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ANTIOXIDANTS, NFκB ACTIVATION AND THE DEVELOPMENT OF INSULIN DEPENDENT DIABETES MELLITUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

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ABSTRACT

Although many risk factors can trigger the development of insulin-dependent diabetes (IDDM), it is likely that reactive oxygen species (ROS) play a central role in β cell death and disease progression. This dissertation work focuses on the role of antioxidant defense systems in the susceptibility to IDDM and the cellular mechanisms by which ROS and antioxidants regulate the expression of genes leading to β cell death. Accumulating evidence indicates that increased antioxidant defense systems reduce the susceptibility to IDDM in animal models or in human studies. We hypothesize that pancreas-specific ROS production plays a critical role in signaling the cellular autoimmune/inflammatory response in IDDM by activating the transcription factor, NFκB. Various diabetogenic factors may lead to an increase in ROS production, which activates the redox-sensitive NFκB. This may be the initial event for the expression of cytokines and chemotactic agents involved in the autoimmune/inflammatory response. To test this hypothesis we have examined pancreatic NFκB activation in two different chemically induced models for IDDM using the drugs alloxan and streptozotocin (STZ). We have also investigated the role of various antioxidants, including N-acetylcysteine (NAC), α-phenyl-t-butyl-nitrone (PBN), zinc and vitamin E, on the ability to inhibit pancreatic NFκB activation and prevent the development of diabetic symptoms. We
found a specific activation of NFκB in the pancreas with both diabetogenic agents. In addition, classes of antioxidants which effectively inhibited NFκB activation were potent in inhibiting the development of the disease. The specificity of antioxidants to inhibit NFκB activation and the hyperglycemic response emphasizes the importance of selectivity in antioxidant therapy. Research in this area will contribute significantly to our understanding of the cellular and mechanistic role of ROS in the etiology of IDDM and will lead to the development of better prevention strategies.
Dedication

This dissertation is dedicated with love to my family and husband for their love and support through the years.
ACKNOWLEDGMENTS

I wish to express my gratitude and thanks to my adviser and mentor, Dr. Tammy Bray for the training, guidance and inspiration that has made this research and my development as a scientist possible. Dr. Bray's dedication and enthusiasm for science is infectious. I thank many members of the Bray laboratory, both past and present, for all their help over the years. Dr. Gouman Chen was instrumental in introducing me to EPR techniques and helped to guide and encourage me when I first starting working in the lab. Dr. Mike Noseworthy and Dr. Karen Iles were also key in pointing me in the right directions during those early years in the lab. I would also like to thank Mark Levy and Dr. Yu-Hwai Tsai for all their help and support both personally and scientifically over the years. For their comradery and assistance during my time in grad school, I would like to thank Shumin Mao, Jun (Jordan) Li, Lu-te (Luther) Chang, Briana Durica and Yunsook (Yoony) Lim.

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TABLE OF CONTENTS

Dedication ................................................................................................................................. iv
Acknowledgments ..................................................................................................................... v
Vita............................................................................................................................................ vii
List Of Abbreviations ............................................................................................................... x
List Of Tables ........................................................................................................................... xi
List Of Figures ........................................................................................................................ xii

Chapters:

1. Introduction.......................................................................................................................... 1
   1.1. References ................................................................................................................. 21

2. Supplementation Of N-Acetylcysteine Inhibits NFκB Activation And Protects Against Alloxan-Induced Diabetes In CD-1 Mice ....................................................................... 30
   2.1 Summary.................................................................................................................... 31
   2.2 Introduction ............................................................................................................... 32
   2.3. Materials And Methods ............................................................................................ 35
   2.4. Results ....................................................................................................................... 40
   2.5 Discussion .................................................................................................................. 44
   2.6 Acknowledgments ..................................................................................................... 50
   2.7. References ................................................................................................................ 61

3. α-Phenyl-T-Butyl-Nitrone (PBN) Inhibits NFκB Activation Offering Protection Against Chemically-Induced Diabetes ........................................................................... 66
   3.1. Summary ................................................................................................................... 67
   3.2. Introduction ............................................................................................................... 68
   3.3. Materials And Methods ............................................................................................ 71
   3.4. Results ....................................................................................................................... 76
   3.5. Discussion .................................................................................................................. 80
4. Dietary Zinc Supplementation Inhibits NFκB Activation And Protects Against The Development Of Insulin-Dependent Diabetes Mellitus

4.1 Summary ....................................................................................................................
4.2 Introduction .............................................................................................................
4.3 Materials And Methods .........................................................................................
4.4 Results ......................................................................................................................
4.5 Discussion ............................................................................................................... 
4.6 References .................................................................................................................

5. The Effect Of Dietary Vitamin E Supplementation On NFκB Activation And The Development Of Chemically-Induced Insulin-Dependent Diabetes Mellitus

5.1 Summary ..................................................................................................................
5.2 Introduction .............................................................................................................
5.3 Materials And Methods .........................................................................................
5.4 Results ......................................................................................................................
5.5 Discussion ............................................................................................................... 
5.6 References ................................................................................................................

6. Conclusions .....................................................................................................................

6.1 List Of References .....................................................................................................
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB rat</td>
<td>Biobreeding rat</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>I.U.</td>
<td>International units</td>
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<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
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<td>NFκB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>PBN</td>
<td>α-phenyl-t-butyl-nitrone</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>86</td>
</tr>
</tbody>
</table>

1.1 Genes Regulated by NFκB

3.1 Comparison of Products obtained from Tissue Homogenate or Photolysis
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Linking various risk factors to ROS generation in the development of IDDM</td>
<td>18</td>
</tr>
<tr>
<td>1.2.</td>
<td>Schematic illustration of ROS-mediated NFκB activation</td>
<td>19</td>
</tr>
<tr>
<td>1.3.</td>
<td>Initiation and amplification of the immune/inflammatory response by ROS-induced NFκB activation in ( \beta ) cell death and IDDM</td>
<td>20</td>
</tr>
<tr>
<td>2.1.</td>
<td>Dose dependent increase in hyperglycemic response with alloxan administration</td>
<td>51</td>
</tr>
<tr>
<td>2.2.</td>
<td>EPR spectra of PBN spin adducts of alloxan-induced free radicals in pancreas trapped \textit{in vitro} and \textit{in vivo}</td>
<td>52</td>
</tr>
<tr>
<td>2.3.</td>
<td>Relative concentrations of alloxan-induced free radicals trapped by PBN in pancreatic tissue homogenate</td>
<td>53</td>
</tr>
<tr>
<td>2.4.</td>
<td>Time course of NF-κB activation in the pancreas and liver of mice injected with alloxan</td>
<td>54</td>
</tr>
<tr>
<td>2.5.</td>
<td>Administration of NAC inhibits alloxan-induced NF-κB activation in pancreas</td>
<td>55</td>
</tr>
<tr>
<td>2.6.</td>
<td>NAC supplementation inhibits alloxan-induced iNOS expression in the pancreas</td>
<td>56</td>
</tr>
<tr>
<td>2.7.</td>
<td>NAC supplementation inhibits alloxan-induced nitric oxide (NO) formation in the pancreas</td>
<td>57</td>
</tr>
<tr>
<td>2.8.</td>
<td>The inhibitory effect of GSH on alloxan-induced free radical production \textit{in vitro}</td>
<td>58</td>
</tr>
<tr>
<td>2.9.</td>
<td>Dietary supplementation of NAC reduces alloxan-induced hyperglycemia</td>
<td>59</td>
</tr>
</tbody>
</table>
4.9. Pancreatic metallothionein (MT) expression is induced with high zinc supplementation ........................................126

5.1. Vitamin E supplementation inhibits alloxan-induced NFκB activation ..........................................................148

5.2. Vitamin E supplementation has no effect on STZ-induced NFκB activation in the pancreas of CD1 mice .........................................................149

5.3. Supplementation with vitamin E reduces alloxan-induced hyperglycemia and weight loss .................................................................150

5.4. Supplementation with vitamin E does not reduce STZ-induced hyperglycemia .................................................................151

5.5. Serum insulin levels in vitamin E supplemented mice following exposure to STZ ..........................................................152

5.6. Pancreatic nitrite levels in vitamin E supplemented mice following exposure to STZ ..........................................................153
CHAPTER 1

INTRODUCTION

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is a complex, multifactorial disease involving severe destruction of the insulin-producing β cells. Occurring predominantly in young children, IDDM has an acute onset and often progresses to numerous secondary health complications. Multiple risk factors such as genetics, environmental stresses, viral infection, and diet can predispose an individual to IDDM. Regardless of the triggering factors, the disease is always characterized by a progressive destruction of the insulin-producing β cells in the pancreas. Although the sequence of events at the cellular level is still unknown, a growing body of research suggests that reactive oxygen species (ROS) are involved in β cell destruction [1-3]. The finding that ROS production is associated with the same risk factors that cause susceptibility to IDDM increases the likelihood that ROS play an important role in the pathogenesis of IDDM.

The link between multiple trigger factors for IDDM and the production of ROS is illustrated as a unified pathway in our proposed model (Figure 1.1). Our model allows us to predict that certain risk factors will result in increased ROS production, which in turn will lead to the destruction of β cells. Involvement of free radicals in the etiology of
IDDM and in the pathophysiology of Type 2 diabetes has been extensively reviewed by others [4-11]. This review will focus on the role of antioxidant defense systems in the susceptibility to IDDM and on ROS as cellular messengers that regulate the expression of genes leading to β cell death.

**ROS and the Development of IDDM**

There is growing evidence that ROS are involved in the pathogenesis of IDDM. As illustrated in Figure 1.1, several known diabetogenic factors can be linked to ROS generation. For example, although IDDM has traditionally been described as an autoimmune disorder, the autoimmune response may be mediated by ROS. Insulitis, a pre-diabetic infiltration of the islet with cells of immune responses, is accompanied by the release of cytotoxic ROS, such as superoxide (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$) and reactive nitrogen species (RNS) such as nitric oxide (NO·)[12]. It is known that certain viruses are associated with the onset of IDDM [13, 14]. Viral infections, either through general inflammation or direct damage to the β cells, also initiate an immune response that culminates in the release of ROS. In addition to immunological factors, accidental chemical induction of diabetes, particularly via N-nitroso compounds, is well documented in humans [15]. Many investigators have demonstrated that poor nutritional status and diets high in nitrates and nitrites are correlated with an increased incidence of IDDM [5, 16, 17]. Poor nutritional status produces an environment in which antioxidant defenses are often low resulting in a higher susceptibility to oxidative damage. In all these cases, the link between ROS and pathogenesis of IDDM is apparent, however, it is
still unclear if ROS production directly causes β cell death or is simply the consequence of the disease progression.

**Antioxidant Status in IDDM Patients.**

Patients with IDDM appear to have significant defects in antioxidant protection compared to healthy, non-diabetic controls. 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 [18]. Diabetic children have also shown significant decreases in the erythrocyte glutathione peroxidase (GPx), total glutathione, plasma α-tocopherol and plasma β-carotene [19]. This reduction in antioxidant activity is coupled with significant increases in lipid hydroperoxides, conjugated dienes and protein carbonyls, which are markers for oxidative stress [20]. This suggests that low antioxidant defenses predispose IDDM patients to enhanced oxidative stress. More recent studies have established that in the pre-diabetic condition, antioxidant status appears to be compromised [21]. Islet cell antibodies (ICA) serve as a serological marker for risk for IDDM and have been used in population studies for the prediction of 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, disturbances in antioxidant defenses appear even in the pre-diabetic state. These observations suggest that antioxidant status is another contributing risk factor in the development of IDDM.
In the past, the proposed genetic component of IDDM has focused on diabetes-susceptible HLA-haplotypes found in the major histocompatibility complex (MHC) [22, 23]. Individuals who possess these susceptible alleles are more predisposed to developing the autoimmune response resulting in IDDM. However, not all diabetics possess a susceptible haplotype, and not all individuals with this haplotype go on to develop the disease [24]. In fact, twin studies have only shown 30% concordance in developing diabetes [25]. This suggests that both environmental and genetic factors play a role in the development of IDDM. If ROS contribute to β cell death and dysfunction, individuals that possess relatively lower endogenous levels of key antioxidant enzymes may be more susceptible to development of IDDM.

**ROS Production in Animal Models of IDDM.**

In animal models of IDDM, the involvement of free radicals in the development of the disease has been clearly demonstrated. For example, alloxan, a derivative of uric acid, is a known diabetogenic agent and has been used for over 50 years to induce diabetes in laboratory animals [26]. Alloxan-induced diabetes was the first described chemical model for the disease, yet still little is known about its precise cytotoxic mechanism or its selectivity for the pancreatic β cells. Research has suggested that alloxan-induced β cell damage is mediated through the generation of ROS [27, 28]. During the metabolism of alloxan, it is reduced to dialuric acid. ROS species are produced during alloxan metabolism via auto-oxidation of dialuric acid back to alloxan. The production of $\text{O}_2^\cdot$ can lead to the production of other ROS such as $\text{H}_2\text{O}_2$ and hydroxyl radical (·OH) via Fenton reactions [29].

4
Another commonly used diabetogenic agent is the N-nitroso compound, streptozotocin (STZ). Several lines of research suggest that NO* production contributes to the cytotoxicity of STZ to the pancreatic β cells. Biochemical evidence for nitric oxide formation in pancreatic islets has also been demonstrated [6, 30, 31]. An increase in nitrite, a stable endproduct of NO*, can be detected in islets treated with STZ. In STZ-induced diabetes, the incidence of hyperglycemia is decreased when NO synthase (NOS) inhibitors are used [32]. In addition, transgenic mice deficient in the inducible isoform of NOS (iNOS) have also demonstrated a reduced sensitivity to STZ-induced diabetes [33], suggesting that NO* is involved in the mechanism of the disease.

In diabetes-prone animals such as the non-obese diabetic (NOD) mouse and the Biobreeding (BB) rat, islet cell infiltration of immune and inflammatory cells occurs prior to β cell death. Both of these strains are thought to mimic the autoimmune destruction of β cells seen in the human disease. Destruction of the insulin-producing β cells may result from direct exposure to free radicals produced by the immune cells [34]. In addition, free radicals may be produced due to production of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and γ-interferon (IFN-γ), which can be cytotoxic to cells through mechanisms involving free radical production. [12, 35].

**Antioxidant Defense and Protection Against Diabetogenesis.**

ROS and RNS scavengers have been shown to prevent β cell death induced by diabetogenic drugs such as alloxan and STZ and prevent β cell death induced by
proinflammatory cytokines. In vitro, addition of scavenging agents such as SOD, catalase, hydroxyl radical scavengers and metal chelators prior to alloxan [36-38], STZ [39-42] and cytokine exposure [43] has prevented β cell death in isolated islets. Our lab has demonstrated in vivo, that transgenic animals overexpressing CuZnSOD are protected against the development of alloxan- and STZ-induced diabetes [44, 45]. Transgenic strains that either ubiquitously overexpress CuZnSOD (TgHS mice) or contain β cell specific CuZnSOD overexpression (RIPSOD mice) have a significantly lower fasting blood glucose levels following injection of alloxan or STZ compared to non-transgenic control mice. Supplementation with antioxidants such N-acetylcysteine (NAC) and α-phenyl-t-butylnitrone (PBN) also protects against the development of experimentally-induced diabetes. Dietary supplementation of NAC, a glutathione precursor, effectively attenuated the hyperglycemic response and weight loss associated with alloxan-induced diabetes [46]. Co-injection of PBN with multiple STZ injections similarly prevented β cell death and attenuated hyperglycemia and weight loss in STZ-induced diabetes [47]. Similarly, antioxidant supplementation has also shown benefit in reducing spontaneous β cell loss and hyperglycemia in NOD mice and BB rats. A variety of antioxidants including metal-chelators, nicotinamide, SOD and α-tocopherol also provide some protection against the development of IDDM in these diabetes-prone animals [48-54].

**Pancreatic Islets Are Highly Susceptible To Oxidative Stress**

Several key enzymes in ROS defense are unusually low in pancreatic islets compared to other tissues, suggesting that the islet cells are uniquely susceptible to oxidative stress-induced damage. Gene expression and activity of several key antioxidant
enzymes such as CuZnSOD, MnSOD, GPx and catalase are all markedly decreased in islets compared to other tissues such as liver [55]. Studies find that the gene expression levels of the cytoplasmic CuZnSOD and the mitochondrial MnSOD in the pancreatic islet cells are 30-40% of levels found in liver. GPx expression is 15% and catalase gene expression was not detectable in pancreatic islets [56]. Corresponding protein and activity levels of these antioxidant enzymes were also found to be markedly lower in the islet cells. The low levels of antioxidant enzyme gene expression may account for the exquisite sensitivity of the β-cells to ROS and free radical-induced damage leading to β cell death and IDDM. For example, the uptake of alloxan by both liver and pancreatic islets has been observed [57]. However, cytotoxicity of alloxan is apparent only to pancreatic islets. Research suggests that most chemical-induced β cell damage is mediated through the generation of ROS [2, 58, 59]. If increased ROS production is a causative factor in the destruction of the β cells, why is a generalized oxidative stress solely detrimental to the pancreatic islet cells and not other tissues? Some of this specificity may be attributed to the low antioxidant capacity of the pancreatic islet cells.

**ROS as Signaling Molecules**

What is the mechanism that causes ROS to destroy these cells? The missing piece in the pathway lies in the connection between ROS production and β cell death. Whereas higher concentrations of ROS may result in massive, indiscriminate oxidative damage, it is possible that low concentrations of ROS are sufficient to activate specific genes and cause the inappropriate activation of autoimmune or inflammatory responses leading to β cell death. We will present some evidence that production of ROS can activate the
transcription factor NFκB. ROS-mediated activation of NFκB may be the key modulator in the pathway that begins with the triggering of ROS production by multiple factors and leads to the ultimate destruction of β cells and the development of IDDM.

The majority of the research has concentrated on how ROS production causes direct cellular damage by oxidizing nucleic acids, protein and membrane lipids to induce the disease. However, there is increasing evidence that ROS also play roles as physiologically important cellular messengers. ROS appear to play a critical role in regulating the expression of genes that encode for proteins involved in inflammation, immune response and cell death [60, 61]. Excess ROS production may be involved in disease progression through inappropriate activation of genes involved in cellular defenses, excess NO* production and apoptosis. These observations have launched a revolution in the understanding of the role of ROS in many human inflammatory and autoimmune diseases. Although IDDM is largely considered to be an autoimmune disease, this link between ROS and cellular response is a relatively unexplored area of research, leaving a large gap in the understanding of the mechanisms that signal β-cell death.

NFκB, a Transcription Factor Sensitive to Oxidative Stress

ROS are produced during normal cellular metabolism. However, under certain stresses, an increase in the production of oxygen free radicals may overwhelm our antioxidant defenses, resulting in oxidative stress. Several researchers have now discovered that there are several “redox sensitive” biological molecules important in cell
signaling that are sensitive to low concentrations of ROS. One potential target of ROS activation is the nuclear transcription factor, NFκB.

NFκB was first discovered by Baltimore and Sen as a B cell specific nuclear protein that bound to a site in the immunoglobulin κ light chain gene enhancer [62]. Since then, NFκB has gained widespread attention in many fields of research. It is now known that NFκB is present in many cell types and controls the expression of numerous gene products. NFκB appears to play a central role in regulating immune and inflammatory responses [63]. For example, many inflammatory response factors such as pro-inflammatory cytokines, chemokines, adhesion molecules, colony-stimulating factors and inflammatory enzymes (Table 1.1) are products of genes regulated by NFκB. Thus, dysregulation or aberrant activation of NFκB could initiate inappropriate autoimmune and inflammatory responses.

**Activation of NFκB, Inflammatory Response And β Cell Death**

NFκB is usually stored in the cytosol in its “inactive” form bound to the inhibitory unit IkBα, which prevents DNA binding and nuclear uptake of the factor. Degradation of IkBα, is critical for NFκB activation. Extracellular stimuli such as ROS, signal the degradation and the release of the inhibitory unit IkBα, through a complex, but rapid cascade of events resulting in a rapid translocation of “active” NFκB to the nucleus (Figure 1.2) [64-66]. NFκB inducing agents will initiate the phosphorylation of IkBα on its N-terminal serine residues (Ser32 and Ser36) [67, 68]. Currently, the kinase cascade that directly phosphorylates IkBα has not been clearly identified. Recently, and
NFκB-inducing kinase (NIK) and IκBα kinase (IKK) have been identified [69-72], but the precise pathway from inducing agent to phosphorylation of IκBα still remains unknown.

This phosphorylation of IκBα induces polyubiquination of IκBα at multiple sites, tagging the subunit for degradation by the 26S proteosome complex. The free NFκB unit is now able to translocate into the nucleus and bind to consensus DNA binding sites in target genes. In the nucleus, NFκB binds to DNA and modulates the expression of several genes, including the genes controlling inflammatory and autoimmune process [66, 73, 74]. Unlike many other systems, NFκB is already present in the cytosol, thus no new protein synthesis is required for its activation. This unique activation system allows NFκB to regulate immune and inflammatory processes in a rapid and very efficient manner.

NFκB activation is responsible for both the initiation and amplification of immune and inflammatory responses in the cell. An increase in NFκB activation is followed by an increase in the release of cytokines and other chemotactic factors involved in inflammation [75]. NFκB also up-regulates the expression of adhesion molecules critical in leukocyte migration into target tissues [76]. NFκB binding sites are also present in region of the HLA class 1 genes and other immunoreceptors involved in cell-mediated immune responses. At the same time, more ROS and other reactive species such as NO· are generated as a result of the infiltration of immune cells such as macrophages and leukocytes which produce ROS and NO· as a killing or defense mechanism [63]. NFκB has also been shown to stimulate the production of NO· by
directly activating the inducible form of NO synthase (iNOS)[75]. Many of these inflammatory cytokines in turn also activate NFκB, thus amplifying ROS production and creating a vicious cycle. In the development of IDDM, it is possible that ROS-mediated NFκB activation in the pancreas is the central signal that initiates and propagates the inflammation and autoimmune processes responsible for β cell death (Figure 1.3). If this is true, agents that can inhibit this process should protect against the development of the disease.

Although IDDM is largely considered to be an autoimmune disease, this link between ROS and cellular response is a relatively unexplored area of research, leaving a large gap in the understanding of the mechanisms that signal β-cell death. More recently, NFκB activation has been detected in rat insulinoma cell lines following exposure to cytokines such as IL-1 [77, 78]. These studies confirm the possible link between NFκB activation, inflammation and iNOS production in a cell culture model system of diabetes.

Several lines of evidence suggest that NFκB is also a redox-sensitive transcription factor. Many different agents can activate NFκB, including phorbol esters, inflammatory cytokines, UV light, γ rays, viral and bacterial proteins and lipopolysaccharide [75, 79]. All of these agents produce oxidative stress. Thus, despite the diverse stimuli, ROS appear to serve as the common intracellular agents involved in the activation of NFκB [80]. Secondly, direct exposure to oxidants, such as H2O2 activates NFκB [81].

Antioxidants have also been known to have the ability to inhibit NFκB activation, both in vitro and in vivo. In vitro, NFκB activation can be inhibited by addition of various antioxidants including lipoic acid [61], NAC [82], vitamin E derivatives [83], pyrroliidine
dithiocarbamate (PTDC) [84], selenoproteins [85] and vitamin C [86]. Antioxidant supplementation in animals has also been shown to inhibit NFκB activation in vivo. Administration of NAC can inhibit NFκB activation by systemic endotoxin treatment in the lung of rats [87]. NAC has also shown to inhibit NFκB activation by the diabetogenic drug alloxan and the pancreas of CD1 mice [46]. The production of ROS appears to be the key factor in initiating NFκB activation. Thus, strategies to enhance antioxidant status may be beneficial in inhibiting inappropriate activation of NFκB and prevent any downstream pathological events.

We have demonstrated in our laboratory, that in alloxan-induced diabetes, there appears to a specific activation of NFκB in the pancreas, not in the liver, within 30 minutes of alloxan injection. In addition, supplementation with NAC can inhibit NFκB activation in pancreas by alloxan and concurrently provide some protection against the development of the disease. NAC supplemented animals have significantly reduced hyperglycemia and weight loss compared to unsupplemented controls. NAC supplementation also inhibited the expression of the inducible nitric oxide synthase (iNOS), a downstream inflammatory enzyme under regulation by NFκB. These results confirm that the role of ROS in the pathogenesis of IDDM may be far more complex than originally thought. The benefit of antioxidant therapy may not be simply to protect the β cells from oxidative damage as a result of ROS produced in the immune response. Instead antioxidant therapy is used to directly stop the initiation and propagation of immune and inflammatory responses through inhibition of NFκB activation. If this is true, the efficacy of antioxidant therapy will depend on the ability of antioxidants to
modulate cellular response pathways that have been inappropriately activated by excess ROS.

The Future of Antioxidant Therapy in IDDM

*In vitro* studies using defined chemical or enzymatic sources of NO· or ROS have demonstrated that islet cells are very susceptible to free-radical induced islet cell death [88] [89, 90]. Furthermore, addition of antioxidants and NO· inhibitors protect against this damage[38, 91]. Because this inhibition of β cell death occurs *in vitro* with antioxidants and NO· inhibitors, we expect to see a protective effect *in vivo*. However, the translation of this phenomenon to *in vivo* studies is less clear. *In vivo* antioxidant intervention studies in both human and animals models have shown conflicting results [92]. For example, in some studies, supplementation with NO synthase inhibitors such as N-nitro-L-arginine-methylester (NAME) has reduced the hyperglycemic response in STZ-induced diabetes [6, 93]. However, other studies show no effect with inhibitors specific to the iNOS [94, 95]. It is clear that excess production of free radical species, (both ROS and RNS) is detrimental to the pancreatic islet cells. However, attempts to use antioxidant therapy in a clinical setting have shown questionable results [96-99]. Currently, large scale, multinational randomized placebo-controlled intervention studies are underway to test the efficacy of nicotinamide to prevent IDDM. Generation of free radicals, DNA strand breaks, activation of poly ADP-ribose polymerase (PARP) and depletion of NAD appear to be common events in β cell death [96]. Nicotinamide, at high doses is a free radical scavenger, an inhibitor and protects against depletion of intracellular NAD. Based on these functions, and promising *in vitro* and animal studies,
nicotinamide appeared to be an excellent candidate for clinical studies in ICA-positive first-degree relatives and children at high risk for IDDM. So far, results indicate only modest decrease or delay in diabetes development with nicotinamide[97, 98]. In some cohorts, no benefit in disease progression was seen at all [99]. It is possible that nicotinamide supplementation targets the repair of oxidative damage that occurs late in the diabetogenic process. A more effective therapy may need to inhibit oxidative signaling processes much earlier in the diabetogenic pathway to be effective.

An important research goal is to understand how the balance between ROS and antioxidants determines susceptibility to the disease. We hypothesize that this critical balance is defined by the ability of ROS to activate cellular responses, such as inflammation and other immune responses. Specifically, an imbalance between ROS and antioxidants (i.e. an excess of ROS or inadequacy of antioxidants) induces the activation of the transcription factor, NFκB. This activation results in an increase in inflammatory and immune responses and leads to an amplification of ROS and NO production which, in turn, ultimately leads to the destruction of the β cells, hyperglycemia and the development of IDDM.

Currently, inhibition of NFκB activation to diminish the expression of pro-inflammatory and immune response genes have been discussed as a therapeutic approach in several other immune or inflammatory-related diseases including inflammatory bowel disease, inflammatory response syndrome, septic shock, asthma and rheumatoid arthritis [100, 101]. Despite the obvious link of NFκB to regulation of immune responses and the link between immune dysregulation and the development of IDDM, the link between NFκB activation and diabetogenesis has still not been fully established. Strategies to
limit inappropriate activation of NFκB may prove to a very effective approach in preventing the disease. We have focused primarily on the role of antioxidants in preventing NFκB activation. There are several other compounds that also have the ability to inhibit NFκB activation. These include proteosome inhibitors, corticoidsteroids and agents that can block NIK and IKK. However, use of these compounds may have limited therapeutic effect. Proteosome degradation is critical for normal protein turnover and regulates normal cellular function and cell cycling. Thus, the use of proteosome inhibitors clinically may have serious side effects in vivo. Long-term glucocorticoid therapy also has limited benefit due to side effects associated with its effects on endocrine function and metabolism. Currently, agents that can selectively block NIK and IKK, and thus block IκBα degradation, have not been identified. Phosphorylation cascades are a widespread regulatory modification throughout cellular metabolism. So far, less specific kinase inhibitors have shown conflicting results.

The key to successful antioxidant therapy in IDDM will rely both on effective targeting to the islet cells and on dose. Studies investigating the effect of NAC in animal models of acute respiratory disease (ARDS) have found that NAC is beneficial in ARDS possibly through inhibition of NFκB activation. However, effect of NAC only proved to be beneficial at lower supplementation levels, while at high doses lung injury was exacerbated [102]. Thus, dose response relationships with antioxidant therapy will also need to be considered.

The overall goal of this research is to determine the mechanism by which ROS are involved in the development of IDDM and to determine if antioxidant supplementation
strategies can alter the development of the disease. More specifically my research has identified increased free radical production and NFκB activation in animal models for IDDM. I have also focused on examining the ability of different classes of antioxidants to inhibit NFκB activation and protect against the development of IDDM. This line of research will help confirm that free radicals and NFκB activation are central signals in β cell destruction and move us closer to understanding the cellular events in IDDM.
<table>
<thead>
<tr>
<th>Class</th>
<th>Target Genes</th>
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<tr>
<td><strong>Cytokines</strong></td>
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<td></td>
<td>TNF-β</td>
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<td></td>
<td>Interferon-β( IFN-β)</td>
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<td>Interleukin-1β (IL-1β)</td>
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<td><strong>Chemokines</strong></td>
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<td>Gro α,β,γ</td>
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<td>RANTES</td>
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<td></td>
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<td><strong>Adhesion Molecules</strong></td>
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<tr>
<td></td>
<td>E-selectin</td>
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<tr>
<td></td>
<td>V-CAM</td>
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<tr>
<td><strong>Colony Stimulating Factors</strong></td>
<td>Granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
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<td>Macrophage colony stimulating factor (M-CSF)</td>
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<tr>
<td><strong>Immunoreceptors</strong></td>
<td>Ig κ light chain</td>
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<td></td>
<td>Major histocompatibility complex (MHC) class I</td>
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<td>T cell receptor β chain</td>
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<tr>
<td><strong>Inflammatory Enzymes</strong></td>
<td>Inducible nitric oxide synthase (iNOS)</td>
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<td></td>
<td>Cyclooxygenase-2 (COX-2)</td>
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<td>12-Lipoxygenase</td>
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Table 1.1 Genes Regulated by NFκB
Figure 1.1. Linking various risk factors to ROS generation in the development of IDDM; ROS, reactive oxygen species; IDDM, insulin-dependent diabetes mellitus.
Figure 1.2. Schematic illustration of ROS-mediated NFκB activation. In response to extracellular inducers, such as ROS, IκB kinase (IKK) is activated and phosphorylates the IκBα subunit associated with the NFκB p50/p65 heterodimer. The phosphorylated IκBα then becomes ubiquinated. This results in subsequent degradation of the IκBα subunit by proteosomes. Degradation of IκBα releases the p50/p65 complex, allowing the complex to translocate into the nucleus where it binds to the κB-binding sites of gene promoters and induces their transcription. Many of the target genes regulated by NFκB include several immune and inflammatory factors.
Figure 1.3. Initiation and amplification of the immune/inflammatory response by ROS-induced NFκB activation in β cell death and IDDM. ROS production induced by diabetogenic factors causes the activation of NFκB. This in turn induces the transcription of autoimmune and inflammatory factors. Initiation of immune or inflammatory responses results in the production of more ROS and further activation of NFκB. This pathway acts as a positive loop, amplifying ROS production and the immune response, ultimately destroying the pancreatic β cells.
1.1. REFERENCES


CHAPTER 2

SUPPLEMENTATION OF N-ACETYLCYSTEINE INHIBITS NFκB ACTIVATION AND PROTECTS AGAINST ALLOXAN-INDUCED DIABETES IN CD-1 MICE

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2.1 SUMMARY

Reactive oxygen species (ROS) are involved in the destruction of pancreatic β cells and the development of insulin-dependent diabetes mellitus (IDDM). However, the cellular mechanism responsible for β cell death is still unclear. We hypothesize that activation of NFκB by ROS is the key cellular signal in initiating a cascade of events leading to β cell death. Thus, enhancement of pancreatic GSH, a known antioxidant and key regulator of NF-κB, should protect against IDDM. Weanling CD1 mice (n=5) were injected with alloxan (50 mg/kg i.v.) to induce IDDM. Using EPR spin trapping techniques, we demonstrated that alloxan generated ROS in the pancreas 15 min after administration. Activation of NFκB in pancreatic nuclear extracts was observed 30 min after alloxan injection, as assessed by an electrophoretic mobility shift assay. Fasting blood glucose levels were monitored for 14 days. Supplementation with N-acetylcysteine (NAC, 500 mg/kg), a GSH precursor, inhibited alloxan-induced NFκB activation and reduced hyperglycemia. Thus, NFκB activation by ROS may initiate a sequence of events leading to IDDM. Inhibition of NF-κB activation by NAC attenuated the severity of IDDM. This research will contribute to the understanding of the etiology of IDDM and may lead to the development of better strategies for disease prevention.
2.2 INTRODUCTION

Type 1 diabetes or insulin dependent diabetes mellitus (IDDM) is known to be a multifactorial disease (1-3). Despite different triggering factors, the final outcome is characterized by profound destruction of the insulin-producing β cells. The precise cellular mechanisms leading to pancreatic β cell death have not yet been fully elucidated. However, there is growing evidence implicating reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the destruction of β-cells in the pathogenesis of IDDM (4-7). Recently, ROS/RNS have gained increasing attention as physiologically and pathologically important cellular messengers (8). Evidence suggests that ROS/RNS act as signal molecules in the regulation of gene expression, cell proliferation and cell death (9). Moreover, ROS/RNS are now known to play a critical role in upregulation of the expression of genes involved in the inflammatory and autoimmune responses (10).

Nuclear transcription factor κB (NFκB) is a transcription factor that can be activated by a variety of stresses such as oxidants, viruses, metals, xenobiotics and pro-inflammatory cytokines (11;12). NFκB is usually stored in the cytosol in its inactive form bound to the inhibitory unit iκB. Activation of NFκB in response to extracellular stimuli involves the release of iκB, resulting in a rapid translocation of NFκB to the nucleus (13;14). ROS/RNS appear to be one of the key factors in initiating NFκB activation (15-17). Once activated, NFκB binds to nuclear DNA and modulates the
expression of several genes for adhesion molecules such as selectins, ICAM-1 and VCAM-1 (18) and upregulates the production of various proinflammatory cytokines such as IL-2 (19), IL-6 (20), TNF-α (21) and inducible NO synthase (iNOS) (22).

IDDM is generally considered as an autoimmune disease of the pancreas (23). The inherent lack of antioxidant protection in the pancreas (24;25), may increase its sensitivity to diabetogenic agents that trigger ROS/RNS production. We propose that the critical determinant in β cell destruction in IDDM may be the inappropriate activation of NFκB, which starts a cascade of events that results in the upregulation of genes involved in the inflammatory and autoimmune response. Once an autoimmune/inflammatory response is launched, the invading immune cells amplify ROS/RNS production, which ultimately destroys the β cells. Thus, ROS are not simply cytotoxic agents that damage β cells, but are key modulators of the cellular response pathways that initiate β cell death and the development of IDDM.

GSH, a cysteine-containing tripeptide, is the most abundant non-protein thiol in mammalian cells (26). It is a substrate of the ROS defense enzyme GSH peroxidase and the GSH transferase family of detoxification enzymes. GSH also protects sulfhydryl groups from oxidation (26; 27). GSH is not only an effective antioxidant, but it also modulates cellular metabolism and gene expression by affecting cellular thiol redox status (28-30). Intracellular redox status appears to be a critical determinant of NFκB activation (28; 29; 31). Hence, if ROS-induced NFκB activation modulates and amplifies the processes leading to β cell death, inhibition of NFκB activation by GSH should prevent this damage and the development of IDDM.
The objectives of this study were to determine free radical-mediated NFκB activation in the pancreas is a key event leading to the onset of IDDM in an \textit{in vivo} model, and to assess the efficacy of supplementation of the GSH precursor, N-acetylcysteine (NAC) on NFκB activation and the development of alloxan-induced diabetes in CD-1 mice.
2.3. MATERIALS AND METHODS

Animals. Weanling male CD-1 mice (Harlan, Indianapolis, IN), weighing 20-25 g were used in all experiments. All animals were housed in individual cages in a temperature controlled environment (22 ± 2°C) with a light period between 0600 to 1800 hrs. Animals were fed their respective experimental diets and allowed free access to the diet with the exception of fasting periods for blood glucose determination. Animal protocol was approved by the OSU Institutional Laboratory Animal Care and Use Committee.

Hyperglycemic Effect of Alloxan. To determine the dose-response of alloxan-induced diabetes, fasting blood glucose levels were monitored in CD1 mice after administration of varying doses of alloxan. Following an overnight fast, weanling CD1 mice were injected with 50, 100, 150, 200 or 300 mg/kg alloxan (i.p). Control animals received a sham saline injection. Fasting blood glucose levels were monitored for 9 days. Blood was obtained from the intra-orbital sinus using a 10uL capillary tube. Glucose concentrations were measured using the ONE-TOUCH ™II complete blood glucose monitoring kit. To minimise the effects of diurnal fluctuations, blood samples were collected at the same time every day.

In vitro and In vivo EPR Spin Trapping of Free Radicals.

In vitro studies: Pancreatic tissue (0.5g) was homogenized in 150mM phosphate buffer. Homogenates were then incubated with 0-80 mg/ml alloxan and 14mg of spin
trap, \( \alpha \)-phenyl-\( \tau \)-butyl-nitrone (PBN, Sigma, St. Louis, MO) at 37\(^\circ\)C for 90 minutes. Control samples contained PBN only. Following the incubation period, PBN spin adducts were extracted with 6ml of benzene and were concentrated to a 0.2ml sample and de-gased with nitrogen gas before EPR analysis. Samples were placed in quartz round cell and analyzed at room temperature with a Bruker X band EPR spectrometer operating at 9.78 GHz. To study the effect of GSH on free radical formation in pancreatic homogenate, reduced GSH (0, 2.5, 5, 10 or 20 mM) was added to pancreatic homogenates. All samples were then incubated with 10 mg alloxan and 14 mg PBN at 37\(^\circ\)C for 90 minutes. Extractions and concentration was performed as previously described and PBN adducts were analyzed by a Bruker X band spectrometer.

**In vivo studies:** Weanling CD1 mice were injected with 150 mg/kg PBN dissolved in saline. Fifteen minutes later, animals were injected (i.v.) with 50 mg/kg alloxan or saline control. Fifteen minutes following alloxan injection, animals were sacrificed and pancreatic tissue was removed and immediately frozen in liquid N\(_2\). Five pancreas samples were pooled and homogenized in phosphate buffer. PBN adducts were extracted and concentrated as previously described and analyzed by EPR spectrometer.

**Determination of NFkB Activation In Vivo.** For NFkB activation analysis, mice were injected with 50 mg/kg alloxan (i.v.) and sacrificed 0, 15, 30 and 60 minutes after injection (n=5 in each time interval). A lower dose of alloxan is required to produce similar hyperglycemic effect when it is injected intravenously. It has been established in our laboratory that an i.v. injection of 50mg/kg BW of alloxan produces similar hyperglycemic profile as an i.p. injection of 200 mg/kg BW (data not shown). Pancreas and liver tissues were immediately removed. NFkB activation was determined using an
electrophoretic mobility shift assay (EMSA). To determine the effect of NAC supplementation on alloxan-induced NFκB activation, animals were injected with NAC (500mg/kg i.p.) 90 minutes prior to alloxan administration.

Crude nuclear extracts were prepared from pancreatic and liver tissues as described by Deryckere and Gannon (32). Double stranded synthetic oligonucleotides probes for NFκB (5'-AGTGAGGGGACTTTCCCAGGC-3') (Promega, Madison, WI) were end-labeled using [γ-32P] (Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Boehringer Manneheim, Indianapolis, IN). Binding reactions containing equal amounts of protein (~7 ug) and labeled oligonucleotide probes were performed for 20 minutes at room temperature in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris, 50 ug/ml poly[dI-dC]. Specific binding was confirmed using 100-400 fold excess unlabelled NFκB oligonucleotide as a specific competitor. Protein-DNA complexes were separated using 6% nondenaturing polyacrylamide gel electrophoresis followed by radiography to detect the level of retardation produced by binding to NFκB probe.

Effect of Dietary Supplementation of NAC on Alloxan-induced Hyperglycemia.

Mice were randomly allocated to one of 3 treatment groups (n=6), Control, Alloxan or Alloxan + NAC. Control and Alloxan grouped animals were fed purified 76A1N rodent diet. NAC supplemented animals were fed a modified 76A1N purified rodent diet containing 0.4% NAC (w/w). All animals were fed their respective diets for one week prior to alloxan administration. To induce diabetes, alloxan monohydrate (200 mg/kg BW) dissolved in saline was injected i.p. following an overnight fast. Control animals received a sham saline injection. Animals were allowed free access to diets, with the exception of
fasting periods for blood glucose determination. Severity of hyperglycemia was measured by monitoring fasting blood glucose levels for 14 days using the ONE-TOUCH™II system, as previously described. On day 14, animals were anaesthetised and sacrificed via cervical dislocation. Pancreas and liver were removed and immediately frozen in liquid nitrogen and were stored at -80°C for further analysis.

Effect of Dietary Supplementation of NAC on Pancreatic GSH Concentration.

The effect of NAC supplementation on pancreatic GSH concentration was assessed by comparing total GSH content in NAC supplemented and unsupplemented control using the method of Tietze (33). Pancreatic tissue was removed from mice after the two-week feeding period. Due to the small size of the tissue, four pancreases were pooled and homogenized in 5% trichloroacetic acid (TCA). Samples were centrifuged at 10,000 x g and supernatant was used for GSH analysis.

Expression of iNOS Protein: Expression of iNOS protein in the pancreas in Control, Alloxan and Alloxan+ NAC (ALX+NAC) groups were determined by Western blot analysis. Pancreatic homogenates taken 30 minutes following alloxan injection were mixed with an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, bromphenol blue) and boiled for 5 minutes. SDS electrophoresis was carried out under standard condition (34). Protein was transferred from the SDS gel to nitrocellulose membranes (Biorad, Hercules, CA) at 50 mA overnight. iNOS blots were blocked with in 5% non-fat dry milk for 1 hour at 37°C. Blots were incubated for 3 hours at 37°C with rabbit anti-mouse iNOS (Alexis Biochemical, San Diego, CA) at a dilution of 1:2000. The blots were washed 5 times with PBS+0.2% Tween 20, then incubated with horseradish peroxidase-conjugated
donkey anti-rabbit antisera (Sigma, St. Louis, MO) at a dilution of 1:5000, for 1 hour at 37°C. iNOS was detected by enhanced chemiluminescence using Hyperfilm and ECL reagents (Amersham, Piscataway, NJ).

**Nitrite Analysis:** Pancreatic tissue was homogenized in 100mM HEPES buffer and then centrifuged at 100,000 x g for 60 min. Nitrite formation was used as an indirect measure of nitric oxide (NO) production. Nitrite concentration was determined using the Griess reaction as described by Hevel & Marletta (35). Protein concentration was determined using the Lowry method (36).

**Statistics.** All data was analyzed using SAS analytical software. Two way analysis of variance (ANOVA) was performed to assess differences between groups and time. Differences in means between and within treatments was tested by using Tukey’s test ($\alpha=0.05$). For nitrite analysis, one way analysis of variance was used to detect differences between groups. Differences in means between groups was tested using Tukey’s test ($\alpha=0.05$).
2.4. RESULTS

Mice injected intraperitoneally (i.p.) with alloxan concentrations of 150 mg/kg BW or higher display symptoms characteristic of diabetes including decreased plasma insulin levels (data not shown), elevated fasting blood glucose levels (hyperglycemia), polyuria, and weight loss. Figure 2.1 demonstrates the dose of alloxan required to induce hyperglycemia to be at or above 150 mg/kg BW when it is i.p. injected. Dose-dependent increase in hyperglycemic response following injection (i.p.) of increasing doses of alloxan is observed. EPR spin trapping studies were performed to assess free radical concentration in the pancreas after exposure to alloxan. Figure 2.2 shows the EPR spectra of PBN spin adducts of alloxan-induced free radicals in pancreatic homogenate (in vitro) and in the pancreas (in vivo) of CD1 mice. We were able to trap identical alloxan-induced free radicals in both in vitro and in vivo as shown by the identical hyperfine splitting constants, $A_N = 14.61$ and $A_H = 3.2$. These splitting constants suggest that the radical detected is a carbon centered radical. However, further chemical analysis is needed to specifically identify the chemical structure of free radical species.

We were also able to establish a dose-dependent increase in free radical generation with alloxan in the pancreas using in vitro EPR spin trapping techniques. Signal peak height is a relative measure of free radical concentration. Figure 2.3 shows that signal peak height increases when increasing concentrations of alloxan are added to
pancreatic homogenates. Thus, these experiments demonstrate that alloxan can generate free radicals in a dose dependent manner.

**Figure 2.4A** is a representative EMSA radiograph that depicts the $^{32}$P-labeled DNA/NFκB complex present in the nuclear extracts of the pancreas following injection of alloxan (50 mg/kg i.v.) at various time points in CD1 mice. Lane 1 is a positive control using HeLa cells which contain activated NFκB. The presence of binding is indicative of the translocation and activation of NFκB (lane 5 and 7) in pancreas. The specificity of NFκB binding was confirmed by using an excess of unlabeled DNA oligo which contains NFκB binding sites as a specific competitor (lane 6 and 8). Significant binding of NFκB was seen 30 minutes following alloxan injection in pancreatic nuclear extracts. Presence of NFκB in nuclear extracts disappeared by 60 minutes. The result demonstrates that administration of alloxan activates NFκB rapidly in the pancreas of CD1 mice. Identical EMSA experiments were performed with liver extracts, however, no evidence of NFκB activation could be seen with alloxan administration in the liver (Figure 2.4B). These experiments illustrate both the time course of alloxan-induced NFκB activation and the specificity of NFκB activation to the pancreas in the *in vivo* system.

**Figure 2.5** illustrates that the supplementation of NAC, a GSH precursor, inhibits alloxan-induced NFκB activation in pancreas *in vivo*. Similar to Figure 2.4A, NFκB activation was observed 30 min following alloxan administration (lane 2). However, pretreatment of NAC (90 min prior to alloxan administration) fully inhibited the activation of NFκB (lane 3). These results clearly demonstrate that pretreatment with
NAC effectively inhibited alloxan-induced NFκB activation in the pancreas compared to non-supplemented animals.

To verify NFκB activation resulted in the activation of gene transcription of downstream targets, expression of proteins with NFκB binding site in the promoter region needs to be measured. One downstream target of NFκB activation is iNOS. Induction of this protein can be directly measured by Western blot analysis. Figure 2.6 depicts a significant increase in pancreatic iNOS expression from animals following alloxan exposure (ALX) when compared with that of saline injected (Saline). Mice treated with NAC prior to alloxan exposure (ALX+NAC) show a significant reduction in iNOS protein expression. LPS, a known iNOS inducer is used as a positive control to confirm iNOS expression in vivo. Increases in nitric oxide (NO) concentration in pancreas are expected following an induction of iNOS protein expression. To determine the effect of alloxan and NAC on NO production, nitrite levels were determined in the pancreas from animals that completed the alloxan/NAC supplementation trial. Similar to the results obtained from iNOS protein expression (Figure 2.6), Figure 2.7 shows that pancreatic nitrite levels in animals injected with alloxan and fed a control diet (Alloxan) was significantly elevated when compared to saline-injected controls (Control). Animals that were fed the supplemented 0.4% NAC diet and injected with alloxan (ALX + NAC) showed no increase in nitrite levels compared to controls (Control). Thus, NO levels were high after alloxan exposure, but supplementation with NAC can inhibit this elevation.
The ability of NAC supplementation to inhibit NFκB activation in the pancreas may be due to its ability to enhance GSH levels and subsequently inhibits alloxan-induced free radicals in the pancreas. When pancreatic homogenates are incubated with alloxan and increasing doses of GSH, there is a dose dependent decrease in EPR signal peak height (Figure 2.8). This demonstrates that GSH can directly decrease alloxan-induced free radical generation and may account for the ability for NAC to inhibit alloxan-induced NFκB activation in the pancreas.

If NFκB activation is the critical step in the development of diabetes, then NAC, which is able to inhibit NFκB activation, should also prevent the onset of the disease. To determine the efficacy of NAC supplementation to inhibit alloxan-induced diabetes, the severity of hyperglycemia and weight loss were compared between NAC supplemented and non-supplemented groups (Figure 2.9A and 2.9B). Alloxan treatment (Alloxan) caused a significant elevation of blood glucose levels and body weight loss compared to baseline saline-injected controls (Control). Animals that were fed a NAC supplemented diet (ALX+NAC) had significantly lower blood glucose levels compared to the non-supplemented group (Alloxan) (Figure 2.9A). NAC supplementation also reduced the degree of weight loss observed in alloxan-induced diabetes (Figure 2.9B). Therefore, NAC supplementation proved to be effective in attenuating the severity of both the hyperglycemia and weight loss associated with the development of diabetes.

Figure 2.10 confirms that NAC is an effective precursor of GSH to elevate pancreatic GSH level in vivo. After two-weeks of NAC supplementation, pancreatic GSH concentration in NAC supplemented group is significantly increased when compared to that of the unsupplemented group.
2.5 DISCUSSION

This is the first study to use an in vivo model, to demonstrate the role of free radicals in mediating cellular signal transduction pathways leading to the development of IDDM. In contrast to the majority of studies investigating ROS/RNS signaling in vitro using cell culture, we have been able to characterize the link between free radicals and NFκB activation in the development of diabetes in a well established in vivo diabetogenic model. In the present study, we have systematically documented the range of alloxan toxicity and the dose-responsiveness of alloxan-induced hyperglycemia. We also demonstrated the specificity of NFκB activation in the development of alloxan-induced diabetes. Exposure to the alloxan, induced NFκB activation specifically in the pancreas, not in the liver, in CD1 mice (Figure 2.4A & 2.4B). It is well known that alloxan is specifically cytotoxic to the pancreatic β cells. However, the precise mechanism for this selectivity is still not clear. The structure of alloxan is very similar to glucose, hence there is some selective uptake of alloxan via glut-2 transporters to glucose-metabolizing tissues, such as pancreatic islets and liver, but not skeletal muscle (37; 38). Alloxan is also thought to produce free radicals during its metabolism (39). The pancreatic islets are known to have much lower antioxidant defense enzymes (24) compared to other tissues such as liver, thus they are highly susceptible to oxidative damage. This vulnerability to free radicals may, in part, account for the exquisite sensitivity of the pancreatic islets to alloxan. Since uptake of alloxan in the pancreas is known to be highly specific to islet
cells, it is assumed that alloxan-induced activation of NFκB is also islet cell-specific. This specificity of NFκB activation in the pancreas may be the key factor in increased free radical production and helps to explain the selective cytotoxicity of alloxan. Furthermore, we have also shown that the antioxidant NAC can effectively and specifically inhibit pancreatic NFκB activation and is able to attenuate the severity of the disease.

Recent breakthroughs with the discovery of ROS-induced activation of NFκB and its role in amplifying inflammatory and immune processes have launched a revolution in the understanding of the role of ROS in many human inflammatory and autoimmune diseases such as inflammatory bowel disease (40), atherosclerosis (41) and rheumatoid arthritis (42). Although IDDM is largely considered to be an autoimmune disease, this link between ROS and cellular response is a relatively unexplored area of research, leaving a large gap in the understanding of the mechanisms that signal β-cell death. More recently, NFκB activation has been detected in rat insulinoma cell lines following exposure to IL-1 (43;44). These studies confirm the possible link between NFκB activation, inflammation and iNOS production in a cell culture model system of diabetes. Although these previous studies suggest a role for NFκB in IDDM, the current study using an in vivo model for IDDM helps us to understand the role of NFκB in a physiological progression of the disease.

In this study, free radical production was detected in the pancreas approximately 15 minutes after injection of alloxan in CD1 mice. Pancreatic activation of NFκB occurred within 30 minutes of alloxan injection. Typically, symptoms of hyperglycemia and weight loss appeared 24–48 hours following injections of alloxan. Peak severity of
hyperglycemia was observed 4-5 days after injection. Based upon these observations, we can envision the sequence of events that lead to the development of diabetes. Early initiation events begin with increased free radical production, which in turn activates NFκB. This activation of NFκB both initiates and amplifies inflammatory responses through upregulation of cytokines and proinflammatory proteins such as iNOS (17;45). This amplification cascade results in increased free radical production and eventually leads to β cell death.

If free radical production and NFκB activation are central to the development of IDDM, then agents that can inhibit these processes should inhibit the disease. We have clearly demonstrated that administration of the GSH precursor, NAC, inhibited alloxan-induced NFκB activation (Figure 2.5) and attenuated the severity of alloxan-induced hyperglycemia and weight loss (Figure 2.9). The ability of NAC to inhibit alloxan-induced NFκB activation may be attributed to its role as a GSH precursor and the ability of GSH to decrease alloxan-induced free radical concentration (Figure 2.10 and Figure 2.8). Indeed, our in vitro EPR spin trapping experiments directly demonstrated the ability of GSH to decrease alloxan-induced free radicals. We were unable to determine the effect of NAC on alloxan-induced radicals in vivo due to constraints of spin trapping techniques. Although EPR is a method for directly detecting free radicals, it is not highly sensitive. Thus, the ability to discern differences in alloxan-induced free radical concentration between NAC-supplemented and unsupplemented animals is difficult because in vivo EPR signals are weak. Nonetheless, NAC’s ability to inhibit NFκB activation attenuated the severity of diabetic symptoms in CD1 mice (Figure 2.9). However, there are other possible mechanisms by which enhanced tissue GSH may
attenuate the toxicity of alloxan. Since GSH is the substrate of GSH-transferase and GSH-peroxidase, increases in tissue GSH may alter the metabolism and detoxification of alloxan as well as reduce the oxidative damage of target tissue. This possibility needs to be further investigated.

NAC supplementation also inhibited iNOS protein expression and decreased NO concentration (Figures 2.6 & 2.7). NFκB has been shown to stimulate the production of NO by directly activating iNOS (11). The induction of iNOS and NO production has been implicated in mediating β cell death and IDDM in other models for IDDM and in cell culture (46-48). Although iNOS is known to be stimulated during inflammatory and immune responses, we currently are unable to determine the source of NO. Increased NO production may be a result of the induction of iNOS in the pancreatic islets themselves, or from infiltrated immune cells. To determine the source of NO, histopathological studies need to be pursued.

To delineate the proposed role of NFκB in the sequence of events leading to inflammation in alloxan-induced IDDM, we need to establish that NFκB activation is indeed inducing the transcription of other downstream target genes, in addition to iNOS. However, transactivation assays using transfection methods and reporter gene assays are usually used in cell culture system to answer this question. These assays are near impossible to perform in an in vivo system. In addition, many of the downstream targets of NFκB activation, such as cytokines are relatively short-lived proteins, making their detection very difficult in an in vivo setting. Future studies, using more sensitive methods such as quantitative RT-PCR assay, are needed to investigate the expression of various
cytokines, chemokines and adhesion molecules to directly confirm the link between free radicals, NFκB, inflammation and IDDM.

Although elucidation of the detail of signal transduction pathways is somewhat limited using an in vivo model, the results of this study highlight the key molecular events in IDDM at the physiological level. The present study has helped reinforce the concept that ROS modulate disease processes via complex signal transduction pathways. We have demonstrated that NFκB activation may be a critical determinant in the progression of the disease. We have established that GSH and NAC are able to inhibit NFκB activation and concurrently decrease ROS and NO production. Both excess ROS and NO production have been implicated as cytotoxic agents to the β cells. However, this observation is only one small piece contributing to the understanding of the complex pathways leading to β cell death in IDDM. More recently, the role of ROS as signaling molecules in cell death pathways such as apoptosis has been gaining significant attention (49; 50). Further studies investigating the role of ROS and NFκB in signal cascades leading to β cell death via necrosis and apoptosis still need to be examined.

Not all antioxidants can prevent the onset of IDDM. In the past, antioxidant intervention studies in both human and animal models show conflicting results. Although it is clear the excess free radical species are detrimental to the pancreatic islet cells, why does it appear that antioxidant supplementation is of limited benefit (51-53)? It appears that the efficacy of antioxidant therapy in IDDM is still a question of specificity. Only those antioxidants that can effectively target critical pathways in pancreatic tissue appear to be effective. If NFκB activation is the critical step, this knowledge will act as a useful tool for screening different classes of antioxidant for their
value in IDDM therapy or prevention. The identification of the pathways leading to β cell death in IDDM is essential before effective treatment and prevention strategies can be developed. A focus on the control of cellular response pathways will bring the field to a level of understanding that is needed to develop effective preventative strategies. This research is the first step in unifying the many factors in the diabetogenic processes in a relevant in vivo system and will help move us closer to finding effective treatment or prevention strategies for IDDM.
2.6 ACKNOWLEDGMENTS

We would like to thank the technical support of Dr. Denis Medeiros for his assistance in establishing the EMSA, Dr. Russ Hille for the use of EPR spectrometer in his laboratory, and Miss N’Diris Z. Barry “Sam” for her assistance in the animal studies. Financial support from Central Ohio Diabetes Association (CODA) is gratefully acknowledged.
Figure 2.1. Dose dependent increase in hyperglycemic response with alloxan administration. On day 0, CD1 mice were injected with 50, 100, 150, 200 or 300 mg/kg alloxan i.p. following an overnight fast. Control animals received saline injection. Fasting blood glucose levels were monitored for 9 days. Values are expressed as mean ± SE (n=4).
Figure 2.2. EPR spectra of PBN spin adducts of alloxan-induced free radicals in pancreas trapped in vitro and in vivo. (A) In vitro study, alloxan monohydrate (10 mg/ml) and PBN (14 mg/ml) were incubated in 0.5 g pancreatic tissue for 90 min at 37°C. PBN spin adducts were extracted and analyzed by EPR. (B) In vivo study, mice were injected i.p. with PBN (150 mg/kg body weight) 15 min prior to injection i.v. of alloxan (50 mg/kg body weight). Pancreas was removed 15 min after alloxan injection. PBN spin adducts were extracted and analyzed by EPR. Instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, $5 \times 10^5$; time constant 100 ms, scan range, 100 G and sweep time, 50 s.
Figure 2.3. Relative concentrations of alloxan-induced free radicals trapped by PBN in pancreatic tissue homogenate. The relative concentration of free radicals was assessed according to relative peak height of the EPR spectra. Alloxan monohydrate (0-80 mg/ml) and PBN (14 mg/ml) were incubated in 0.5 g pancreatic tissue homogenate for 90 min at 37°C. PBN spin adducts were extracted and analyzed by EPR. Instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, 5 x 10^5; time constant 100 ms, scan range, 100 G and sweep time, 50 s.
Figure 2.4. Time course of NF-κB activation in the pancreas and liver of mice injected with alloxan. Mice were injected with 50 mg/kg alloxan i.v. In Figure (A), a total of 5 pancreas samples were pooled together for nuclear extract preparation at each time point. Lane 1 is a positive control using HeLa cells (Promega) which contain activated NFκB. Lanes 2, 4, 6, 8 and 10 represent pancreatic extracts incubated with specific competitor (unlabelled NFκB oligo) to confirm the specificity of NFκB binding. In Figure (B), nuclear extracts were prepared from single liver of each mouse at each time point. Lane 1 is a positive control. Lane 2 (COMP) is the competitor reaction which contains 100 fold excess of unlabelled NFκB oligonucleotide. This confirms the specificity of NFκB binding. These Figures are representative of 3 individual experiments.
Figure 2.5. Administration of NAC inhibits alloxan-induced NF-κB activation in pancreas. Control group received only sham saline injections. Alloxan group received alloxan only (50 mg/kg i.v.). ALX+NAC group was injected with 500 mg/kg NAC i.p. 90 minutes prior to alloxan (50 mg/kg BW i.v.). Pancreatic tissue was removed from mice 30 minutes following alloxan injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This Figure is a representative of 2 individual experiments.
Figure 2.6. NAC supplementation inhibits alloxan-induced iNOS expression in the pancreas. iNOS expression was determined by Western blot analysis in saline-injected (Control), alloxan-injected (Alloxan) and alloxan-injected/NAC supplemented (500 mg/kg NAC i.p. 90 minutes prior to alloxan injection). SDS-PAGE was performed as outlined in Methods. Each sample contains 5 pooled pancreases from each group. Results are representative of 2 individual experiments.
Figure 2.7. **NAC supplementation inhibits alloxan-induced nitric oxide (NO) formation in the pancreas.** Nitrite production was used as an indirect measure of NO production. Nitrite levels were assessed in saline-injected (Control), alloxan-injected (Alloxan) and alloxan-injected/NAC supplemented (0.4% NAC in the diet) (ALX+NAC) animals. Pancreas was removed on day 14 following saline or alloxan injection. Values are expressed as mean ± SE (n=6). Level of significance was evaluated using Tukey’s test at p<0.05.
Figure 2.8. The inhibitory effect of GSH on alloxan-induced free radical production in vitro. Alloxan monohydrate (10 mg/ml), PBN (14 mg/ml) and increasing concentrations of GSH (0-20 mM) are incubated in 0.5 g pancreatic tissue homogenate for 90 min at 37°C. PBN spin adducts were extracted and analyzed by EPR. Instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, 5 x 10^5; time constant 100 ms, scan range, 100 G and sweep time, 50 s.
Figure 2.9. Dietary supplementation of NAC reduces alloxan-induced hyperglycemia. On day 0, animals received 200 mg/kg alloxan i.p. following an overnight fast. Fasting blood glucose levels (A) and body weights (B) were monitored for 14 days. Control (▲) animals received saline injection only. Alloxan (●) treated animals were fed a control diet. NAC-supplemented animals injected with alloxan (ALX+NAC) (■) received 0.4% w/w of NAC in the diet for 7 days prior to alloxan injection and were maintained on this diet for another two weeks. Values are expressed as mean ± SE (n=6). Alloxan treated groups are significantly different from Control groups. ALX+NAC groups are not significantly different from Control group. Level of significance was evaluated using a Tukey's test at p<0.05.
Figure 2.10. **Effect of NAC supplementation on pancreatic GSH level.** Pancreatic tissue was removed from mice after the two-week feeding period of a basal and NAC supplemented diet. Due to the small size of the tissue, four pancreases were pooled for GSH analysis. Values represent the mean ± SE of triplicate measures of four pooled pancreas samples. Differences in means are determined by student’s t test with significance level p<0.05.
2.7. REFERENCES


63


CHAPTER 3

α-PHENYL-T-BUTYL-NITRONE (PBN) INHIBITS NFκB ACTIVATION OFFERING PROTECTION AGAINST CHEMICALLY-INDUCED DIABETES

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3.1. SUMMARY

PBN is an effective spin trapping agent by reacting with and stabilizing free radical species. Reactive oxygen species (ROS) have been implicated in pancreatic β cell death and the development of insulin-dependent diabetes mellitus (IDDM). We speculate that treatment with the PBN, will protect against diabetes development in two distinct chemically-induced models for IDDM. Pre-treatment with PBN (150 mg/kg i.p.) significantly reduced the severity of hyperglycemia in both alloxan- and streptozotocin (STZ)-induced diabetes. To determine the mechanism by which PBN prevents hyperglycemia, we examined the ability of PBN to inhibit NFκB activation and to stabilize alloxan- and STZ-induced radicals. Both alloxan and STZ induced NFκB activation in the pancreas 30 min after their injection (50 mg/kg i.v.). PBN pre-treatment inhibited both alloxan- and STZ-induced activation of NFκB and nitric oxide production. EPR studies showed that PBN could effectively trap alloxan-induced free radicals. It is clear that PBN can inhibit NFκB activation in the pancreas and reduce hyperglycemia in two distinct diabetogenic compounds. This research indicates that NFκB activation may be a key signal leading to β cell death and IDDM. Understanding the cellular pathways leading to β cell death may help in developing effective preventive or therapeutic targets for IDDM.
3.2. INTRODUCTION

Type 1 diabetes or insulin dependent diabetes mellitus (IDDM) is known to be a multifactorial disease [1-3]. Despite different triggering factors, the final outcome is characterized by profound destruction of the insulin-producing β cells. There is growing evidence implicating free radical species in the destruction of β-cells [4-7], however the precise cellular mechanisms leading to pancreatic β cell death have not yet been fully elucidated. Although IDDM is generally considered to be an immune disease of the pancreas, there are several known chemicals used to induce IDDM. Two commonly used diabetogenic agents are alloxan and streptozotocin (STZ). These structurally diverse compounds have a long history of use in diabetes research and are known to be specifically toxic to the pancreatic β cells. Alloxan is thought to produce reactive oxygen species (ROS) during its metabolism [8]. STZ is thought to release nitric oxide (NO) in its metabolism [9-11]. The inherent lack of antioxidant protection and the selectivity of uptake in the pancreatic islets [12, 13], may increase their sensitivity to diabetogenic agents. However, there is no direct evidence to demonstrate that the initial concentrations of ROS produced by alloxan or STZ are high enough to induce massive oxidative damage to islet cells. It is possible that other factors may be involved in amplifying their cytotoxicity. We propose that a critical determinant in β cell death leading to IDDM may be the activation of nuclear transcription factor κB (NFκB). NFκB
is a transcription factor that can be activated by a variety of stresses such as oxidants, xenobiotics, viruses and pro-inflammatory cytokines [14, 15]. Once activated, NFkB translocates to the nucleus and binds to DNA and upregulates the expression of several genes involved in inflammatory and immune responses. Target proteins include adhesion molecules such as selectins, ICAM-1 and VCAM-1 [16] and various proinflammatory cytokines such as IL-2 [17], IL-6 [18], TNF-α [19] and inducible NO synthase (iNOS) [20]. It is speculated that NFkB activation initiates and propagates the immune/inflammatory response, resulting in the invasion immune cells that produce and amplify free radical production, which ultimately destroys the β cells. NFkB activation may serve as a common mechanism for the selective cytotoxicity of structurally diverse diabetogenic compounds. Thus, compounds that can inhibit NFkB activation induced by oxidative stress may modulate the severity of IDDM.

α-Phenyl-t-butyl nitrone (PBN) is a commonly used spin trapping agent used in electron paramagnetic resonance (EPR) studies. PBN acts as an effective trapping agent by reacting with and stabilizing free radical species so they can be visualized by an EPR spectrometer. However, this stabilization property may also reduce the reactivity of the free radical species, consequently making PBN an effective antioxidant. The potential therapeutic value of PBN has been examined in other free-radical mediated diseases such as ischemia-reperfusion damage, septic shock and aging [21-23].

The objectives of this study were 1) to determine the efficacy of PBN supplementation in modulating the development of chemically induced diabetes in CD-1 mice, and 2) to investigate the ability of PBN in inhibiting NFkB activation and
stabilizing free radicals produced by diabetogenic chemicals. Alloxan and STZ's specificity to the pancreatic islets, combined with their ability to produce free radicals, provides us excellent models for delineating the role of oxidative stresses on β cell damage and the development of IDDM.
3.3. MATERIALS AND METHODS

**Animals.** Weanling male CD-1 mice (Harlan, Indianapolis, IN), weighing 20-25 g were used in all experiments. All animals were housed in individual cages in a temperature controlled environment (22 ± 2°C) with a light period between 0600 to 1800 hrs. Animals were allowed free access to the diet with the exception of fasting periods for blood glucose determination. Animal protocol was approved by the OSU Institutional Laboratory Animal Care and Use Committee.

**Effect of PBN on Alloxan-induced Diabetes.** To determine the dose-response of alloxan-induced diabetes, weanling CD1 mice were injected with 150, 200 or 300 mg/kg alloxan (i.p) following an overnight fast and the fasting blood glucose levels were monitored daily for 9 days. To examine the effect of PBN on the development of alloxan-induced diabetes, PBN (150 mg/kg body weight) dissolved in saline was administered 30 min prior to alloxan injection. Control animals received sham saline injection. Blood was obtained from the intra-orbital sinus following an 8 hr fast using a 10 uL capillary tube. Glucose concentrations were measured using the ONE-TOUCH ™II complete blood glucose monitoring kit. To minimise the effects of diurnal fluctuations, blood samples were collected at the same time every day.

**Effect of PBN on STZ-induced Diabetes.** Multiple low doses of STZ, a commonly used method to induce diabetes were employed [24]. STZ (40 mg/kg) dissolved in citrate buffer (pH 4.0), was immediately injected (i.p.) into mice (n = 5) for 5 consecutive
days. Control animals received a sham saline injection. Fasting blood glucose levels were monitored over 25 days. Glucose concentrations were measured using the ONE-TOUCH™ complete blood glucose monitoring kit.

To examine the effect of PBN on the development of STZ-induced diabetes, PBN (150 mg/kg body weight) dissolved in saline was administered 30 minutes prior to STZ injections for 5 consecutive days. Fasting blood glucose levels were monitored over 25 days.

**Determination of NFκB Activation *In Vivo.*** For NFκB activation analysis, mice were injected with 50 mg/kg alloxan or 50 mg/kg STZ (i.v.) and sacrificed at 30 min after injection (n=5 in each time interval). Pancreas was immediately removed. NFκB activation was determined using an electrophoretic mobility shift assay (EMSA). To determine the effect of PBN on alloxan- or STZ-induced NFκB activation, animals were injected with PBN (150 mg/kg) 30 min prior to alloxan or STZ administration.

Crude nuclear extracts were prepared from pancreatic tissue as described by Deryckere and Gannon [25]. Double stranded synthetic oligonucleotides probes for NFκB (5'-AGTGAGGGGACTTTCCCAGGC-3') (Promega, Madison, WI) were end-labeled using [γ-32P] (Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). Binding reactions containing equal amounts of protein (~7 ug) and labelled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (4% glycerol, 1mM MgCl₂, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris, 50 ug/ml poly [dl-dC]. Specific binding was confirmed using 100-400 fold excess unlabelled NFκB oligonucleotide as a specific competitor. Protein-DNA complexes were separated using 6% nondenaturing
polyacrylamide gel electrophoresis followed by radiography to detect the level of retardation produced by binding to NFκB probe.

**Nitrite Analysis.** Pancreatic tissue was homogenized in 100 mM HEPES buffer and then centrifuged at 100 000 x g for 60 min. Nitrite formation was used as an indirect measure of nitric oxide (NO) production. Nitrite concentration was determined using the Griess reaction as described by Hevel & Marietta [26]. Protein concentration was determined using the Lowry method [27].

**Ability of PBN to Stabilize Alloxan- and STZ-induced Radicals.**

Pancreatic or liver tissue (0.5g) was homogenized in 150 mM phosphate buffer. Homogenates were then incubated with 20 mg/ml alloxan or STZ and 14 mg of spin trap, PBN (Sigma, St. Louis, MO) at 37°C for 90 min. Control samples contained PBN only. Following the incubation period, PBN spin adducts were extracted with 6 ml of benzene. These adducts were concentrated to a 0.2 ml sample and de-gased with nitrogen gas before EPR analysis. Samples were placed in quartz round cell and analyzed at room temperature with a Bruker X band EPR spectrometer operating at 9.78 GHz. Extractions and concentration was performed as previous described PBN adducts were analyzed by a Bruker X band spectrometer. For identification of PBN-adduct, solutions were transferred to hexane for normal phase HPLC or to acetonitrile/water (70/30) for reverse phase HPLC.

**Identification of PBN-adduct by HPLC.** HPLC analyses were performed on a Hewlett Packard 1050 Liquid Chromatographic Series HPLC. Two HPLC systems were used for analyses.
A) **Normal phase system**: A variable UV absorbance detector set at 289 nm, a bondclone 10 silica column (300 x 3.9 mm; Phenomenex, Torrance, CA) were employed. The mobile phase was a mixture of hexane/isopropanol (100/3). The flow rate was 1.0 ml/min and the UV spectrum was recorded at a chart speed 0.25 cm/min.

B) **Reverse phase system**: An ODS Spheri-5 (4.6 mm x 22 cm) column was utilized and acetonitrile-water (70/30) containing 0.02 M phosphate buffer (pH 7.4) was selected as the mobile phase. A flow rate of 0.7 ml/min and chart speed 0.5 cm/min was used. An electrochemical detector with a pyrolytic graphite-working electrode at 0.7 V vs. Ag/AgCl reference electrode was connected for simultaneous monitoring.

**Synthesis Of Alloxan Free Radical Adduct By Photolysis.**

PBN (50 mg) and alloxan (20 mg) were added to 2 ml benzene and degassed with nitrogen for 10 min. The solution was illuminated by UV light under stirring and nitrogen degassing for 12 min. The UV light was generated from 200 W Hg, using 5.2 amperes by an Arc lamp power supply. The adduct produced was transferred to hexane for normal phase HPLC or to acetonitrile/water (70/30) for reverse phase HPLC.

**Subcellular Distribution of Alloxan Free Radical Adduct.**

Mouse tissue (3 g) was homogenized in 3 ml of 150 mM phosphate buffer containing 100 mM EDTA. Homogenates were incubated with 60 mg/ml alloxan and 14 mg/ml PBN for 90 min at 37°C. Following incubation period, differential centrifugation was performed to separate subcellular components. To remove the nuclear fraction, samples were centrifuged at 600xg, the pellet was removed, washed and resuspended in phosphate buffer. The first supernatant was again centrifuged at 10 000xg to separate the mitochondrial fraction. The mitochondrial pellet was removed, washed and resuspended in phosphate buffer.
The second supernatant was centrifuged at 100,000xg for 60 min. The pellet containing the microsomal fraction was removed, washed and resuspended in phosphate buffer. The cytosolic supernatant was also removed. PBN spin adducts were extracted with 6 ml of benzene. These adducts were concentrated to a 0.2 ml sample and degased with nitrogen gas before EPR analysis. Samples were placed in quartz round cell and analyzed at room temperature with a Bruker X band EPR spectrometer operating at 9.78 GHz.

**Statistics.** One way analysis of variance (ANOVA) was performed to assess the differences between diabetic, PBN treated and control groups at various endpoints. Difference in means among treatments was tested by using Duncan’s test. Level of significance was evaluated at p<0.05.
3.4. RESULTS

Hyperglycemia, a commonly used marker for the prevalence of diabetes was used to assess the severity of IDDM. Mice injected intraperitoneally (i.p.) with alloxan at the level or higher than 150 mg/kg BW display symptoms characteristic of diabetes including hyperglycemia, weight loss and polyuria. Animals injected with alloxan alone showed significantly elevated fasting blood glucose levels compared to controls (Figure 3.1A, 3.1B and 3.1C). A dose-dependent increase in the severity of hyperglycemic following injection (i.p.) of increasing doses of alloxan was observed. Pretreatment of PBN attenuated the severity of alloxan-induced hyperglycemia at all three doses (Figure 3.1). Treatment with PBN significantly reduced fasting blood glucose levels in mice injected with 200 and 300 mg/kg alloxan (Figure 3.1A and 3.1B). At a dose of 150 mg/kg alloxan (Figure 3.1C), fasting blood glucose levels were brought back to that of control with PBN treatment.

Multiple low-dose STZ model was used to induce hyperglycemia. In mice injected with multiple doses of STZ, fasting blood glucose levels begin to rise shortly after last injection on Day 5 and continues to stay elevated through Day 25 (Figure 3.2). Similar to alloxan-induced diabetes, pre-treatment of PBN prior to STZ injections attenuated the hyperglycemic response.

Figure 3.3 illustrates that the pre-treatment of PBN inhibits alloxan-induced NFκB activation in pancreas in vivo. Similar to previous studies, maximal NFκB
activation was observed 30 min following alloxan administration (lane 2). However, pre-treatment of PBN (30 min prior to alloxan administration) fully inhibited the activation of NFκB (lane 4). N-acetylcysteine (NAC) is known to inhibit NFκB activation. Thus NAC treatment (500 mg/kg i.p.) with alloxan acts as an external control for comparison. This result clearly demonstrates that pretreatment with PBN effectively inhibited alloxan-induced NFκB activation in the pancreas compared to non-treated animals.

**Figure 3.4A** illustrates the time course of NFκB activation following administration of STZ. STZ-induced activation of NFκB *in vivo* has not been previously demonstrated. Lanes 2, 4, 6 and 8 confirm the specificity of NFκB binding using a cold specific competitor reaction (see Methods). At time 0, no NFκB activation is observed, and maximal activation of NFκB is evident 30 min following injection of STZ. **Figure 3.4B** demonstrates that similar to alloxan, PBN pre-treatment inhibits STZ-induced NFκB activation in the pancreas. Saline injected animals show no activation (Lane 1) and STZ-injected animals show significant activation at 30 min following injection (Lane 3). However, PBN-treated animals show markedly lower levels of activation with STZ (Lane 5). Thus, PBN is also effective inhibiting STZ-induced NFκB activation.

One downstream target of NFκB activation is the inducible form of nitric oxide synthase (iNOS). Induction of this protein can be indirectly assessed through the measurement of changes in NO production. To determine the effect of alloxan and PBN on NO production, pancreatic nitrite levels were determined in the pancreas from animals on Day 9 following alloxan injection. **Figure 3.5** demonstrates that animals injected with alloxan (Alloxan) show a significant elevation in pancreatic nitrite levels compared to saline-injected controls (Control). Animals that were pre-treated with PBN and injected
with alloxan (ALX + PBN) showed no increase in nitrite levels compared to controls (Control). Thus, the results suggest indirectly that iNOS, a down stream target of NF\(\kappa\)B, is induced after alloxan exposure. Pre-treatment with PBN can inhibit this induction, possibly through inhibition of NF\(\kappa\)B.

EPR spin trapping studies were performed to assess the ability of PBN to trap and stabilize alloxan-induced free radicals. Figure 3.6 shows the EPR spectra of PBN spin adducts of alloxan-induced free radicals in pancreatic and liver homogenate. A more intense signal can be detected in pancreas compared to liver homogenates when identical amounts of tissue are used. Hyperfine splitting constants are \(a^N = 14.46\) and \(a^H = 2.38\). These splitting constants suggest that the radical detected is a carbon centered radical.

To identify the primary radical trapped with PBN, we compared the hyperfine splitting constants of the EPR spectra obtained from tissue homogenate (\(a^N = 14.46, a^H = 2.38\)) and from a photolysis reaction (\(a^N = 14.50, a^H = 2.31\)). These similar splitting demonstrates that PBN-alloxan adduct generated from tissue homogenates and photolysis reactions are most likely identical (Table 3.1 and Figure 3.7). HPLC studies demonstrate that retention times of the PBN adduct both in the homogenate and photolysis reaction, are also identical (Table 3.1). This confirms that the primary free radical produced is most likely an alloxan radical.
Subcellular distribution of alloxan adduct was investigated in order to find the possible site of metabolism of the alloxan or target of alloxan radical in the cells. The strongest signal of alloxan radical was detected in the nuclear fraction (Figure 3.8). Markedly smaller EPR signals are also present in the mitochondrial, microsomal and cytosolic fractions. This suggests that the alloxan radical is either being produced primarily in the nuclear region of the cell or the nuclear region is the main target of the alloxan radical.
3.5. DISCUSSION

The results clearly demonstrate that pretreatment of the spin-trapping agent, PBN is capable of reducing the severity of hyperglycemia induced by two structurally diverse diabetogenic compounds in CD-1 mice (Figures 3.1 and 3.2). To determine the mechanism that PBN protects against alloxan- or STZ-induced diabetes, we examined the ability of alloxan and STZ to induce the activation of NFκB and determined if PBN could inhibit NFκB activation and its downstream target. There is no doubt that both alloxan and STZ induce NFκB activation in vivo (Figures 3.3 and 3.4). This is the first time that STZ administration has been shown to induce NFκB activation in pancreas in an in vivo model. It is also interesting to note that both alloxan [28] and STZ (Figure 3.4A) rapidly induce NFκB activation in pancreas within 30 min after i.v. injection. In another commonly used model for IDDM using IL-1 in culture, NFκB activation is apparent in isolated islets following IL-1 exposure in a similar time frame [29].

Pretreatment with PBN 30 min prior to alloxan or STZ injection effectively inhibits NFκB activation in the pancreas in both models (Figures 3.3 and 3.4B). Previous work has shown that PBN is maximally distributed to tissues within 30 min [30]. The mechanism by which PBN inhibits alloxan-induced diabetes and NFκB activation may be through its ability to trap and stabilize free radicals. We have previously demonstrated that another antioxidant, N-acetylcysteine (NAC), can also inhibit alloxan-induced NFκB activation and significantly reduce the severity of
hyperglycemia and weight loss associated with IDDM development. EPR spin trapping
studies suggest that PBN can stabilize alloxan-induced free radicals (Figure 3.6) and the
radical species trapped is an alloxan radical (Table 3.1). In the metabolism of alloxan,
both an alloxan radical and ROS can be produced. The ability of alloxan to produce free
radicals has been determined by several investigators [8, 31-33]. For example,
production of an alloxan radical has been demonstrated by EPR experiments [34].
Although we did not detect ROS-PBN adducts, it is possible that these adducts are simply
not stable enough to be detected. The detection of various EPR signals largely depends
on the rate of production and decay. Carbon-centered radicals such as CCl₄ radical, are
highly stable PBN adducts [35]. Thus the prevalence of the carbon-centered alloxan
radical may be due to an increased stability compared to ROS adducts. There also exist
several lines of indirect evidence for ROS production during alloxan metabolism. For
example, addition of ROS scavengers such as superoxide dismutase, catalase and
hydroxyl scavengers [36, 37] protect against alloxan cytotoxicity in vitro which suggests
that ROS are in fact produced with alloxan. A second possibility is that although PBN
does not trap ROS, PBN may indirectly reduce ROS production by trapping the alloxan
radical and subsequently blocking dialuric acid cycling that produces ROS.

In attempts to understand the sites of alloxan metabolism or cytotoxicity, we
performed EPR experiments following subcellular fractionation in an in vitro system.
We expected to find the alloxan radical in the microsomal fraction, the site of mixed
function oxidase (MFO) systems. However, subcellular distribution studies revealed that
the alloxan radical resides predominantly in the nucleus. Peak height is an indicator of
free radical concentration. Figure 3.8 clearly shows that signal intensity (peak height) is
much greater in the nuclear fractions. It is unlikely such a marked difference can be due to contamination or solubility differences. Thus, it appears the alloxan radical either is largely produced in the nucleus, or the nuclear region is the primary site of radical attack. This leads us to believe that alloxan metabolism may not be through classic xenobiotic metabolism pathways. It is currently unknown why the alloxan radical is concentrated in the nucleus and what precise functional consequence it has. It has been shown that alloxan induces islet DNA strand breaks [38, 39]. In vitro, there is evidence for increased 8-oxodG formation, a marker for oxidative DNA damage, in calf thymus DNA with alloxan [34]. Future studies that precisely identify subcellular distribution and localization using radiolabeled alloxan are needed. The functional consequences of the nuclear distribution of the alloxan radical, including the determination DNA adduct formation, should be also examined in our IDDM models.

The mechanism by which PBN inhibits STZ-induced hyperglycemia is not clear. It is well known that STZ releases NO in its metabolism [10]. We were however unable to detect the presence of PBN-STZ or NO adducts in the pancreas. Other investigators, have shown using N-methyl-D-glucamine dithiocarbamate have (MGD) as a spin trap, that PBN can reduce MGD-NO production by STZ [40]. The peak height of NO radicals trapped with MGD is significantly reduced when PBN is co-injected with STZ compared to STZ alone. The mechanism by which PBN inhibits NO production may be through the direct inhibition of NFκB activation (Figure 3.4B). This concept is supported by other disease models that are known to produce NO. In a model for septic shock, PBN has shown to inhibit NFκB activation and both mRNA and protein expression of iNOS.
induction following LPS treatment [21, 41]. Thus, the effect of PBN on STZ-induced hyperglycemia may primarily be through inhibition of NFκB activation and the downstream regulation of iNOS transcription. These observations are consistent with our hypothesis that NFκB activation is a central signal in the diabetogenic process.

The role of ROS and NFκB in the pathogenesis of many human inflammatory and autoimmune diseases has been gaining widespread attention [42]. The use of pharmacological agents that target NFκB activation has become a new therapeutic strategy for many of these diseases including inflammatory bowel disease [43], atherosclerosis [44] and rheumatoid arthritis [45] and septic shock [46]. Although IDDM is largely considered to be an immune disorder, this link between ROS and cellular response is a relatively unexplored area of research, leaving a large gap in the understanding of the mechanisms that signal β-cell death. NFκB is a ubiquitous rapid response transcription factor in cells involved in immune and inflammatory reactions. In our laboratory, we have confirmed that alloxan and STZ specifically activates NFκB in the pancreas within 30 min of injection. No activation is seen in other tissues such as liver [28]. It is speculated that NFκB contributes to immunologically mediated diseases by exerting its effect on the regulation of the expression of genes that participate in pathways involving inflammation or susceptibility to apoptosis. We have not determined any specific expression of cytokines, chemokines, cell adhesion molecules, and immunoreceptors after NFκB activation induced by alloxan or STZ in this study. However, it has been shown that NO production in the pancreas is elevated by alloxan [28] and STZ [10]. An increase in NO production is most likely through the induction of
iNOS, another downstream target of NFκB. Although iNOS is known to be stimulated during inflammatory and immune responses, we currently are unable to determine if iNOS is being induced in the pancreatic islets themselves, or from infiltrated immune cells. To determine the source of NO, in situ experiments that cross-stain for iNOS and infiltrated immune cells can be performed. Future studies investigating the expression of various cytokines, chemokines and adhesion molecules to directly confirm the link between free radicals, NFκB, inflammation and IDDM also need to be pursued.

The NFκB activation process can be inhibited by antioxidants or pharmacologic agents at several different points of the activation pathway. For example, glucocorticoids inhibit NFκB by directly upregulating IκB expression, thereby trapping NFκB in inactive cytoplasmic complexes or by interfering with NFκB transactivation [47]. Cyclosporine and protease inhibitors prevent NFκB activation by inhibiting IκB degradation [48]. Deoxyspergualin inhibits NFκB activation by blocking its nuclear translocation [49]. Aspirin and salicylates inhibit upstream events inducing IκB phosphorylation [50]. Some antioxidants inhibit NFκB activation by influencing the redox state of the cell. We have previously shown that N-acetylcysteine (NAC) supplementation increased pancreatic glutathione levels, inhibited alloxan-induced NFκB activation and concurrently reduced the severity of diabetes [28]. In this study, PBN appears to have more than one possible mechanism to inhibit NFκB activation, e.g. through a direct inhibitory effect on the activation cascade or indirectly through a reduction of oxidative stress or both. Regardless of the mechanisms, the inhibition of alloxan- and STZ-induced NFκB activation in pancreas and concurrently reduced the severity of hyperglycemia with PBN.
treatment is consistent with our hypothesis that NFκB activation is a critical signal in the development of IDDM.

If NFκB activation is a critical step in the diabetogenic process, this knowledge will act as a useful tool for screening different classes of compounds, such as antioxidants, for their value in IDDM therapy or prevention. Selective modulation of NFκB activation or its downstream effect could lead to the attenuation of the disease outcomes. A focus on the control of cellular response pathways will bring the field to a level of understanding that is needed to develop effective preventative strategies. This research is the first step in unifying the many factors to ROS signaling in the diabetogenic processes in a relevant in vivo system and will help move us closer to finding effective treatment or prevention strategies for IDDM.
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**Table 3.1:** Comparison of Products obtained from Tissue Homogenate or Photolysis
Figure 3.1. Pre-treatment with PBN reduces alloxan-induced hyperglycemia at several three doses of alloxan. Mice (n = 5 at each dose) were injected with A) 300 mg/kg alloxan, B) 200 mg/kg alloxan, or C) 150 mg/kg alloxan (-♦ - Alloxan + Sham). PBN (150mg/kg i.p.) was injected 30 min prior to alloxan injections (i.p.) (-■- Alloxan-injected + PBN). Controls (-▲-) were injected with saline only. Mean values were compared at each day using one-way ANOVA. Level of significance was evaluated using Duncan’s test at p<0.05.
Figure 3.2. Pre-treatment with PBN reduced STZ-induced hyperglycemia. Mice (n = 5) were injected with 40mg/kg STZ for 5 consecutive days (-●-, STZ + Sham). PBN (150 mg/kg i.p.) was injected 30 min prior to STZ injections (-■-, STZ-injected + PBN). Controls (-△-) were injected with saline only. Mean values were compared at each day using one-way ANOVA. PBN treated groups are significantly different than untreated groups at all days, with the exception of day 0, 1 and 2. Level of significance was evaluated using Duncan’s test at p<0.05.
Figure 3.3. Administration of PBN inhibits alloxan-induced NFκB activation in pancreas. Control group received only sham saline injections. Alloxan group received alloxan only (50 mg/kg i.v.). ALX+PBN group was injected with 150 mg/kg PBN i.p. 30 min prior to alloxan (50 mg/kg BW i.v.). N-acetylcysteine (NAC) is known to inhibit NFκB activation. ALX+NAC group was used for external comparison. Pancreatic tissue was removed from mice 30 min following alloxan injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This Figure is a representative of 2 individual experiments.
Figure 3.4: (A) Time Course of NFkB Activation following STZ injection. Mice were injected with 50 mg/kg STZ i.v. A total of 5 pancreas samples were pooled together for nuclear extract preparation at each time point. Lanes 2, 4, 6, 8 are specific competitor reactions (unlabelled NFkB oligo) to confirm the specificity of NFkB binding. These figures are representative of 2 individual experiments.

(B) Pre-treatment with PBN inhibits STZ-induced NFkB activation in pancreas. Control group received only sham saline injections. STZ group received STZ only (50 mg/kg i.v.). STZ+PBN group was injected with 150 mg/kg PBN i.p. 30 min prior to STZ(50 mg/kg BW i.v.). Lanes 2, 4 and 6 are specific competitor reactions (unlabelled NFkB oligo) to confirm specificity of NFkB binding. Pancreatic tissue was removed from mice 30 min following STZ injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This Figure is a representative of 2 individual experiments.
Figure 3.5. Pretreatment of PBN inhibits alloxan-induced NO formation in the pancreas. Nitrite production was used as an indirect measure of NO production. Nitrite levels were assessed in saline-injected (Control), alloxan-injected (Alloxan) and alloxan-injected/PBN supplemented (150 mg/kg PBN) (ALX+PBN) animals. Pancreas was removed on day 14 following saline or alloxan injection. Values are expressed as mean ± SE (n = 6). Comparison of means was performed using one-way ANOVA. Level of significance was evaluated using Duncan’s test at p<0.05.
Figure 3.6. EPR spectra of PBN spin adducts of alloxan-induced free radicals trapped in pancreatic and liver homogenates. Alloxan monohydrate (10 mg/ml) and PBN (14 mg/ml) were incubated in 0.5 g pancreatic or liver tissue for 90 min at 37°C. PBN spin adducts were extracted and analyzed by EPR. Instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, $5 \times 10^3$; time constant 100 ms, scan range, 100 G and sweep time, 50s.
Figure 3.7. Identification of PBN adducts generated from alloxan.

(A) PBN-alloxan adducts generated in tissue homogenate: alloxan monohydrate (20 mg/ml) and PBN (14 mg/ml) were incubated in tissue homogenate for 90 min at 37°C. PBN spin adducts were extracted and analyzed by HPLC and EPR.

(B) PBN- alloxan radical produced by photolysis: alloxan monohydrate (20 mg/ml) and PBN (50 mg/ml) were incubated in 2 ml benzene exposed to UV light for 12 min. PBN spin adducts were extracted and analyzed by HPLC and EPR.

A* and B*: normal phase HPLC of products from tissue homogenate or photolysis reaction.
A^ and B^: reverse phase HPLC of products from tissue homogenate and photolysis reaction.

EPR instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, 5 x 10^3; time constant 100 ms, scan range, 100G and sweep time, 50s.
Figure 3.8. Subcellular Distribution of Alloxan-induced Free Radical Adducts. Tissue (3 g) was homogenized in 0.15 M phosphate buffer and incubated with 60 mg/ml alloxan and 14 mg/ml PBN for 90 min at 37 °C. Samples were then sequentially centrifuged at 600xg, 10 000xg and 100 000xg to retrieve nuclear (A), mitochondrial (B), microsomal (C) and cytosolic (D) fractions. In each fraction, PBN spin adducts were extracted and analyzed by EPR. EPR instrument settings were: microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, $5 \times 10^2$; time constant 100 ms, scan range, 100G and sweep time, 50s.
3.6. REFERENCES


CHAPTER 4

DIETARY ZINC SUPPLEMENTATION INHIBITS NFκB ACTIVATION AND PROTECTS AGAINST THE DEVELOPMENT OF INSULIN-DEPENDENT DIABETES MELLITUS

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4.1 SUMMARY

Zinc status in patients with insulin-dependent diabetes mellitus (IDDM) is significantly lower than healthy controls. However, the role of zinc in the etiology of IDDM is still unknown. Recent studies have suggested that the generation of reactive oxygen species (ROS) is a cause of β cell death leading to IDDM. NFκB is a redox sensitive transcription factor that regulates immune responses. NFκB activation may be the key cellular oxidative signal in initiating and amplifying the cascade of events leading to β cell death. Zinc is a known antioxidant and is important in immune function. Therefore, this study is designed to test the hypothesis that NFκB activation in the pancreas is the initial signal leading to β cell death, and that an increase in dietary zinc inhibits NFκB activation and prevents the onset of IDDM. The results show that high zinc intake significantly reduced the severity of IDDM (based on hyperglycemia, insulin level and islet morphology) in alloxan and streptozotocin-induced diabetes. Electromobility shift assays (EMSA) show that zinc supplementation inhibited NFκB activation. Western blot revealed a decrease in the expression of inducible NO synthase, a downstream target gene of NFκB, with higher zinc intake.

It is concluded that antioxidants such as zinc that modulate key cellular signals in the diabetogenic pathway may be better nutritional strategies for IDDM prevention.
4.2 INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a devastating disease that occurs most often in children or young adults. This disease is characterized by the profound destruction of the β cells in the pancreatic islets, resulting in the inability to produce insulin. Without insulin, severe disturbances in glucose metabolism result, causing intracellular starvation and a dramatic elevation in blood glucose levels (hyperglycemia). Patients are dependent on exogenous insulin therapy for life. However, even with insulin therapy, numerous life-threatening complications can result in these patients, such as cardiovascular disease, neuropathy, retinopathy and kidney failure.

Although it is known that the pancreatic islets cells are destroyed in IDDM, it is unclear the exact mechanism which causes their death. There is increasing evidence that excess free radical production may contribute to the death of the β cells (1-4). It is hypothesized that the inherent lack of antioxidant protection in the pancreatic islets (5, 6) may render the islets more susceptible to damage by free radicals produced during inflammatory and immune processes, which lead to β cell death. The overall objective of the present study is to examine the effect of the antioxidant nutrient, zinc, in the development of IDDM and explore the cellular mechanisms by which zinc exerts its effects.
Recently, the efficacy of several antioxidant agents in preventing or delaying the onset of IDDM in pre-diabetic patients (7-10) and animals (11-15) has been tested. Zinc supplementation shows promise in defending the islet cells from damage due to its antioxidant capabilities and its unique relation to carbohydrate metabolism, insulin and immune function (16-20). In addition, decreases in zinc status have been documented in both IDDM patients and in animal models for the disease (21-25). Zinc deficiency is also associated with impairment in glucose tolerance (26, 27) and an increased sensitivity to diabetogenic agents. It is possible that compromised zinc status in diabetics may predispose these individuals to external triggers for IDDM.

In our laboratory, we have shown that free radical induced activation of NFκB may be a key cellular signal in initiating the sequence of events leading to β cell destruction (28). NFκB is an important redox sensitive transcription factor in modulating immune and inflammatory response (29). Zinc is known to have significant impact on immune function (30), however, zinc's effect on NFκB activation remains relatively unexplored. If NFκB is a central signal in the diabetogenic pathway, then strategies that can limit this activation should prove to be beneficial for disease prevention.

The aims of this study were to investigate the effect of dietary zinc supplementation on NFκB activation in two in vivo models for IDDM and to test the efficacy of dietary zinc supplementation in protecting against the development of IDDM.
4.3 MATERIALS AND METHODS

**Animals.** Weanling male CD-1 mice (Harlan), weighing 20-25 g were used in all experiments. All animals were housed in individual cages in a temperature controlled environment (22 ± 2°C) with a light period between 0600 to 1800 hrs. Mice were randomly allocated to one of 3 zinc groups; normal (50 ppm zinc) or high zinc (500 or 1000 ppm zinc). All animals were fed diets based on a modified AIN93G rodent diet (31) with additional zinc as zinc carbonate (Dyets). Animals were allowed free access to the diet with the exception of fasting periods for blood glucose determination. Animal protocol was approved by the OSU Institutional Laboratory Animal Care and Use Committee.

**Serum and Pancreatic Zinc and Copper Levels.** Serum and pancreatic zinc and copper levels were assessed according the method by Luterotti et al. (32). Briefly, serum or pancreatic homogenates were digested in 2N hydrochloric acid for 24 hours at room temperature. Samples were then centrifuged at 7000 x g for 25 minutes and the supernatant was used for direct measurement of metal concentrations using an atomic absorption spectrometer (Model AA-5, Varian Techtron, Australia).

**Determination of NFκB Activation In Vivo.** For NFκB activation analysis, mice were fed for 2 weeks on respective zinc diets and were then injected with 50 mg/kg alloxan or 50 mg/kg STZ (i.v.) (Sigma). Control animals received saline injections. All mice were sacrificed 30 min after injection (n=5) and pancreas was
immediately removed. NFkB activation was determined using an electrophoretic mobility shift assay (EMSA). Crude nuclear extracts were prepared from pancreatic tissue as described by Deryckere and Gannon (33). Double stranded synthetic oligonucleotides probes for NFkB (5'-AGTGAGGGGACTTTCCCAGGC-3') (Promega) were end-labeled using [γ-32P] (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim). Binding reactions containing equal amounts of protein (~10 μg) and labelled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP-40, 50 μg/ml poly [dl-dC]. Specific binding was confirmed using 100-400 fold excess unlabelled NFkB oligonucleotide as a specific competitor. Protein-DNA complexes were separated using 6% nondenaturing polyacrylamide gel electrophoresis followed by radiography to detect the level of retardation produced by binding to NFkB probe.

Effect of Zinc Supplementation on Alloxan and STZ-induced Diabetes.

To examine the effect of zinc on the development of alloxan and STZ-induced diabetes, mice were started on unsupplemented and zinc supplemented diets for two weeks prior to alloxan or STZ injections. Alloxan was administered i.v. (50 mg/kg) to induce diabetes. For STZ-induced diabetes, multiple low doses of STZ, a commonly used method to induce diabetes were employed (34). STZ (40 mg/kg) dissolved in citrate buffer (pH 4.0), was immediately injected (i.p.) into mice (n = 5) for 5 consecutive days. Control animals received sham saline injection. Blood was obtained from the intra-orbital sinus following an 8 hr fast using a 10 uL capillary tube. Glucose concentrations were measured using the ONE-TOUCH ™II complete blood glucose monitoring kit. To minimize the effects of diurnal fluctuations, blood samples were collected at the same
time every day. Hyperglycemia was monitored for 9 days after injections. On Day 9, plasma insulin levels were assessed using ELISA (CrystalChem).

**Histology**
Pancreas from each group were removed on Day 10 and immediately immersion-fixed in neutral buffered formaldehyde (4%) for 24 hours. Tissues were then paraffin embedded using standard histological techniques. Serial sections were cut (4 μM) and stained with hematoxylin and eosin (H & E) for pathology studies. Adjacent sections were used for immunocytochemical studies as detailed below.

**Immunocytochemistry.** Immunocytochemical localization of insulin by polyclonal anti-insulin antibody (Dako) was used. Cellular localization was determined in paraffin-embedded sections by an indirect labeled avidin-biotin-peroxidase method with diaminobenzidine as the substrate.

**iNOS and MT Protein Expression.** iNOS and MT protein expression in the pancreas in was determined by Western blot analysis. Pancreatic homogenates containing equal protein were mixed with an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, bromphenol blue ) and boiled for 5 minutes. SDS electrophoresis was carried out under standard conditions (35). Protein was transferred from the SDS gel to nitrocellulose membranes (Biorad) at 50 mA overnight. iNOS blots were blocked with in 5% non-fat dry milk for 1 hour at 37°C. Blots were incubated for 3 hours at 37°C with rabbit anti-mouse antibody (Alexis Biochemical) at a dilution of 1:2000. The blots were washed 5 times with PBS+0.2%Tween 20, then incubated with horseradish peroxidase-conjugated donkey anti-rabbit antisera (Sigma) at a dilution of 1:4000, for 1 hour at 37°C. iNOS was detected by enhanced chemiluminescence using Hyperfilm and ECL reagents (Amersham). of 1:2000. For MT, the primary antibody used was a mouse anti-human
MT antibody (Zymed) at a dilution of 1:2000. For secondary antibody, blots incubated with horseradish peroxidase-conjugated donkey anti-mouse antisera (Sigma) at a dilution of 1:4000.

Statistics. One way analysis of variance (ANOVA) was performed to assess the differences between control and zinc treatments. Difference in means among treatments was tested by using Duncan's test. Level of significance was evaluated at p<0.05.
4.4 RESULTS

To establish the influence of our diets on zinc status, serum and pancreatic zinc determinations were taken following a 2-week feeding period of diets containing 50, 100, 500 or 1000 ppm zinc. There was also a concern that high zinc supplementation may compromise copper status, thus we concurrently measured serum and pancreatic copper levels. Figure 4.1 shows both serum and pancreatic zinc and copper levels following the 2 week feeding period with each zinc diet. A significant enhancement in serum and pancreatic zinc could only be seen in animals fed a diet containing 500 ppm and higher. The diet containing 50 ppm zinc closely approximates the level of zinc recommended by the 93AIN guidelines. Supplementation with high amounts of zinc did not appear to compromise copper status in these mice. No significant change in both serum and pancreatic copper levels were found with high zinc supplementation. Zinc supplementation also did not affect body weight gain or food intake in these mice (data not shown). Since zinc status was only improved at levels of 500 ppm and higher, we chose to use 500 ppm and 1000 ppm zinc levels for our diabetic intervention studies.

To determine if dietary zinc supplementation could inhibit pancreatic NFκB activation, electromobility shift assays (EMSA) were performed. Figure 4.2 shows the EMSA experiment in alloxan-induced diabetes. Zinc supplementation itself had no impact on NFκB activation (lanes 3-5). In contrast, we could see significant activation of
NFκB in the pancreas of mice injected with alloxan. Animals that were injected with alloxan and fed the normal dietary zinc level (50 ppm) show significant activation of NFκB (lane 5). In contrast animals that are fed higher zinc levels, demonstrate a significant inhibition of alloxan-induced NFκB activation (lane 6 and 7). Animals supplemented with 1000 ppm zinc have an almost complete inhibition of NFκB (lane 7). These findings demonstrate that zinc supplementation was able to effectively inhibit alloxan-induced NFκB activation.

A similar inhibitory effect was seen with zinc supplementation in the STZ-induced diabetic model (Figure 4.3). Lane 1 shows no activation of NFκB in the pancreas of animals injected with saline. However, we can see significant activation of NFκB in the pancreas of animals injected with STZ but fed the normal 50 ppm zinc diet (lane 2). In contrast, animals that are fed the 1000 ppm zinc diet show a significant inhibition of this activation. Thus, high zinc supplementation also appears to effectively inhibit STZ-induced NFκB activation, as shown by EMSA. To verify the effect of zinc supplementation on NFκB transactivation, we assessed the expression of NFκB responsive genes. One protein, which is downstream of NFκB is the inducible form of nitric oxide synthase (iNOS). Figure 4.4 represents the Western blot for iNOS following STZ injection in unsupplemented and zinc supplemented animals. A significant increase in iNOS expression is apparent with STZ injections in the pancreas of unsupplemented animals (50 ppm). Mice supplemented with 500 ppm zinc prior to STZ exposure also show significant iNOS protein expression. In contrast, mice supplemented with 1000 ppm zinc prior to STZ exposure show a significant reduction in iNOS
expression. Thus, high zinc supplementation effectively inhibited iNOS induction with STZ. These results correspond with the effect of zinc supplementation on NFκB translocation.

If zinc supplementation is able to inhibit alloxan and STZ-induced NFκB activation, we can predict that supplementation will also inhibit the development of IDDM. Figure 4.5 illustrates the ability of zinc to inhibit both alloxan and STZ-induced hyperglycemia. In figure 4.5A, we can see that injection with alloxan (50 mg/kg i.v.) causes a significant elevation in fasting blood glucose levels in mice fed the normal 50 ppm zinc diet compared to saline injected controls. Alloxan treated animals supplemented with 1000 ppm zinc show a marked reduction in blood glucose levels compared to unsupplemented animals fed 50 ppm zinc. Reduced hyperglycemia was only seen in the initial days in animals supplemented with 500 ppm zinc. By Day 9, no difference in blood glucose levels was detected between 500 ppm supplemented and 50 ppm unsupplemented animals. Figure 4.5B shows that zinc supplementation had a similar protective effect in STZ-induced diabetes. Multiple low dose injections of STZ caused marked hyperglycemia in unsupplemented animals fed 50 ppm zinc. Supplementation with 500 ppm zinc had little effect on reducing STZ-induced hyperglycemia. On the other hand, supplementation with 1000 ppm zinc significantly reduced fasting blood glucose levels. Thus, high zinc supplementation does appear to have a profound effect on both alloxan and STZ-induced hyperglycemia.

To determine the effect of zinc supplementation on pancreatic islet cell death and islet cell functioning, histology, immunocytochemistry and serum insulin measurements were performed. Figure 4.6 shows representative islet cells from mice fed 50, 500, or
1000 ppm zinc following alloxan or STZ injections and stained with conventional H&E stains. Controls are islets from saline injected mice. In **figure 4.6**, panel marked represents a normal healthy islet cell control. The first sets of slides are H&E stained pancreatic sections from alloxan-injected mice. In panel B, we can see several areas of cellular necrosis are obvious, and there is a marked reduction in islet cell mass. In mice supplemented with 500 ppm zinc (panel C), there appears to be some improvement, however, there are still several areas of cellular necrosis. In panel D, we can see that animals that are supplemented with 1000 ppm zinc show a marked reduction in cellular death. The second sets of slides are pancreatic sections from STZ-injected mice. In the STZ-induced diabetic, we can also demonstrate a profound effect with zinc supplementation. In panel B, we can see marked infiltration of leukocytes into pancreatic islets (insulitis). When animals are supplemented with 500 ppm or 1000 ppm zinc, there appears to be a marked decrease in islet cell infiltration. Animals supplemented with 1000 ppm zinc show the most dramatic improvement. Thus, a significant reduction in both islet cell death and islet infiltration is apparent with zinc supplementation in STZ-induced diabetes.

To examine β cell function, both serum insulin and immunocytochemical staining for insulin in islet cells were performed. **Figure 4.7** depicts serum insulin levels in zinc supplemented animals following alloxan or STZ exposure. Animals injected with saline act as controls. Animals injected with either alloxan or STZ show a dramatic decline in circulating serum insulin, suggesting that pancreatic β cell function is severely compromised. Supplementation only at the 1000 ppm zinc level caused a significant increase of insulin levels and restoration of β cell function. Immunocytochemical stains
also effectively demonstrate that zinc supplementation protects islet cells from alloxan and STZ-induced loss of insulin (Figure 4.8). In panels A, control islets show dramatic staining for insulin (in dark brown). In unsupplemented animals (50 ppm zinc), a clear loss of insulin is evident in both alloxan and STZ-treated mice (panel B). However, with higher zinc supplementation we see a gradual restoration of insulin (panels C and D) in both diabetes models. The most dramatic change is in the 1000 ppm zinc supplemented groups (panel D). In this group, insulin stains resemble closely to that of controls. Therefore, using hyperglycemia, insulin and islet cell death as indices of IDDM, we can clearly demonstrate that zinc supplementation has a profound effect of the development of IDDM to two distinct models for the disease.
4.5 DISCUSSION

In the present study, we were able to demonstrate that dietary supplementation with zinc effectively protected against the development of IDDM in two distinct animal models for the disease. We were also able to demonstrate in vivo that zinc supplementation modulates the activation of the redox sensitive transcription factor NFκB and consequently modulates downstream expression of iNOS and insulitis associated with the development of IDDM. Other studies have also shown a protective effect of zinc in models for IDDM (12), however the current study is the first to examine the molecular mechanisms by which zinc may exert its effects.

More recently, there has been an increased interest in understanding how free radicals act as cellular messengers in disease pathways. In particular, a focus on the oxidative stress responsive transcription factor, NFκB has become the target in several disease models. In this research, we have investigated the role of NFκB activation in two distinct chemically induced models for IDDM. Injection of alloxan is known to cause selective degeneration of the β cells and has been used to induce diabetes in animals for several decades. Alloxan is thought to produce free radicals in its metabolism (36, 37), although the precise mechanisms explaining for its selective cytotoxicity to the islet cells is still relatively unknown. Although STZ is also thought to produce free radicals in its metabolism, STZ destroys the islet cells by a different mechanism. However, unlike
alloxan, STZ induces pronounced immune and inflammatory processes prior to cell
death. Following the injection of multiple low doses of STZ, pancreatic islets show
obvious insulitis with infiltrating lymphocytes and macrophages, architectural distortion
and β cell necrosis (34). Despite different mechanisms, both compounds also specifically
activate pancreatic NFκB (11, 38). Furthermore, supplementation with zinc both inhibits
NFκB activation (Figure 4.2 and 4.3) and islet cell death (Figure 4.6) in both alloxan
and STZ models. We find it most striking that zinc can not only mitigate the direct
cytotoxic effects of free radical inducing compounds, but it can also mitigate the immune
response induced by streptozotocin. Thus, zinc cannot be exerting its effects simply by
protecting the islet cells from oxidative damage. Instead, zinc could be functioning by
altering redox sensitive signal cascades leading to inflammatory responses and β cell
death.

These results reinforce the hypothesis that increased free radical production can
be deleterious due to effects on signal transduction, not simply through massive oxidative
damage. Supplementation with zinc also inhibited iNOS protein expression (Figure 4.4).
The induction of iNOS has been implicated in mediating β cell death in IDDM (4, 39).
More importantly, although iNOS is known to be stimulated during inflammatory and
immune responses, it is also a downstream target of NFκB activation. Thus, the increase
in iNOS expression acts as indirect evidence for the transactivation of NFκB. To further
confirm the role of zinc in NFκB activation and the events leading to inflammatory and
immune responses in IDDM, many other downstream target such as cytokines,
chemokines and adhesion molecules still need to be examined. Additional studies
investigating β cell apoptosis also needs to be examined. It is possible that zinc may

114
have profound influences on NFκB and activation of cell death via apoptotic pathways. Regardless, these results so far are consistent with the hypothesis that free radical induced NFκB activation plays a central role in the signaling the events leading to the destruction of the pancreatic β cells.

The mechanism by which zinc inhibits NFκB activation and islet cell death may be through several different mechanisms. First, zinc is known to have several unique antioxidant properties. It acts to stabilize membrane structure and protects sulphydryl groups from oxidation. Zinc also may compete with iron (Fe), a potent inducer of Fenton reactions, which result in the production of hydroxyl radicals. Zinc is also an essential cofactor in antioxidant enzyme copper/zinc superoxide dismutase (CuZnSOD), one of the first line defense enzymes in scavenging reactive oxygen species. Transgenic animals that overexpress CuZnSOD are also protected against developing alloxan and STZ-induced diabetes (40, 41). However, we have found that high zinc supplementation does not significantly affect CuZnSOD activity in the pancreas (data not shown). Yang and Cherian have also demonstrated that STZ-induced lipid peroxidation can be improved with zinc without any changes in SOD activity (42).

Zinc may also exert its antioxidant activity through the induction of metallothionein (MT). MT is a small molecular weight, cysteine-rich protein with significant antioxidant activity (43). We can show a significant induction of pancreatic MT expression when animals are fed a diet containing 1000 ppm zinc (Figure 4.9). Little induction was seen at lower supplementation levels (100 ppm and 500 ppm). Other investigators, have established the regulatory role of MT on NFκB activation in vitro (44, 45). Thus, it becomes difficult to determine if the inhibitory effects we see are due to zinc
effects or MT effects. Apostolova et al. (46) has shown that zinc pretreatment has a unique inhibitory effect on STZ-induced diabetes using MT-null mice. Pre-treatment with zinc (1 mg/kg) had a protective effect in MT-null mice, while there was no effect in non-transgenic mice. These data suggest that zinc ions, free from MT molecules exerted a protective effect. More extensive analysis, using MT-null animals or agents that block MT expression will need to be performed before effects of zinc itself and MT can be differentiated.

From these studies, it is possible that zinc supplementation may have a significant impact on the development of IDDM. Inadequate nutrition and or inherently low antioxidant nutrient status may predispose individuals to developing the disease. Inadequate zinc intake has been reported in IDDM children (47) and a correlation between areas with low groundwater zinc with an increased incidence for IDDM have been documented (48). However, before recommendations for supplementation can be made, issues of dose and safety need to be addressed. In these studies, zinc supplementation at 1000 ppm zinc, most dramatically inhibited NFκB activation (Figure 4.2 and 4.3) and prevented symptoms of hyperglycemia (Figure 4.5), cell death (Figure 4.6) and loss of insulin (Figure 4.7 and 4.8) in both alloxan and STZ-induced diabetes. This suggests that very high doses of zinc (20 times that normal level) are required to inhibit NFκB activation and the development of the IDDM. In the human population this level of supplementation may be difficult, since issues of compliance, gastrointestinal absorption and toxicity will come into play. Regardless, these studies lay down the model for understanding the role of free radicals and antioxidants in the development of IDDM. If NFκB is the critical pathway in β cell destruction, we can use this as a means
for screening potential preventive agents. It is becoming increasingly clear that the role of free radicals and antioxidants in disease processes involves far more complex pathways than previously thought. Not all antioxidants act the same, thus the use of relevant \textit{in vivo} systems to understand these pathways will move the field closer to finding effective prevention strategies.
Figure 4.1. Serum and pancreatic zinc and copper levels with dietary zinc supplementation. Zinc and copper levels in A) serum and B) pancreas following 2 week feeding period with diets containing 50 ppm (normal), 100 ppm, 500 ppm and 1000 ppm zinc. Level of significance was evaluated using Duncan’s test at p<0.05. “*” denotes significantly different from normal zinc level (50 ppm)
Figure 4.2. High zinc supplementation inhibits alloxan-induced NF-κB activation in the pancreas. +ve control were HeLa nuclear extracts. Competitor reactions include cold specific NFκB oligonucleotides. Samples in Lane 3-5 received only sham saline injections. Alloxan treated groups received alloxan (50 mg/kg i.v.) (Lanes 6-8). Pancreatic tissue was removed from mice 30 minutes following alloxan or saline injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This Figure is a representative of 2 individual experiments.
Figure 4.3. **High zinc supplementation inhibits STZ-induced NF-κB activation in the pancreas.** Control (Lane 1) received only sham saline injections. STZ-treated groups received STZ (50 mg/kg i.v.) (Lanes 2-4). Pancreatic tissue was removed from mice 30 minutes following STZ or saline injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This Figure is a representative of 2 individual experiments.
+ STZ (40 mg/kg for 5 days)

Figure 4.4. High zinc supplementation inhibits STZ-induced iNOS expression in the pancreas. iNOS expression was determined by Western blot analysis in mice injected with STZ and fed 50, 500 or 1000 ppm zinc. Control animals were injected with saline. SDS-PAGE was performed as outlined in Methods. Each sample contains 5 pooled pancreases from each group. Results are representative of 2 individual blots.
Figure 4.5. High zinc supplementation reduces alloxan and STZ-induced hyperglycemia. Mice (n = 5 at each dose) were injected with alloxan (A) (50 mg/kg i.v.) or STZ (B) (40 mg/kg i.p. for 5 days). Controls (○ -) were injected with saline only. Fasting blood glucose measurements following alloxan or STZ exposure were taken in animals fed 50 ppm (◆ -), 500 ppm (■ -) and 1000 ppm (▲ -). Day 1 is first day after alloxan or last STZ injection. Mean values were compared at each day using one-way ANOVA. Level of significance was evaluated using Duncan’s test at p<0.05. “*” denotes significantly different from control.
Figure 4.6. Islet cell pathology in zinc supplemented mice following alloxan and STZ exposure. On day 9, pancreas were removed, fixed and paraffin embedded. Conventional H & E stains were performed to assess islet cell pathology. In panel 1, animals were injected with alloxan (ALX, 50 mg/kg i.v.): A) Control islet (saline injected); B) 50 ppm + ALX; C) 500 ppm + ALX; D) 1000 ppm + ALX. Arrows point to obvious areas of cellular necrosis. In panel 2, animals were injected with low multiple doses of streptozotocin (STZ, 40 mg/kg i.p. for 5 days): A) Control islet (saline injected); B) 50 ppm + STZ; C) 500 ppm + STZ; D) 1000 ppm + STZ.
Figure 4.7. Plasma insulin levels of zinc supplemented mice following alloxan or STZ exposure. Plasma insulin levels were assessed 9 days after alloxan injection (50 mg/kg i.v.) or STZ (40 mg/kg i.p. for 5 days) in mice fed 50 (normal zinc level), 500 and 1000 ppm zinc diets. Control animals received saline injection and were fed 50 ppm zinc diet (normal diet).
Figure 4.8. Immunocytochemical stains for insulin in zinc supplemented mice following alloxan and STZ exposure. On day 9, pancreas were removed, fixed and paraffin embedded. Tissue sections were stained with hematoxylin and the immunohistochemically stained for insulin. In panel 1, animals were injected with alloxan (ALX, 50 mg/kg i.v.): A) Control islet (saline injected); B) 50 ppm + ALX; C) 500 ppm + ALX; D) 1000 ppm + ALX. In panel 2, animals were injected with low multiple doses of streptozotocin (STZ, 40 mg/kg i.p. for 5 days): A) Control islet (saline injected); B) 50 ppm + STZ; C) 500 ppm + STZ; D) 1000 ppm + STZ.
Figure 4.9. Pancreatic metallothionein (MT) expression is induced with high zinc supplementation. MT protein expression in the pancreas was determined by western blot analysis following 2-week dietary treatment with 50, 100, 500 and 1000 ppm zinc diets. SDS-PAGE was performed as outlined in Methods. Results are representative of 4 individual blots.
4.6 REFERENCES


CHAPTER 5

THE EFFECT OF DIETARY VITAMIN E SUPPLEMENTATION ON NFκB ACTIVATION AND THE DEVELOPMENT OF CHEMICALLY-INDUCED INSULIN-DEPENDENT DIABETES MELLITUS

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5.1 SUMMARY

There is growing evidence implicating free radicals as a final common mediator in the development of insulin dependent diabetes mellitus (IDDM). Vitamin E is a potent biological antioxidant, and vitamin E status may be compromised in IDDM patients. The goal of this study was to determine if dietary vitamin E supplementation could prevent the development of either alloxan or streptozotocin (STZ)-induced diabetes. Secondly, we investigated the possible cellular mechanisms by which vitamin E exerts its effect by examining the ability of vitamin E to inhibit NFκB activation. Weanling CD1 mice (n=10) were fed diets containing 75 IU (normal vitamin E level), 500 IU, 1000 IU or 2000 IU vitamin E for a 2 week period. After this initial feeding period, animals were injected with either alloxan (50 mg/kg i.v.) or STZ (40 mg/kg i.p. for 5 days). Control animals were fed the normal vitamin E diet (75 IU vitamin E) and were injected with saline. Pancreatic NFκB activation was assessed by electromobility shift assays (EMSA) in a subgroup of animals. The remaining animals were monitored for weight loss and hyperglycemia for another 2 weeks. At the end of the trial, animals were sacrificed and blood and pancreas samples were taken for insulin measurements and nitrite determination. We found that vitamin E supplementation, at some doses, inhibited NFκB activation and the development of alloxan-induced diabetes. Supplementation at 500 IU significantly inhibited alloxan-induced NFκB activation and markedly reduced the
severity of diabetes. Higher doses of vitamin E (1000 IU and 2000 IU), did not inhibit alloxan-induced NFκB activation and were less protective. Vitamin E supplementation had no effect on STZ-induced NFκB activation and did protect against the development of STZ-induced diabetes. Vitamin E did however, decrease pancreatic nitrite levels in the STZ-induced model. These results suggest that vitamin E supplementation may not be beneficial in preventing the immune-mediated destruction of the β cells. However, these results do give further support to our hypothesis that NFκB activation may be a critical signal in the development of IDDM. Understanding these cellular pathways leading to IDDM will move us closer to finding potential preventive agents for disease.
5.2 INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a disorder caused by the selective immune destruction of the pancreatic β cells resulting in a lack of endogenous insulin secretion. It has been proposed that the disease begins with a genetic susceptibility, followed by a external triggering factor which initiates a cascade of events leading to the immune destruction of the islet cells [1]. Several of these triggering factors, such as dietary factors, viral attack and chemical exposure, can illustrate a link to increased free radical production [2-4]. In addition, the antioxidant capacity of the islet cells is relatively low compared to other tissues [5, 6], making them highly susceptible to oxidative damage. Thus, it has been hypothesized that free radicals may be a key factor in the destruction of the β cells in IDDM.

Several studies, including those done in our laboratory, have supported this hypothesis. Isolated pancreatic islets in culture were protected against cell damage caused by diabetogenic agents with addition of free radical scavengers such as superoxide dismutase, catalase and dimethylurea [7-9]. Free radical scavengers administered prior to diabetogenic agents can also protect rodents from the onset of diabetes [10, 11]. Administration of free radical scavengers to genetically diabetic rats and mice has also decreased the incidence of diabetes [12-15]. The goal of the current study was to investigate the effect of dietary vitamin E supplementation in two distinct chemically
induced models of IDDM and examine the cellular mechanisms by which vitamin E exerts its effects.

Vitamin E is an important biological antioxidant that limits oxidative damage to membrane structures by preventing the propagation of lipid peroxidation reactions [16, 17]. Administration of vitamin E to animals has ameliorated the cytotoxic effect of several free radical-inducing agents such as carbon tetrachloride [18, 19], adriamycin [20], ozone [21] and irradiation [22, 23]. Supplementation of vitamin E has also been shown to reduce the onset of diabetes in the genetically diabetic BB rat [24]. Alterations in vitamin E status have also been reported in diabetic children [25]. In addition, Vitamin E has demonstrated the ability to inhibit NFκB activation in vitro [26]. We have previously postulated that NFκB activation may be a key signal initiating the cascade of events leading to islet cell destruction. Together, these data suggest that vitamin E supplementation may be effective in preventing the onset of IDDM. To examine this possibility, we have used alloxan and streptozotocin (STZ) as two distinct chemically-induced models for IDDM and asked the following questions. Can dietary vitamin E supplementation limit NFκB activation in alloxan or STZ-induced diabetes? Secondly, can vitamin E supplementation protect against the diabetogenic effect of these agents?
5.3 MATERIALS AND METHODS

**Animals.** Weanling male CD-1 mice (Harlan), weighing 20-25 g were used in all experiments. All animals were housed in individual cages in a temperature controlled environment (22 ± 2°C) with a light period between 0600 to 1800 hrs. Mice were randomly allocated to one of 4 vitamin E groups; normal (75 IU vitamin E) or supplemented (500 IU, 1000 IU or 2000 IU vitamin E). All animals were fed diets based on a modified AIN93G rodent diet [27] with additional vitamin E as α-dl tocopherol acetate (Dyets). Animals were allowed free access to the diet with the exception of fasting periods for blood glucose determination. Animal protocol was approved by the OSU Institutional Laboratory Animal Care and Use Committee.

**Determination of NFκB Activation In Vivo.** For NFκB activation analysis, mice were fed for 2 weeks on respective vitamin E diets and were then injected with 50 mg/kg alloxan or 50 mg/kg STZ (i.v.) (Sigma). Control animals received saline injections. All mice were sacrificed 30 min after injection (n=5) and pancreas was immediately removed. NFκB activation was determined using an electrophoretic mobility shift assay (EMSA). Crude nuclear extracts were prepared from pancreatic tissue as described by Deryckere and Gannon [28]. Double stranded synthetic oligonucleotides probes for NFκB (5'-AGTGAGGGGACTTTCCCAGGC-3') (Promega) were end-labeled using [γ-32P] (Amersham) and T4 polynucleotide kinase (Boehringer Manneheim). Binding reactions containing equal amounts of protein (~10 ug) and
labelled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.05% NP-40, 50 ug/ml poly [dI-dC]). Specific binding was confirmed using 100-400 fold excess unlabelled NFκB oligonucleotide as a specific competitor. Protein-DNA complexes were separated using 6% nondenaturing polyacrylamide gel electrophoresis followed by radiography to detect the level of retardation produced by binding to NFκB probe.

Effect of Vitamin E Supplementation on Alloxan and STZ-induced Diabetes.

To examine the effect of vitamin E on the development of alloxan and STZ-induced diabetes, mice were fed vitamin E diets for two weeks prior to alloxan or STZ injections. Alloxan was administered i.v. (50 mg/kg) to induce diabetes. For STZ-induced diabetes, multiple low doses of STZ, a commonly used method to induce diabetes were employed [29]. STZ (40 mg/kg) dissolved in citrate buffer (pH 4.0), was immediately injected (i.p.) into mice (n = 5) for 5 consecutive days. Control animals received sham saline injection. Blood was obtained from the intra-orbital sinus following an 8 hr fast using a 10 uL capillary tube. Glucose concentrations were measured using the ONE-TOUCH ™II complete blood glucose monitoring kit. To minimize the effects of diurnal fluctuations, blood samples were collected at the same time every day. Hyperglycemia was monitored for 9 days after injections. On Day 9, plasma insulin levels were assessed using ELISA (CrystalChem).

Nitrite analysis. Pancreatic tissue was homogenized in 100 mM HEPES buffer and centrifuged at 100 000 x g for 60 minutes. Nitrite formation was used as an indirect measure of nitric oxide (NO) production. Nitrite concentration was
determined using the Griess reaction, as described by Hevel and Marletta [30]. Protein concentration was determined using the Lowry method [31].

Statistics. One way analysis of variance (ANOVA) was performed to assess the differences between control and zinc treatments. Difference in means among treatments was tested by using Duncan's test. Level of significance was evaluated at p<0.05.
5.4 RESULTS

Mice injected with the diabetogenic drugs alloxan or STZ show a specific activation of NFκB approximately 30 minutes following an i.v. injection [10, 37]. Figures 5.1 and 5.2 illustrate the effect of vitamin E supplementation on alloxan and STZ-induced NFκB activation in CD1 mice. Mice were fed either a normal vitamin E diet (75 IU) or were supplemented with 500, 1000 or 2000 IU vitamin E for two weeks prior to injections. In Figure 5.1, we see no activation of NFκB in control animals injected with saline (lane 3). Mice fed a normal diet (75 IU) had significant activation of NFκB (lane 4). Animals supplemented with 1000 IU vitamin E (lane 6) and 2000 IU (lane 7) also showed significant activation. On the other hand, supplementation with 500 IU vitamin E markedly inhibited alloxan-induced NFκB activation (lane 5). Thus, it appeared that vitamin E supplementation was effective in inhibiting NFκB activation. However, only certain doses were capable of inhibition. As vitamin E dose became higher, inhibition of NFκB became less effective.

When we examined the efficacy of vitamin E to inhibit STZ-induced NFκB activation, we saw different results. In this model, vitamin E had no effect on STZ-induced NFκB activation. Similar to figure 1, in figure 5.2 we can see very little activation of NFκB in pancreas of mice injected with saline. Animals fed a normal diet
(75 IU) and injected with STZ showed significant activation of NFκB (lane 4). All supplemented doses also showed marked activation (lanes 5-7). Therefore, vitamin E supplementation at all levels, could not inhibit NFκB activation induced by STZ.

**Figure 5.3** illustrates the effect of vitamin E supplementation on the severity of alloxan-induced diabetes. Typically, mice injected with alloxan display symptoms characteristic of IDDM, including elevated fasting blood glucose levels (hyperglycemia), polyuria and weight loss. In figure 5.3, we can demonstrate the varying efficacy of vitamin E on reducing alloxan-induced hyperglycemia and weight loss. Animals fed the normal diet (75 IU), showed marked hyperglycemia and weight loss following alloxan injection. Vitamin E supplemented animals were somewhat protected against these symptoms, depending on dose. Mice that are supplemented with 500 IU vitamin E show little signs of weight loss, and fasting blood glucose levels are not significantly different from control. As vitamin E dose increases, there was a progressive decrease in the efficacy of vitamin E supplementation. Animals fed 1000 IU were more hyperglycemic than those as those fed 500 IU. Animals supplemented with 2000 IU vitamin E demonstrated even more hyperglycemia. Thus, the efficacy of vitamin E in protecting against the development of IDDM largely depends on dose. Moreover, there appears a threshold effect, where more vitamin E is not necessarily more effective.

We have previously postulated that NFκB activation is a key event in the signal cascades leading to β cell death. If NFκB activation is a critical step in the diabetogenic pathway, then we can predict that agents that inhibit NFκB will be effective in preventing the disease. In figure 5.2, we demonstrated that vitamin E could not inhibit STZ-induced NFκB activation. **Figure 5.4** shows the effect of vitamin E on STZ-induced
hyperglycemia. No protection was seen at any vitamin E dose. Both unsupplemented and supplemented animals (at all doses) show significant elevation in blood glucose levels. When we examine serum insulin levels (Figure 4.5), we see a similar phenomenon. Animals that were injected with STZ show a dramatic drop in serum insulin. Vitamin E supplementation at all doses had no effect in preventing this insulin loss. Therefore, vitamin E provided no protection against the destruction of the pancreatic β cells in the STZ-induced diabetic model.

To determine the effect of STZ and vitamin E on nitric oxide (NO) production, nitrite levels were evaluated in the pancreas of supplemented and unsupplemented mice. Increased NO production has been implicated in β cell death, and the inducible form of nitric oxide synthase (iNOS) is a known downstream target of NFκB and inflammatory enzyme. Figure 5.6 shows that pancreatic nitrite levels are significantly elevated in animals injected with STZ and fed the normal vitamin E diet (75 IU) compared to saline injected controls. Animals that were supplemented with either 500, 1000, or 2000 IU vitamin E and injected with STZ showed no increase in nitrite levels compared to controls. Thus, NO levels were high after STZ exposure, but supplementation with vitamin E could inhibit this elevation.
5.5 DISCUSSION

The efficacy of vitamin E to protect against chemically induced diabetes had varied results. Vitamin E did help protect against the development of alloxan-induced diabetes, while vitamin E had little protective effect in the STZ-induced diabetic model. In addition, vitamin E supplementation inhibited alloxan-induced NFκB activation, yet could not inhibit STZ-induced NFκB activation. Doses of vitamin E that effectively inhibited NFκB activation were the only doses that effectively protected against IDDM. These results suggest that supplementation strategies that effectively target NFκB activation will be capable of protecting against β cell destruction.

Low vitamin E status has been documented in both animal and humans prior to developing IDDM. The diabetic-prone BB rat has shown to have lower levels of vitamin E in the thymus and pancreas prior to the onset of diabetes [32]. Researchers have also found that serum α-tocopherol concentration is inversely associated with the occurrence of IDDM 4-14 years later [33]. From these data, it was hypothesized that vitamin E may have a protective effect against the development of IDDM. The protective effect of vitamin E has been confirmed, to some extent, in this study and in previous studies. Previously, vitamin E supplementation prior to alloxan or streptozocin protected against the diabetogenic effect of these compounds [34]. We have found in this study that vitamin E supplementation offered protection in the alloxan-induced model for IDDM. However, the most effective supplementation level was at 500 IU (Figure 4.3).
dietary level of vitamin E became higher, the efficacy of vitamin E to protect against IDDM decreased. In this case, higher doses of vitamin E were less effective. In the multiple low dose STZ-induced model for IDDM, there vitamin E had no effect at any dose (figure 5.4). When STZ is administered at one high dose, it has a similar pathology to animals given alloxan. However, when the same amount of STZ is administered in multiple low doses, the destruction of the islet cells is accompanied by infiltration of lymphocytes and macrophages (insulitis) [29]. There is an immune component to this model that more closely the immune-mediated destruction of the islet cells in humans. Previous studies which have found that vitamin E prevented the disease have only looked at the high dose STZ model. We have found that in the multiple low dose model, vitamin E is not effective. Thus, it is possible that vitamin E supplementation may have little effect on the immunologically-based destruction of the pancreatic β cells.

The inability of vitamin E to mitigate immune responses associated with IDDM has been found in other diabetic models. The NOD mouse spontaneously develops diabetes with insulitis occurring approximately at 4 weeks of age. Progressive destruction of the β cells occurs and the outward signs of diabetes are seen from 10 weeks of age [35]. Vitamin E supplementation could not reduce the incidence of diabetes in the mice, but could delay its onset [15, 36]. Furthermore, vitamin E supplementation could not reduce insulitis in these mice. These results corroborate the hypothesis that vitamin E supplementation may not affect immune-mediated destruction of the β cells.

The precise reason why vitamin E has differing effects on alloxan and STZ-induced diabetes is unclear. However, it is known that vitamin E predominantly acts as an antioxidant by preventing the propagation of lipid peroxidation and preventing...
oxidative damage to lipid membranes. If increased free radical production by immune responses targets other macromolecules, such as proteins or DNA, then vitamin E may not be effective. In addition, vitamin E must act only at later stages of the disease. Simple protection from oxidative damage may not be enough to overcome the diabetogenic effects of STZ. Compounds that can mitigate the initial cascade of events leading to the immune response may prove to be more effective.

One striking observation we found in these studies was that an inhibition of NFκB activation consistently correlated with a protective effect. That is, vitamin E supplementation was beneficial only when NFκB activation was inhibited. We have previously postulated that NFκB activation is the central signal in the cascade of events leading to IDDM. We can clearly see in both alloxan and STZ-induced diabetes that inhibition of NFκB activation was an excellent predictor of success of supplementation. In alloxan-induced diabetes, only those doses that effectively inhibited NFκB activation significantly reduced the symptoms of diabetes. In STZ-induced diabetes, vitamin E supplementation at all doses could not inhibit NFκB activation nor could it inhibit the development of diabetes. In previous studies, we have also demonstrated that antioxidants such as n-acetylcysteine (NAC) and a-phenyl-t-butylnitrone (PBN) that inhibited NFκB activation could also inhibit the development of IDDM [10, 37]. Thus, the varying effects of vitamin E, could be centered around NFκB activation as the central signal leading to β cell death.

Vitamin E was able to inhibit increased nitric oxide production in STZ-induced diabetes. However, this drop in nitric oxide production did not correlate with a protective
effect. The inducible form of nitric oxide synthase (iNOS) is a downstream target of NFκB, thus is curious why vitamin E can decrease nitric oxide production without inhibition of NFκB. It is possible that the source of nitric oxide may not due to an induction of iNOS. Instead the nitric oxide may be released during the metabolism of STZ itself. The ability of STZ to release nitric oxide has been documented by other researchers [38]. Therefore, future studies using other inflammatory downstream targets for NFκB will be necessary to confirm the role of NFκB in these diabetogenic pathways.

As we learn more about the role of free radicals and antioxidants in disease processes, it is becoming increasingly apparent that these pathways leading to disease are highly complex. Not all antioxidants are the same and dose is highly important. Although a wide body of research supports the idea that antioxidants may be beneficial in IDDM, before recommendations can be made we need to understand the critical pathways leading to disease. If NFκB activation is this critical signal, we can use this model to predict potential successful preventive agents and establish dose relationships.

In conclusion, we have found that vitamin E prevents the development of alloxan-induced diabetes, but not STZ-induced diabetes. These effects could be centered on the ability of vitamin E to inhibit NFκB activation. The inability of vitamin E to inhibit STZ-induced NFκB activation and development of diabetes makes the value of vitamin E as a preventive agent in the human disease questionable.
Regardless, this research supports the hypothesis that NFκB activation may be a key signal in the cascades leading to β cell death. If this is true, then we can use this information as a screening tool to identify potential preventive agents. A clearer understanding of the cellular pathways in the development of IDDM will move the field closer to targeting effective prevention or therapeutic strategies for this disease.
Figure 5.1. Vitamin E supplementation inhibits alloxan-induced NFκB activation in the pancreas of CD1 mice. In lanes 3, mice are injected with 0.2 ml saline i.v. In lanes 4-7, mice are injected with 50 mg/kg alloxan i.v. 75 IU vitamin E is the normal recommended level of vitamin E for rodents. 500, 1000 and 2000 IU vitamin E are supplemented groups. Lane 1 is a positive control using Hela cells (Promega) that contain activated NFκB. Lane 2 a specific competitor reaction using unlabeled NFκB oligo to confirm specificity of NFκB binding.
Figure 5.2. Vitamin E supplementation has no effect on STZ-induced NFκB activation in the pancreas of CD1 mice. In lane 3, mice are injected with 0.2 ml saline i.v. In lanes 4-7, mice are injected with 50 mg/kg streptozotocin (STZ) i.v. 75 IU vitamin E is the normal recommended level of vitamin E for rodents. 500, 1000 and 2000 IU vitamin E are supplemented groups. Lane 1 is a positive control using Hela cells (Promega) that contain activated NFκB. Lane 2 a specific competitor reaction using unlabeled NFκB oligo to confirm specificity of NFκB binding.
Figure 5.3. Supplementation with vitamin E reduces alloxan-induced hyperglycemia and weight loss. Animals were fed 75 IU (○), 500 IU (■), 1000 IU (▲) or 2000 IU (×) vitamin E diets for 2 weeks prior to alloxan injection (200 mg/kg i.p.). Fasting blood glucose levels (A) and body weight (B) were monitored for 14 days. Control animals (●) received a sham saline injection and were fed the normal dietary level of vitamin E (75 IU). Values are expressed as means (n=5). Level of significance was evaluated using Duncan's test at p < 0.05. Groups fed 500 IU and 1000 IU are not significantly different from control at all time points.
Figure 5.4. Supplementation with vitamin E does not reduce STZ-induced hyperglycemia. Animals were fed 75 IU (●), 500 IU (■), 1000 IU (▲) or 2000 IU (×) vitamin E diets for 2 weeks prior to streptozotocin injections (40 mg/kg i.p. for 5 days). Fasting blood glucose levels were monitored for 10 days. Control animals (●) received a sham saline injection and were fed the normal dietary level of vitamin E (75 IU). Day 1 is the first day after the final STZ injection. Values are expressed as means (n=5). Level of significance was evaluated using Duncan's test at p < 0.05. All levels are significantly different from control, with the exception of day 1.
Figure 5.5. Serum insulin levels in vitamin E supplemented mice following exposure to STZ. Animals were fed 75 IU, 500 IU, 1000 IU or 2000 IU vitamin E diets for 2 weeks prior to STZ injections (40 mg/kg for 5 days). Control animals received a sham saline injection and were fed the normal dietary level of vitamin E (75 IU). On day 10, animals were sacrificed and blood serum samples were taken for insulin determination. Values are expressed as mean ± SE (n=5). Level of significance was evaluated using Duncan's test at p < 0.05.
Figure 5.6. Pancreatic nitrite levels in vitamin E supplemented mice following exposure to STZ. Animals were fed 75 IU, 500 IU, 1000 IU or 2000 IU vitamin E diets for 2 weeks prior to STZ injections (40 mg/kg for 5 days). Control animals received a sham saline injection and were fed the normal dietary level of vitamin E (75 IU). On day 10, animals were sacrificed and pancreas was removed for nitrite determination. Values are expressed as mean ± SE (n=5). Level of significance was evaluated using Duncan’s test at p < 0.05.
5.6 REFERENCES


CHAPTER 6

CONCLUSIONS

Although there is substantial evidence that free radicals are involved in the etiology of diabetes, the precise cellular mechanisms leading to β cell death and the development of IDDM remains unknown. The understanding of the pathways leading to IDDM is essential before effective treatment and prevention strategies can be developed. Since IDDM is primarily a childhood disease, developing effective preventative strategies will produce considerable savings in terms of long-term health care costs, and immeasurable savings in the human anguish associated with this disease. Currently, there is little understanding of the cellular events leading to the development of diabetes. Activation of NFκB by oxidative stress and free radicals may be the critical signal initiating the cascade of events leading to β cell death and IDDM.

I have examined the effect of antioxidant supplementation on the development of IDDM using several different classes of antioxidants, including n-acetylcysteine (NAC), α-phenyl-t-butyl-nitrone (PBN), zinc and vitamin E. Not all antioxidants function in the same manner, thus becomes important to examine several types of antioxidants to better understand possible mechanisms. For example, NAC is a glutathione (GSH) precursor. Glutathione protects –SH groups of proteins from oxidation and serves as a substrate for
the enzymes GSH peroxidase and GSH transferases. PBN was originally developed as a free radical trapping agent for the use in electron paramagnetic resonance. PBN's ability to trap and stabilize free radical species, making them less reactive, gives PBN its antioxidant effect. Zinc is an essential trace mineral nutrient which is an essential cofactor in the antioxidant enzyme CuZnSOD. Vitamin E is lipid soluble-vitamin and is known to be the major chain-breaking antioxidant in the lipid peroxidation cycle. Vitamin E has been proven to be effective in protecting against lipid oxidation of membranes. Obviously, each antioxidant has its own unique properties and not all classes of antioxidants may be effective in preventing IDDM. We have found that NAC, PBN and zinc effectively reduced the severity of diabetes in our chemically-induced models, while vitamin E has little protective effect. Understanding the mechanisms by which these antioxidants exert their effects will be essential to identify other potential preventative agents.

A major finding in these studies was that the induction of IDDM \textit{in vivo} was strongly associated not only with the generation of free radicals, but also with the activation of NFκB. NFκB is a central regulatory factor that controls the transcription of genes involved in inflammatory responses, autoimmune responses and cellular responses to oxidative stress. Because the pathogenesis of IDDM has been associated with all these responses, the activation of NFκB is likely to be a pivotal process leading to the death of the β cells. In addition, only the antioxidants that could inhibit NFκB activation significantly reduced the symptoms of IDDM. From these results, it seems likely that NFκB activation is a key early player in the etiology of IDDM. Early triggering factors may begin with increased free radical production, which in turn activates NFκB. This
activation of NFκB both initiates and amplifies inflammatory and immune responses, which ultimately results in β cell death. Although the present research supports these hypothesized pathways, more rigorous experiments are needed to absolutely confirm that NFκB activation is necessary for the development of IDDM. Experiments using specific inhibitors for NFκB and a more in depth analysis of inflammatory and immune events need to be performed to ultimately answer these questions.

This research has moved us forward in understanding the key cellular mechanisms involved in the development of IDDM and will lead to the development of better prevention strategies. Ideally, interventions need to be performed before initiation of the disease. We are now able to identify high-risk individuals using islet-cell autoantibodies as a serological marker for the pre-diabetic state. Antioxidant therapies that can target NFκB activation may prove to be an effective tool in screening for effective treatments to prevent IDDM. Thus, finding effective antioxidant intervention strategies for these high-risk individuals before they develop the disease may become a reality.
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164


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