mice, as the presence of ICA and IAA have been reported. However, the role of these autoantibodies in the pathogenesis of the disease appears to be minor, and cell-mediated responses are considerably more relevant. In islet-cell infiltrates, Th cells, CTLs, NK and other immune cells are found [43]. Initially, there is a non-destructive perinsular infiltrate, which is characterized by a T helper type 2 (Th2)-associated cytokine pattern. This progresses to an intra-islet infiltration and β-cell destruction, associated with Th1 type immune reaction [62]. In addition, GAD-autoreactive T cells have been detected in NOD mice [63]. Of the immunologic questions under study in

NOD mice, the putative autoantigens, the effector cells (macrophages, Th, CTLs), and the role of cytokines are of chief interest.

A.1.4.1.1.2. NOD-mouse Genetics

Research into the genetics of IDDM in NOD mice is very active, and it is hoped that it will clarify some aspects of the genetics of human IDDM [64]. The genes involved have been provisionally designated Idd genes, pending precise categorization. As in humans, the contribution of the MHC is strong. There is expression of a unique MHC class II A-beta locus (histidine as residue number 56 and serine as residue number 57), homologous to diabetogenic HLA-DQβ non-aspartic acid 57 containing alleles [65]. Moreover, the contribution of several non-MHC genes has been demonstrated, and they are located on several chromosomes. These genes appear to interact with a diabetogenic MHC haplotype to create the threshold of susceptibility required for IDDM development [64].

A.1.4.1.1.3 Cyclophosphamide (CYP) accelerated diabetes

Cyclophosphamide (CYP) is widely used clinically as an immunosuppressive agent, particularly in autoimmune diseases. However, although it is known to be an alkylating agent that kills dividing cells, the mechanisms of its therapeutic effect are not always clear, and in some situations it augments immune responses. Thus, the administration of CYP before sensitization increases delayed type of hypersensitivity

(DTH) responses [66], antibody-dependent cell-mediated cytotoxicity (ADCC) [67], and enhances the expression of experimentally-induced diabetes [68]. CYP also accelerates the development of diabetes in NOD mice [69]. The diabetogenic effect of CYP dose not appear to be mediated by direct toxicity on β-cell. However, CYP administration induces a burst of IFNγ release, with an increase in both the circulating levels and intra-islet production. This is associated with the rapid onset of insulitis and diabetes and blocked by neutralizing IFNγ with mAbs or soluble receptors [70-72]. IFNγ induces macrophages to express an inducible form of nitric oxide synthase (iNOS), and release large amounts of NO which appears to be one of the major toxic products for islets [73]. IFNγ production is associated with other features of Th1 mediated immunity.

A.1.4.1.2 BB rats

The BB rat was discovered in 1974 in a commercial breeding company (Bio Breeding Laboratories, Ottawa, Ca). The diabetic syndrome of this animal has many aspects in common with the human disease. Clinical onset usually occurs at the age of 3 months, equally in both sexes, often coinciding with puberty. Weight loss, glucosuria, hyperglycemia and hypoinsulinemia are reported. This progresses to diabetic ketoacidosis, which is fatal if exogenous insulin is not administered [74]. The onset of the disease is associated with insulitis which is similar to human IDDM, with infiltration of islets with Th cells, CTLs, macrophages, NK cells, and B lymphocytes [43].

Genetic susceptibility is associated with the MHC, just as in humans and NOD mice. There are also at least two non-MHC genes that participate in the pathogenesis of the disease [43]. Similar to humans and mice, autoantibodies against islet cell antigens have been described. As well, there is an increased frequency of autoantibodies against antigens in other organs such as thyroid and stomach [75]. Interestingly, GAD antibodies and GAD-reactive T-cells have been reported in BB rats, as in NOD mice [63]

One characteristic of BB rats is lymphopenia, i.e. a lack of T-lymphocytes mainly of the CD8+/RT6+ subtype [75-77,63]. These appear to be regulatory T cells which protect against autoimmune response.

A.1.4.2 Induced animal models of IDDM

A number of chemicals have been shown to induce diabetes, with alloxan and streptozotocin being the most widely used. Alloxan and streptozotocin can interfere with several β-cell functions, e.g., cell-membrane glucose transport mechanisms, glucose kinase activity, and mitochondrial activity. These drugs induce the formation of toxic free radicals [43].

Multiple low doses of streptozotocin administered to genetically susceptible mice induce IDDM [78]. It has been recently demonstrated that streptozotocin induces apoptosis in β-cells [79].

B.1. Cytokines

B.1.1. General overview

Cytokines are low molecular weight proteins that are important mediators of inflammatory and/or autoimmune diseases [80-82]. Most cell types produce one or more cytokines, but leukocytes are distinguished by producing a wide variety. Cytokines, unlike endocrine hormones, are usually short range mediators that operate through paracrine and/or autocrine signals. They are pleiotropic molecules and, as such, are considered as a network, with the function of one cytokine commonly overlapping that of another [83]. Cytokines are produced mostly by activated cells. Moreover, molecular receptor expression is often low or absent unless target cells are activated [84]

B.1.2. The JAK-STAT pathway

The majority of cytokines act through specific membrane-bound hetero- or homodimeric receptors which lack intrinsic kinase activity and rely on the activation and recruitment of intracellular tyrosine kinase proteins [85,86]. The best described are the Janus Kinase (JAK) family [87]. JAK-related transcription factors are termed 'signal transducers and activators of transcription' (STATs). Upon ligand binding and dimerization of the cytokine receptor subunits, the JAK kinases induce tyrosine phosphorylation of the receptor, creating docking sites for the associated STATs. These STATs are phosphorylated and then, as dimers, translocate to the nucleus.

STAT dimers bind their respective DNA response elements and thereby initiate cytokine-directed gene transcription [86,88]

B.1.3. Cytokines and T cell immunoregulation

Mosmann and colleagues in the mid 1980s, observed that many mouse Th clones consistently expressed one of two cytokine profiles [89]. The Th1 cells expressed IL-2, IFNγ,and TNFβ; while Th2 cells expressed IL-4 and IL-5 [90]. No phenotypic differences were found between these subsets, and they were distinguished by their cytokines expression profile. More recently, some markers such as IL-12 receptor or chemokine receptor expression have been found to differentiate these cells. A third type, Th0 cells, are not restricted in their cytokine profile, and produce a mixture of Th1 and Th2 cytokines.

Recently, it has been proposed that there is an additional subset of CD4+ T cells, Th3 cells, which are generated during oral administration of autoantigens, produce variable amounts of IL-4 and IL-10, but differ from Th2 cells in their capacity to produce large amounts of TGF-β1 [91]. Th3 cells have a regulatory function, and are protective in some autoimmune diseases.

B.1.4 Cytokines and their role in IDDM

IDDM, like other organ-specific autoimmune diseases, results from a dysregulation of immune responses. T cells directed against islet β-cells are activated, expand clonally, activate the macrophages, and provoke a cascade of immune and/or inflammatory processes in the islet [92]. The current paradigm is that the pathogenic immune response is mediated by the Th1 subset of T cells, whereas a protective immune response is mediated by Th2 subset [92]. However, recent evidence suggests that Th3-like cells may be the protective subset [93]

B.1.4.1 Cytokine regulation of immune responses

Th1 and Th2 subsets have distinct patterns of cytokine secretion that lead to strikingly different T cell action [94c]. Th1 cells and their cytokine products (IL-2, IFNγ, TNFα, TNFβ) are the mediators of delayed-type hypersensitivity. Th1 cell-derived cytokines activate vascular endothelial cells, recruit circulating leukocytes into the tissue at the site of antigen challenge, and activate macrophages. In addition, Th1-derived IL-2 and IFNγ activate CTLs and NK cells. In contrast, Th2 cytokines stimulate the humoral immune response, especially IgE production by B lymphocytes. Furthermore, Th1 and Th2 cells are mutually inhibitory. Thus, IFNγ inhibits the production of Th2 cytokines and IL-4 and IL-10 inhibit Th1 cytokine production [94-96].

B.1.4.2. Cytokine effects on isolated islets

IL-1, TNFγ, TNFβ, and IFNγ in very low concentrations (pico- to nanomolar concentrations) are toxic to β-cells [97-98]. Each of these individual cytokines inhibits insulin secretion in response to glucose stimulation. That effect is largely recovered after cytokine removal. These cytokines can also be cytocidal, particularly when added in combination, to β-cells of both rodents and humans. However, the cytodestructive effects of cytokines on β-cells in vitro are not specific to β-cells, since α islet cells are also damaged.

B.1.4.3. Cytokines studies in vivo

Several cytokines have been found to be expressed at the gene and/or protein level in islet lesions in NOD mice, BB rats, or IDDM patients [93]. However, the simple presence of cytokines does not identify their role in IDDM. These cytokines may be proinflammatory, or alternatively they may be regulatory, suppressing the inflammatory process.

IFNγ, which is an inflammatory cytokine produced by Th1 cells, CTLs and NK cells, has an important role in IDDM. For instance, IFNγ mRNA expression correlates with β-cell destructive insulitis [99], and monoclonal antibodies to IFNγ protect against diabetes in NOD mice [100] and BB rats [101]. Transgenic expression of IFNγ by islet β-cells in normal mice leads to immune-mediated insulitis, β-cell destruction and IDDM [102]. In addition, recent gene therapy experiments have shown that

intramuscular injection of a nonviral vector encoding an IFNγ receptor/IgG1 fusion protein prevented NOD-mouse diabetes and multiple low-dose streptozotocin-induced diabetes in CD-1 mice [103]. IFNγ has been also detected in the islets of human subjects with recent-onset IDDM [104].

Another proinflammatory cytokine that seems to have a role in the pathogenesis of IDDM is IFNα, which has been detected in recent-onset diabetic patients [105]. IFNα mRNA expression was increased significantly in the pancreata of IDDM patients as compared with control human pancreata [106]. In addition, islet expression of IFNα preceded insulitis and diabetes in BB rats [107]. Moreover, transgenic expression of IFNα by islet β-cells in normal mice elicited an autoimmune destruction of β-cells, which was prevented by anti-IFNα antibodies [108]. IFNα is a product of many cell type that are virally infected or otherwise stressed, suggesting that initial β-cell stress or viral infection may induce the production of this cytokine by β-cells. IFNα may recruit immune cells and initiate β-cell destruction.

Several studies in transgenic mice have demonstrated the proinflammatory role of TNFα [109], TNFβ [110], and IL-2 [111] produced locally in the islets. On the other hand, IL-4, which is produced by Th2 cells, has been shown to have protective effects. Administration of either IL-4 [112], or a plasmid encoding IL-4/IgG1 [113], protected NOD mice from diabetes.

Systemic administration of other cytokines to NOD mice and BB rats in vivo has also revealed protective effects, though mechanisms are unclear. This includes IL-1, IL-2, IL-10, TNFα and TNFβ [reviewed in 114]. Deficiencies in the endogenous

production of IL-1, IL-2, IL-4, TNFα and TNFβ also have been reported [114] in diabetes-prone NOD mice and/or BB rats. It is possible that the preventive effects of chronic administration of these cytokines result from a correction of specific deficits in cytokine production. In addition, systemically administered cytokines may act on targets outside the immune system, e.g., IL-1 and TNF can stimulate the hypothalamic-pituitary axis, leading to secretion of adreno-corticotropic hormone and adrenal corticosteroids which suppress inflammatory cells [115].

B.1.5. Effector mechanisms

β-cell destruction is mediated by both immune cells (T-cells and macrophages) and their mediators (cytokines, free radicals, and others). The current concept is that certain islet β-cell proteins act as autoantigens (β-Ag), after being processed by antigen presenting cells (APC), such as macrophages and dendritic cells. These cells present the antigen in the context of MHC-II molecules on the surface of APC. β-Ag-MHC-II complexes, accessory molecules on APC (B7 molecule), APC-derived IL-12 and possibly other signals will direct the immune response toward a $CD4^+$ Th1 response. These Th1 cells stimulate effector mechanisms involving macrophages, CTLs or NK cells. Effector cells may kill islet β-cells by at least three mechanisms: 1. Direct interactions of antigen-specific CTLs with β-cell autoantigen-MHC-I complexes on the β-cell membrane; 2. Non-antigen-specific killing by NK or macrophages; 3. The toxic action nonspecific inflammatory mediators such as

cytokines (IL-1, TNFα, TNFβ, IFNγ), nitric oxide (NO) and other free radicals (Reviewed by Rabinovitch et al 1998) [114].

B.1.6. Molecular mechanisms of cytokine actions on β-cells

The exact mechanisms of cytokine cytotoxic effects on islet β-cells are not totally elucidated. Some authors have proposed that the cytotoxic effects of cytokines on β-cells are mediated at least in part by NO and other free radicals [116, 117]. In addition cytokines may sensitize β-cells to T-cell mediated cytotoxicity. For example, IFNγ up-regulates MHC-I expression on β-cells, and IL-1 (possibly also IFNγ and TNFα) induces Fas (CD95) expression on β-cells [118]. The Fas molecule can deliver an apoptosis-induced signal to islet cells.

C.1. Nitric oxide (NO)

C.1.1 Overview

NO is the smallest molecule, and the first gas, known to act as a biological messenger in mammals. The molecule has one unpaired electron, making it a free radical that avidly reacts with other molecules. In the presence of oxygen, NO may vanish a few second after it forms, although its life span in the human body is unknown. NO was chosen by Science as a molecule of the year in 1992 [119]. In the atmosphere, NO is a noxious chemical; while in the body, in small-controlled doses, it is a physiological mediator.

Indeed, NO has been identified as a pleiotropic intercellular messenger molecule regulating a variety of diverse cellular functions in many tissues. Initial reports characterized NO as an endothelium-derived relaxation factor (EDRF) important for the maintenance of vascular tone and the regulation of blood pressure. In the brain, it participates in the action of excitatory neurotransmitters such as glutamate, and seems to have important roles in learning and memory processes. In males, it is a mediator of penile erection. In macrophages, it is a cytotoxic mediator and contributes to the antimicrobial and tumoricidal activity of these cells [120]. Moreover, NO has been linked to pathophysiological effects, and it may account for the destruction of islet β-cells and the development of IDDM.

C.1.2 Nitric oxide synthase

NO is produced by the enzyme nitric oxide synthase (NOS), in a reaction where arginine and oxygen are converted into citrulline and NO. There are two major classes of the enzyme [121]: the constitutive isoforms present either in neurons (nNOS or type 1) or in the endothelial cells (eNOS or type 3); and the inducible isoform (iNOS or type 2) which was originally described in macrophages but now known to be expressed in a large variety of cells. This includes renal mesangial cells, islet cells, hepatocytes, chondrocytes, endothelial cells, smooth muscle cells, megakaryocytes and thyrocytes [122].

The constitutive isoforms are calcium and calmodulin dependent, and produce small amounts of NO for physiological activities. On the other hand, iNOS expression

requires de novo synthesis following cellular stimulation by cytokines or bacterial lipopolysaccharide (LPS). This results in the production of large amounts of NO independent of the calcium or calmodulin levels. NO exerts lethal effects on several pathogens including protozoa, fungi, bacteria and viruses [122]. It has been proposed that this free radical is part of an "innate" defense system against invading microorganisms [123]. This system appears to be activated rapidly at the target cell level, even before the immune system responds. If this is the case, it may explain why so many nucleated cells express iNOS.

It is noteworthy that there are important species and tissue differences in the requirements for iNOS induction. Human hepatocytes [124] and pancreatic islet cells [125,126] produce large amounts of NO in response to cytokines. In human macrophages iNOS is produced in lower amounts than in rodent macrophages [127].

C.1.2.2 NOS genes and proteins

The nNOS (NOS1) gene, which is one of the two constitutively expressed forms, was cloned from neuroectodermal cells. It is a 161-kDa protein translated from a 10 kilobase (kb) mRNA [128, 129]. The other constitutively expressed NOS was cloned from endothelial cells (eNOS or NOS3), is transcribed as an approximately 4.7 kb mRNA and translated into a 133-kDa protein [130,131]. nNOS has been associated with neuronal signal transduction [132], and eNOS with the regulation of vascular tone [133]. They share approximately 60% amino acid sequence similarity [130]. The inducible form (iNOS) was cloned from IFNγ and LPS-stimulated mouse macrophages

[134-136], and shows only 50% amino acid sequence identity with the constitutive NOS isoforms. An approximately 4.4 kb mRNA translates into a 131-kDa protein. In their active forms, the three NOS are homodimers. They contain flavine adenine dinucleotide (FAD), and flavine mononucleotide (FMN), and require tetrahydrobiopterin and reduced nicotinamide adenine nucleotide phosphate (NADPH) for their activity [137].

iNOS is mostly regulated at the transcription/translation level by a variety of inflammatory stimuli [138]. On the other hand, the two-dimensional gel analysis of recombinant rat islet iNOS expressed in human fibroblast suggests the possibility of post translational control by phosphorylation [139]. Different patterns of stimulation are required to induce iNOS gene expression in different cells. For example, in rat, IFNγ and LPS are required to induce macrophage iNOS, while IL-1β is the main stimulant for islet β-cells [135, 136]. Human islet cells require a combination of IL-1β, IFNγ and TNFα for iNOS induction [125, 126]. Cloning of the iNOS gene from rat islets and a rodent β-cell line [139], and recently from human islets [140], revealed that iNOS is the same in macrophages and islet cells [141].

C.1.2.3 Chromosomal localization of NOS

In humans, the chromosomal localization of the three NOS genes has shown that the nNOS (NOS1) maps to the position q24.2-24.31 on chromosome 12 [142], eNOS (NOS3) to position q35-36 on chromosome 7 [143], and iNOS (NOS2) to position p11.2-q12 on human chromosome 17 [144]. In mice, the iNOS gene has been mapped

to chromosome 11, which has been previously associated with IDDM in the spontaneously diabetic NOD mouse through a polymorphic region (Idd4) [145]. Interestingly, the iNOS gene is located in the middle of this Idd4 region [146]. Furthermore, iNOS mRNA has been detected in the islets from both NOD mice [147] and BB rat [148] around the time of spontaneous diabetes onset.

The possible existence of iNOS gene polymorphism was studied in rats, but none was found [149]. On the other hand, minor differences in human iNOS cDNA sequences, potentially representing polymorphisms in the gene, have been reported. However, it is unknown if this influences susceptibility to diabetes.

Most of the available data suggests the presence of only one iNOS gene within the genome. However, the presence of at least three iNOS-related genes in murine and human genomes has been proposed [150-153]. It remains to be determined whether any of these multiple iNOS-like sequences are pseudogenes or authentic, transcriptionally active genes.

C.1.2.4 5' Region and promoter elements in the iNOS gene

Several agents such as cytokines, cAMP, LPS, glucocorticoids, prostaglandin E2, picolinic acid, etc., can up- or down-regulate iNOS gene expression in different tissues. In the mouse iNOS promoter regions, there are transcription factor motifs for IFNγ-response element (γ-IRE), γ-activated site, nuclear factor $_{K}$B ($NF_{K}B$), IFNα-stimulated response element (ISRE), activator protein 1 (AP1), TNF response element

(TNF-RE), nuclear factor interleukin-6 (NF-IL-6), shear stress response element (SSRE), X box, and hypoxia-responsive enhancer (HRE) [154-157].

The macrophage iNOS gene promoter contain two major positively regulating regions, 48 to 209 (region 1) and 913 to 1029 (region 2), upstream of a putative TATA box [155]. Region 1 contains LPS-related responsive elements, including binding sites for NF-IL-6 and NF_KB, and promotes the transcription activity. Region 2 potentiates/augments the promoting activity of region 1, and contains motifs of IFN-related transcription factors.

C.1.2.5. 3' Region and mRNA stability

Both murine and human iNOS mRNA have a conserved region in the 3' untranslated end with high homology to an octanucleotide sequence UUAUUUAU [158]. This sequence has been shown to decrease mRNA stability in the TNF gene, probably by allowing binding of labile cycloheximide-sensitive proteins (cycloheximide is an inhibitor of protein synthesis) [159]. On the other hand, cycloheximide prolongs iNOS mRNA half life in smooth muscle cells and chondrocytes by inhibiting the synthesis of proteins required for mRNA degradation [160]. The iNOS activity in IL-1-treated human chondrocytes was inhibited by elevating intracellular calcium and reducing iNOS mRNA stability [161]. Similarly, TGFβ decreases iNOS content in macrophages by increasing iNOS mRNA degradation [162]. In islet cells, IL-1-induced iNOS transcription is arrested by

blockers of gene transcription, e.g. actinomycin D [163], which suppress 50% of iNOS mRNA content within 2h.

These findings suggest a role for mRNA stability in the regulation of cellular iNOS mRNA content. This may represent one of several factors responsible for the different iNOS mRNA kinetics reported among different species and different tissues.

C.1.3.2. iNOS gene expression and regulation in pancreatic islets

Rat islets express iNOS mRNA after 4h of exposure to IL-1β [164]. An insulinoma cell line of rat origin (RIN) also expresses iNOS and produces NO after exposure to IL-1β or a combination of TNFα and IFNγ. iNOS expression rises after 4h, peaks after 6h, and progressively decreases after 12-48 h, in spite of the continuous presence of IL-1β [163]. This is possibly due to negative feedback by NO on iNOS transcription, as suggested by the observation that NO donors inhibit cytokine-induced iNOS expression in insulin-producing cells [163], macrophage-like RAW 264.7 cells [165], and glial cells [166]. There is evidence that NO decreases iNOS expression by preventing NF_KB activation [167], and this effect is mediated by induction and stabilization of $I\text{-}_KB$ (see below). NO has been shown also to inhibit iNOS enzymatic activity in macrophages by interacting with enzyme-bound heam [168]. Thus, NO-mediated negative feedback on its own production could represent a cellular protective mechanism against the deleterious effects of this radical.

Pancreatic islets contain a heterogeneous cell population, making it difficult to identify the cellular source(s) of NO production. However, there is evidence that

differentiated β-cells [169] and clonal insulin-producing hamster [170] and rat cells [163] can be induced to produce a large amount of NO. Immunohistochemical staining of islet sections, and Western blot analysis of proteins from sorted cells have shown that the iNOS gene is expressed in β-cells but not in α-cells [171, 172]. Thus, IL-1β alone induces iNOS expression in both intact whole islets and isolated β-cells, but not in α-cells [170]. TNFα alone induces a low level of iNOS expression in rat insulin producing RIN cells, but neither IFNγ nor LPS by themselves induce iNOS expression in these cells [163,173]. A combination of LPS and TNFα induces iNOS expression in intact isolated islets but not in single β-cells, suggesting that LPS + TNFα stimulate intra-islet IL-1β release, which subsequently induces iNOS expression in the β-cells [170]. Both TNFα and IFNγ potentiate IL-1β-induced iNOS expression in a rodent insulin-producing cell line [163, 173]. Of note, IFNγ increases NO production by mouse pancreatic islet cells, similar to the IL-1β effect [173]. As described for other cell types [141], iNOS mRNA expression in β-cells is mainly regulated at the transcriptional level [174, 175].

IL-4 and IL-10, two cytokines known to suppress iNOS mRNA in macrophages [176, 177], do not have the same effect on insulin-producing cells [178]. Another potential suppressor cytokine in this context is TGFβ, previously shown to prevent iNOS activation and NO production by macrophages [176, 177]. TGFβ has also been reported to decrease IL-1β-induced NO production by β-cells [179].

C.1.3 IL-1 induced signal transduction and iNOS activation

Several investigators have presented indirect evidence for the presence of IL-1 receptors (IL-1Rs) on islet cells [180-182]. Recently, islet cell receptors for IL-1 were characterized in NOD mice [183]. Insulin producing cells express mRNA for both type I and type II IL-1Rs. The type I IL-1 receptor appears to be the main mediator of the biological effects of IL-1 in these cells, as suggested by the observation that both IL-1 R antagonist protein [184] and monoclonal antibodies against type I IL-1R [185] prevented the effects of IL-1α and IL-1β on rat and mouse islet-cell function. IL-1R appears to associate with a putative serine/threonine protein kinase.

IL-1-induced signal transduction involves at least three major signaling pathways, namely the transcription factor NF_KB, the stress-activated protein kinases (SAPK/JNK) and PKC. The three pathways seem to activate specific sets of transcription factors that may interact to induce iNOS and possibly other genes in insulin-producing cells. The signaling pathway involving NF_KB is the most studied and will be reviewed.

C.1.4. Nuclear factor $_KB$ (NF_KB)

NF_KB is a nuclear transcription regulatory factor, present in most cells, and acts mainly as an early immune and inflammatory mediator [186]. It is activated by various factors such as viruses, bacteria, oxidative stress and cytokines, and induces the transcription of genes encoding defense and signaling proteins. NF_KB is sequestered in the cytosol of non-stimulated cells as an inactive trimer consisting of p50, Rel-A (p65)

and I_KB. Upon activation, I_KB is phosphorylated and proteolysed, which leads to the release and translocation of p50 and Rel-A dimer to the nucleus where it binds to the B motif (a decameric DNA sequence motif) [187].

In insulin producing-cells, IL-1 induces a rapid translocation of NF_KB from the cytosol to the nucleus. This process has been shown to be inhibited by protease inhibitors [188]. Recent findings show that NF_KB inhibitors such as pyrrolidine dithiocarbamate (PDTC) suppress iNOS expression in IL-1-stimulated insulin-producing cells [188,189], suggesting that NF_KB activation is necessary for IL-1 to induce iNOS. PDTC is an antioxidant, and might counteract free radical-induced activation of NF_KB. Furthermore, radical scavengers such as N-acetyl cysteine prevent NF_KB activation and iNOS expression in RINm5F cells [190]. In addition, PDTC also inhibits cytokine- (IL-1β + IFNγ +TNFα) induced iNOS expression in human islets [191], suggesting that NF_KB plays an important role in the induction of iNOS transcription in these cells.

The fact that different cell types require different patterns of stimulation to induce iNOS expression suggests that NF_KB is necessary but not sufficient to evoke iNOS expression in some cells, such as human islet cells. Recently, it was found that macrophages from knock-out mice defective in the interferon response factor-1 (IRF-1) do not produce NO in response to immunostimulants [192]. It is conceivable that activation of both NF_KB and IRF-1 are needed to induce iNOS transcription. Other transcription factors of potential relevance are AP-1 and c-fos. However AP-1

activation is not sufficient for iNOS expression in insulin-producing cells [189], and increased c-fos expression did not affect iNOS mRNA [189].

C.1.5 NO as a mediator of β-cell destruction

NO may damage the β-cell plasma membrane and also diffuse intracellularly to cause further damage. It can inactivate the iron-sulfur centers of iron-containing enzymes such as mitochondrial aconitase, leading to decreased oxidative phosphorylation required for glucose oxidation and insulin release [193]. This results in decreased glycolysis, and consequently decreased ATP levels and impaired insulin secretion. Higher levels and/or long-lasting production of NO may increase damage to cellular constituents, e.g., membrane phospholipids, cellular enzymes, and DNA. These biochemical mechanisms ultimately lead to β-cell death [194].

C.1.6. Pancreatic islets are highly susceptible to oxidative stress

Several key enzymes involved in the defense against reactive oxygen species are unusually low in pancreatic islets compared with other tissues, suggesting that the islet cells are uniquely susceptible to oxidative stress-induced damage. Thus, gene expression and activity of several key antioxidant enzymes such as CuZnSOD, MnSOD, glutathione peroxidase (Gpx) and catalase are all markedly decreased (30-40% of normal level) compared with other tissues such as liver [195]. For instance, GPx expression was only 15% of liver levels, and catalase gene expression was not

detectable in islets [196]. This may account for the exquisite sensitivity of β-cells to free radicals including NO-induced damage. The uptake of alloxan by both liver and pancreatic islet cells was studied [197], and the cytotoxicity of alloxan was apparent only in pancreatic islets.

Interestingly, several studies have demonstrated a significant reduction in total antioxidant status in both plasma and serum samples from IDDM patients compared to age-matched controls [198]. Diabetic children also have a significant decrease in erythrocyte glutathione peroxidase (Gpx), total glutathione, plasma α-tocopherol, and plasma β-carotene [199]. More recent studies have established that in the prediabetic condition, antioxidant status appears to be compromised [200]. ICA serve as a serological markers for risk of developing IDDM. Total plasma antioxidant status was assessed in both ICA-positive and ICA-negative first-degree relatives of patients with IDDM. Antioxidant status was significantly lower in ICA-positive subjects compared to ICA-negative relatives and healthy unrelated subjects. Hence, antioxidant status is probably another contributing risk factor in the development of IDDM.

C.1.7. Efficacy of NOS inhibitors in animal models of IDDM

Guanidines such as N^G-nitro-L-arginine, L-N^G-monomethyl-arginine (L-NMMA), N-nitro-L-arginine-methylester (L-NAME) and aminoguanidine (AG) have the ability to inhibit NOS, probably due to their structural similarity to L-arginine.

In animal models of IDDM, activated islet-infiltrating macrophages secrete a number of cytokines, including IL-1β that either alone or in combination, can elicit

iNOS expression. On the basis of this observation, it has been suggested that therapeutic intervention using these NOS inhibitors might confer a protective effect against the development of IDDM. Several studies have shown that treatment of mice with L-NMMA [201] and L-NAME [202, 203] suppressed multiple low dose streptozotocin-induced-diabetes (MDSD). On the other hand AG failed to decrease the incidence of IDDM in NOD mice [204], BB rats [205] or MDSD in C57BL/Ks mice [206]

AG has been shown to be an effective iNOS inhibitor, but a less potent eNOS or nNOS inhibitor. However, the other NOS inhibitors (L-NMMA, L-NAME, N^G-Nitro-L-arginine) are less potent iNOS inhibitors, and more effective nNOS and eNOS inhibitors [207-209]. Inhibition of eNOS markedly increases blood pressure and leads to changes in the circulation [210], and inhibition of nNOS leads to neurologic disorders and influences the memory process. These undesirable effects will limit the potential benefit of these inhibitors in preventing diabetes. In addition, the most specific iNOS inhibitor, AG, has been found to inhibit catalase and generate free oxygen radical hydrogen peroxide in rat liver cells and human erythrocytes. [211]. AG has also been shown to inhibit insulin production by human pancreatic islets in vitro [212].

D. Therapeutic perspectives

Evidence suggests that IDDM results from an immunoregulatory imbalance in which Th1 cells and their cytokines dominate over Th2 or Th3 cells and their

cytokines. Therefore, the current notion is that therapies aimed at IDDM prevention should be directed at increasing Th2/Th3 function and/or decreasing Th1 function. Bacterial products and immune adjuvants can prevent diabetes in NOD mice and BB rats by stimulating a type 2 cytokine response [213]. However, these reagents are immunostimulatory, and have undesirable side effects. More selective immunostimulation can be obtained by administration islet β-cell autoantigens such as GAD65 which prevents diabetes in NOD mice. This protection is associated with induction of specific tolerance to this antigen [214, 215], with suppression of GAD65-responsive Th1 cells [215]. In addition, a strong humoral response to GAD correlates with slow progression to IDDM. Administration of other β-cells autoantigen (e.g., insulin) has been reported to prevent diabetes in NOD mice, BB rats, and human subject at high risk for IDDM [216].

Another approach to shifting the balance of cytokines production in favor of Th2 may be by manipulating the expression of costimulatory molecules, such as B7 on APC. Treatment of NOD mice with CTLA-4 immunoglobulin (CTLA4-Ig), which binds to B7 molecules on APCs and prevent their binding to CD28 on T cells, has been reported to protect NOD mice from diabetes [217].

Cytokine-based therapies for IDDM prevention may take several forms. This includes blocking type 1 cytokines by administering antibodies to these cytokines or to the corresponding cytokine receptors. Alternatively, cytokines can be blocked with soluble cytokine receptors, or receptors antagonists. In addition, Th2 cytokine such as IL-4 can be directly administered.

Another approach to protect β-cell against proinflammatory cytokines is by blocking the production or the action of oxygen and nitrogen-based free radicals. A variety of antioxidants, such as nicotinamide, deferoxamine, SOD, α-tocopherol, probucol and lazaroid, have been shown to provide protection in NOD mice and BB rats [218-220]. However, in this thesis, we are presenting a novel approach to prevent diabetes in NOD mice, i.e., the administration of phosphodiesterase inhibitors.

E.1 The phosphodiesterase inhibitors.

E.1.1 Overview of Cyclic-3', 5'-adenosine monophosphate

More than 35 years ago, Robinson, Butcher and Sutherland [221] discovered that hormones, autocoids, and neurotransmitters exert their regulatory effect on cell functions through intracellular production of a specific adenine nucleotide, cAMP. Shortly after that, cyclic-3', 5'-GMP (cGMP) was also identified as a messenger [222]. Since this pioneering era, numerous intracellular signaling pathways have been discovered. However, the intricate mechanisms by which cAMP acts in cellular signaling are far from being completely understood. Indeed, novel aspects of cAMP signaling function are still being revealed. cAMP is synthesized by a membrane-bound adenylate cyclase enzyme family. The biosynthesis is regulated by hormonal agents.

Of relevance to our work, as discussed below, cAMP is degraded by a family of enzymes, i.e., the phosphodiesterases (PDE).

E.1.2 cAMP and T cell subsets

T cell activation by specific antigens, mitogens, or monoclonal antibodies against the T cell receptor (TCR) induces hydrolysis of phosphatidylinositol 4'5' biphosphate by phospholipase C. This hydrolysis leads to production of second messengers, such as diacylglycerol (DAG), and inositol triphosphate (InsP3). DAG can activate protein kinase C (PKC), and InsP3 increases the intracellular calcium concentrations. It is thought that these second messengers lead to increased production of lymphokines, expression of lymphokine receptors, cellular proliferation, and proto-oncogene mRNA expression. Another pathway involves activation of adenylate cyclase and accumulation of intracellular cAMP which activate a cAMP-dependent protein kinase A (PKA) pathway. The PKA- and PKC- pathways interact in certain cells.

In 1990, Munoz and his colleagues [223] found that Th1 and Th2 cells use different signaling pathways after TCR-mediated stimulation. They reported that PKC was the major pathway of activation in Th1 cells, whereas Th2 cells were less dependent on this pathway. Th2 cells may use a different second messenger, probably cAMP. Recently, PKA activation pathway was found to play a prominent role, as high levels of cAMP favored the production of type 2 cytokines [224], and Th2 cells maintained significantly higher levels of intracellular cAMP than Th1 cells [225]. The

accumulation of cellular cAMP blocks Th1 cytokine but not Th2 cytokine production (see below).

E.1.3. cAMP and cytokine production

Cholera toxin, which promotes accumulation of intracellular cAMP, inhibits the production of IL-2 but not IL-4, and also inhibits the proliferation of Th1 but not Th2 cells in response to TCR-mediated stimulation [223]. This correlates with increased IL-4 mRNA and decreased in IL-2 mRNA levels. A study in the mouse thymoma cell line EL-4, revealed that cAMP activated the IL-5 (Th2 cytokine) promoter through the PKA pathway [226].

The inhibitory effects of elevated cAMP on TNFα production are well documented [228, 229]. In addition, there are recent studies examining the relationship between cAMP and IL-12, which is a potent inducer of Th1 responses, and alters the Th1/Th2 balance in the favor of Th1 cells. Prostaglandin E2 (PGE2) elevates intracellular cAMP levels via stimulation of adenylate cyclase, and has been found to inhibit LPS-induced IL-12 production. The inhibitory effect of PGE2 on IL-12 is independent of IL-10, since neutralization with anti-IL-10 antibodies does not reverse this inhibition. The inhibitory effect is correlated with elevated cAMP levels, and is mimicked by other agents which elevate cAMP [227]. Rolipram (ROL) and pentoxifylline (PTX), PDE inhibitors which increase intracellular levels of cAMP, also suppress IL-12 production by activated peritoneal macrophages [228].

Dibutyryl cAMP (db-cAMP), an active cAMP analogue that is lipid soluble and crosses the cell membrane, has been reported to inhibit IFNγ mRNA expression without affecting IL-10 [224]. Cholera toxin and PGE2 have also been reported to inhibit the expression of IFNγ mRNA, while the expression of IL-4 and IL-13 mRNA were not inhibited. At the protein level, IFNγ was also inhibited by PGE2 or db-cAMP, whereas IL-10 was enhanced. ROL and PTX have also been shown to inhibit IFNγ production by activated T cells [228].

E.1.5 cAMP and JAKs/STAT activation

In mammalian cells there are four members of the JAK family (JAK1, JAK2, JAK3, JAK4) and several member of the STAT family (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6). Generally, stimulation by a cytokine results in the activation of a distinct set of JAKs.

These JAKs activate particular STATs with subsequent activation of several important genes [230]. For example, IFNγ preferentially activates STAT1 [231], IL-4 activates STAT6 [232] and IL-12 activates STAT4 [233]. It was reported that several signaling pathways could interrupt JAK-STAT signal transduction and modulate cytokine activity during inflammatory and immune responses [234]. Notably, the elevation of cAMP levels have been shown to inhibit STAT1 at both the mRNA and protein levels [230]. Therefore, cAMP may skew cytokine production by inhibiting STAT1.

A.1.6. cAMP and NO production

The role of cAMP on NO production is not yet clear. However, Bulett et al. [235] reported that elevation of cAMP by a combination of PGE_2 and IBMX (a general PDE inhibitor), suppressed NO production by the murine macrophage cell line J774. They also reported that this suppression correlated with prolonged, but not transient, elevation of cAMP. Furthermore, other studies showed that different cAMP elevating agents such as Forskolin, PGE_2, PGI_2, 8-bromo-cAMP and IBMX, reduced iNOS expression and NO production by the same cell line (J744) [236]. On the other hand, elevated cAMP induces iNOS expression by rat vascular smooth muscle cells [237], while controversial results were reported in other cell types such as chondrocytes [238]. Pentoxifylline (PTX) (a general PDE inhibitor) has been found to suppress NO production by mouse [239] and rat [240c] peritoneal macrophages, and to enhance iNOS in rat astrocytes [240]. Rolipram (ROL) (a type IV PDE inhibitor) was reported to increase NO production by the murine macrophage cell line RAW 264.7 at low concentrations, while at higher concentrations the effect was reversed [241]. Contradictory results may occur due to differences in promoter elements in different cells, and/or because cAMP dependent and independent pathways are involved [242].

E.2 Phosphodiesterases

E.2.1 The Superfamily of PDE isozymes

For many years, PDEs were identified by their catalytic and regulatory properties [243]. All PDEs inactivate their cyclic nucleotide targets by hydrolytic cleavage of the 3'-phosphodiester bond, resulting in the formation of the corresponding inactive 5'-monophosphate. Although it was initially thought that cAMP and cGMP are hydrolyzed by a single PDE, it is now known that the PDEs constitute a large superfamily of related isozymes. Mammalian PDEs were initially grouped into five families based on a variety of biochemical characteristics including their substrate specificity, mode of regulation, kinetic properties and response to selective inhibitors [244].

Mammalian PDEs are now known to constitute at least ten gene families (PDE1 - 10). Table 1 summarizes the characteristics of the PDE families. Some PDE gene families are very diverse whereas others, such as PDE2, PDE5, PDE7, PDE8 and PDE9, have no documented isogenes. In some families, several single gene products (subtypes, PDE4A, 4B, 4C, etc.), and numerous isoform-splice variants (isoforms PDE4A1A, PDE4A4B, PDE4A5, etc.) have been described [245-249]. PDE isozymes (products of different genes) and splice-variant isoforms confer great diversity and specificity to the PDE superfamily system. The various PDE types are selectively expressed in various tissues and cell types [248-252] (Table 1).

E.2.2. Molecular structure of PDE isozymes.

Vertebrate PDEs are usually dimers of linear 50-150 kDa proteins. An exception is the PDE6 family (a photoreceptor-specific PDE), which is a tetramer [248, 249, 253]. The PDE monomer consists of three domaims, i.e., the N-terminal domain, the central catalytic core, and the C-terminal domain, connected by hinge regions. The central catalytic core region is highly conserved in all vertebrate PDEs. It consists of 270 amino acids and is positioned close to the COOH terminus. This core region is more similar in terms of amino acids identity within one individual PDE gene family (more than 80%),

TABLE 1:

PDE family	Isogenes	Isozymes* Isoforms	Characteristics	Tissue distribution	Examples of inhibitors
PDE1 [271, 272]	3	> 9	Ca2+/CaM-stimulated	Heart, brain, lung, smooth muscle	Vinpocetine KS-505a
PDE2 [273- 275]	1	3	GMP-stimulated	Adrenal gland, heart lung, liver, platelets	EHNA
PDE3 [276-279]	3	>5	cGMP-inhibited cAMP>cGMP-inhibited	β-cells, platelets, heart liver, brain, lung, immunocytes	Cilostazol Cilostamide Milrinon Vesnarrinon
PDE4 [280-284]	4	>15	cAMP-specific	Sertoli cells, kidney, brain, liver, β-cells immunocytes	ROL CDP480 RP 73401 SB207499 CP 80-633 RS 33793
PDE5 285,249]	2	2	cGMP-specific	Lung, platelets	Dipyridamole Sildenafil Zaprinast MY-5445
PDE6 [248,249]	3	4	cGMP-specific	Photoreceptors	Dipyridamole Zaprinast
PDE7 [286]	1		cAMP-specific	Skeletal muscles, heart, kidney, brain T lymphocyte	none available
PDE8 [287, 288]	2		cAMP-specific	Testis, ovary, intestine	Dipyridamole
PDE9 [289]	1	4	cGMP-specific	Spleen, small intestine, kidney, thymus,	SCH 51866 Zaprinast
PDE10 [290,291]	1		cAMP inhibited cGMP	non available	IBMX

*Includes all known enzymes produced in a PDE family.

than between two distinct gene family (25-40%) [248,250,254]. The core region also contains signature consensus sequences and motifs that are common among all vertebrates [255]. Furthermore, it contains two consensus Zn^{2+} binding motifs [256]. In contrast to the catalytic core, the structure of the NH2- terminal (regulatory domain) of PDE molecules diverges widely in structure and size among PDE isozymes and isoforms[245]. The N domain contains sequences that are target sites for various modes of regulation; including ligand-binding sites, phosphorylation sites, and sites for several modes of protein-protein interaction. These include binding site for Ca2+-calmodulin (CaM) and non-catalytic sites for cGMP and phosphatidic acid. There are also several consensus sequences that are phosphorylation site for various kinases; Se/Thr protein kinases (PKs), Ca2+ -CaM PKs, PKA, PKG, PKC, and insulin stimulate PK [256]

The functional significance of the C-terminal domain of PDEs is largely unknown. Perhaps it has some regulatory or localization functions, since recent report showed that mitogen-activated protein kinase (MAPK) can phosphorylate serine in the C-terminal portion of PDE4B2B [257]. This is a possible point of direct interaction between cAMP and MAPK.

E.2.3 Regulation of the activity of PDE isoforms

PDE gene products demonstrate selective cellular and subcellular localizations [258 - 260]. Thus, compartmentalization of PDE activity may play an important role in the local regulation of intracellular cyclic nucleotide content. Moreover, a variety of

mechanisms allow rapid short-term regulation of the activity of PDE isoforms. First, a local shift in cyclic nucleotide content through the activity of one PDE family may affect the activity of other families through allosteric regulation or active site competition [261,262]. Second, during cellular signaling, individual splice variants are differentially expressed. Since these isoforms may be confined to specific compartments, local cyclic nucleotide homeostasis may be affected [263, 264]. Third, the activity of specific PDE isoforms may be regulated by protein kinase (A, G, or tyrosine kinase) which mediates protein phosphorylation [265].

E.2.4. PDE expression in immune cells

B and T lymphocytes contain PDE3 and PDE4, and in addition, T lymphocytes possess PDE1, PDE5 and PDE7 [266c]. PDE3 and PDE4 activity is similar in Th and CTLs, [266, 267c]. Interestingly, differential expression of PDE4 isoforms has been reported in Th1 versus Th2 clones [266].

In general, PDE4 inhibitors down regulate antigen- or mitogen-induced T cell proliferation and/or proinflammatory cytokine production. Moreover, PDE4 inhibitors have been reported to up-regulate the anti-inflammatory cytokines, such as IL-10 [267, 268]. On the other hand, PDE3 inhibitors do not appear to affect lymphocytes [266], suggesting a dominant role for PDE4.

Freshly isolated circulating monocytes/macrophages express PDE4, but little or none of the other PDEs. Some macrophages, such as alveolar macrophages, have been

shown to express PDE4, PDE3 and PDE1 [269]. Thus, it appears that PDE isozyme expression depends on the biologic state and environment of the macrophage. However, PDE4 inhibitors have been shown to down-regulate LPS-induced TNFα and IL-12 production. PDE3 inhibitors are less efficacious in down-regulating TNFα production and demonstrate variable efficacy in modulating macrophage function [270].

E.3. Characteristics of the main PDEs expressed by lymphoid cells and islet cells

E.3.1. The PDE3 family

The PDE3 family (cGMP-inhibited) displays a high affinity for both cAMP and cGMP, but has a far higher affinity for cAMP. The products of two isogenes, PDE3A and PDE3B, have been found to differ in their organ and tissue distribution [276], as well as in their regulation [249, 277]. PDE3A is more sensitive to inhibition by cGMP than PDE3B. Both subtypes, PDE3A and PDE3B, are activated by Ser/Thr phosphorylation on Ser in the N-regulatory domain, which is catalyzed by PKA [277c]. Insulin-dependent phosphorylation and activation of PDE3B, catalyzed by PDE3IK, was reported [277]. However, the whole sequence of steps in signaling by which the insulin receptor activates PDE3IK is not elucidated. It is likely that an

essential step in this pathway involves activation of phosphatidylinositol-3 kinase [277]. Thus PDE3 is a target for negative cross-talk with cGMP pathways, and positive cross-talk between insulin, as well as cAMP, signaling pathways. It is postulated that phosphorylation of PDE3A or PDE3B by PKA represents a short-term negative feed back regulation. PDE3 isozymes are expressed in different tissues and cells including lymphocytes, alveolar macrophages, and islet β-cells. Both PDE3A and PDE3B are sensitive to inhibition by a number of synthetic inhibitors including cilostamide, cilostazol, milrinone, amrinone. Both cilostazol [278] and milrinone [279] have been extensively investigated and approved for clinical use.

E.3.1.2. PDE4 family

The PDE4 family is the most diverse and, with high affinity, selectively hydrolyzes only cAMP. Therefore, PDE4 is also sometimes referred to as the "cAMP-specific PDE" [254c]. PDE4 isozymes comprise four subfamilies (subtypes) encoded by isogenes PDE4A to PDE4D. These, in turn are transcribed and expressed as numerous splice-variants of PDE4 isoforms [254]. Splice-variant isoforms of PDE4 are distinguished by the structure in the N-terminal domain, which confers diverse regulatory properties and the capacity of interactions with other cellular structures. Some isozymes of PDE4 can be up-regulated by cAMP via two possible mechanisms. Short isoforms, which are less than 72 kDa, such as PDE4D1 or PDE4D2, are subject to long-term up-regulation by cAMP via transcriptional control mechanisms [280-281]. An increase in the cellular cAMP level enhances synthesis of mRNA and de

novo synthesis of the enzyme molecule [280- 282], and this induction can be blocked by actinomycin D and cycloheximide [283]. Interestingly, elevated cAMP in T cells resulted in an induction of PDE4D and simultaneous decrease of PDE4A [283].

On the other hand, long isoforms of PDE4, which are more than 93 kDa, such as PDE4D3, are activated by cAMP through reversible phosphorylation by PKA [284c]. This phosphorylation causes a several fold increase in the catalytic activity of PDE4 and greatly increases the sensitivity to selective inhibitors such as ROL [284]. Other selective inhibitors for PDE4 isozymes are RO-20-1727, RS-33793 and denbufylline. However, recently a large number of newer PDE4 inhibitors have been synthesized such as CP77059, CP80,633, RS33793, RP73401, and CDP840, and they display up to 1000 times more affinity to PDE4 than ROL (reviewed in [256]).

E.4. PDE inhibitors used in our studies

E.4.1 PTX

PTX [3,7-dimethyl-1-(5-oxohexyl) xanthine], a non-specific PDE inhibitor, has been used clinically for many years under the trade name Trental. It is used to treat peripheral vascular disorders, such as intermittent claudication [292], and diabetes-induced peripheral vascular disease [292, 293]. It increases erythrocyte [294] and leukocyte [295] flexibility, thus lowering the blood viscosity, and improving

microcirculatory blood flow. PTX also inhibits platelet aggregation [296], and improves mobility and viability of spermatozoa [297].

PTX has been found to suppress the inflammatory cytokines, i.e., TNFα, IFNγ, and IL-12 [228]. It is therapeutically useful in many infammatory and/or autoimmune disorders, such as scleroderma [299], vasculitis [300], rheumatoid arthritis [301], and septic shock [302]. Interestingly, PTX has also been reported to decrease the requirement of insulin in children with recent onset diabetes [303]. In this trial, 21 childen with new onset type 1 diabetes, aged 6 to 20 years, received PTX (1200-2400 mg/d) in addition to routine treatment with insulin. The daily insulin dose of the PTX-treated group was significantly lower at 3-12 months of PTX treatment compared to an age-matched control group. However, PTX failed to prevent the disease. Notably, the drug was administered after the diagnosis of diabetes, which is probably too late to interfere with disease expression. However, PTX treatment prolonged the honeymoon period, or non-insulin required period, in the treated vs the control group.

Pharmacokinetics studies of PTX showed that it is almost completely absorbed after oral administration. It reaches a peak plasma concentration of less than 3 μg/ml 2-3 hours after oral administration of 400 mg, and this level is maintained for a few minutes [304]. The plasma concentration has been found to reach a higher level when PTX is administered parentally [305]. The drug is metabolized and its biotransformed products are eliminated almost exclusively by the kidney [306]. PTX has limited adverse effects. The most frequent are nausea (14%), dizziness (9.4%), headache (4.9%), and vomiting (3.4%). Flushing, abdominal discomfort, bloating,

diarrhea, dyspepsia and malaise are reported in 1-3% of the cases. Other rare side effects, such as chest pain, arrhythmia, hypertension, drowsiness, tremor, abdominal pain, decreased serum fibrinogen, pruritus, blurred vision, scotoma, etc., are reported in less than 1% of the cases [306].

E.4.2. ROL

ROL, [(±)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone], a specific PDE4 inhibitor, was first studied as an anti-depressant [307]. It was reported that ROL at a dose between 0.25 and 3 mg t.d. was more effective as an antidepressant than drugs such as the tricyclic antidepressants [308]. Recently, immunologists found that ROL could be useful as an anti-inflammatory agent. ROL has been reported to have inhibitory effects in asthma [309], rheumatoid arthritis [310], glomerulonephritis [311] multiple sclerosis [312], acute respiratory distress syndrome [313] and septic shock [314]. ROL has also been reported to suppress proinflammatory cytokines such as, TNFα, IFNγ, and IL-12 [228], and to prevent diabetes in NOD mice (our studies described later).

Pharmacokinetics studies in healthy volunteers reveal that ROL is rapidly absorbed after oral administration. It is reported that oral administration of 1 mg of ROL gives a peak of plasma concentration of 16 ng/ml after half an hour. Thereafter, the plasma levels decline in three phases, with half-lives of 0.1h, 0.5 h, and 9.4 h respectively [315].

The reported adverse effects of ROL include nausea, vomiting, increased gastric secretion and psychotropic activity [316, 317]. The toxicity of repeated exposure to ROL (up to 100 mg/kg/day) has been studied in rats [318], and a number of histopathological alterations, involving the liver, heart, salivary glands, mesentry and stomach were observed, but only at high doses. In clinical trials, it was reported that patients tolerated therapeutic doses, and there was no evidence casting doubt on the drug's safety at these doses [319].

III. Hypothesis, rationale, and objectives of research

PDE inhibitors augment intracellular cAMP levels and have been shown to be therapeutically effective in animal models of autoimmune and/or inflammatory diseases, such as experimental allergic encephalomyilitis, rheumatoid arthritis, septic shock, and asthma (as described before). Both PTX and ROL have suppressive effects on the production of the proinflammatory cytokines TNFα, IFNγ, and IL-12, which are important mediators of β-cell destruction. On the other hand, these drugs show either no suppressive effect or even stimulatory effects on Th2 cytokines such as IL-4 and IL10. The latter cytokines seem to have protective effects on diabetes. Moreover, PDE inhibitors might inhibit the production of NO, a β-cell toxic mediator. However, the effects of these drugs on NO production have not been extensively studied, and contradictory results have been reported.

Our hypotheses were:

1) PDE inhibitors, such as PTX and ROL would prevent insulitis and diabetes in NOD mice by suppressing inflammatory cytokine production or by other anti-inflammatory effects.

2) PDE inhibitors would protect islets, at least in part, by suppressing iNOS expression and NO production by macrophages.

3) PDE inhibitors would also protect by suppressing iNOS/NO expression in islet β-cells.

4) Selective PDE3 or PDE4 inhibitors would be effective since these isoforms are expressed by islet cells and macrophages.

5) The anti-inflammatory and immuno-protective effects of PDE inhibitors are dependent on elevated intracellular cAMP levels.

This study is divided into three main parts. In the first part, we investigate the effectiveness of two PDE inhibitors, PTX and ROL, in preventing insulitis and diabetes in NOD mice. In the second part of the study, we examine the effect of PTX and ROL on NO production by a macrophage cell line (RAW246.7) and normal peritoneal macrophages, and we correlate these effects with elevated cAMP levels. In the third part of the study, we examine the effects of PDE inhibitors on NO production by an insulinoma cell line (NIT-1) and normal mouse islet cells. We also correlate these effects with cAMP levels.

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CHAPTER 2

THE PHOSPHODIESTERASE INHIBITORS PENTOXIFYLLINE AND ROLIPRAM PREVENT DIABETES IN NOD MICE

by

Evette Beshay and Gerald J. Prud'homme *

from

Department of Pathology, McGill University

3775 University St. Montreal, Qc, Canada H3A 2B4

* Address correspondence and reprint requests to:

Dr. Gerald J. Prud'homme

3775 University St. Room B13

Montreal, Qc, Canada H3A 2B4

Tel. 514-398-7192 (ext. 7237); Fax 514-398-7446

E-mail: gprudh@po-box.mcgill.ca

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ABSTRACT

The phosphodiesterase (PDE) inhibitors, pentoxifylline (PTX), a general inhibitor, and rolipram (ROL), a type 4 specific inhibitor, have been shown to suppress

the production of proinflammatory cytokines such as TNFα, IFNγ, and IL-12, which are implicated as mediators of β-cell destruction and diabetes development. Recently, both PTX and ROL were reported to prevent insulitis and diabetes in cyclophosphamide-accelerated-diabetes in NOD mice.

In this study, we examined the effects of PDE inhibitors in preventing insulitis and diabetes in the natural course of disease in NOD mice. We found that treatment of female NOD mice (12 weeks) with either PTX (80 mg/kg i.p.,b.i.d.) or ROL (7-14 mg/kg i.p.b.i.d.), significantly reduced the severity of insulitis and prevented diabetes in NOD mice ($P < 0.005$ treated vs control group). Thus a relatively short course of treatment between weeks 12 to 16 of life had a lasting protective effect, which was observed over 10 weeks after withdrawal of the drug treatment. At optimal doses, there was no statistically significant difference in the effectiveness of these two inhibitors. It appears that blocking the activity of PDE type 4 is sufficient to mediate the effects reported, since ROL inhibits only this isoform. PTX and ROL may be effective in the treatment of autoimmune diabetes and/or other conditions characterized by excessive production of inflammatory cytokines.

INTRODUCTION

NOD mice spontaneously develop a form of IDDM similar to human disease, caused by an autoimmune destruction of islet β-cells. The precise mechanisms of islet destruction are not totally elucidated, but there is substantial evidence for a Th1 mediated response with IFNγ production associated with macrophages activation and production of TNFα, IL-1, 1L12, and nitric oxide (NO) [1-7]. Immunosuppressive drugs, such as cyclosporin A, can prevent IDDM in rodent and may be of benefit in human [8] but have substantial adverse effects.

Pentoxifylline (PTX) [3,7-dimethyl-1-(5-oxohexyl) xanthine], is a general phosphodiesterase inhibitor (PDE) that has been available for many years to treat vascular disorders [9,10]. Rolipram (ROL) [(±)-4-(3 cyclopentyloxy-4-methoxy-phenyl)-2-pyrrolidone] is a specific PDE4 inhibitor (the major isoform in macrophages) that has been tested extensively as an antidepressant [11,12]. Both drugs have anti-inflammatory properties [13-15], can be administered orally, and are well tolerated. PDE inhibitors induce increased intracellular cAMP levels that have been reported to suppress TNFα secretion by macrophages [13-15], and to lesser degree, T-cell secretion of IL-2 and other cytokines [13-16]. A recent study has shown that PTX and ROL also suppress IL-12 and IFNγ production by macrophages and T cells, respectively [17]. In addition, PTX and ROL prevented insulitis and diabetes in cyclophosphamide-accelerated diabetes in NOD mice [17].

In this study, we examined the capacity of PDE inhibitors, PTX and ROL, in preventing insulitis and diabetes, in the natural course of disease in NOD mice.

MATERIALS AND METHODS

Mice.

Female NOD mice (10-12 weeks) were obtained from Taconic Farms (Germantown, NY). These mice exhibit insulitis as early as 4 weeks of age. Diabetes appears at about 12 weeks of age and reaches a level of ~ 80% at 27 weeks. The mice were kept under specific pathogen-free condition throughout the experiments.

Drugs.

PTX was a gift of Hoechst-Marion-Roussel Canada (Montreal, Quebec, Canada), and ROL was a gift of Shering (Berlin, Germany). PTX was dissolved in phosphate-buffered saline (PBS), and ROL was dissolved in PBS containing 100 g/l cremophor EL (Sigma, St. Louis, MO). Both drugs were administered by the intra-peritoneal route.

Treatment of the mice with PTX or ROL

Female NOD mice received PTX (80 mg/kg, i.p.,b.i.d.), ROL (7-14 mg/kg, i.p.,b.i.d.) or PBS, from week 12 to week16 of life and remained untreated thereafter. The mice were sacrificed when diagnosed as diabetic, or by 27 weeks of age. The

pancreata were removed and fixed in 10% formalin, and histological slides were prepared with hematoxylin and eosin staining.

Diagnosis of diabetes

Mice were diagnosed as having diabetes when random blood glucose measurements equaled to or exceeded 16 mmol/l for 3 consecutive days. Statistical analysis was performed with Fisher's exact test.

Grading of insulitis

Insulitis was graded as described by Charlton et al. [18], based on lymphocytic infiltration, as follows: grade 0, normal islet free of any peri-islet mononuclear cells; grade 1, focal peri-islet infiltrate <25% of the islet circumference; grade 2, peri-islet infiltrate >25% of islet circumference; grade 3, intra-islet infiltration with good retention of islet cells; and grade 4, extensive intra-islet infiltration with gross distortion or destruction of islet morphology. Three randomly obtained and nonadjacent levels of each pancreas were examined independently by two observers with coded slides, and all islets were scored. Statistical analysis was performed with the X^2 test.

Immunohistochemical staining of insulin producing β-cells

Immunohistochemistry was performed on formalin-fixed tissue with the Histo-Mouse SP kit (Inter-Medico, Markham, Ontario). The primary antibody was polyclonal guinea pig anti human insulin which cross reacts with murine insulin (Cedarlane, Ontario, CA), followed by biotinelated secondary antibody, streptavidin-peroxidase, conjugate and ABC substrate.

RESULTS

PTX and ROL prevent diabetes in NOD mice

We treated female NOD mice with either PTX or ROL, and we found that a relatively short course of treatment, from week 12 to week 16 of life, has a lasting protective effect (Fig. 1). While diabetes appeared at 13 weeks of age in control mice, the PTX- or ROL-treated mice only started to develop diabetes in small numbers at week 19. At week 27, 80% of control mice had diabetes, while only 20-25% of PDE inhibitor-treated mice were hyperglycemic (Fisher's exact test, $P < 0.005$ vs control mice).

Thus 11 weeks after withdrawing drug therapy, the incidence of disease was still three to four times lower than in non-treated mice. At optimal doses, there was no statistically significant difference in the effectiveness of these two PDE inhibitors. Rolipram was effective at doses ranging from 7 to 14 mg/kg b.i.d. (Fig.2), but at lower doses, larger numbers of mice developed diabetes after withdrawing ROL therapy

FIGURE 1

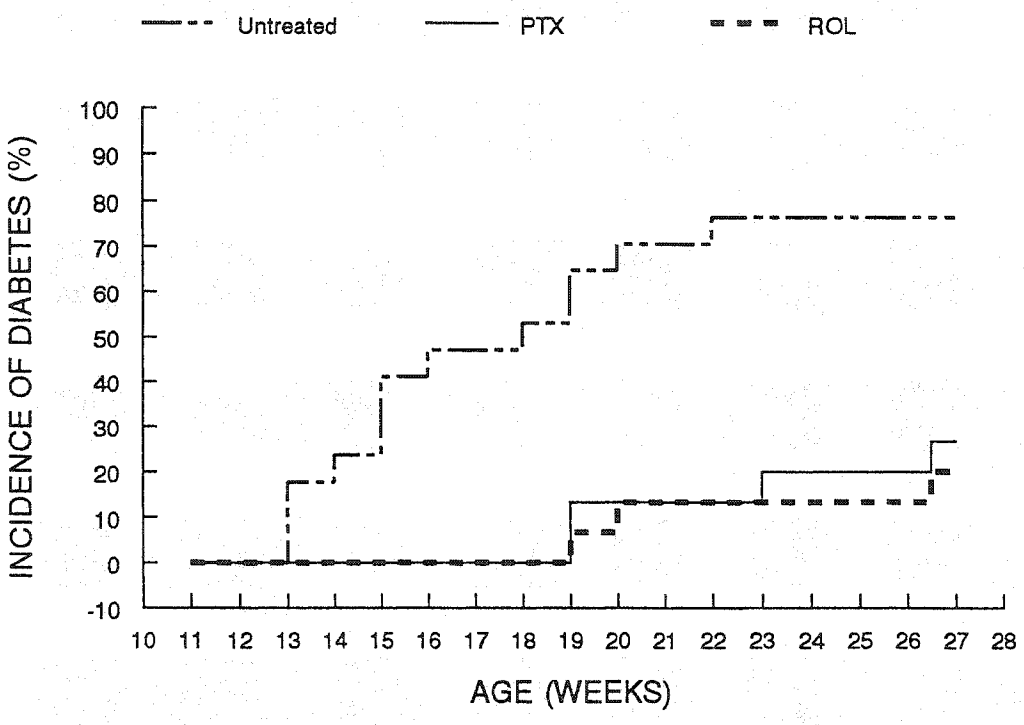
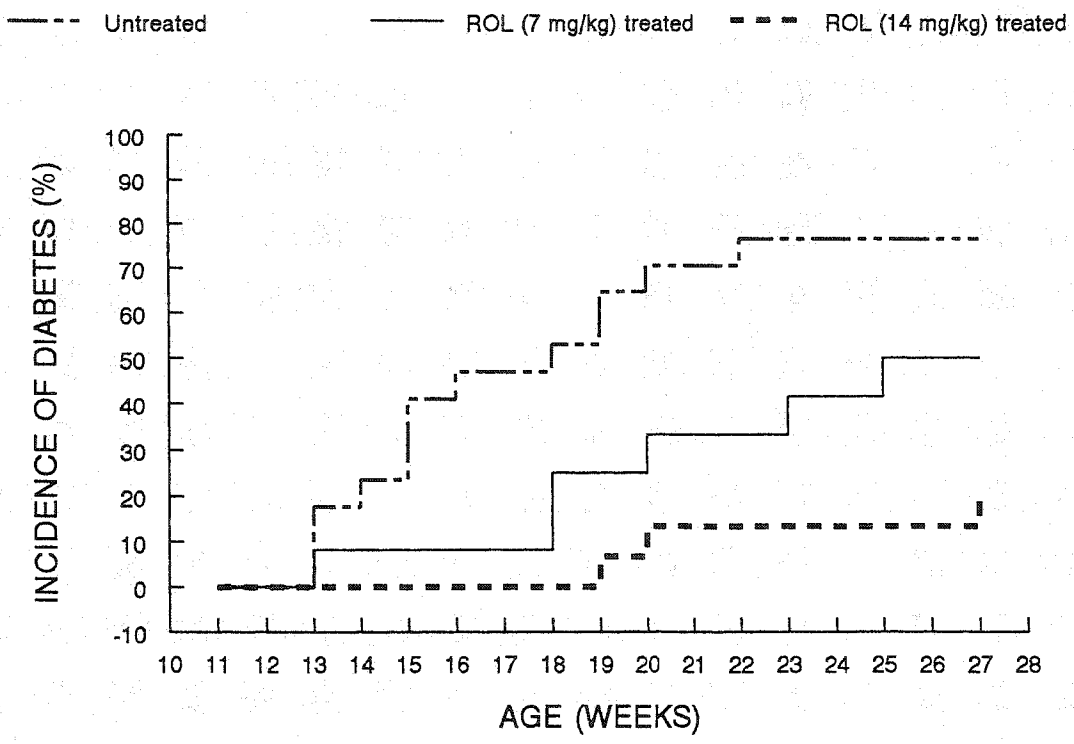

Untreated
PTX
ROL
INCIDENCE OF DIABETES (%)
100
90
80
70
60
50
40
30
20
10
0
-10
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
AGE (WEEKS)

FIGURE 2


Untreated
ROL (7 mg/kg) treated
ROL (14 mg/kg) treated
INCIDENCE OF DIABETES (%)
100
90
80
70
60
50
40
30
20
10
0
-10
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
AGE (WEEKS)

(Fig.2). On the other hand, PTX was effective at a dose of 80 mg/kg b.i.d., but not at lower doses.

PDE inhibitor treatment reduces the severity of insulitis

Histological studies were performed on the pancreata after the onset of diabetes (3 consecutive days of hyperglycemia) or after planned death in normoglycemic mice (week 27 of life). These studies revealed that both PTX- and ROL- treated mice had less severe insulitis (Table 1). The results represent either the mean scores of a whole group (nondiabetic plus diabetic, denoted ND + D) or nondiabetic mice only (denoted ND). All diabetic mice had an insulitis score above 3. In the drug- treated groups of NOD mice, consistent with the lower incidence of diabetes, there were much larger number of either normal islets of Langerhans (grade 0) or islets with only peri-insulitis (grade 1 or 2), i.e., lacking true insulitis (Fig. 3A). The mean grade of insulitis was significantly higher in non-treated control group (3.4) vs PDE inhibitors-treated group (1.6 for ROL and 1.8 for PTX) (Fig. 3B) ($P<0.0001$ vs untreated control mice)

Preservation of insulin producing cells by PDE inhibitor treatment

The immunohistochemical studies to detect insulin producing cells showed that both PTX and ROL treatment preserved insulin producing β-cells. On the other hand, in the control group most of the islets were totally destroyed and it was difficult to find preserved insulin producing cells.

FIGURE 3A

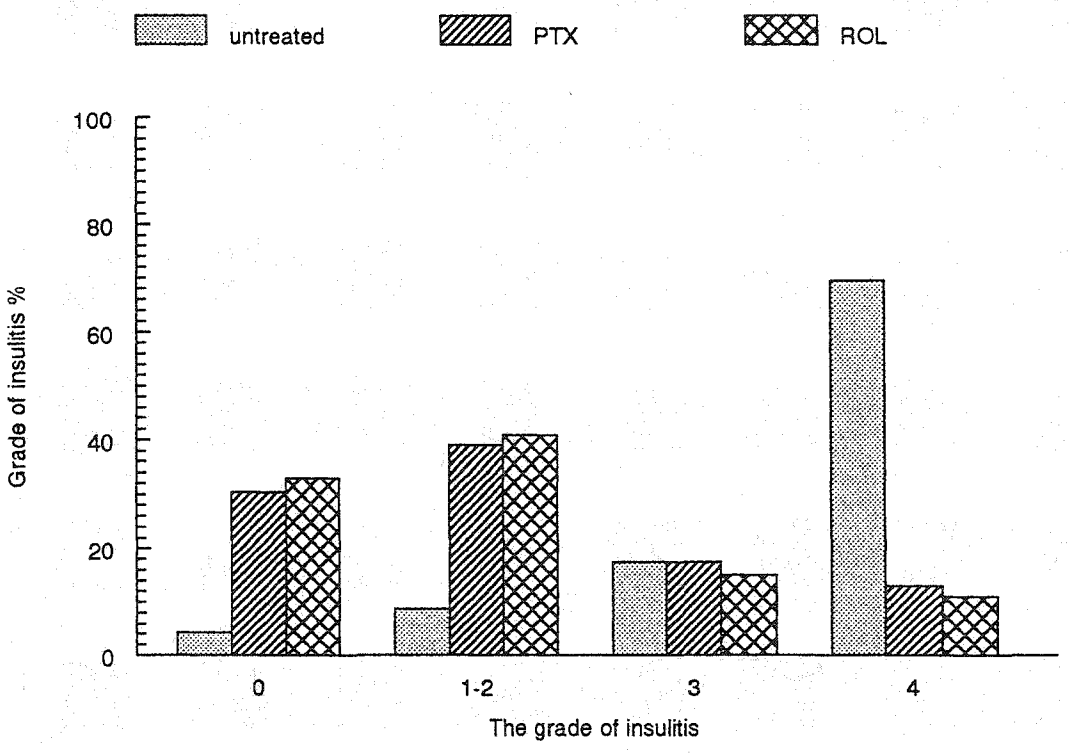
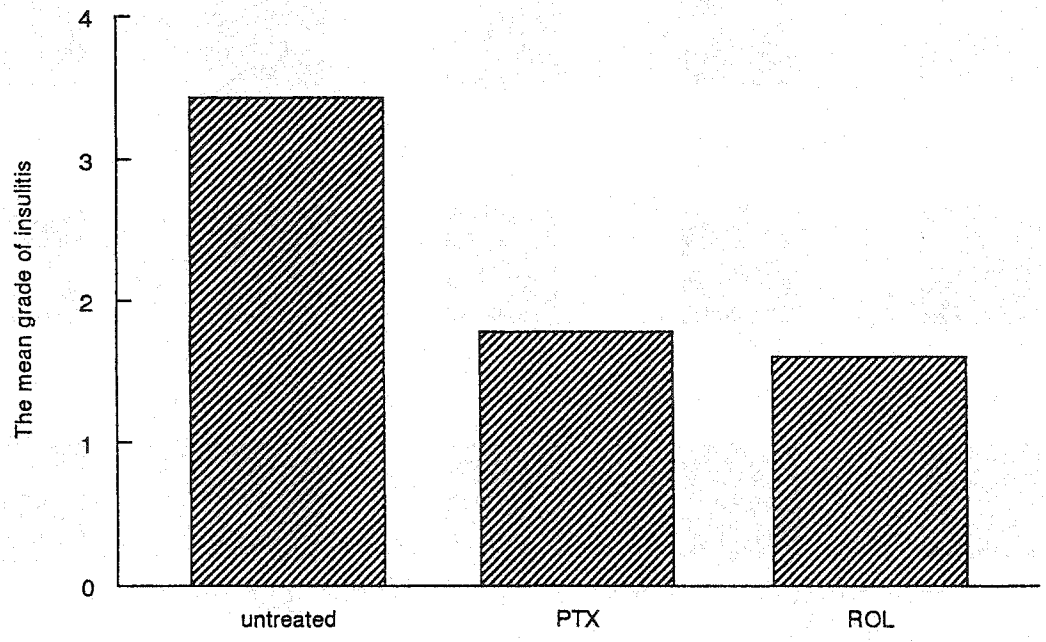
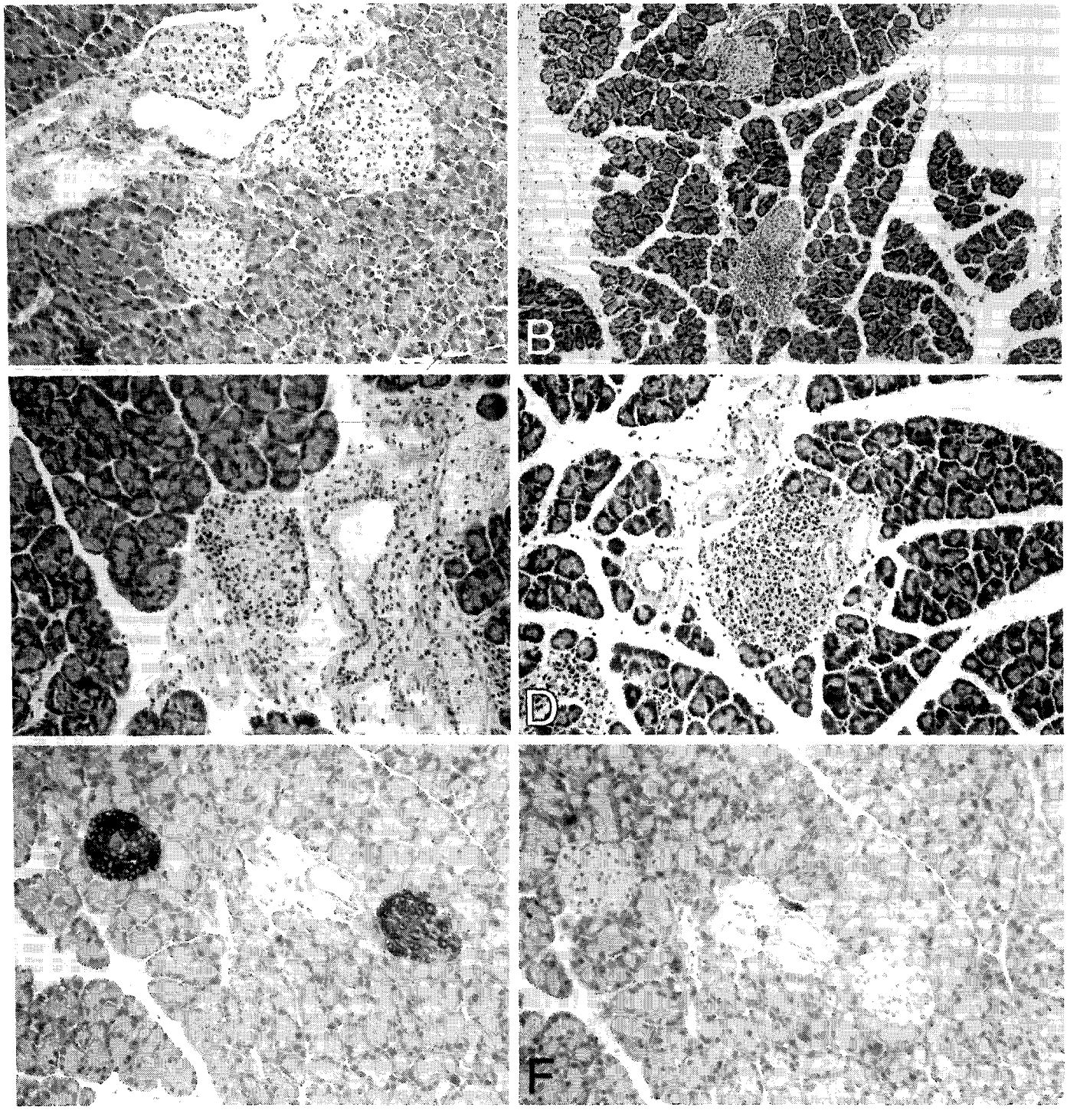

untreated
PTX
ROL
100
80
60
40
20
0
Grade of insulitis %
0
1-2
3
4
The grade of insulitis

FIGURE 3B




B
D
F

DISCUSSION

In this study, we present evidence that the PDE inhibitors PTX (a general inhibitor) and ROL (a type 4 specific inhibitor) prevent insulitis and diabetes in NOD mice. At optimal doses both drugs were equally effective in preventing diabetes. A 4 week treatment with either PTX (80 mg/kg i.p.b.i.d.) or ROL (14 mg/kg i.p.b.i.d.) had a strong protective effect that was still apparent 11 weeks after withdrawing the drugs. This was associated with a significant reduction in the severity of insulitis. Thus PDE inhibitors exert an anti-inflammatory or immunosuppressive effect. After withdrawal of treatment, there was a slow rise in the incidence of disease over the length of the experiments. Whether this could be prevented by a longer course of therapy is unknown. Because these drugs have a short half-life in plasma after intraperitoneal injection [19] it is likely that increasing the frequency of administration would be even more effective.

Diabetes in NOD mice is an autoimmune process that is dependent on inflammatory cytokine production. Several cytokines have been implicated as effector molecules involved in β-cell destruction. T- helper (Th)-1 cytokines (IFNγ, IL-2, TNFα) have been shown to have a pathogenic effect, while Th2 cytokines (IL4, IL10) are protective. The most studied pathogenic cytokines are IFNγ, TNFα, and IL-12. Thus treatment of the mice with an anti-IFNγ monoclonal antibody prevented diabetes in NOD [7]. Transgenic mice expressing IFNγ in their islets developed insulitis and diabetes [20]. Moreover, gene therapy with a nonviral vector encoding IFNγR/IgG1 fusion protein protected from autoimmune diabetes [21]. IL-12 also has been shown to

accelerate diabetes by enhancing Th1-dependent response [6]. Administration of an IL-12 antagonist, i.e., IL-12 (p40) 2, suppressed cyclophosphamide-accelerated-diabetes [22].

PDE inhibitors induce an increase in intracellular cAMP levels and alter cytokines production [13-15]. Their effect on suppressing TNFα production by macrophages is particularly well documented, where they block transcription and translation (13-15, 23-25). In addition, a recent study [17] showed that the PDE inhibitors PTX and ROL also suppressed IFN γ production by T cells, and IL-12 production by macrophages, while IL-4 was less sensitive [17]. Thus suppressing proinflammatory cytokines production by PDE inhibitors may represent a possible mechanism preventing insulitis and diabetes in NOD mice.

cAMP modifies the activity of many protein kinases and the transcription of numerous genes, and we cannot exclude the possibility that PDE inhibitors protect islet cells by mechanisms unrelated to their anti-inflammatory effects. PDE3 and 4 are expressed in islet cells [26], but while inhibition of PDE 3 can enhance insulin secretion, the inhibition of type 4 does not [26]. Therefore, the antidiabetic effect of ROL is unlikely to be related to an effect on insulin secretion. Inasmuch as this drug is a specific inhibitor of PDE4, it appears that inhibition of this isoform is sufficient to mediate all the effects reported in this study.

PTX and ROL prevent experimental allergic encephalomyelitis (EAE) in rodent [13,14]. PTX has been found to inhibit contact dermatitis in normal mice [24], and to diminish proteinuria and anti-ds DNA autoantibody production in lupus-prone MRL-

lpr/lpr mice [25]. Not surprisingly, these PDE inhibitors can be beneficial in conditions characterized by massive TNFα release [15,27]. In vitro, PTX reduces the toxicity of TNFα for islet cells [28]. In addition, PDE inhibitors can probably exert anti-inflammatory activity by acting on T-cells.

The effects of cAMP elevation on various signaling pathways in both lymphocytes (expressing type 3, 4 and 7) and macrophages (expressing mainly type 4) have not been fully elucidated. However, recent reports indicate that cAMP interrupts cytokine-triggered JAK-STAT signals [29]. In particular cAMP suppresses STAT 1 activity, which may skew cytokine production to Th2 pattern [30]

Interestingly, in an EAE model, PTX appears to favor Th2 over Th1 differentiation [13], possibly because Th1 cells are more sensitive to high levels of cAMP [16]. We have no evidence that a similar shift occurs in NOD mice, although it was reported that IL-4 was suppressed by PDE inhibitors at higher drug concentrations than either IL-12 or IFNγ [17]

We have not studied the effects of PDE inhibitors in other species. However, Rabinovitch and Sumoski [31] found that the PDE inhibitor theophylline had a protective effect in diabetes-prone BB rats and potentiated cyclosporin A protection. In an unmasked study of 21 children with new onset type 1 diabetes, MacDonald et al. [32] reported that PTX could reduce (but not abrogate) the insulin requirements or lengthen the "honeymoon" (non-insulin-requiring) period. Unfortunately, by the time the diagnosis of diabetes is made, islet-cell destruction is extensive, and it is perhaps too late to apply this form of treatment. However, in patients known to be at risk of

developing diabetes, the administration of PDE inhibitors could be considered to arrest the progression of insulitis and prevent disease. Because PTX and ROL are usually well tolerated, as demonstrated in clinical trials [9,12,33], they could probably be administered for prolonged periods of time without serious adverse effects. Moreover, these inhibitors could be of benefit in a large number of inflammatory disorders in which there is excessive production of inflammatory cytokines.

Acknowledgments

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Table 1

Insulitis score in PDE inhibitor-treated mice

		Grade of insulitis (%)				
Treatment group	Mice (n)	0	1-2	3	4	Mean grade
PBS	ND + D (15)	4.3	8.7	17.4	69.5	3.4
	ND (3)	5.9	17.6	23.5	52.9	3.05
PTX	ND + D (15)	30.4	39.1	17.4	13.0	1.78*
	ND (12)	31.3	46.9	15.6	9.4	1.63*
ROL	ND + D (15)	33.0	40.8	15.1	11.1	1.6*
	ND (11)	34.4	40.6	18.8	6.3	1.53*

NOD mice received PTX (80mg/kg i.p.b.i.d.) or ROL (14 mg/kg i.p.b.i.d.) from week 12 to week 16 and remained untreated thereafter.They were killed after the onset of diabetes or, in normoglycemic mice, at week 27. The pancreases were examined histologically and scored for insulitis. ND+D, analysis of a whole group, including nondiabetic and diabetic mice; ND, analysis of non diabetic mice only. Statistical analysis was performed by χ^2 test; ND +D treated group vs ND + D untreated control group $P < 0.0001$; ND treated group vs ND control group, $P < 0.001$.

Figure Legends

Figure 1. PTX and ROL treatment reduces the incidence of diabetes in NOD mice. Mice (n = 15 per group) received PTX (80 mg/kg i.p., b.i.d.) or ROL (14 mg/kg i.p.,b.i.d.) from week 12 to week 16 of life and remained untreated thereafter. The results represent the percentage of diabetic mice in the PBS-treated control group (— --), the PTX-treated group (—), and the ROL-treated group (----) at various time points. Fisher exact test was performed ($P < 0.005$ treated vs control group). At these doses, there was no statistically significant difference in the effectiveness of PTX and ROL.

Figure 2: ROL treatment reduces the incidence of diabetes in NOD mice. Mice (n = 15 /group) received ROL (7 mg/kg i.p.,b.i.d.), ROL (14 mg/kg i.p., b.i.d.), or PBS (untreated control group), from week 12 to 16 of life and remained untreated thereafter. The result represent the percentage of diabetic mice in PBS treated control group (—), ROL 7mg/kg treated group (----), and ROL 14 mg/kg treated group (— -). Treatment of mice with ROL at a dose 7 mg/kg prevented diabetes in NOD mice, but a large number of mice developed diabetes after withdrawal of the drug. Fisher exact test ($P < 0.01$ treated vs control group).

Figure 3: PTX or ROL treatment reduces the severity of insulitis in NOD mice. 12 week-old mice (n = 15 mice per group) received PTX (80 mg/kg i.p., b.i.d.) or ROL (14 mg/kg i.p., b.i.d.) or PBS (control group), from week 12 to 16 and remained untreated thereafter. They were killed after the onset of diabetes, or in normoglycemic mice, at week 27. The pancreata were examined histologically and scored for insulitis. A. The percent of islets of various grades in control (PBS-treated group), PTX treated group, or ROL treated group ($P < 0.0001$ treated vs control group). B. The mean grade of insulitis in control (PBS-treated) mice, PTX-treated mice, or ROL-treated mice

Figure 4: Insulitis grading and immunohistochemical staining. Female NOD mice (12 week) were treated for four weeks with either PTX (80 mg/kg), ROL (14 mg/kg) or PBS (non treated control). The mice were killed and the pancreases were recovered and fixed in 10% formalin. H&E stained slides were used for grading insulitis. Immunohistochemical staining for insulin was performed in formalin fixed tissue with Histo-Mouse-SP kit. The primary antibodies were guinea pig anti-human insulin polyclonal antibodies which cross react with murine insulin

A. Grade 0 insulitis, normal islet from PTX treated group.

B. Grade 1 and Grade 2 insulitis (peri-insulitis) from ROL treated group.

C. Grade 3 insulitis from non-treated control group.

D. Grade 4 insulitis from non-treated control group.

E. Insulin staining showed preserved insulin producing cells from PTX treated group.

F. Negative control for insulin staining.

CHAPTER 3

ROLIPRAM SUPPRESS MACROPHAGE ACTIVATION AND NITRIC OXIDE PRODUCTION *IN VITRO* AND *IN VIVO*

by

Evette Beshay, France Croze and Gérald J. Prud'homme*

from

The Department of Pathology, McGill University,

3775 University St., Montreal, Qc, Canada H3A 2B4

*Address correspondence and reprint requests to:

Dr. Gérald J. Prud'homme

3775 University St., Room B13

Montreal, Qc, Canada H3A 2B4

Tel. 514-398-7192 (ext. 7237); Fax 514-398-7446

E-mail : gprudh@po-box.mcgill.ca

Running title: Phosphodiesterase inhibitors suppress nitric oxide production

Keywords: Phosphodiesterase inhibitor, pentoxifylline, rolipram, nitric oxide, macrophage, cytokines, inflammation.

Abstract

We studied the effects of the phosphodiesterase inhibitors pentoxifylline (PTX) and rolipram (ROL) on nitric oxide (NO) production by macrophages and correlated this with cellular cAMP levels. The RAW 264.7 cell line or mouse peritoneal macrophages were activated with lipopolysaccharide (LPS) and interferon γ (IFNγ), with or without ROL, PTX, cAMP analogues or Forskolin. *In vivo*, peritoneal macrophages were stimulated with staphylococcal enterotoxin B (SEB) with or without administration of ROL. Nitrite levels in culture and the total cellular cAMP levels were measured. ROL and PTX suppressed NO production of LPS/IFNγ stimulated macrophages. ROL (IC_{50}=68-74 μM) was about 40 times more potent than PTX (IC_{50}=2.4-2.9 mM). The suppression paralleled increased total cellular cAMP level (EC_{50} = 68-72 μM) and was mimicked by other cAMP elevating agents. ROL and PTX suppressed inducible NO synthase at the mRNA level. The inhibition of NO production of macrophages by ROL or PTX could be beneficial in NO-mediated inflammatory and/or autoimmune disorders

.

INTRODUCTION

Nitric oxide (NO) is a free radical found to be a potent and pleiotropic mediator with physiologic and toxic activity [1, 2, 3]. NO is synthesized by various isoforms of nitric oxide synthase (NOS) which catalyze the oxidation of L-arginine to form L-citrulline and NO [2]. NOS exists in constitutive isoforms present in endothelial cells (eNOS) or neurons (nNOS), and a cytokine or endotoxin inducible form (iNOS) [3]. The iNOS isoform was first identified in macrophages, but now has been found in many nucleated cells (hepatocytes, islet cells and others). In contrast to other isoforms, iNOS is Ca^{2+} independent and produces large amounts of NO which appears to mediate in large part the cytotoxic action of macrophages on some pathogens and target cells.

cAMP and protein kinase A (PKA) are involved in the regulation of cellular activation. Phosphodiesterase (PDE) inhibitors, which elevate intracellular cAMP levels, are potent regulators of various immune processes. There are ten different PDE families [4]. PDE type IV is the major type in macrophages while lymphocytes express both the type III and type IV isoforms [5]. We have previously shown that rolipram (ROL), a selective type IV PDE inhibitor, and pentoxifylline (PTX), a general PDE inhibitor, can both prevent insulitis and diabetes in diabetes-prone NOD mice [6]. The role of PTX and ROL in inhibiting tumor necrosis factor-α (TNFα) production by macrophages is well documented [6, 7]. However we have found that these drugs also inhibit interleukin-12 (IL-12) and interferon-γ (IFNγ) production by macrophages and lymphocytes, respectively. The effects of PTX and ROL on NO

production and iNOS stimulation have not been extensively studied. Some studies have shown inhibitory effects [8] and others stimulatory effects [9]. In this study, we report on the inhibitory effects of PTX and ROL on NO production in IFNγ/lipopolysaccharide (LPS) costimulated macrophages, and the relationship to cellular cAMP levels. We find that ROL suppresses NO production independently of its inhibitory effects on the secretion of TNFα or IL-12 by macrophages, since addition of these cytokines to cultures had no effect. We show that ROL and PTX supress iNOS at the mRNA level. Moreover, the inhibitory effects of ROL and PTX on macrophage activation are demonstrated *in vivo* in superantigen-treated mice. Our results suggest that PDE inhibitors could have a role in the treatment of NO-dependent inflammatory disorders.

MATERIAL AND METHODS

Mice.

Female NOD mice (8-10 weeks) were purchased from Taconic farms (Germantown, NY). Male CD-1 mice (8-10 weeks) were purchased from Charles River Canada (St. Constant, Quebec, Canada). Mice were housed under pathogen-free conditions.

RAW 264.7 cell culture and stimulation.

The murine macrophage cell line RAW 264.7 (ATCC, Rockville, MD), henceforth referred to as RAW, was used for nitric oxide production. Briefly, RAW cells were plated in 96 well plates in DMEM (GIBCO BRL, Grand Island, NY, USA) containing 10% heat inactivated fetal bovine serum (FBS) at a density of 2 x 10^4 cells /100 μL /well, at 37 °C for 24 h. In all experiment which required nitrite measurements, phenol red free DMEM media was used. Once the supernatant was discarded, medium containing IFNγ (0.8 ng/ml) and LPS (30 ng/ml) was added to adherent cells. Where indicated cultures were supplemented with ROL (Shering, Berlin, Germany), PTX (Sigma, St.Louis, MO), the adenylate cyclase stimulator Forskolin (Sigma), cAMP analogues (dibutyryl cAMP or 8-bromo-cAMP [both from Sigma]), dibutyryl cGMP (Sigma), recombinant murine IL-12 and TNFα (Pepro Tech INC., Rocky Hill, USA). After a further 24 h of culture supernatant was collected for nitrite measurement.

Isolation of peritoneal macrophages.

Peritoneal macrophages were isolated as previously described [10]. Briefly, nonactivated peritoneal wash cells were added to 96 well plates at a density of 2 x 10^5 cells/100 μl/well, and incubated for 2 h at 37 °C in RPMI-1640 containing 10% FBS. Nonadherent cells were then removed by gentle washing. Over 90% of these adherent

cells are macrophages, as determined by phagocytosis of fluorescent microbeads. Adherent cells were incubated as described above for RAW cells.

Superantigen injection and recovery of peritoneal macrophages.

The mice were injected i.p. with either PBS, or ROL (14 mg/kg body weight) at five time points (0, 15 min., 4 h, 10 h, 18 h). Staphylococcal enterotoxin B (SEB) (Sigma) was injected (100 μg in 200 μl PBS) i.p. once at 15 min. 24 h after SEB injection, peritoneal wash cells were recovered, and adherent peritoneal macrophages prepared as described above. The adherent cells were incubated with or without LPS, and culture supernatants were collected 24 h later for nitrite measurement.

Nitrite measurement.

Nitrite accumulation, as an indicator of NO production, was measured using the Griess reagent as we have described [11]. Absorbance was measured at 540 nm in a microplate reader (Titertek Instruments, Hunstville, AL). Nitrite concentration was calculated from a $NaNO_2$ standard curve.

$$\text{Percent inhibition of nitrite production} = 100 - \frac{(\text{the } NO^-_2 \text{ level with inhibitor}) \times 100}{(\text{the } NO^-_2 \text{ level without inhibitor})}$$

IC_{50} values were calculated from the dose response percent inhibition curve for each treatment.

DNA content determination by fluorometric assay.

We followed the protocol described by Rago [12] with some modifications. Briefly, culture supernatants were removed from wells containing adherent macrophages (96 well plates). The plates were frozen at -80 C and thawed until they reached room temperature, and 100 µl of TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) was added to lyse the cells. The lysate was transferred to microtubes with 1 ml of Hoechst (Sigma) 1:2000 dilution of stock solution (200 µg/ml). The fluorescence emission of the Hoechst dye was measured with a fluorescent spectrophotometer (Hoefer) at a wave length 457 nm. DNA content was measured from a standard curve obtained with calf thymus DNA (Sigma).

Measurement of cAMP level.

RAW cells were seeded in 24 well plates at a density of 6×10^4 in DMEM with 10% FBS for 24 hours. The total cAMP from both the supernatant and cell lysate, referred to as total cellular cAMP, was measured for each well using an enzyme immune assay (EIA) kit from Amersham Pharmacia Bioteck (Quebec, Canada), following the manufacturer's instructions.

Determination of iNOS mRNA by reverse transcription-polymerase chain reaction (RT-PCR).

RNA extraction and RT-PCR were performed as we have previously described [11]. PCR amplification of cDNA with primers specific for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out and the products were visualized by electrophoresis in 2% agarose gel containing ethidium bromide. iNOS primers were: sense, 5'-CTTCCGAAGTTTCTGGCAGCAGCG-3'; antisense, 5'-GAGCCTCGTGGCTTTGGGCTCCTC-3' and the PCR product was 487 bp. GAPDH primers were : sense, 5'-TCCACCACCCTGTTGCTGTA-3'; antisense, 5'-ACCACAGTCCATGCCATCAC-3' and the PCR product was 450 bp. PCR reactions were run for 25-35 cycles, and were terminated in the linear portion of the amplification reaction.

Statistical Analysis.

Statistical analysis was performed using Student's t test, and $p < 0.05$ was considered significant.

RESULTS

ROL and PTX inhibit NO production by Macrophages.

A combination of IFNγ (0.8 ng/ml) and LPS (30 ng/ml) stimulates RAW cells and peritoneal macrophages to produce a considerable amount of NO, which is rapidly transformed to nitrite in culture. We measured nitrite levels as an indication of NO production. In the absence of LPS and IFNγ the cells produce an undetectable amount of nitrite (not shown). We find that ROL suppresses nitrite production in a dose dependent manner (Fig. 1A). A concentration of 3.1 μM ROL is sufficient to significantly inhibit NO production ($p = 0.01$), and a concentration of 200 μM inhibits by over 95 % (IC_{50} = 68 - 74 μM). PTX also inhibited NO production (Fig. 1B), but at higher concentrations (IC_{50} = 2.4 - 2.9 mM). Thus, on a molar basis ROL is about 40 times more potent than PTX at inhibiting NO production. Similar results were obtained when peritoneal murine macrophages were used instead of RAW cells (Fig. 2A and data not shown). In these experiments, suppression of NO production cannot be attributed to cell death since adherent-cell total DNA content per well was not significantly reduced by either PTX or ROL (data not shown).

cAMP elevating factors inhibit NO production.

FIGURE 1A

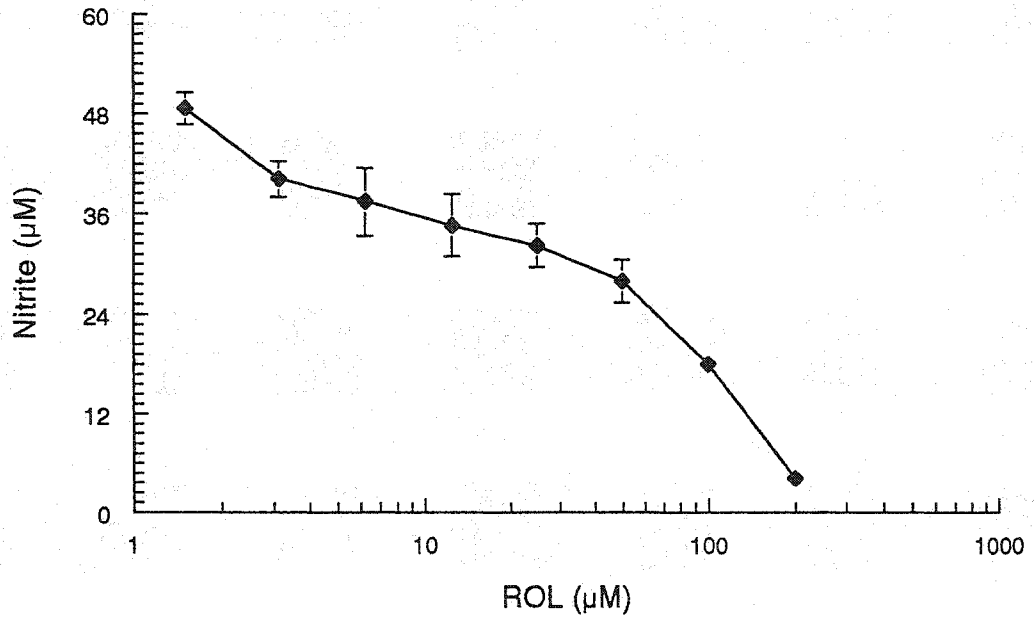
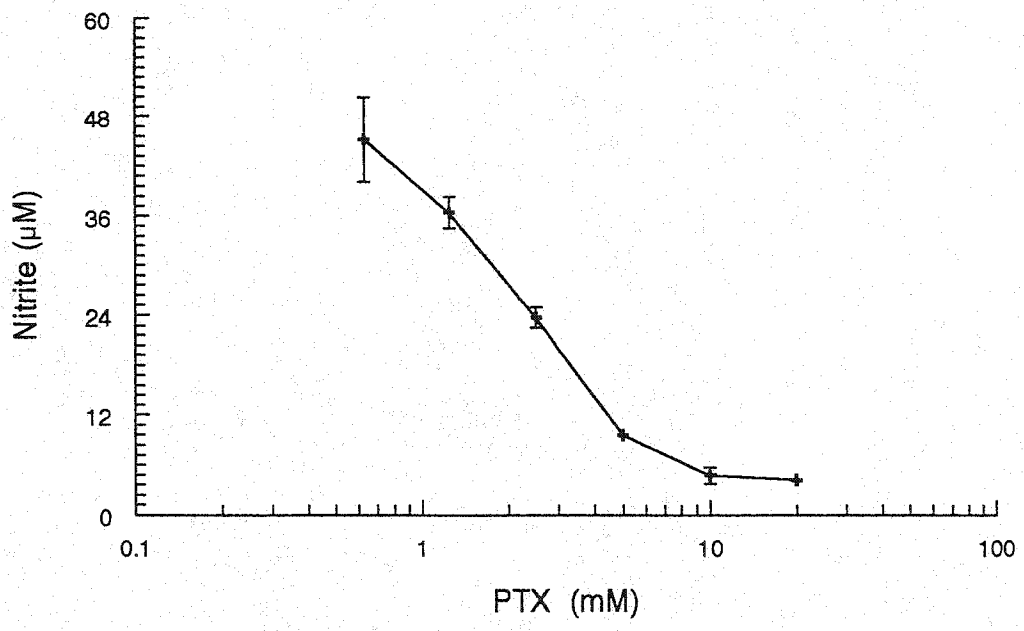


FIGURE 1B



PTX, ROL, 8-bromo-cAMP (br-cAMP), dibutyryl-cAMP (db-cAMP), and Forskolin all increase intracellular cAMP activity. These agents all significantly inhibited NO production by stimulated peritoneal murine macrophages (Fig. 2A). Similar inhibition was observed with RAW cells (not shown). Dose response curves for 8-bromo-cAMP and dibutyryl-cAMP are shown in (Fig. 2B). Our results show that dibutyryl cAMP, 8-bromo cAMP and Forskolin all supress NO production, but even at high concentrations the suppressing effect is only modest. These data are suggestive that cAMP elevation plays a role in inhibiting NO production, but it is not the only contributor to this effect. Dibutyryl-cGMP has no effect on NO production of either RAW cells or peritoneal macrophages (data not shown).

Total DNA content of adherent cells per well was not significantly reduced with any of these agents, and no DNA fragmentation was observed by electrophoresis in DNA extracted from stimulated macrophages (not shown). Thus, it is unlikely that reduced NO production was secondary to cell death by apoptosis or necrosis.

ROL increases the intracellular cAMP level in RAW cells.

To examine whether the suppression of NO production by ROL was associated with elevated cAMP, we measured total cellular cAMP levels (total cAMP in cells and supernatant) in LPS/IFNγ-costimulated RAW cells. Our data shows that ROL increases total cellular cAMP within 10 minutes, peaking at 30 minutes, remaining at a plateau level before declining after 4 h (Fig. 3A). ROL increases total cellular cAMP levels in a dose dependent manner (Fig. 3B), and this occurs at drug concentrations

FIGURE 2A

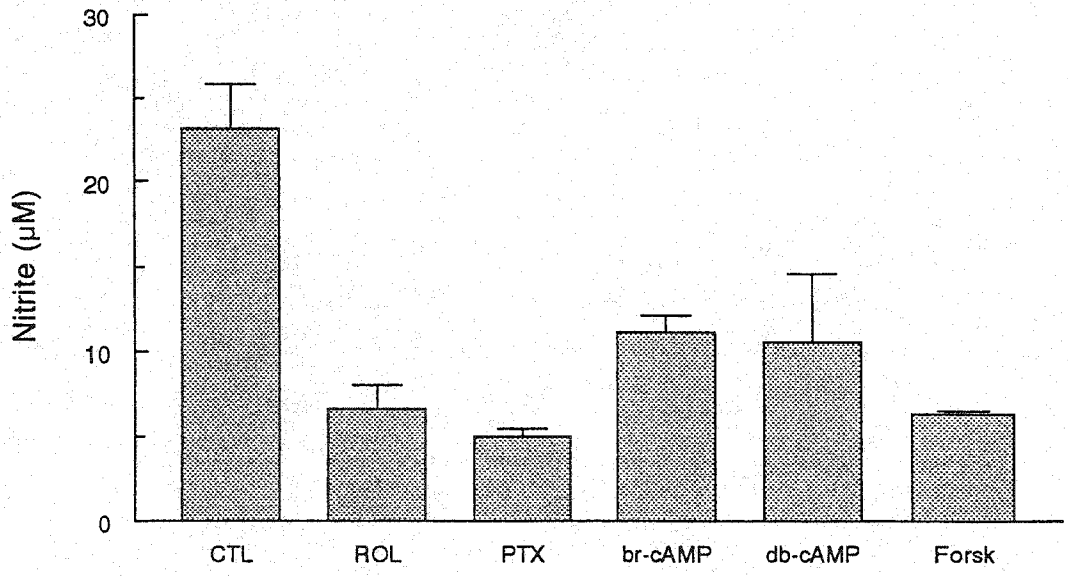


FIGURE 2B

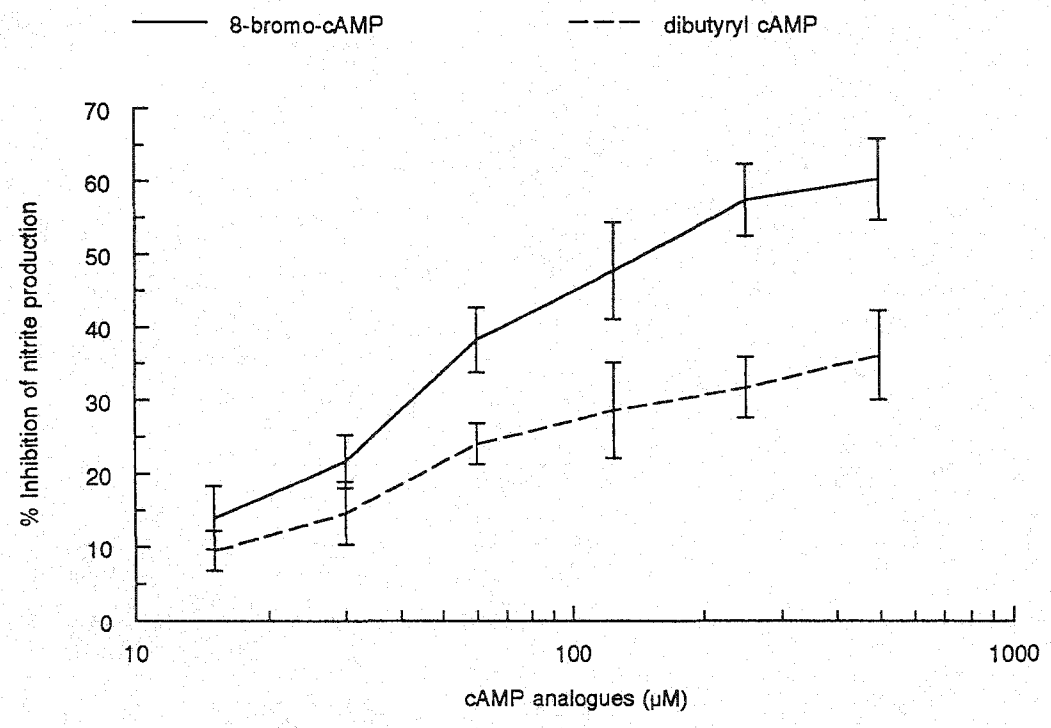

8-bromo-cAMP
dibutyryl cAMP
% Inhibition of nitrite production
70
60
50
40
30
20
10
0
10
100
1000
cAMP analogues (μM)

FIGURE 3A

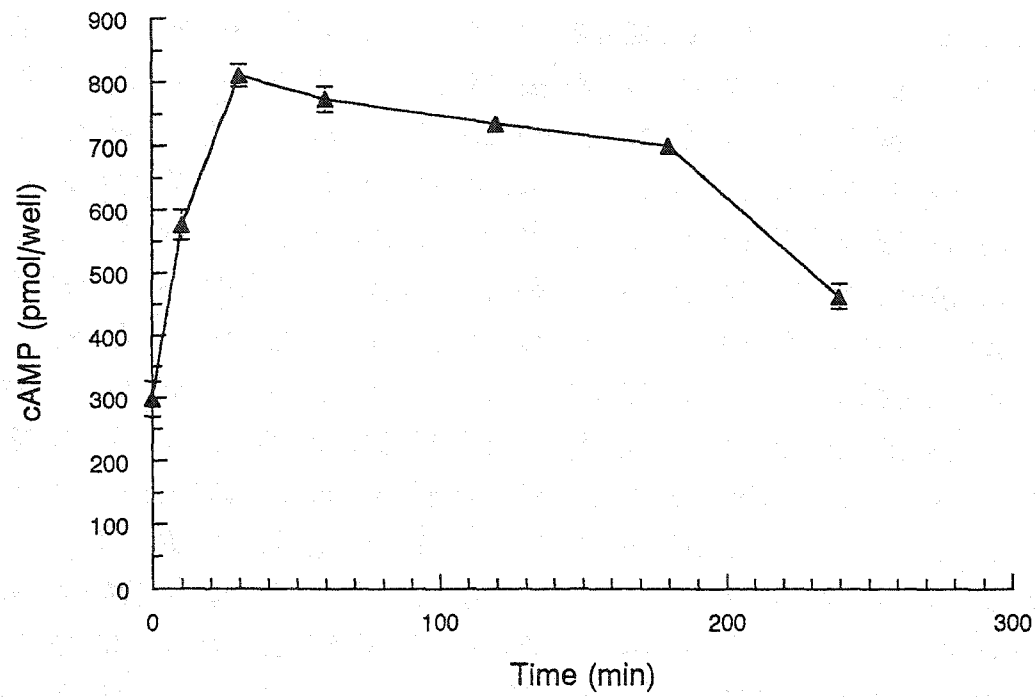
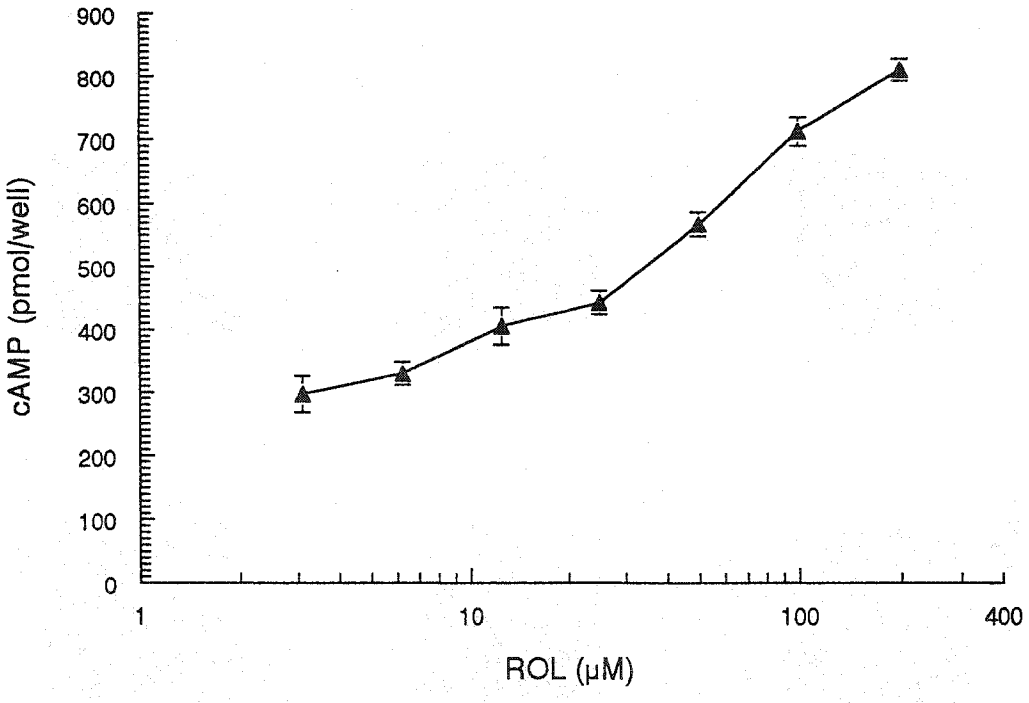

900
800
700
600
500
400
300
200
100
0
cAMP (pmol/well)
0
100
200
300
Time (min)

FIGURE 3B


900
800
700
600
500
400
300
200
100
0
cAMP (pmol/well)
1
10
100
400
ROL (μM)

that block NO production (Fig. 1A). The correlation of these data suggests that ROL suppresses NO production at least in part by elevating cAMP level.

ROL and PTX suppress the expression of iNOS mRNA by macrophages.

To determine if ROL and PTX-mediated suppression of NO production is a pretranslational event, we investigated the expression of iNOS mRNA in IFNγ/LPS stimulated macrophages in the presence or absence of these drugs. iNOS mRNA expression by macrophages is maximal after 4 hours stimulation with IFNγ and LPS, and either ROL (200 μM) or PTX (5 mM) suppressed iNOS mRNA expression (Fig. 4).

Administration of ROL inhibits macrophage activation in vivo.

Macrophages collected from mice 24 h after i.p. injection of the SEB superantigen (100 μg) produce a high amount of NO when stimulated with LPS (without any added IFNγ). This reflects *in vivo* macrophage activation, and may be mediated at least in part by IFNγ inasmuch as it is associated with systemic release of this cytokine (our unpublished observations). Treatment of the mice with ROL (14 mg/kg body weight) i.p. at different time points prevented any increase in nitrite production from recovered peritoneal macrophages (Fig. 5). Similar results were obtained in CD-1 mice which, unlike NOD mice, are not prone to autoimmunity (not shown).

FIGURE 4

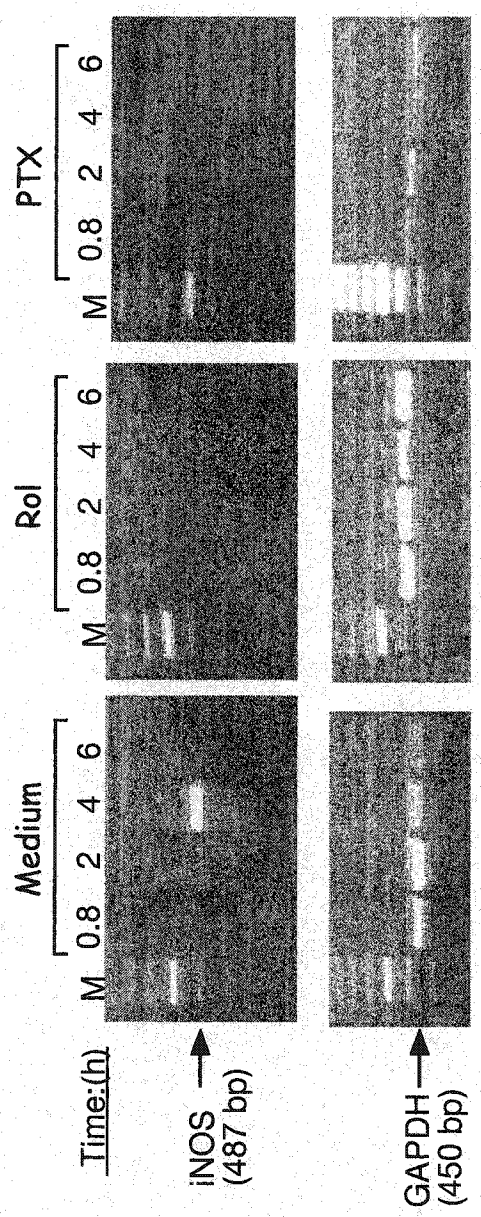
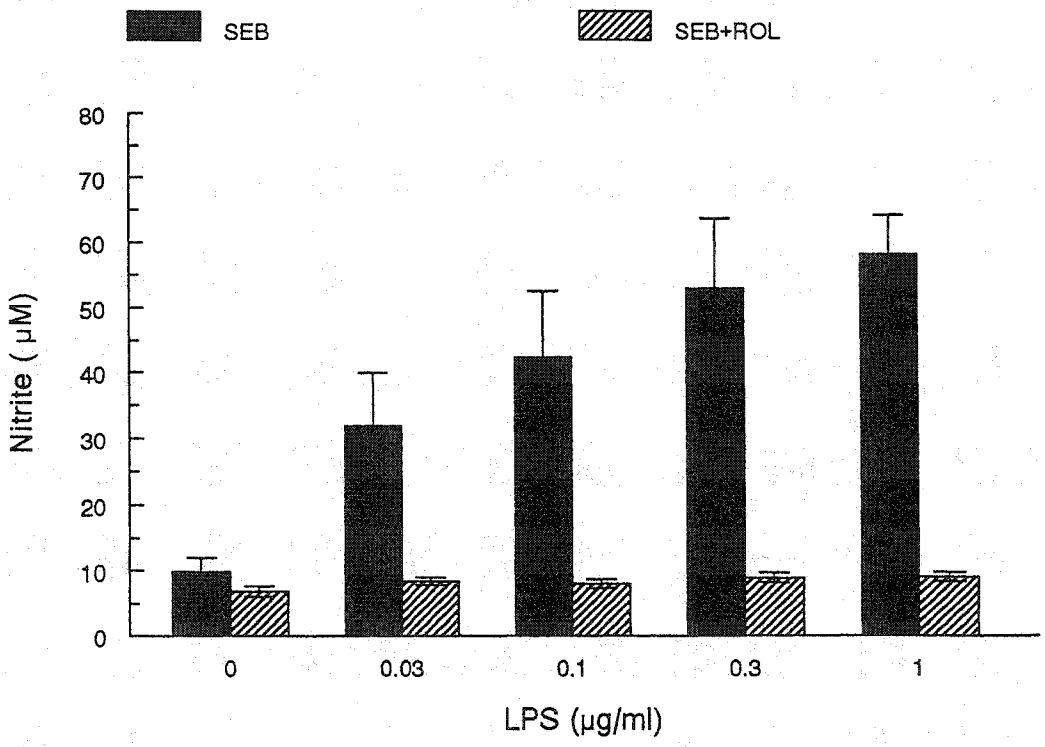


FIGURE 5


SEB
SEB+ROL
Nitrite (μM)
80
70
60
50
40
30
20
10
0
0
0.03
0.1
0.3
1
LPS (μg/ml)

TNFα and IL-12 do not restore NO production.

Recombinant murine IL-12 and TNFα were added to the cultures to determine whether suppression of NO production is secondary to reduced production of these cytokines by macrophages. IL-12 and TNFα alone did not stimulate nitrite production in our assay, and ROL suppressed nitrite production with unchanged efficiency when IL-12 (Fig. 6A), TNFα (Fig. 6B) or both (not shown) were added to culture at various concentrations. This suggests that ROL does not act indirectly by suppressing these cytokines.

FIGURE 6A

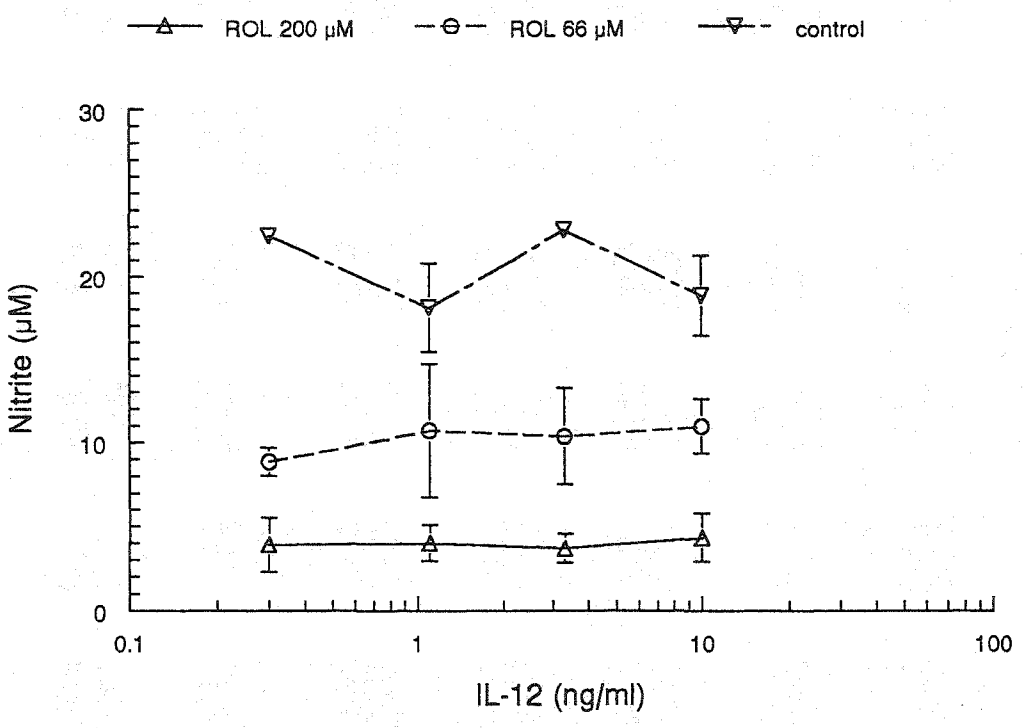
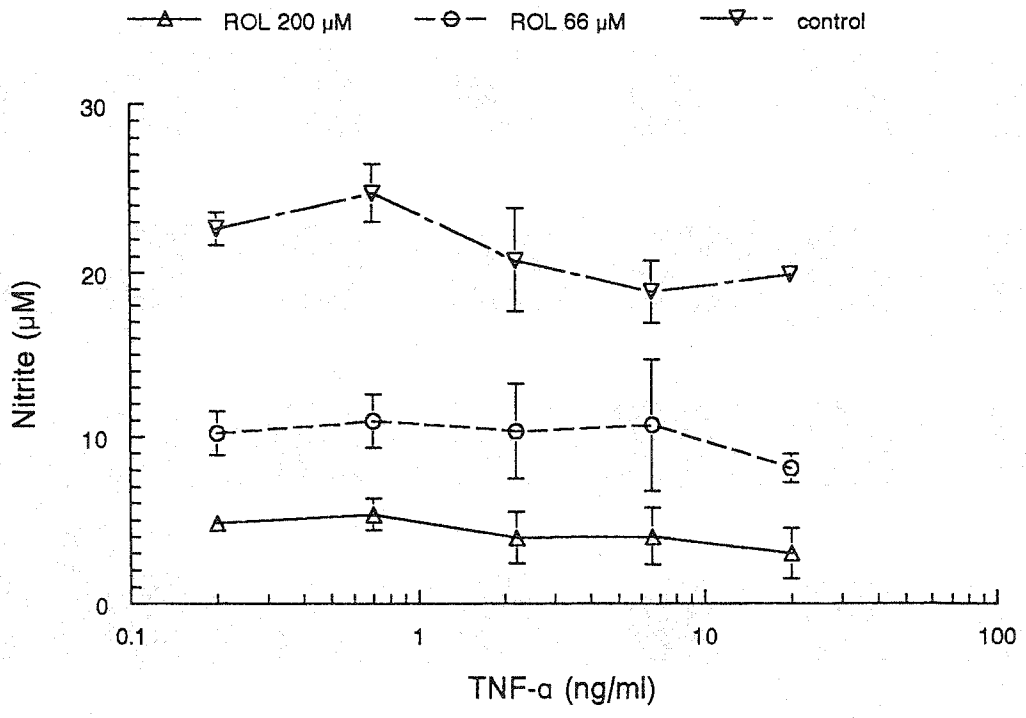

ROL 200 µM
ROL 66 µM
control
Nitrite (µM)
30
20
10
0
0.1
1
10
100
IL-12 (ng/ml)

FIGURE 6B


ROL 200 µM
ROL 66 µM
control
Nitrite (µM)
30
20
10
0
0.1
1
10
100
TNF-α (ng/ml)

Discussion

We present evidence that two PDE inhibitors, ROL and PTX, suppress NO production by IFNγ and LPS costimulated macrophages. This was equally apparent with the RAW macrophage cell line and normal murine peritoneal macrophages. Inhibition of NO production is associated with elevated total cellular cAMP. Moreover, it is mimicked by raising the intracellular cAMP level by different mechanisms, either by cell membrane permeable cAMP analogues (dibutyryl-cAMP and 8-bromo-cAMP), or an adenylate cyclase activator (Forskolin).

Agents that elevate cAMP have been previously reported to inhibit NO production. For example, Bulut et al.[13] found that a combination of prostaglandin E2 (adenylate cyclase activator) and isobutyl methyl-xanthine (IBMX; a general PDE inhibitor), induced prolonged elevation of cAMP level and suppressed iNOS activity in an IFNγ/LPS costimulated murine macrophage cell line (J774). However, Greten et al. [9] reported that while ROL inhibited TNFα production by RAW cells, NO production was increased. It should be noted that these authors activated the cells with high levels of LPS (over 300 fold higher than us) in the absence of IFNγ. We find that these conditions are not optimal for stimulating macrophages and result in low NO production. At any rate, at high concentrations of ROL these authors did observe mild NO suppression. A caveat is that cell lines may mutate in culture and alter their response to various stimuli. We obtained similar results with both RAW cells and normal peritoneal macrophages. Therefore, our findings cannot be attributed to the particularities of a cell line. We could also demonstrate inhibition of macrophage NO

production *ex vivo* in ROL-treated mice (see below). Thus, our data suggest that ROL can inhibit NO production under physiologically relevant conditions.

There is a time delay between the peak cAMP levels induced by ROL in culture (< 3 h) and the maximal induction of iNOS mRNA (4 h). However, under the influence of ROL, cAMP levels remained significantly elevated for at least 24 h. Moreover, cAMP may be blocking early events related to iNOS gene transcription. The mechanism(s) by which cAMP suppresses iNOS mRNA expression following LPS/IFNγ stimulation have not been elucidated, but this presumably related to alterations in the levels or activity of transcription factors that bind to the *iNOS* promoter (see below). For instance, elevated cAMP levels could inhibit the expression of other genes that regulate iNOS gene transcription, and that are only expressed early (< 3 h) after LPS/ IFNγ stimulation.

At any rate, our data show that both ROL and PTX suppress iNOS mRNA expression, and this appears secondary to increased cAMP levels. In some cells, increased cAMP is associated with reduced activation of NF-κβ, a transcription factor that plays an important role in regulating iNOS gene transcription [14]. Interestingly, some cytokines inhibited by ROL or PTX also have NF-κB binding elements in their promoters or enhancers [15]. We speculate that this could represent a common factor leading to inhibited expression of these genes. Moreover, macrophage iNOS has a consensus sequence for protein kinase A (PKA) [16], and perhaps phosphorylation of iNOS reduces its activity. On the other hand, the rat iNOS promoter has a cAMP response element [17], such that rat iNOS could be induced also by elevating cAMP

level in an NF_KB independent pathway [18]. However, the signaling pathways involved in regulation of iNOS expression are distinct in different cell types [18], perhaps explaining some contradictory reports on the regulatory effect of elevated cAMP level on NO production. Our data suggest that both the murine macrophage cell line RAW 264.7 and murine peritoneal macrophages have the same regulatory pathway.

PTX and ROL suppress inflammatory cytokine production and they are therapeutically effective in several experimental inflammatory and/or autoimmune diseases. ROL is generally more effective than PTX on a molar basis, and it inhibits PDE IV which is the major isoform found in macrophages. Both PTX [19] and ROL [20] prevent experimental allergic encephalomyelitis (EAE) in rodents. PTX has been found to inhibit contact dermatitis in mice [21], and to diminish proteinuria and anti-ds DNA autoantibody production in lupus-prone MRL-*lpr/lpr* mice [22]. In addition, these PDE inhibitors can reduce inflammatory cytokine production, especially TNFα, in septic shock [7, 23, 24]. Since ROL is a specific PDE IV inhibitor, it appears that inhibition of this isoform is sufficient to block inflammatory cytokine production by T cells and macrophages, as well as NO production by macrophages.

We have recently shown that PTX and ROL prevent insulitis and diabetes in spontaneously diabetes-prone NOD mice and cyclophosphamide (CYP)-accelerated diabetes in this strain [6]. These mice develop a form of insulin-dependent diabetes (IDDM) similar to the human disease, due to an autoimmune destruction of islets β cells [25]. The precise mechanisms of islet destruction are not totally elucidated, but

there is evidence for a T helper-1 (Th-1)-mediated response with IFNγ production, associated with macrophage stimulation and production of IL-12, TNFα, IL-1, and NO. We have shown that PTX and ROL suppress IFNγ production by T cells, as well as both IL-12 and TNFα production by macrophages [6]. TNFα, like IFNγ, can induce NO production by macrophages [26]. Inhibition of these cytokines could be a mechanism by which PTX and ROL suppress NO production in macrophages. However, this is unlikely to be the only mechanism, since we find that addition of TNFα and IL-12 to stimulated RAW cells in the presence of ROL does not restore NO production. Inasmuch as IFNγ is always added to the medium in our NO production assays, and it is equally improbable that ROL was acting by suppressing that cytokine.

Notably, our results are applicable *in vivo* since ROL suppresses macrophage activation in SEB-injected mice. In this model, SEB (a potent superantigen) stimulates both T-cell and macrophage activation by simultaneously binding to T-cell receptor Vβ elements (of some families) and class II MHC molecules of macrophages or other antigen presenting cells [27]. Bacterial superantigen-induced lymphocyte responses have been reported to be NO dependent and mediated by IFNγ and IL-12 [27]. Moreover, *ex vivo* the peritoneal macrophages of SEB-treated mice produce markedly increased amounts of NO when stimulated with LPS, without addition of IFNγ to cultures [28]. We found that ROL suppresses NO production of peritoneal macrophages collected 24 h after SEB injection. This effect mimics our observations *in vitro*; however, in this case the drug may be acting by suppressing IFNγ or other

cytokine production and this question was not addressed in our study. Since the NOD peritoneal macrophage has an abnormal phenotype, we examined the effect of ROL in CD-1 mice, but the results were similar in both strains.

Suppression of NO production by macrophages could be an important mechanism by which ROL and PTX protect from autoimmune diseases. In autoimmune diabetes, cytokines have been implicated as immunological effector molecules that induce dysfunction and destruction of pancreatic β cells. Interestingly, the cytotoxicity of inflammatory cytokines such as IL-1 on islet cells *in vitro* is at least partially mediated by NO [29-31]. Thus, inhibition of NOS by L-arginine analogues (N^G-monomethyl-L-arginine[NMMA] and N_ω-nitro-L-arginine methyl ester [L-NAME]) blocks IL-1-induced NO formation and prevents IL-1 induced inhibition of insulin release in rat islets [32]. NO has been shown to inactivate important enzymes in β cells by nitrosylation of target iron sulfur proteins e.g., mitochondial aconitase, required for glucose oxidation and insulin release [33, 34]. This inactivation impairs β-cell metabolism, diminishes the capacity of insulin secretion, and may eventually cause β-cell death. NO is likely to be produced by islet-infitrating macrophages, and such infiltration is an early event in insulitis and essential for the development of diabetes. However islet cells can express iNOS [35], and it is possible that ROL and PTX inhibit nitric oxide production by islet cells.

Our findings suggest that the PDE inhibitors such as PTX and ROL may be effective in the therapy of autoimmune and/or inflammatory NO-mediated disorders. PTX (Trental) is already in clinical use for the treatment of vascular diseases, and may

find a new application in this context.

Acknowledgments

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Figure Legends

Figure 1. Suppression of NO production by PDE inhibitors. NO production from IFNγ (0.8 ng/ml) and LPS (30 ng/ml) costimulated RAW cells was determined by measuring nitrite (a stable product derived from NO) using the Griess reagent, in 24 h culture supernatants. **A.** Suppression of NO production by ROL. **B.** Suppression of NO production by PTX. The results represent the mean of triplicate cultures ± 1 SD. A representative experiment is shown for each drug and three independent experiments yielded similar results in each case.

Figure 2. Agents which increase intracellular cAMP suppress NO production. **A.** Peritoneal wash macrophages from NOD mice (10-12 week old) were collected without prior activation. The adherent cells were stimulated and supernatants analyzed as in Fig. 1. Cells were incubated with IFNγ and LPS in the presence or absence of cAMP elevating agents, i.e., ROL (200 μM), PTX (5 mM), dibutyryl cAMP (db-cAMP; 400 μM), 8-bromo-cAMP (br-cAMP; 250 μM), or Forskolin (Forsk; 200 μM). The results represent mean levels of nitrite ± 1 SD in triplicate cultures in a representative experiment, and 2 experiments yielded similar results. **B.** Suppression of NO production of IFNγ and LPS costimulated RAW cells versus the concentration of 8-bromo-cAMP (ÄÄÄ) or dibutyryl cAMP(------). The data represent percent

inhibition of nitrite production ± 1 SD of triplicate wells in a representative experiment, and 3 experiments yielded similar results.

Figure 3. Time response curve of ROL effect on total cellular cAMP levels. **A.** RAW cells were seeded at a density of 6 x 10^4 per well in 24 well plates. The cells were stimulated as in Fig. 1 with a combination of IFNγ and LPS in the presence or absence of ROL (200 μM). The total cellular cAMP concentrations were measured at different time points, and the results represent the mean of triplicate cultures ± 1 SD. The 24 h level of cAMP was 438.75 ± 17.3 pmol/well, which is significantly elevated over the time 0 level ($p < 0.05$). A representative experiment is shown, and two independent experiments yielded similar results. **B.** ROL elevates the total cellular cAMP level in a dose dependent manner. RAW cells were stimulated with a combination of IFNγ and LPS with or without ROL (3.1 μM - 200μM). The total cellular cAMP levels were measured after 30 minutes, and the results represent the mean of triplicate cultures ± 1 SD.

Figure 4. ROL and PTX suppress iNOS mRNA expression. RAW cells were stimulated with LPS and IFNγ as described in the legend to Fig1, with either medium alone (left panels), ROL (200 μM; middle panels), or PTX (5 mM; right panels). RT-PCR with GAPDH and iNOS primers was performed on total RNA extracted at 45 min, 2 h, 4 h, and 6 h after initiation of culture. Agarose gels were stained with ethidium bromide. Stimulation times are reported in h at the top of the panels. Upper

panels, iNOS RT-PCR; lower panels, GAPDH RT-PCR; M, molecular size markers (DNA 100 bp ladder).

Figure 5. Administration of ROL suppresses macrophage activation *in vivo*. The peritoneal wash macrophages of NOD mice injected i.p. with SEB (100 μg) without ROL treatment (SEB) or with ROL treatment (SEB + ROL), were recovered and stimulated in culture with LPS (see material and methods). Supernatants were collected after 24 h in culture for nitrite measurement. The results represent the mean nitrite levels ± 1 SD (n = 6 mice/group).

Figure 6. Suppression of NO production is not secondary to decreased TNFα and IL-12 production. RAW cells were stimulated with IFNγ (0.8 ng/ml) and LPS (30 ng/ml) with or without ROL (200 or 66 uM). **A.** Recombinant IL-12 was added at the start of cultures. **B.** Recombinant TNFα was added at the start of cultures. In both panels, supernatants were collected after 24 h for nitrite measurement. The results represent the mean of triplicate cultures ± 1 SD. A representative experiment is shown, and three experiments yielded similar results.

CHAPTER 4

INHIBITORS OF PHOSPHODIESTERASE ISOFORMS III OR IV SUPPRESS ISLET-CELL NITRIC OXIDE PRODUCTION

by

Evette Beshay and Gérald J.Prud'homme*

from

The Department of Pathology, McGill University

3775 University St., Montreal, Qc, Canada H3A 2B4

*Address correspondence and reprint requests to:

Dr.Gérald J.Prud'homme

3775 University St., Room B13

Montreal, Qc, Canada H3A 2B4

Tel. 514-398-7192 (ext. 7237); Fax 514-398-7446

E-mail: gprudh@po-box.mcgill.ca

Running title: Phosphodiesterase inhibitors suppress nitric oxide production by islet cell

Keywords: cAMP, cilostamide, cytokines, inflammation, insulinoma cells, islet-β cells, nitric oxide, pentoxifylline, phosphodiesterase inhibitors, rolipram.

ABSTRACT

The general phosphodiesterase (PDE) inhibitor pentoxifylline (PTX), and the PDE type IV inhibitor rolipram (ROL), both increase intracellular cAMP levels and suppress inflammatory cytokine production by T cells and macrophages. We have previously shown that PTX and ROL protect from autoimmune diabetes in NOD mice. These drugs may mediate some of their anti-inflammatory effects by blocking nitric oxide (NO) production by macrophages. In this study, we investigated the effect of PDE inhibitors in blocking NO production by insulin secreting NIT-1 insulinoma cells and mouse islet cells *in vitro* and *in vivo*. Insulinoma cells and islet cells produced NO when stimulated with a combination of inflammatory cytokines and LPS. We found that both PTX and ROL markedly suppressed this induced NO production. Islet cells express PDEs III and IV and, accordingly, a PDE III inhibitor cilostamide (CIL) also suppressed NO production, and a combination of ROL and CIL had a synergistic effect. This suppression appeared to be mediated at least in part by elevating cAMP level, and was mimicked by other cAMP elevating agents, i.e., membrane permeable cAMP analogues (dibutyryl cAMP and 8-bromo cAMP), and an adenylate cyclase stimulator (Forskolin). PDE inhibitors suppressed the expression of inducible nitric oxide synthase (iNOS) mRNA. *In vivo*, treatment with PTX or ROL prevented iNOS protein expression in the islets of NOD mice with cyclophosphamide-accelerated disease. Our findings suggest that PDE inhibitors can protect islets against autoimmunity.

INTRODUCTION

The isozymes of cyclic-3',5' nucleotide phosphodiesterase (PDE) are critically important components of the cyclic-3',5' adenosine monophosphate (cAMP) protein kinase A (PKA) signaling pathway. PDE inhibitors elevate intracellular cAMP levels, and can regulate many processes including immune responses. There are at least ten PDE families [1], several subtypes and numerous isoform splice variants. PDE isozymes differ in molecular structure, catalytic properties, intracellular regulation and location, sensitivity to selective inhibitors, as well as selective expression in various cell types.

PDE type IV (PDE4) is the major type in macrophages while lymphocytes express both type III (PDE3) and PDE4 [2]. Islet β cells, like lymphocytes, have been shown to express these two types [3]. Rolipram (ROL), a specific PDE4 inhibitor, and pentoxifylline (PTX), a general PDE inhibitor, suppress inflammatory cytokines production and are therapeutically effective in several autoimmune and/or inflammatory diseases. For example, ROL [4] and PTX [5] prevent experimental allergic encephalomyelitis in rodents, and PTX has been found to inhibit contact dermatitis in mice [6]. Suppression of TNFα production by these drugs is well documented [7, 8]. We have shown that ROL and PTX prevent insulitis and spontaneous diabetes in NOD mice, as well as cyclophosphamide (CYP)-accelerated diabetes in the same strain [8]. In addition, we showed that these drugs suppress interferon gamma (IFNγ) and interleukin-12 (IL-12) production.

Cytokines appear to be important mediators of islet β cell dysfunction and destruction. Interleukin 1 beta (IL-1β), IFNγ and TNFα, particulary in combination, are toxic to β cells in culture [9, 10]. The molecular mechanisms of toxicity are not well understood. However, cytokines augment inducible nitric oxide synthase (iNOS) expression and consequently nitric oxide (NO) production, and some investigators have proposed that this contributes to islet cell damage [11].

We recently reported that ROL and PTX suppress NO production by macrophages *in vitro* and *in vivo* [12]. However, the effects of these PDE inhibitors on NO production by islet cells have not been reported. In this study, we demonstrate that PDE3 or PDE4 inhibitors block NO production by islet cells, and that combined inhibition is most effective. This inhibitory effect is apparent *in vivo*, and provides an important new avenue for the prevention or treatment of inflammatory/autoimmune diseases.

RESEARCH DESIGN AND METHODS

Mice.

Female NOD mice (5-6 weeks) were purchased from Taconic farm (Germantown, NY). Male CD-1 mice (5-6 weeks) were purchased from Charles River Canada (St. Constant, Quebec, Canada). Mice were housed under pathogen-free conditions.

NIT-1 cell culture and stimulation.

The NIT-1 insulinoma cell line is of transgenic NOD mouse origin (ATCC, Rockville, MD). To stimulate NO production, NIT-1 cells were seeded in 96 well plates in Ham's F12K medium containing 10% heat-inactivated dialyzed fetal bovine serum (FBS), at a density of 0.5 X 10^6 cells /100 μL/well, at 37°C for 40 h. Then, the supernatant was discarded and replaced with medium containing IFNγ (0.4 ng/ml), LPS (30 ng/ml), (Sigma, ST. Louis, MO), IL-1β (5 ng/ml), and TNFα (10 ng/ml) (Pepro Tech INC., Rocky Hill, USA). Where indicated, cultures were supplemented with either ROL (Shering AG, Berlin, Germany), PTX (Sigma), Cilostamide (CIL) (Biomol), Forskolin (Sigma), or cAMP analogues (dibutyryl cAMP or 8-bromo-cAMP) (Sigma). After a further 40 h of culture, the supernatant was collected for nitrite measurement.

Islet isolation and culture.

Mouse islets were isolated from the pancreas of CD-1 mice (5-6 weeks) as previously described [13], with Liberase RI enzyme digestion (Roch Diagnostic, Montreal), according to the manufacturer's instructions. The isolated islets (pooled from 15-18 mice) were isolated on a Ficoll density gradient and cultured overnight in CMRL 1066 media supplemented with 10% FBS, soya bean trypsin inhibitors, fungison and penicillin streptomycin (Gibco). Subsequently, the islets were seeded at a density of 400 islets/100 μL/well in 96 wells plates in conditioned medium containing

IL-1β, IFNγ, TNFα, and LPS (as described above). After a further 40 h, the supernatants were collected for nitrite measurement and the plates were frozen at -80 °C prior to DNA content measurement.

Determination of islet cells purity.

We trysinized the isolated islets into single cells using trypsin solution (0.25% in PBS). For flow cytometry analysis, the cells were incubated with FITC anti-mouse CD11b (Mac-1; a macrophage marker) monoclonal antibody (Cederlane, Ontario, CA). To identify macrophages on the basis of phagocytic activity, we incubated the cells with fluorescent microbeads (Cederlane, Ontario, Ca.) for 30 min at 37°C. By fluorescent microscopy, we counted the number of positive cells (3 or more fluorescent microbeads inside the cell) in 10 μL cell suspention containing 10^3 cells. At least 12 x 10^3 cells were examined from each islet preparation.

Nitrite measurement.

Nitrite accumulation as an indicator of NO production was measured using the Griess reagent as we have described [14]. Absorbance was measured at 540 nm in a microplate reader (Titertek Instruments, Hunstville, AL). Nitrite concentration was calculated from $NaNO_2$ standard curve.

Percent inhibition of nitrite production = 100 - $\frac{\text{(the } NO^-_2 \text{ level with inhibitor) X 100}}{\text{(the } NO^-_2 \text{ level without inhibitor)}}$

IC_{50} values were calculated from the dose response percentage of the inhibition curve for each treatment.

DNA content determination by fluorometric assay.

We followed the protocol described by Rago et al. [15] with some modifications. Briefly, culture supernatants were removed from the wells containing islet cells or adherent NIT-1 cells (96 well plates). The plates were frozen in -80 °C and thawed until they reached room temperature, and 100 µL of TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) was added. The cell lysate was transferred to a microtube with 1 ml of Hoechst (Sigma) 1:2000 dilution of stock solution (200 µg/ml). The fluorescent emission was measured with a fluorescent spectrophotometer (Hoefer) at a wave length 457 nm. DNA content was determined from a standard curve obtained with calf thymus DNA (Sigma).

Determination of iNOS mRNA by reverse transcription-polymerase chain reaction (RT-PCR).

PCR amplification of cDNA with primers specific for iNOS and glyceraldehyde -3-phosphate dehydrogenase (GAPDH) was carried out, and the products were visualized by electrophoresis in 2% agarose containing ethidium bromide. iNOS primers were: sense, 5'-CTTCCGAAGTTTCTGGCAGCAGCG-3'; antisense, 5'-GAGCCTCGTGGCTTTGGGCTCCTC-3' and the PCR product was 487 bp. GAPDH primers were: sense, 5'- TCCACCACCCTGTTGGTGTA-3'; antisense,5'-ACCACAGTCCATGCCATCAC-3' and the PCR product was 450 bp. PCR reactions were run for 25-35 cycles, and reactions were terminated in the linear portion of the amplification.

Cyclophosphamide-accelerated diabetes in NOD

Female NOD mice (5-6 weeks) were injected i.p. with cyclophosphamide (CYP) (250 mg/kg). The mice were treated with either ROL (14 mg/kg) or PTX (80 mg/kg) or PBS, i.p.,b.i.d., for 10 days. The mice were killed and the pancreata were recovered and fixed in 10% formalin, and histological slides were prepared.

Immunohistochemical detection of iNOS in the pancreas.

Immunohistochemistry was performed on formalin-fixed tissue with the Histo-Mouse SP kit (Inter Medico, Markham, Ontario). The primary antibodies were either polyclonal rabbit anti-mouse iNOS (Biomol Research Laboratory, PA, USA), or guinea pig antihuman insulin (which cross reacts with mouse insulin), followed by biotinelated secondary antibody, streptavidin-peroxidase conjugate and ABC substrate.

Grading of insulitis.

Insulitis was graded as described as described by Charlton et al. [16], based on lymphocytic infiltation, as follows; grade 0, normal islet totally free of any peri-islet mononuclear cells; grade 1, focal peri-islet infiltrate <25% of islet circumference; grade 2, peri-islet infiltrate > 25% of islet circumference; grade 3, intra-islet infiltration with good retention of islet cells; and grade 4, extensive intra-islet infiltration with gross distortion or destruction of islet morphology. Coded slides were examined independently by two observers, and statistical analysis was performed with the χ^2 test.

RESULTS

PTX, ROL and CIL inhibit NO production by NIT-1 cells and islet cells.

NO is rapidly transformed to nitrite in culture (breakdown stable product), and we measured nitrite levels as an indication of NO production. In the absence of stimulation, NIT-1 cells produced an undetectable amount of nitrite (not shown). When IL-1β was added alone, a small amount of nitrite was detected, but when either IFNγ, TNFα, or LPS were added alone, nitrite was undetectable (not shown). However, NIT-1 cells produced a considerable amount of NO when stimulated with a combination of IL-1β (5 ng/ml), TNFα (10 ng/ml), IFNγ (0.4 ng/ml), and LPS (30 ng/ml) (Fig.1A- C). This cytokine combination and these concentrations were found optimal in preliminary experiments, and used subsequently.

We found that ROL suppresses nitrite production in a dose dependent manner (Fig.1A). A concentration of 6.25 μM ROL was sufficient to significantly suppress nitrite production ($p= 0.009$), while 200 μM concentration suppressed 87 % of nitrite production. PTX also suppressed nitrite production but at a higher concentration (ROL IC_{50} = 96.5 μM and PTX IC_{50} = 1.48 mM) (Fig. 1B). Thus, on a molar basis ROL was 15 times more potent than PTX. The suppression of NO production cannot be attributed to cell death since there was no increase in dead cells as determined by

FIGURE 1A

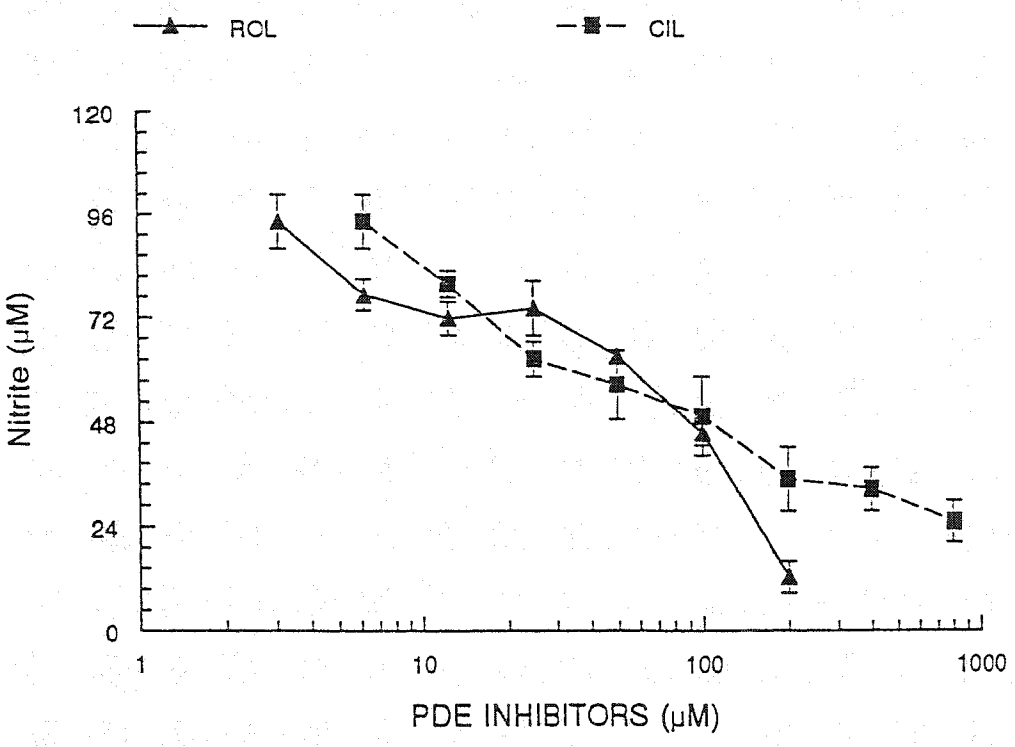
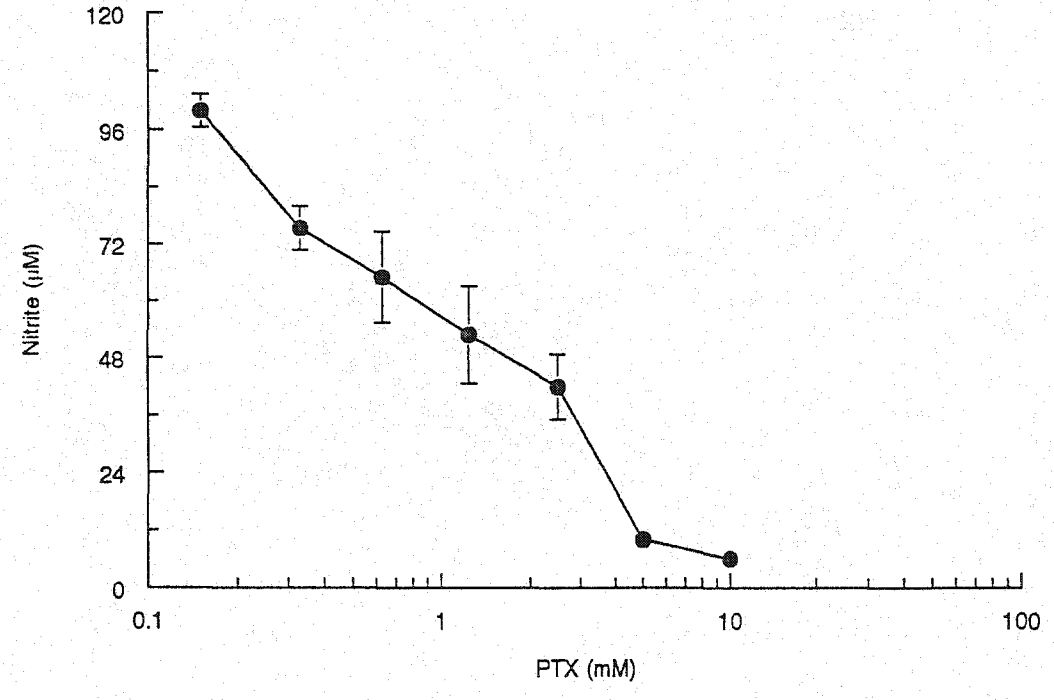

ROL
CIL
120
96
72
48
24
0
Nitrite (μM)
1
10
100
1000
PDE INHIBITORS (μM)

FIGURE 1B



Trypan blue dye exclusion, and no decrease in adherent-cell DNA content (data not shown).

CIL, a specific PDE3 inhibitor, also suppressed NO production (IC_{50} = 327.5 μM) (Fig.1A). Notably, a combination of ROL and CIL has a strong suppressive effect on NO production (Fig. 1C), much higher than either drug alone. Thus, ROL at 12.5 μM + CIL at 12.5 μM markedly inhibited NO production, but not 25 μM of each inhibitor alone. This was greater than a simple additive effect, suggesting a synergistic effect.

Mouse islet cells also produced NO when stimulated with the same cytokine/LPS mixture as with NIT-1 cells and, similarly to insulinoma cells, ROL and PTX suppressed NO production ($P < 0.001$) (Fig. 2). Macrophages constituted less than one cell per 2000 islet cells as determined by flow cytometry analysis with Mac-1 antibody staining, and ingestion of fluorescent microbeads in culture (data not shown). We found that the amount of nitrite produced by this number of peritoneal macrophages was less than 2 μM (not shown), which is much less than the values produced by islet-derived cells. Thus, NO does not appear to be produced by contaminating macrophages.

ROL and PTX block iNOS mRNA expression.

We analyzed the expression of iNOS mRNA at different time points, in stimulated (IL-1β, TNF-α, IFN-γ and LPS) NIT-1 cells, in the presence or absence of ROL or PTX. iNOS mRNA expression was maximal after 4 hours, and severely

FIGURE 1C

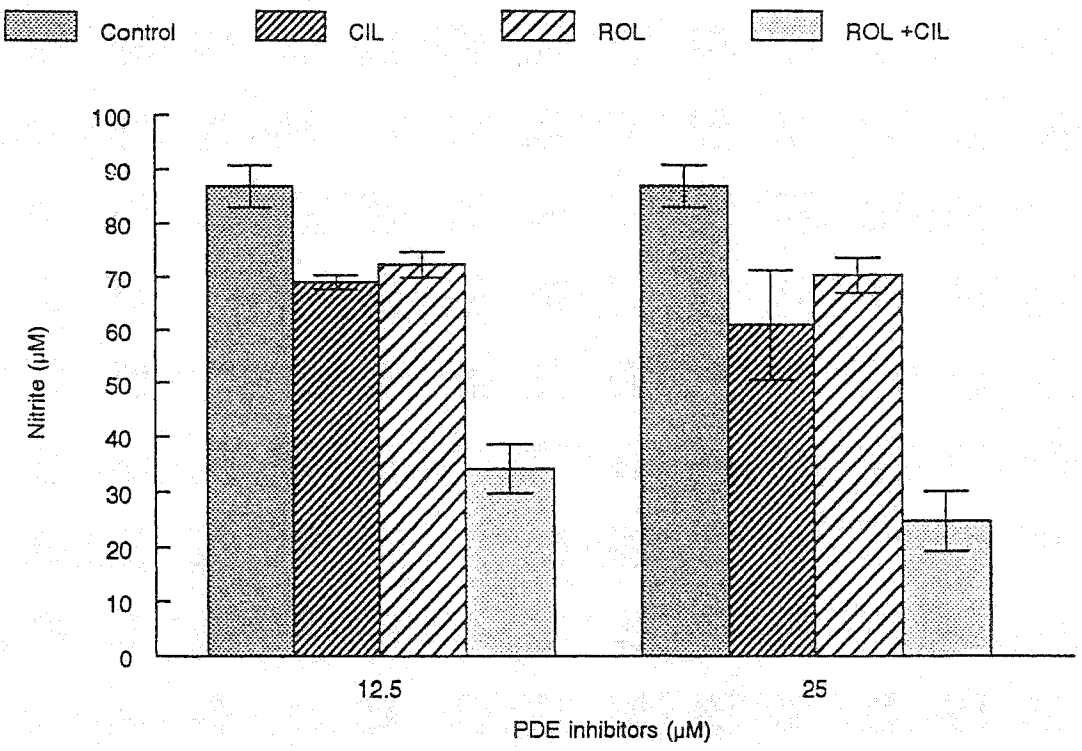
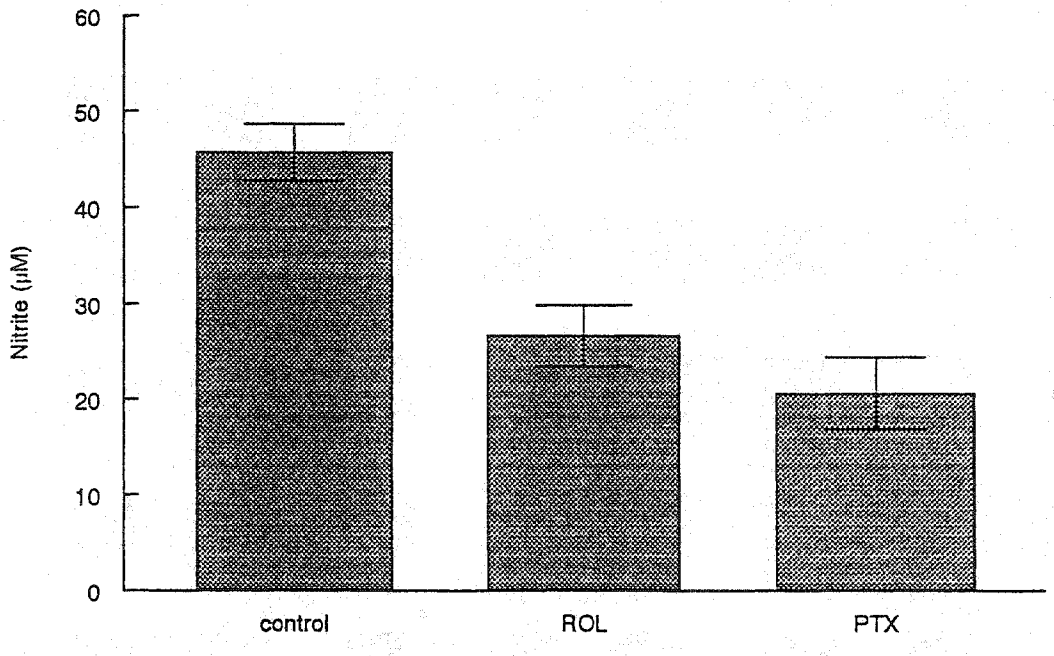

Control
CIL
ROL
ROL +CIL
100
90
80
70
60
50
40
30
20
10
0
Nitrite (μM)
12.5
25
PDE inhibitors (μM)

FIGURE 2



inhibited by either ROL (200 μM) or PTX (5 mM) (Fig.3), as determined by semi-quantitative RT-PCR analysis.

cAMP elevating factors inhibit NO production by NIT-1 cells.

Membrane permeable cAMP analogues, dibutyryl cAMP and 8-bromo-cAMP, both inhibited NO production by stimulated NIT-1 cells (Fig.4A). Similarly, Forskolin, which elevates cAMP levels by stimulating adenylate cyclase, suppressed NO production by NIT-1 cells (Fig.4B). Cell death was not increased by these agents (not shown).

ROL or PTX treatment inhibits iNOS expression in pancreatic islets

Ten days after receiving a dose of 250 mg/kg of CYP, most islets of control NOD mice showed insulitis between grade 3 and 4 with many iNOS-positive cells (Table 1). Even the few remaining islets with low-grade insulitis had visible iNOS-positive cells (Fig. 5A, B), although these cells were clearly found in higher numbers in islets with higher grade lesions, such as grade 3 (Fig. 5C). In groups receiving either ROL (14 mg/kg i.p., b.i.d.) (Table 1; Fig. D-F) or PTX (80 mg/kg i.p., b.i.d) (Table 1; Fig. G, H) for 10 days, the severity of insulitis was significantly reduced. In addition, both ROL and PTX reduced the number of iNOS positive cells ($P < 0.0001$) (Table 1;

FIGURE 3

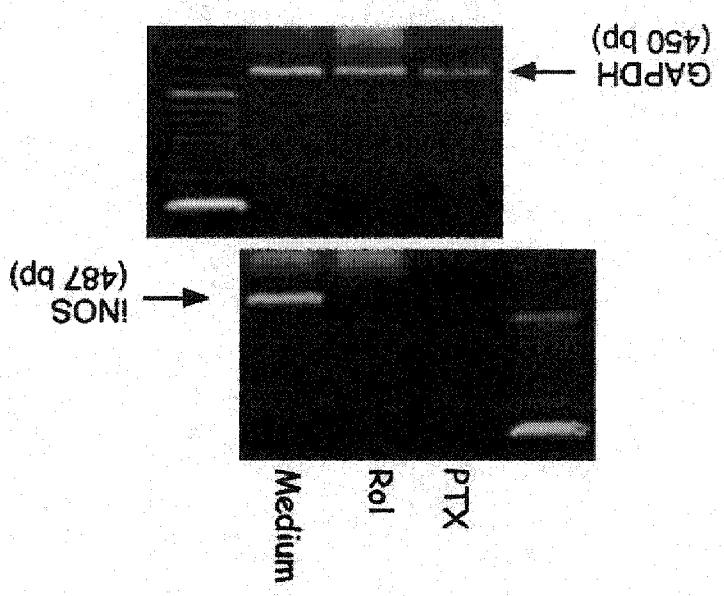


FIGURE 4A

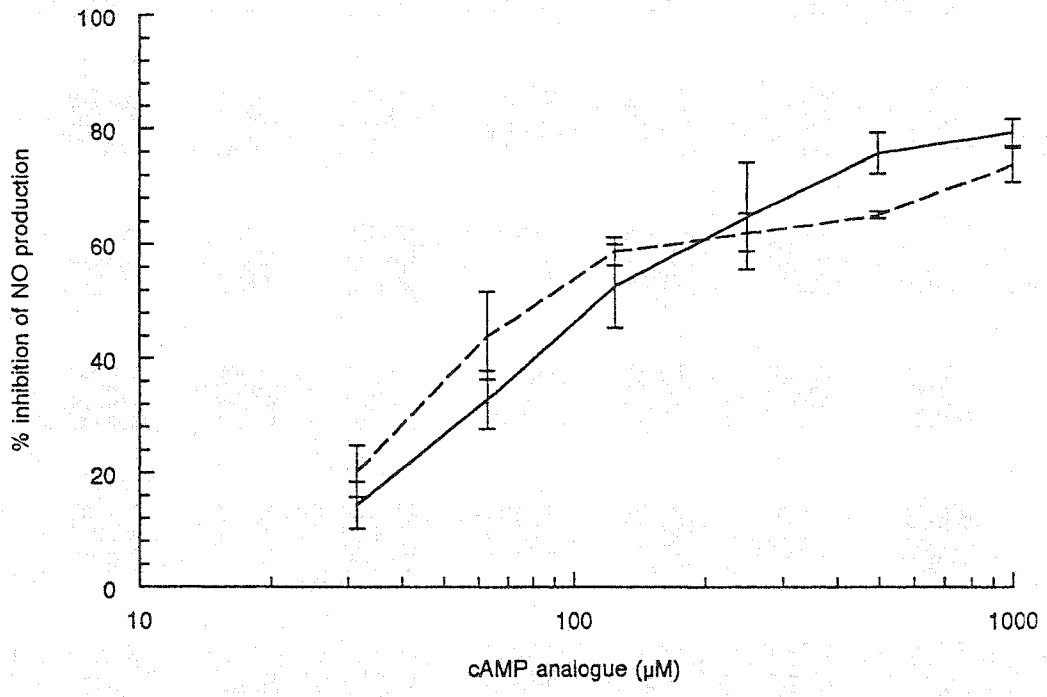
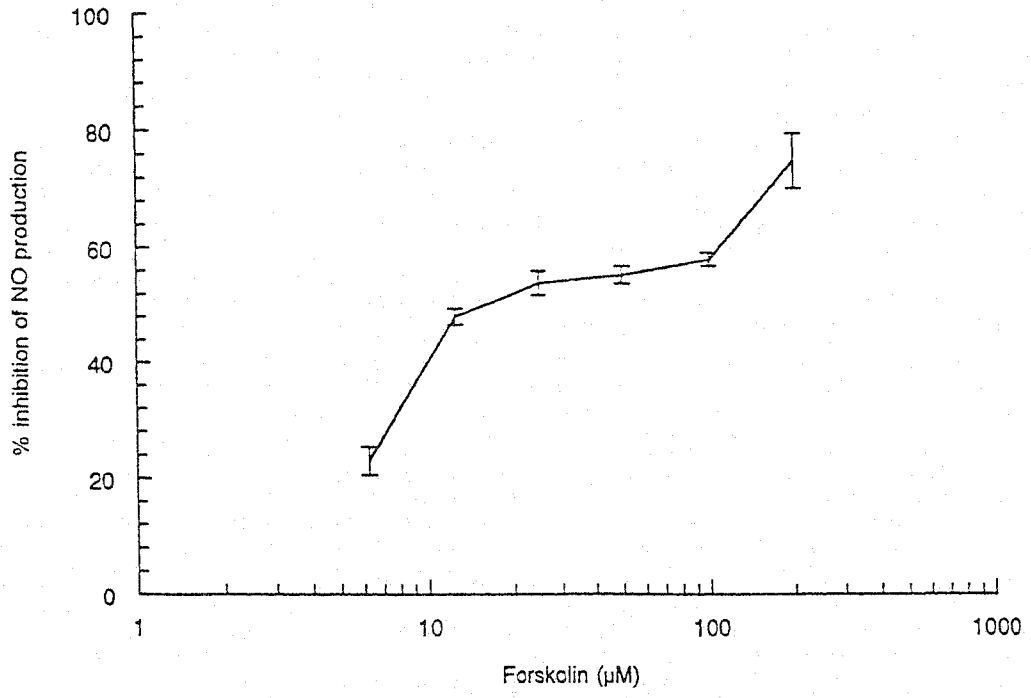

100
80
60
40
20
0
% inhibition of NO production
10
100
1000
cAMP analogue (μM)

FIGURE 4B


100
80
60
40
20
0
% inhibition of NO production
1
10
100
1000
Forskolin (μM)

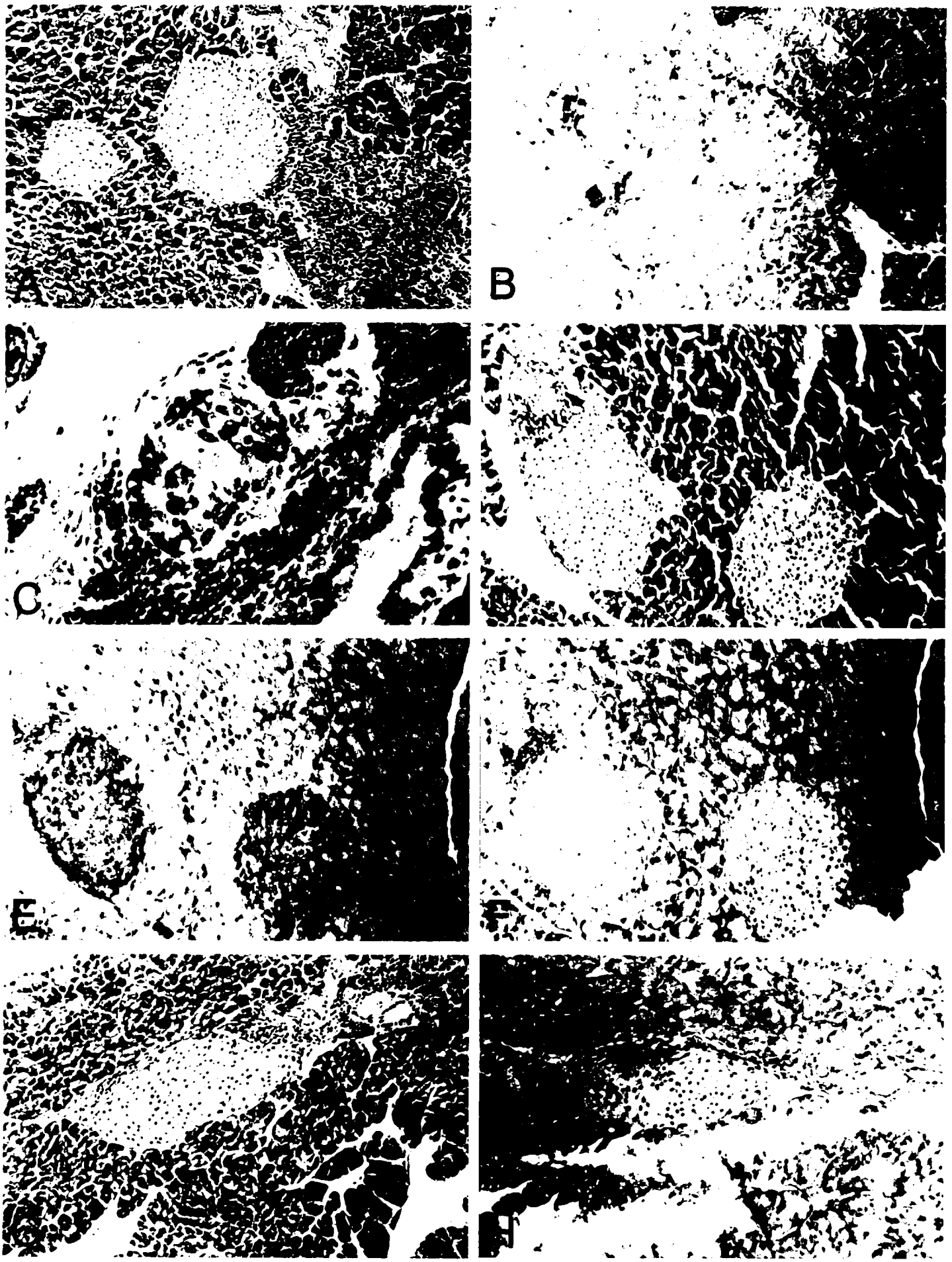

B
C

Fig. 5F, H). Occasional islets of PDE inhibitor-treated mice showed grade 3 lesions, but even these islets expressed very few iNOS-positive cells (data not shown).

DISCUSSION

NO is a potent and pleiotropic mediator with physiologic and toxic activity. NO is synthesized by various isoforms of nitric oxide synthase (NOS) which catalyze the oxidation of L-arginine to form L-citrulline and NO [17]. NOS exists in a constitutive isoforms present in endothelial cells (eNOS) or neurons (nNOS), and a cytokine or endotoxin inducible form (iNOS) [18,19]. The iNOS isoform was first identified in macrophages, but now has been found in many nucleated cells, including hepatocytes, islets cells, chondrocytes, smooth muscle cells, megakaryocytes, thyrocytes, and mesangial cells [20-22]. NO inactivates important enzymes in β cells by nitrosylation of target iron sulphur proteins, e.g., mitochondrial aconitase, required for glucose oxidation and insulin release [23, 24]. This inactivation impairs β-cell metabolism, and diminishes the capacity of insulin secretion and may eventually cause β-cell death. NO is likely to be produced by islet-infiltrating macrophages, and such infiltration is an early event in insulitis and essential for the development of diabetes [25]. However, islet β-cells could be another source for NO production which contributes to β-cell damage and death.

Agents that elevate cAMP have been reported to inhibit NO production by some cells. We have recently reported [22] that both ROL and PTX suppress iNOS

expression and NO production by the RAW 264.7 macrophage cell line and freshly isolated peritoneal macrophages. Bulut et al. [26] found that a combination of prostaglandin E2 (adenylate cyclase activator) and isobutyl methyl-xanthine (IBMX; a general PDE inhibitor) induced prolonged elevation of cAMP level and suppressed iNOS activity in an IFNγ/LPS costimulated murine macrophage cell line (J774). Other studies showed that elevated cAMP levels have a cell-type specific effect in terms of NO production [27, 28].

To our knowledge, the current study is the first to investigate the effect of cAMP elevating agents on NO production by insulin-producing cells. Cytokine stimulation (IL-1β, IFNγ and TNFα), combined with LPS stimulation, induced NO production by both NIT-1 insulinoma cells and normal islet cells. We found that PDE inhibitors, such as the general inhibitor PTX, suppressed NO production in both cell types. Islets of Langerhans were isolated from the pancreas of normal CD-1-strain mice, and NO appeared to be produced by endocrine islet cells, rather than contaminating macrophages. Macrophages constituted less than one cell per 2000 islet cells, and the amount of NO (measured as nitrite) produced by equivalent numbers of macrophages was found to be much less than the values obtained with cultured islets.

Therapeutically, it may be desirable to administer an isoform-specific PDE inhibitor, rather than a general inhibitor like PTX. Since islet cells express both PDE3 and PDE4 [3], it was of interest to compare the effectiveness of selective inhibitors of these enzymes. For this purpose, we inhibited PDE3 with CIL and PDE4 with ROL. We observed that both PDE inhibitors were effective at suppressing NO production

when used alone, and several fold more effective than PTX on a molar basis. Moreover, when used together they had more than an additive effect, suggesting synergism. This is of obvious clinical interest, since potential adverse effects may be reduced by administering these drugs in combination at a reduced dose of each.

The suppression of NO production appeared to be at least partly mediated by elevated cAMP. It was mimicked by raising the intracellular cAMP level by either cell membrane permeable cAMP analogues (dibutyryl-cAMP and 8-bromo-cAMP), or an adenylate cyclase activator (Forskolin). This suppression was at the transcriptional level, since iNOS mRNA expression was markedly inhibited by either PTX or ROL. The mechanism by which elevation of cAMP inhibits iNOS gene transcription, and ultimately NO production, is yet unclear. In some cells, increased cAMP is associated with reduced activation of NF-$_K\beta$, a transcription factor regulating iNOS gene transcription [29]. Interestingly, some cytokines whose production is inhibited by ROL and PTX also have NF-$_K\beta$ binding elements in their promoters or enhancers [30]. We speculate that this could represent a common factor leading to inhibited expression of these genes.

NOD mice develop a form of autoimmune insulin-dependent diabetes mellitus (IDDM) similar to the human disease [10, 31]. The precise mechanisms of islet β cells destruction in these mice are not totally elucidated, but there is evidence for a T-helper type 1 (Th1)-mediated response with IFNγ production, associated with macrophages stimulation and production of IL-12, TNFα, IL-1, and NO. In NOD mice, ROL and PTX prevent diabetes, perhaps in part by suppressing TNFα, IFNγ,

and IL-12 as we have shown in [8], but prior to this study their effect on NO production was not known.

To determine if PDE inhibitors alter NO production *in vivo*, we administered these drugs to CYP-treated NOD mice. CYP precipitates the onset of diabetes in these mice [16] and this is associated with increased intra-islet IFNγ and NO production. We found that the NOD mice injected with CYP (250 mg/kg) developed severe insulitis within 10 days of CYP injection and, unlike control mice, had many iNOS-positive islet cells. ROL or PTX treatment markedly reduced the severity of insulitis, as well as the number of iNOS-positive cells in the islets. Thus, from this and previous studies, we now have evidence that PDE inhibitors block at least four mediators (TNFα, IFNγ, IL-12 and NO) that have been implicated in islet-cell destruction.

Although we have not examined this question in this study, PDE inhibitors could be altering T-helper type 2 (Th2) activity. Indeed, cAMP clearly has immunomodulatory activity, and its elevation by PDE inhibitors favors Th2 responses [5, 32]. Although most cAMP effects are mediated through the PKA pathway, this does not appear to be the case in lymphocytes. Instead, cAMP stimulates activation of p38 mitogen-activated protein kinase in the Th2 cells [32]. Activated Th2 cells produce cytokines (IL-4, IL-10, and IL-13) which inhibit the production of NO and other inflammatory mediators by macrophages [33]. These cytokines also suppress IFNγ-production by Th1 cells [10]. Some of these Th2 cytokines may also act directly on islet cells [34], but this has not been extensively studied. In any case, Th2 bias could be one factor protecting mice treated with PDE inhibitors.

Clearly, the ability of PDE inhibitors to protect islets from autoimmunity may be related to suppression of several inflammatory mediators, and not necessarily NO. Moreover, *in vivo*, decreased iNOS expression may have been secondary to decreased cytokine production, rather than a direct inhibitory drug effect on the transcription of the iNOS gene. In fact, we observed that the great majority of islets in PDE-treated NOD mice had only low-grade inflammatory lesions. Nevertheless, occasional islets had higher grade (grade 3) lesions but still very few iNOS-positive cells, perhaps reflecting noncytokine- related inhibitory effects.

In terms of the pathogenesis of this disease, there is substantial published evidence that NO can contribute to β-cell injury. For instance, incubation of rat islets with IL-1β results in a concentration and time dependent inhibition of glucose-stimulated insulin secretion, followed by cell death after prolonged exposure [35]. The free radical NO appears implicated, inasmuch as β-cell damage can be prevented by the NOS inhibitors N^G-nitro-L-arginine methylester (L-NAME) and N^G-monomethyl-L-arginin (L-NMMA) [36-38]. A recent study in iNOS gene-knockout mice suggests cytokine-induced islet cell apoptosis is NO independent, whereas necrosis required NO formation [39]. Consistent with this, iNOS deficient mice have increased resistance to streptozotocin-induced diabetes [40].

Previous studies have shown that inhibition of NO formation prevents IDDM. Thus, administration of L-NMMA [41] and L-NAME [42] protected against IDDM in mice treated with multiple low doses of streptozotocin. However, when the NOS inhibitor aminoguanidine (AG) was administered to either NOD mice [43], diabetes-

prone Biobreeding rats [44], or in conjunction with low-dose streptozotocin injections in C57BL/Ks mice [45], it failed to decrease the incidence of IDDM. In this context, it may be of relevance that AG augments the free oxygen radical hydrogen peroxide by an inhibition of catalase, in some cell types [46]. In addition, high concentrations of AG ($\geq$ 4.55 mM) impaired β-cell function and inhibited insulin release from human islets [47, 48]. In contrast, PDE3 inhibitors have been reported to enhance insulin secretion while PDE4 inhibitors have no effect [3].

We show that PDE inhibitors have a therapeutic potential against NO- and/or cytokine-mediated immunological disorders. Conveniently, these drugs can be administrated orally. PTX (Trental) is in clinical use for the treatment of intermittent claudication and is well tolerated. ROL has been studied as an anti-depressant drug, with side effects including gastrointestinal disorders, dizziness and headache. Interestingly, a number of newer PDE4 inhibitors have been synthesized, displaying up to 1000 times more affinity for PDE4 than ROL, and perhaps they will have therapeutic advantages. In addition, there are several PDE3 inhibitors available for clinical use, such as cilostamide, cilostazol, milrinone and amrinone. Cilostasol, for instance, was shown to have minimal side effects and was recently approved for the treatment of intermittent claudication [47], due to its vasodilatory and platelet activation inhibitory activities. PDE inhibitors may present a novel therapeutic approach for diabetes and other autoimmune and/or inflammatory diseases

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Table 1: Reduction of iNOS-positive islet cells in CYP-treated NOD mice

Treatment Groups [a]				
CYP	ROL	PTX	Mean grade of Insulitis [b]	iNOS-positive cells/islet [b]
+	-	-	3.4	7.8
+	-	+	1.3[c]	0.5[c]
+	+	-	1.4[c]	0.6[c]

a. Female NOD mice (5-6 weeks) were injected with CYP (250 mg/kg). The mice were treated with either PTX (80 mg/kg), ROL (14mg/kg) or PBS, i.p.,b.i.d, for 10 days (n = 10 mice/group). The mice were killed after 10 days of treatment for examination of tissues.

b. H& E stained slides were used for the grading of insulitis. Immunohistochemical identification of iNOS-positive cells was performed on formalin-fixed tissue with Histo-Mouse SP kit, using a polyclonal rabbit anti-mouse iNOS primary antibody. The results represent the mean number of iNOS-positive cells per islet of Langerhans.

c. $P < 0.001$ (X^2 test) versus control mice not receiving ROL or PTX.

FIGURE LEGENDS

Figure 1. Suppression of NO production by PDE inhibitors. NO production from IL-Iβ (5 ng/ml), TNFα (10 ng/ml), IFNγ (0.4 ng/ml) and LPS (30 ng/ml) costimulated NIT-1 insulinoma cells was determined by measuring nitrite levels (a stable product derived from NO) using the Griess reagent, in 40 h culture supernatants. **A.** Suppression of NO production by ROL and CIL. **B.** Suppression of NO production by PTX. **C.** CIL and ROL combination. The results represent the mean of triplicate cultures ± 1 SD. A representative experiment is shown for each drug or combination and three independent experiments yielded similar results in each case.

Figure 2. Suppression of NO production in mouse islet cells by PDE inhibitors. NO production from IL-1β (5 ng/ml), TNFα (10 ng/ml), IFNγ (0.4 ng/ml) and LPS (30 ng/ml) costimulated mice islets are reported. ROL (200 μM) or PTX (5 mM) significantly suppressed NO production ($P<0.01$). The results represent the mean of triplicate cultures ±1 SD. A representative experiment is shown and three independent experiments yielded similar results.

Figure 3. ROL and PTX suppress iNOS mRNA expression. NIT-1 insulinoma cells were stimulated as described in the legend to Figure 1, with either medium alone, ROL (200 μM), or PTX (5 mM). RT-PCR with GAPDH and iNOS primers was performed on total RNA extracted at 4h after initiation of culture. Agarose gels were stained with

ethidium bromide. Upper panel, iNOS RT-PCR; lower panel, GAPDH RT-PCR; DNA 100 bp ladder, left lane of upper panel and right lane of lower panel.

Figure 4. Agents which increase intracellular cAMP levels suppress NO production. The results represent the percent inhibition of nitrite production by NIT-1 insulinoma cells stimulated as described in the legend to Figure 1. **A**. cAMP analogues, dibutyryl cAMP (solid line) or 8-bromo-cAMP (broken line). **B**. Adenylate cyclase stimulator (Forskolin). Data represent percent inhibition of nitrite production ± 1 SD of triplicate cultures in a representative experiments, and 3 experiments yielded similar results (panels A and B).

Figure 5. Immunohistochemical analysis of iNOS expression. Female NOD mice (5-6 weeks) were injected with CYP (250 mg/kg). The mice were treated with either PTX (80 mg/kg), ROL (14 mg/kg), or PBS, i.p.,b.i.d., for 10 days (n =10 mice/group), and then killed for examination of tissues. H&E stained slides were used for the grading of insulitis. Immunohistochemical staining for iNOS or insulin was performed in formalin fixed tissue with Histo-Mouse SP kit. The primary antibodies were either polyclonal rabbit anti-mouse iNOS or Guinea pig anti human insulin polyclonal antibodies which cross react with mouse insulin. **A**. H&E staining, control CYP-treated mice (no PDE inhibitors). **B.** and **C**. iNOS staining, control CYP-treated mice. **D.** H&E, CYP-and ROL-treated mice. ***E***. insulin staining, CYP-and ROL-treated mice.

F. iNOS staining, CYP-and ROL-treated mice. **G.** H&E, CYP-and PTX-treated mice.

H. iNOS staining, CYP-and PTX-treated mice

CHAPTER 5

GENERAL DISCUSSION

DISCUSSION

The PDE inhibitors PTX (a general inhibitor) and ROL (a type 4 inhibitor), have been reported to have anti-inflammatory effects. They suppress the production of inflammatory cytokines such as TNFα, IFNγ and IL-12. These drugs elevate intracellular cAMP levels and modulate the immune response in favor of Th2 responses. Both PTX and ROL have been reported to be therapeutically effective in various models of autoimmune and/or inflammatory diseases such as EAE, SLE, contact dermatitis and septic shock. Their effects on NO production, an important inflammatory mediator, are not well studied and there are contradictory results in the literature. However, elevated intracellular cAMP levels suppressed NO production in some cells.

Our hypotheses were: 1) PDE inhibitors would prevent insulitis and diabetes in NOD mice by suppressing inflammatory cytokines and modulating the immune response. 2) These inhibitors would have inhibitory effects on macrophage NO production. 3) They would also directly suppress NO production by islet cells. 4) Selective PDE3 and PDE4 inhibitors would be effective since these PDE types are expressed in islet cells and macrophages. 5) The anti-inflammatory effects of PDE inhibitors result from elevated cAMP levels.

In the first part of the study, we found that PDE inhibitors (PTX and ROL) prevent insulitis and diabetes in NOD mice. A 4 week treatment with either PTX or

ROL has a strong protective effect, that was still apparent 11 weeks after withdrawing the drugs. At optimal doses, both drugs were equally effective. These findings are novel and suggest that these inhibitors have a therapeutic potential. PTX is in clinical use and ROL was studied as an antidepressant. Both drugs can be administered orally and they are tolerated with mild undesirable side effects. The target population could be people at high risk of developing diabetes such as monozygotic twins when one of the twins has diabetes, siblings of diabetics, or those with a strong family history of diabetes. These drugs can be useful also in recently diagnosed diabetic, as they may reduce the insulin requirement and prolong the honeymoon (non-insulin-requiring) period. Currently, there is no adequate preventive therapy for these high-risk populations.

The ability to predict diabetes is improving with the use of genetic and immune techniques. Elevated autoantibody titres, such as ICA, IA-2, IAA, and GAD, have been shown to have a considerable predictive value. This is improved by analysis of several autoantibody titers. In addition, the presence of HLA susceptibility genes can be analysed and has a major predictive value.

The exact mechanisms by which PDE inhibitors prevent diabetes are not totally clear. β cell destruction appears to be mediated mainly by T cells and macrophages. These two effector cell types can destroy β cell by direct interaction or by inflammatory mediator production. Cytokines and NO appear to have important roles in β cell dysfunction and destruction. Moreover, several lines of evidence

suggest that the cytotoxic effects of cytokines on β cells are mediated at least in part by the induction of NO production.

The suppression of cytokines using monoclonal antibodies, soluble receptors or receptor antagonists appears to be effective in animal models of diabetes. Cytokine gene therapy also has been reported to be effective, but there is still a question concerning the possibility of human applications. NOS inhibitors have also been shown to prevent diabetes in animal models. However, these are generally non-specific inhibitors which can suppress all forms of NOS. Undesirable side effects, such as hypertension, and learning/memory disorders, will limit the use of these inhibitors in the clinic. The specific iNOS inhibitor AG has been studied, but unfortunately it also has undesirable effects such as increased production of H_2O_2 and suppression of insulin production by β cells.

In the second part of the study, we examined the effects of PTX and ROL on NO production by peritoneal macrophages and RAW 246.7 cells in vivo and in vitro. We also correlated these effects with elevated cAMP levels. We found that both PTX and ROL inhibited NO production by macrophages. These inhibitory effects correlated with elevated cAMP levels and were mimicked by other agents which elevate cAMP levels such as dibutyryl cAMP, 8-bromo-cAMP and Forskolin. The suppression was found to be at the transcriptional level. Macrophages express mainly PDE4, and it was not surprisingly that suppression of PDE4 was sufficient to inhibit NO production. There was no significant difference in the maximum degree of suppression of NO production between ROL and PTX. However, on a molar basis

ROL was about 40 times more effective. Both RAW246.7 cell and peritoneal macrophages responded similarly to these drugs. Thus, it appears that the regulation of iNOS expression is similar in both cell types. Interestingly, the suppression of NO production by macrophages was not secondary to the suppression of TNFα, IL-12 or IFNγ, since addition of these cytokines to the medium did not restore NO production.

Our results are applicable *in vivo* since ROL suppresses macrophage activation in SEB-injected NOD mice. In this model, SEB stimulates both T-cell and macrophage activation by simultaneously binding to T-cell receptor Vβ elements (of some families) and class II MHC molecules of macrophages or other APCs. Bacterial superantigen-induced lymphocyte responses have been reported to be NO dependent and mediated at least in part by IFNγ and IL-12. We found that ROL suppressed NO production of peritoneal macrophages collected 24 h after SEB injection. This effect mimics our observation *in vitro*. In this case, the drug may be acting by suppressing IFNγ or other cytokines. Since, the NOD peritoneal macrophage has an abnormal phenotype, we examined the effect of ROL in CD-1 mice, but the result were similar in both strains.

In the third part of the study, we examined the effect of PDE inhibitors on iNOS expression and NO production by insulin producing NIT-1 insulinoma cells and normal islet cells. We found that PDE inhibitors suppressed NO production by both NIT-1 and islet cells. As with macrophages, this suppression appears to be mediated by elevated cAMP levels, and is observed at the transcriptional level. Therapeutically, given the wide tissue distribution of PDEs, it may be desirable to administer an

isoform-specific PDE inhibitor, rather than a general inhibitor like PTX. Since islet cells express both PDE3 and PDE4, it was of interest to compare the effectiveness of selective inhibitors of these enzymes. For this purpose, we inhibited PDE3 with CIL and PDE4 with ROL. We observed that both PDE inhibitors were equally effective at suppressing NO production when used alone, and several fold more effective than PTX on a molar basis. Moreover, when used together they had more than an additive effect, suggesting synergism. This is of obvious clinical interest, since potential adverse effects may be reduced by administering these drugs in combination at a reduced dose of each.

PDE inhibitors have a therapeutic potential against NO- and/or cytokine-mediated immunological disorders. Conveniently, these drugs can be administrated orally. PTX (Trental) is in clinical use for the treatment of intermittent claudication and is well tolerated. ROL has been studied as an anti-depressant drug, with side effects including gastrointestinal disorders, dizziness and headache. Interestingly, a number of newer PDE4 inhibitors have been synthesized, displaying up to 1000 times more affinity for PDE4 than ROL, and perhaps they will have therapeutic advantages. In addition, there are several PDE3 inhibitors available for clinical use, such as cilostamide, cilostazol, milrinone and amrinone. Cilostasol, for instance, was shown to have minimal side effects and was recently approved for the treatment of intermittent claudication, due to its vasodilatory and platelet activation inhibitory activities.

PDE inhibitors may present a novel therapeutic approach for the prevention of type 1 diabetes Indeed, these inhibitors suppress various mediators implicated in β cell

destruction. They elevate cAMP and modify the activity of many protein kinases and the transcription of numerous genes. However, we cannot exclude the possibility that PDE inhibitors also protect islet cells by mechanisms unrelated to their anti-inflammatory action. For instance, inhibition of PDE3 has been shown to enhance insulin secretion, but inhibition of PDE4 does not appear to have a similar effect.

These studies are original. To our knowledge, we are the first to show:

1) The PDE inhibitors PTX and ROL prevent insulitis and diabetes in NOD mice.

2) Elevation of cAMP levels mediated by PDE inhibitors or other means, correlates with suppression of iNOS transcription and NO production by macrophages.

3) Elevation of cAMP levels suppresses iNOS expression and NO production by islet cells.

4) Clinically relevant selective (PDE3 and PDE4) PDE inhibitors suppress NO production in both macrophages and islet cells.

In conclusion, PDE inhibitors have a therapeutic potential in many immune disorders. These drugs can be considered for long-term preventive therapy in patients at high risk of developing IDDM. Moreover, they may also be relevant in the context of islet transplantation, since both cytokines and NO have been implicated in the rejection process.

Contributions of Authors

I would like to acknowledge the contribution of Dr. France Croze in the studies described in Chapter 3 of this thesis. She provided technical advice and help in RAW cell culture and stimulation. All experiments described in Chapter 2, 3 and 4 were designed, performed and analyzed by the author of this thesis, under the supervision of Dr. Gerald J. Prud'homme