

## **ABSTRACT**

**Pittman, Kristianna Marie. Exploring the regulation of inducible nitric oxide synthase and the effects of nitric oxide production in endotoxin-induced uveitis.**

**(Under the direction of Janice B. Allen and Kenneth Adler).** Anterior uveitis is a painful inflammatory condition of the eye whose complications include cataract, glaucoma, and most alarming, blindness. The molecular mechanisms of the mainly idiopathic disease are largely unknown. The pathogenesis of the condition is linked to the increase in nitric oxide (NO) production and the concomitant decrease in the activity of the cytokine, transforming growth factor beta 2 (TGF $\beta$ 2). During inflammation, increased NO production is regulated by the enzyme inducible nitric oxide synthase (iNOS). iNOS transcription is regulated, in part, by the transcription factor nuclear factor-kappa B (NF- $\kappa$ B). iNOS gene transcription occurs when inflammatory mediators stimulate phosphorylation of NF- $\kappa$ B's bound inhibitory subunit, inhibitory kappa B alpha (I $\kappa$ B $\alpha$ ), targeting it for degradation. As a result, transcription of iNOS is initiated, NO production increases, and cytotoxicity occurs. NO cytotoxicity is associated with its metabolite peroxynitrite (ONOO $^-$ ). ONOO $^-$ , through the addition of a nitrogen dioxide group on key tyrosine residues of proteins, i.e. nitration, can modify and inactivate proteins. The cytokine, TGF $\beta$ 2 has been shown to negatively regulate iNOS transcription and modulate activation of NF- $\kappa$ B. Therefore, inflammatory production of NO and subsequent production of ONOO $^-$  can be regulated by TGF $\beta$ 2. In this study, we hypothesized that during uveitis NF- $\kappa$ B activity and subsequent iNOS gene expression increase as a result of the decrease in TGF $\beta$ 2 activity. Increased iNOS expression leads to an increase in NO activity and production of ONOO $^-$ . ONOO $^-$  nitrates and inactivates

proteins critical to regulation of the inflammatory response. We further proposed that exogenous administration of TGF $\beta$ 2, i.e. increasing TGF $\beta$ 2 activity or scavenging ONOO $^-$  may protect against EIU. Scavenging of ONOO $^-$  was examined by using the ONOO $^-$  scavenger, ebselen. All studies were undertaken using either the *in vivo* rat model of anterior uveitis, endotoxin-induced uveitis (EIU) or the *in vitro* system of rat iris ciliary body cells stimulated with IL-1 $\beta$ . Results demonstrated that TGF $\beta$ 2 decreases iNOS expression during EIU, however, the mechanism is NF- $\kappa$ B independent. Exogenous administration of TGF $\beta$ 2 did not protect against EIU. In addition, these studies also identified ONOO $^-$  as a component of EIU, through the identification of nitrated MnSOD, a ONOO $^-$  target. Scavenging of ONOO $^-$  by the compound ebselen did protect against EIU. Ebselen mediated inhibition of EIU by not only scavenging ONOO $^-$  but also by inhibiting the recruitment of polymorphonuclear (PMN) leukocytes and suppressing NF- $\kappa$ B activation. Overall, this study demonstrated that TGF $\beta$ 2 negatively regulates iNOS expression during EIU and that iNOS, NO, and ONOO $^-$  are participants in the pathogenesis of EIU. Attenuation of uveitis occurs when suppression of these mediators is coupled to PMN suppression and inhibition of NF- $\kappa$ B activation.

**Exploring the Regulation of Inducible Nitric Oxide Synthase and the Effects of  
Nitric Oxide Production in Endotoxin-Induced Uveitis**

by  
**Kristianna Marie Pittman**

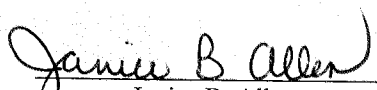
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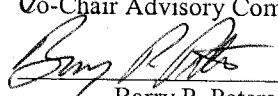
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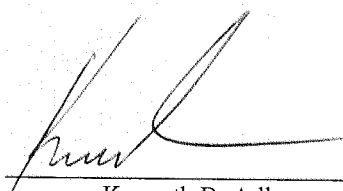
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## **DEDICATION**

This dissertation is dedicated to my mother, Willie Rae Pittman, who sacrificed all including her own desire to receive a doctorate to raise a family. Mom, you sacrificed your financial stability to keep us in private school and you sacrificed your time to keep us in competitive athletics. Through this you instilled in me that nothing good comes without sacrifice, hard work, and no matter how difficult it gets, never give up and never quit until the job is done. I love you, your youngest daughter, Kristi.

## **BIOGRAPHY**

Kristianna Marie Pittman was born Kristi Ann Pittman in Amory, Mississippi on August, 9, 1971. Three weeks after her birth, her parents and four older siblings moved to Nashville, Tennessee, where her parents have since remained. She received 12 years of Catholic school education under the imperious guidance of the Dominican sisters, eight years at St. Pius X elementary school and four years at St. Cecilia Academy all girls' college preparatory high school. She left Nashville to attend college at the University of Rochester in Rochester New York, on an academic scholarship. She graduated from the University of Rochester in May of 1994. After working two years, post-graduate in a dermatology laboratory she decided to pursue a doctoral degree in dermatotoxicology at North Carolina State University in Raleigh, North Carolina. Three years within the toxicology program, her aspirations for a more molecular based dissertation led her to Dr. Janice Allen's laboratory where she completed her degree in summer 2002 studying the inflammatory condition, anterior uveitis. After graduation, she will commence a post-doctoral position at Duke University Medical Center.

## ACKNOWLEDGEMENTS

Anyone that knows me knows of my deep and sincere faith in God and the truth. He has given me the fortitude to persevere despite the difficulties that have arisen as I have traversed this tumultuous path a little less than six years ago. Success would not have been possible without the souls He blessed me with during my doctoral process. I would especially like to acknowledge my committee co-chair, Dr. Janice Allen, who had extreme patience with me, who never stifled my growth as an independent scientist, who pushed me strive for the unattainable and seemingly unimportant, and most importantly, was always there for me as a friend, confidante, and mother...thank you Janice. I would like to thank the following others: my committee members, Dr. Kenneth Adler and Dr. Brian Gilger, who critically reviewed my projects and dissertation.; my committee member, Dr. Barry Peters, who provided me with technical insight and a wonderful ear during my years at NCSU; our collaborator, Dr. Lee Ann Macmillan-Crow from the University of Alabama at Birmingham, who helped me explore the importance of nitration. I would also like to thank my family and friends who have supported me during these years; Jo, Brian, Parker, and Griffin—the Leinbachs, you guys have been there and seen it all. I do not know how I would have eaten without you. My sisters, Karen, Raquel, Michelle and my brother, Jack...we have shown them once again, they can't keep the Pittman's down. And finally, my dad, thank you for the chemistry lessons...the botany lessons...and the fruit fly mating in the kitchen...you were the first real scientist in the family...I just made it through the technicalities. I love you.

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## INTRODUCTION

The uveal tract of the eye consists of the iris, ciliary body and choroid (Figure 1).

These structures function in the following manner: the iris supplies nutrients to the anterior segment and controls the amount of light entering the eye; the ciliary body produces aqueous humor, the nutrient-

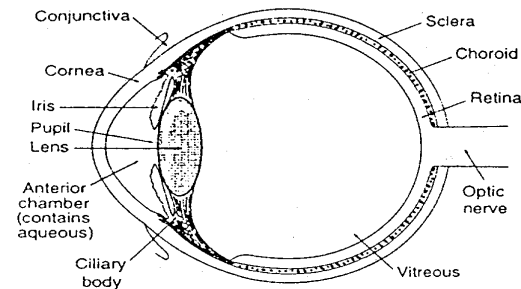


Figure 1 Diagrammatic representation of the eye in cross-section. The uvea consists of the iris, ciliary body and choroid.

filled fluid which bathes the anterior segment, and also regulates refraction by controlling lens zonules; and the choroid is vascularized and is the blood supply for the outer retina. Uveitis is a term used to describe an inflammatory condition which causes tissue damage to these structures, ultimately affecting vision. There are three forms of uveitis: anterior which affects the iris and ciliary body, posterior, which affects the choroid and retina, and panuveitis, describing condition which encompasses both anterior and posterior uveitis.

The most common form of uveitis is anterior uveitis. This form encompasses more than 90% of uveitic cases seen clinically. Anterior uveitis (AU) afflicts mostly those under the age of 65 and accounts for more than 10% of blindness worldwide, thus becoming a very important disease socioeconomically. AU can be precipitated by systemic disease, more commonly autoimmune disorders, an event such as trauma or surgery, or classified as idiopathic. Irrespective of etiology, the preemptive response is always an acute inflammatory response, which then is treated as the acute form of anterior uveitis (AAU). Clinical examination of a patient with AAU reveals the hallmark signs of an acute inflammatory response, “cell and flare”. “Cell” are the leukocytes floating within the aqueous humor and “flare” refers to the liberated protein exudate from

the inflamed iris and ciliary body. With AAU, inflammation generally resolves within six weeks but for two-thirds of the patients, the disease becomes recurrent. Recurrent acute anterior uveitis, i.e. chronic anterior uveitis, may lead to cataract, glaucoma, and in a small percentage of cases, severe legal blindness. Current treatments consist of topical and systemic corticosteroids; notwithstanding, these treatments can induce cataract, high intraocular pressure, and increase susceptibility to microbial infection. In addition, withdrawal of therapy may precipitate another attack of inflammation. Thus, understanding the immune basis of AU will enable one to target therapies related to the pathogenesis of this vision-threatening condition.

The acute inflammatory response is the immediate and early response to an injurious agent. To fend off the injurious agent, the immune system responds by delivering leukocytes to the site of injury. There, through the use of chemotactic and chemical mediators, these cells protect the area from further invasion by microorganisms. Endotoxin and other microbial products are known proponents of the acute inflammatory response and have been implicated in the pathogenesis of AU. Exposure to these products can induce vascular changes. These changes are orchestrated by pro-inflammatory cytokines, such as  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$ . By upregulating cell surface adhesion molecules, these cytokines are responsible for leukocytic activation and extravasation. In response to cytokines acting on the cells, leukocytes extravasate to the site of injury and concomitantly release chemical mediators, which in turn recruit more immune cells to the site of invasion. Once at the site of invasion, leukocytes engulf pathogens and destroy them using oxygen radicals. Oxygen radicals such as nitric oxide (NO), superoxide ( $\text{O}_2^-$ ), and their toxic metabolites destroy pathogens by inducing

cellular damage. If left unchecked, however, these radicals can cause damage to the surrounding tissue and endothelium, prolonging the inflammatory response and generating a chronic inflammatory situation. The NO metabolite,  $\text{ONOO}^-$ , has been regarded as a critical factor in the progression of the inflammatory response. It is generated by high circulating amounts of  $\text{O}_2^-$  and NO produced during inflammation.  $\text{ONOO}^-$  can perpetuate an inflammatory response through its ability to nitrate critical tyrosine residues of key enzymes and inactivate them.

The high levels of NO produced during an inflammatory response are driven by the enzyme inducible nitric oxide synthase (iNOS). iNOS produces NO by catalyzing the conversion of L-arginine to citrulline and NO. Levels of  $\text{O}_2^-$ , produced as a by-product of the mitochondrial electron transport chain, are regulated by the enzyme manganese superoxide dismutase (MnSOD). MnSOD quenches  $\text{O}_2^-$  by converting  $\text{O}_2^-$  to hydrogen peroxide and oxygen gas. Gene regulation of both enzymes can occur through the transcription factor nuclear factor-kappa B (NF- $\kappa$ B). This transcription factor not only regulates transcription of the aforementioned enzymes, but also several pro-inflammatory mediators, including TNF- $\alpha$  and IL-1 $\beta$ . Therefore, it is believed that regulating the induction of this transcription factor, and thus its gene products may attenuate the acute inflammatory response.

Studies using models of acute anterior uveitis have revealed that NF- $\kappa$ B-dependent genes are upregulated during the uveitic response. Moreover, these studies showed that the epithelium of the iris/ciliary body (ICB), along with leukocytes, are participants in the inflammatory response through their production of the cytokines TNF- $\alpha$  and IL-1 $\beta$ , and the enzyme iNOS. iNOS has received much attention as a key mediator

in the progression of the disease. This finding was propelled by studies which showed that the uveitic response could be attenuated in rats when a specific iNOS inhibitor, aminoguanidine (AG), was administered. In contrast, in a subsequent study, Smith et al demonstrated that iNOS deficient mice develop AAU at the same magnitude as control. These studies and others have prompted investigators to explore the manner in which NO and ONOO<sup>-</sup> participate in ocular inflammation.

In addition to producing pro-inflammatory cytokines, the ICB has also been shown to produce anti-inflammatory mediators. In fact, the immune privilege environment of the eye is hypothesized to be maintained by a certain level of immunosuppressive and immunomodulating factors existing in the aqueous humor produced by the ICB. One such factor is the immunosuppressive cytokine, transforming growth factor beta 2 (TGFβ2). Among the factors in the aqueous humor, TGFβ2 is believed to be one of the most important in inhibiting T-cell responses *in vitro*. In addition, the cytokine has also been found to negatively modulate gene expression iNOS and activation of NF-κB. These findings in conjunction with the observation that the activity of the cytokine decreases in the aqueous humor, have lead researchers to speculate that the initiation and progression of the uveitic response may be due to the drop in TGFβ2 levels in the aqueous environment and a concomitant increase in iNOS expression, subsequent NO activity and cytotoxicity in the ICB.

Thus, this study was undertaken to further the understanding of molecular regulation of iNOS gene expression by TGFβ2 and to determine if cytotoxicity as induced by its product NO was involved in the pathogenesis of uveitis. Negative regulation of INOS expression by TGFβ2 was determined by examining *in vitro* mRNA

expression in rat ICB cultures incubated with TGF $\beta$ 2 and subsequently stimulated with IL-1 $\beta$ . Protein expression of iNOS was examined using the rat ICB cultures stimulated with IL-1 $\beta$  and *in vivo* using the rat model of endotoxin-induced uveitis (EIU).

Activation of NF- $\kappa$ B was also explored in the same *in vitro* and *in vivo* systems so that signaling pathways utilized to modulate gene expression could be better defined. NO cytotoxicity was investigated by determining if the NO cytotoxic component, ONOO<sup>-</sup>, was involved in the pathogenesis of the inflammatory condition. Utilizing the *in vivo* rat model of EIU, nitration of the ONOO<sup>-</sup> target, MnSOD was investigated. To further implicate ONOO<sup>-</sup> as a critical mediator, *in vivo* studies were continued using a known ONOO<sup>-</sup> scavenger, ebselen, to determine if the response could be abrogated. *In vitro* studies using rat ICB cultures incubated with ebselen and simultaneously stimulated with IL-1 $\beta$  were conducted to identify molecular mediators and pathways utilized to suppress the response. Collectively, these studies provide insight as to the role iNOS may play in the development of anterior uveitis and introduce new targets for therapeutic intervention.

## **LITERATURE REVIEW:**

**Endotoxin-Induced Uveitis :** Although several animal models exist to study AU, the one most frequently employed to study early mediators involved in the development of the condition is the model of endotoxin-induced uveitis (EIU). First described by Rosenbaum et al in 1980, this model uses components of Gram-negative bacterial cell wall to produce an acute inflammatory response that manifests in the anterior segment of the eye. EIU can be induced in either rats, mice, or rabbits.<sup>1,2</sup> In rats, EIU is induced either by a single endotoxin injection into the peritoneum or in the hind footpad; in mice, by a systemic or intraocular injection of endotoxin; and in rabbits, through an intravitreal injection of endotoxin. Maximal inflammation is seen within 24 hours with resolution of the response within 3 days.<sup>3</sup> The immune cells infiltrating the iris and ciliary body include mononuclear cells and polymorphonuclear leukocytes (PMN). Mononuclear cells infiltrate the iris vasculature within 2 hours of endotoxin injection whereas PMN marginate in iris vessels after 4 hours. The infiltrate in the ciliary body is primarily PMN and maximum levels are noted at 24 hours.<sup>4</sup>

Clinical examination of the inflammatory response is done biomicroscopically through the use of a slit lamp. A slit lamp provides visualization of the anterior chamber and can be used to subjectively grade the extent of inflammation, numerically. Iridal hyperemia, leukocytic infiltrate, and amount of protein circulating within the aqueous humor can be graded on a scale of 0-4, with 0 being normal and 4 the most severe. In mice, inflammation is not as intense and is verified through histopathology. The inflammatory response in mice and rats is also dependent upon strain differences.<sup>5</sup> For

example, Lewis rats have been shown to be the most sensitive/susceptible, whereas Brown-Norway are unresponsive clinically.<sup>1,6</sup>

As with most animal models, similarities must exist between the clinical manifestations within the model and those seen in human medicine. Table I highlights the clinical similarities and differences<sup>7</sup>:

**TABLE I.**

|                                  | <b>Age</b>                              | <b>Clinical Symptoms</b>                                                                                                                          | <b>Time Course</b>        | <b>Gender</b>        |
|----------------------------------|-----------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|----------------------|
| <b>Endotoxin-Induced Uveitis</b> | young animal<br>6-12 weeks of age       | bilateral, keratic precipitates, iridal hyperemia, hypopyon, miosis, redness, aqueous flare, posterior synechiae                                  | resolves within a week    | More severe in males |
| <b>Acute Anterior Uveitis</b>    | affects all ages, more common in adults | unilateral, ciliary injection, keratic precipitates, iridal hyperemia, hypopyon, miosis, redness, aqueous flare, anterior and posterior synechiae | resolves within 6-8 weeks | More common in males |

Although differences do exist, the model of EIU still remains an excellent tool to study early pathogenic mechanisms involved in the development and progression of the AU. Moreover, clinical similarities may suggest parallels at the cellular and molecular level. Investigations using the model of EIU determined that inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) are key mediators in the initiation and progression of AU.

**iNOS:** iNOS is a member of a family of enzymes, nitric oxide synthases (NOSs), which catalyze production of the radical NO. This family of enzymes consists of the isoforms constitutive NOS (cNOS) and inducible NOS, which differ in localization and

dependence on calcium.<sup>8</sup> NOS isoforms are characterized by their bidomain structure and dual monooxygenase reactions to generate NO. The bidomain structure of NOSs consists of an oxygenase and reductase domain linked by a calmodulin (CaM) binding site. CaM binding is required for domain alignment and subsequent activation, therefore these enzymes are sensitive to intracellular levels of calcium.<sup>9</sup> Also required for activation are binding of the cofactors heme iron, tetrahydrobiopterin (BH<sub>4</sub>), the substrate L-arginine and the reducing cofactors, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the reductase domain. CaM binding triggers dimerization of monomers of NOS and concomitant binding of cofactors heme, BH<sub>4</sub>, and L-arginine to the oxygenase domain.<sup>10</sup> These binding events initiate monooxygenase reaction I, i.e the incorporation of dimolecular oxygen into the substrate arginine.<sup>11</sup> In this reaction, NADPH donates electrons sequentially to FAD and FMN, which in turn reduces ferric heme to ferrous heme to produce the substrate intermediate N<sup>o</sup>-hydroxy-L-arginine (NOH-Arg). Enzyme-bound NOH-Arg initiates monooxygenase reaction II<sup>11</sup>, which involves transfer of an electron from FMN to ferrous heme and oxidation of NOH-Arg to L-citrulline and NO. Interestingly, monomers are organized so that the electron flow from the reductase domain of one monomer interacts with the heme iron in the oxygenase domain of the separate monomer.

cNOS includes isoforms neuronal NOS and endothelial NOS. Production of NO by these isoforms occurs following a rise in intracellular calcium. Calcium levels initiate binding of CaM to its site on the enzyme. cNOS produces what is described as “puffs” of NO, i.e. transient, low output levels of NO.<sup>12</sup>

In contrast, NO production by iNOS is considered high output. This is due to the irreversible binding of CaM, as such iNOS is the only isoform that is  $\text{Ca}^{2+}$  independent and once induced, continuously active.<sup>13</sup> Furthermore, iNOS, unlike its other family members, exists in the cell in latent form unless activated by various inflammatory stimuli such as TNF- $\alpha$ , IL-1 $\beta$  and lipopolysaccharide (LPS). Consequently, as a whole, iNOS is considered the isoform responsible for the pathophysiological effects of NO. Therefore, downregulation of iNOS expression has been viewed as a mechanism to control the pathological levels of NO produced by this isoform.

**iNOS and NO in EIU:** The involvement of iNOS and NO in the pathogenesis of uveitis was emphasized through studies that examined their role in EIU. Goureau et al demonstrated that nitrite levels, indicative of NO production, are temporally increased in the vitreous and aqueous humor after endotoxin injection with maximal levels achieved at 16 hours.<sup>14</sup> Moreover, by utilizing NOS inhibitors, a reduction in iNOS activity was seen and clinical and histological signs of the condition diminished. A subsequent study by this group determined that iNOS mRNA was localized in the iris and ciliary body of EIU eyes six hours post injection of endotoxin.<sup>15</sup> At 16 hours, iNOS expression was observed in the stromal cells of the ciliary processess, thus highlighting the importance of the ICB in the inflammatory process. Allen et al further underscored the importance of iNOS in EIU when it was determined that EIU could be suppressed by utilizing a selective iNOS inhibitor.<sup>16</sup> In contrast to these studies, Smith et al have shown that iNOS deficient mice develop EIU identical to that of normal mice, indicating that iNOS is not critical for the initiation or progression of the response.<sup>17</sup> Overall, these studies

emphasize the need to further elucidate the role that iNOS and NO play in anterior uveitis.

**Transcriptional regulation of iNOS:** iNOS gene expression is regulated by the nuclear factor-kappa B (NF- $\kappa$ B)/Rel family of transcription factors.<sup>18</sup> These transcription factors are characterized by their sequestration in the cytoplasm by an inhibitory protein, inducible kappa B (I $\kappa$ B). Transcription of genes regulated under NF- $\kappa$ B control occurs when stimuli such as cytokines, TNF- $\alpha$ , IL-1 $\beta$  or oxidative stress initiate phosphorylation and subsequent degradation of the inhibitory protein. This releases bound NF- $\kappa$ B and unmasks a nuclear localization signal (NLS) which allows binding of NF- $\kappa$ B to sites on target genes.

Within the NF- $\kappa$ B family of transcription factors are five mammalian isoforms, p50, p52, p65 (Rel A), Rel B, c-Rel. These isoforms are able to dimerize to form either homodimers or heterodimers.<sup>19</sup> The p65/p50 heterodimer is the most abundant in cell types and therefore, the term “NF- $\kappa$ B” has been associated with their dimerization. All these proteins share a highly conserved region of 300 amino acids termed Rel homology region (RHR), which enables the proteins to dimerize, bind DNA, and interact with the inhibitory subunit, I $\kappa$ B.

The I $\kappa$ B family of proteins consist of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , and Bcl-3. This family shares a region of six to seven ankyrin repeats, which allows for binding to the RHR and masking of the NLS contained on Rel proteins.<sup>20</sup> The expression of all isoforms, with the exception of I $\kappa$ B $\beta$ , are under NF- $\kappa$ B control. I $\kappa$ B $\alpha$  was the first member characterized and as such the best understood (physiological mechanisms of the

other forms are poorly understood). It was determined that I $\kappa$ B $\alpha$  not only regulates the nuclear translocation of NF- $\kappa$ B, but also causes re-exportation of NF- $\kappa$ B back to the cytoplasm by means of a nuclear export sequence (NES). Thus, NF- $\kappa$ B regulates the transcription of genes by regulating itself, i.e. auto-regulation.

Phosphorylation and ubiquitination are the key events in NF- $\kappa$ B activation. Extracellular or intracellular stimuli initiate a phosphorylation event via the activation of inhibitory kappa kinase (IKK). IKK is a multisubunit kinase consisting of catalytic subunits IKK $\alpha$  and IKK $\beta$ , and regulatory subunit IKK $\gamma$ . IKK phosphorylates serine 32 and 36 of I $\kappa$ B $\alpha$ .<sup>21</sup> Phosphorylation leads to polyubiquitinylation by a SKp1-Cullin-Fbox (SCF)-type E3 of I $\kappa$ B $\alpha$  at lysines 21 and 22.<sup>22</sup> This modification targets I $\kappa$ B $\alpha$  for degradation by the 26S proteasome complex.<sup>23</sup> Degradation leads to exposure of the NLS of NF- $\kappa$ B resulting in binding to karyopherins and translocation of NF- $\kappa$ B to the nucleus.<sup>24</sup>

The role of this transcription factor in iNOS gene expression was first determined in the murine gene coding for iNOS.<sup>25</sup> Within the promoter region, there exist two putative NF- $\kappa$ B binding sites. Subsequent studies, using the NF- $\kappa$ B inhibitors pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine, in murine macrophages, ischemic rat liver, and RAW cells, associated downregulation of iNOS mRNA and protein with inhibition of NF- $\kappa$ B.<sup>18,26,27</sup> Collectively, these studies suggested that activation of the NF- $\kappa$ B pathway can lead to an increase in iNOS expression, and further suggest using agents to inhibit the pathway may uncover alternative therapeutic strategies to regulate inflammation.

**NF- $\kappa$ B and EIU:** Information regarding the role of NF- $\kappa$ B in EIU is limited to a study by Ohta et al using the antioxidant and well-known NF- $\kappa$ B inhibitor PDTC. NF- $\kappa$ B translocation occurred in vivo at 3 –6 hours post-endotoxin injection. Use of PDTC corresponded to a decrease in mRNA expression of the NF- $\kappa$ B dependent gene expression of cytokine, IL-1 $\beta$ , IL-6 and TNF $\alpha$ . The authors concluded that suppression of pro-inflammatory gene expression through the downregulation NF- $\kappa$ B pathway was responsible for the anti-inflammatory effects of PDTC.<sup>28</sup>

**Post-transcriptional and Post-translational Control of iNOS:** Expression of iNOS can be regulated at the level of the gene, as described above, or post-transcriptionally or post-translationally. Transforming growth factor –beta (TGF $\beta$ ), a multifunctional cytokine, has been identified as a post-transcriptional and post-translational modulator of iNOS. TGF $\beta$  is a member of a family of cytokines with multiple biological functions, including inhibition of growth of hematopoietic, epithelial, and endothelial cells, chemotaxis of lymphocytes, macrophages and fibroblasts, and stimulation of extracellular matrix.<sup>29,30</sup> There are three mammalian isoforms, TGF $\beta$ 1,-2,-3,. These isoforms share little homology, and have unique regulatory elements as well as activation signals. Similarities among isoforms lie in the protein processing, receptor binding, and response induction.<sup>29</sup> Once synthesized TGF $\beta$ s are secreted as latent complexes which are bound to a latency associated peptide (LAP), preventing binding to their cognate receptors. Activation is speculated to involve activated cells, cell-cell contact, enzymes, and acidic environment. Signaling is initiated when the mature processed protein binds to its type II

receptor, which recruits the type I receptor to the liganded complex.

Transphosphorylation of a glycine-serine rich domain of the type I receptor by the type II receptor occurs and the signal is propagated. Inactivation of TGF $\beta$  occurs via reassociation of the LAP.<sup>29</sup>

The effect of TGF $\beta$  on iNOS expression has been aggressively studied. Ding et al demonstrated that all three mammalian isoforms are able to suppress the capacity of murine macrophages to produce NO.<sup>31</sup> This phenomenon has since been demonstrated in renal mesangial cells, smooth muscle cells, glial cells, cardiac myocytes, retinal pigment epithelial cells, endothelial cells, osteoblasts and osteoclast, hepatocytes, Kupffer cells, articular chondrocytes, fibroblasts and keratinocytes.<sup>32,33,34,35,36,37,38,39,40,41,42</sup> The manner in which TGF $\beta$  suppresses production of NO has been dissected in several cell types. In macrophages, TGF $\beta$  was shown to reduce the stability and rate of translation of iNOS mRNA, and increase the rate of degradation of iNOS protein. Studies in smooth muscle cells suggested that TGF $\beta$  could reduce transcription, and affect posttranscriptional and translational levels of iNOS. The transcriptional effects of TGF $\beta$  on iNOS expression may include alterations in binding or downregulation of transcription factors which regulate its expression, in particular NF- $\kappa$ B. It has been reported that TGF- $\beta$ 1 can increase the expression of I $\kappa$ B, and thus iNOS gene expression.<sup>43</sup> Conversely, Perrella et al reported a NF- $\kappa$ B independent suppression of iNOS by TGF $\beta$ .<sup>44</sup> Other possible transcription factors affected are AP-1 or IRF-1 which all contain promoter sites on the iNOS gene.<sup>45,46</sup>

Aberrant immune responses have been seen in inflammatory conditions in which TGF $\beta$  is deficient. In models of experimental encephalomyelitis and experimental arthritis, systemic injection of TGF $\beta$  suppressed disease symptoms whereas a local injection of TGF $\beta$  antibodies exacerbated inflammation.<sup>47,48,49</sup> These studies emphasize the importance of TGF $\beta$  as an immunomodulator of inflammatory conditions and the use of the cytokine as a therapeutic modality. Moreover, on a more extensive note, the correlation of increased iNOS expression and loss of TGF $\beta$  both leading to inflammatory conditions strongly suggests deficiencies in TGF $\beta$  activity can lead to a dysregulation of iNOS expression and therefore, an onset of inflammatory conditions.

**TGF $\beta$  in EIU:** In the eye, the predominant isoforms TGF- $\beta$ 1 and TGF- $\beta$ 2 function to suppress immune activity.<sup>50</sup> By inhibiting the activation and proliferation of lymphocytes, these cytokines play a major role in maintaining the immune privilege environment of the eye.<sup>51</sup> Studies have shown that down-regulation of TGF $\beta$  by interferon gamma leaves the ocular environment more susceptible to immunogenic inflammation. In vitro assays of the aqueous humor have shown that active TGF- $\beta$ 2 is the primary isoform in the anterior portion of the eye.<sup>52</sup> Interestingly, studies have also revealed that it is primarily active TGF- $\beta$ 2 that decreases during uveitis as NO production increases.<sup>53,54</sup> In vivo studies utilizing both isoforms, TGF- $\beta$ 1 and TGF- $\beta$ 2, have demonstrated that suppression of uveitis can occur with exogenous administration of the cytokine. Peng et al showed a decrease in leukocyte count, protein concentration, and IL-6 levels in EIU mice injected intraperitoneally with TGF- $\beta$ 1 eight hours post-

induction.<sup>55</sup> In addition, a 50% reduction in cellular infiltrate was noted when TGF- $\beta$ 2 was injected intravitreally in LPS-injected rabbit eyes.<sup>16</sup>

**Pathophysiology of NO:** Several studies have focused on the pathophysiology of NO as related to formation of its reactive intermediates.<sup>56,57,58,59</sup> NO pathophysiology is linked to the presence of an unpaired electron ( $e^-$ ) within the molecule. The manner in which NO acts within the cell is dependent upon the interaction of this unpaired  $e^-$  within the cellular milieu.<sup>60</sup> Depending on the environment, NO can either accept an  $e^-$  to generate nitroxyl anion ( $\text{NO}^-$ ), or lose an  $e^-$  to form nitrosonium cation ( $\text{NO}^+$ ), or undergo a reaction with another radical such as  $\text{O}_2^-$  to form the species, peroxynitrite ( $\text{ONOO}^-$ ).<sup>61</sup> These reactions generate what has been termed ‘reactive nitrogen species (RNS)’. Cytotoxicity elicited through RNS is dictated by local concentrations of NO and concentrations of their biomolecular targets.<sup>13</sup>

The role of  $\text{NO}^-$  in NO toxicity remains unclear and undefined. It is thought in normal cellular function that  $\text{NO}^-$  is a source of NO. A recent controversy has arisen as to the immediate product of NOS activation. Is it  $\text{NO}^-$  or NO? Murphy et al proposed that the primary product is  $\text{NO}^-$  which can then be oxidized to NO by SOD.<sup>62</sup> Still, others have suggested that formation of  $\text{NO}^-$  or NO is condition dependent, i.e. substrate and cofactor availability.<sup>63</sup> The difficulty in addressing this question lies in the difficulties in detecting these species experimentally, therefore, the question may always remain open until better experimental techniques are developed.

Toxic effects of  $\text{NO}^-$  are exerted through its reaction with dioxygen to form the highly reactive radical species ( $\text{ONOO}^-$ , detailed further below).<sup>64</sup> Due to the level of dioxygen within the cell, this reaction has been deemed to be a significant source of  $\text{ONOO}^-$ .

Reactions with  $\text{NO}^+$ , known as nitrosation reactions, are the most well-studied. Nitrosation reactions become biologically relevant through a process called transnitrosation. This process involves the transfer of the nitrosonium cation between a protein centers containing sulfur or nitrogen. Alterations in cellular function via transnitrosation occur when  $\text{NO}^+$  is transferred between nucleophilic centers of proteins.<sup>60</sup> This process will result in cellular damage when  $\text{NO}^+$  interacts with a critical center of a protein, resulting in modification and alteration of protein function. This phenomenon is exemplified with the transnitrosation of amines and sulfur to yield N-nitrosamines and S-nitrosothiols, respectively. N-Nitrosamines have been found to be chemical modifiers of nucleic acids and therefore represent potent mutagens and carcinogens.<sup>13</sup> Studies exposing nucleic acid to high levels of NO in oxygen-dependent deamination reactions implicated NO as a causative agent of DNA strand breaks.<sup>65,66</sup> Additionally, it has been suggested that N-nitrosamines also, via DNA damage, contribute to cancerous states initiated by various inflammatory conditions.<sup>67</sup>

S-nitrosothiols formation, in vivo, can potentially present a problem because of the numerous cellular proteins that contain thiol groups. Candidate molecules include receptor sites in control and signaling molecules, G proteins, protein kinases, transcription activating factors, and antioxidants such as glutathione.<sup>68,69,70,71,72,73</sup> S-nitrosoglutathione is an excellent nitrosating agent, transferring a  $\text{NO}^+$  group to a

variety of nucleophiles.<sup>57</sup> Interestingly, protein nitrosothiols may serve as surrogate forms of NO or as NO reservoirs, decomposing to liberate NO.<sup>74,75</sup>

ONOO<sup>-</sup> is the best understood and most reactive of all the RNS. It is formed by the interaction of NO and O<sub>2</sub><sup>-</sup> at a diffusion-limited rate. At pH 7.4, it exists in either a protonated form, peroxyntrous acid (ONOOH) or ionized form, peroxyntrite anion (ONOO<sup>-</sup>). Under physiological conditions, ONOO<sup>-</sup>, with a lifetime of 1s, is sufficiently stable to diffuse several cell diameters to reach cellular targets before becoming protonated forming ONOOH. ONOOH can decompose to yield species capable of nitrating phenolic rings or hydroxylating aromatic rings.<sup>76</sup> ONOOH has also been shown to oxidize lipids, proteins, and DNA via attack of nucleophilic centers.<sup>61</sup> While most oxidative species are derived from ONOOH, ONOO<sup>-</sup> can undergo direct reactions with nucleophiles like thiols or rapidly within the vasculature with carbon dioxide (CO<sub>2</sub>). The direct reaction of ONOO<sup>-</sup> and CO<sub>2</sub> is of physiological importance, because of high reactivity of the two molecules and the abundance of CO<sub>2</sub> in biological systems.<sup>59</sup> It has been determined that the reactive intermediates formed from the interaction of ONOO<sup>-</sup> and CO<sub>2</sub> are partly responsible for the cytotoxicity elicited by the RNS. These intermediates are capable of acting as nitrating species and, through electrophilic substitution, can add NO<sub>2</sub> moiety to phenolic residues (namely tyrosine) of particular proteins.<sup>77,78</sup> Nitration of tyrosine residues can modify and potentially alter the function of proteins.

**Tyrosine nitration:** ONOO<sup>-</sup>-induced tyrosine nitration is the ONOO<sup>-</sup> pathway of the highest yield and at one point was the hallmark sign of its toxicity. Recently, however,

tyrosine nitration has been shown to occur via other nitrating species. Current literature has proposed that myeloperoxidase, the most abundant protein in neutrophils, may, under inflammatory conditions, produce species capable of nitrating.<sup>79</sup> This mechanism occurs in activated neutrophils when hydrogen peroxide--produced as a result of the respiratory burst-- is converted to hypochlorous acid (HOCL) and then interacts with nitrite ( $\text{NO}_2^-$ ), produced by NO metabolism. The interaction forms the reactive intermediates nitryl chloride ( $\text{Cl-NO}_2$ ) and chlorine nitrite ( $\text{Cl-ONO}$ ).<sup>80</sup> Both species have the ability to nitrate, chlorinate, and dimerize phenolic compounds.<sup>81</sup> However, to complicate matters further, much speculation has arisen as to whether neutrophils actually produce NO directly or not. iNOS expression has been reported in human blood neutrophils stimulated with cytokines.<sup>82,83</sup> Furthermore, NOS activity has been detected in neutrophil-enriched fractions of patients with urinary tract infections.<sup>84</sup> In contrast, Miles et al reported that extravasated neutrophils from peritonitis patient expressed no iNOS mRNA, protein, or exhibited enzymatic activity.<sup>85</sup> Therefore, whether neutrophils play a significant part in tyrosine nitration is controversial.

Tyrosine nitration, similar to S-nitrosation, can pose several problems in vivo due to the prevalence of tyrosine residues in proteins critical to cell function. Potential mechanisms of tyrosine nitration include 1) “tagging” a protein for proteolysis, 2) nitration of proteins important in cell signaling which may either mimic phosphorylation or prevent dephosphorylation, 3) nitration may alter protein conformation and function.<sup>61</sup> The first protein to be unambiguously identified, in vivo, as tyrosine nitrated was manganese superoxide dismutase (MnSOD). MnSOD, the mitochondrial enzyme important in regulating  $\text{O}_2^-$  levels in the cell, was found to be tyrosine nitrated and

inactivated during human chronic renal allograft rejection.<sup>86</sup> Additional studies demonstrated that complete inactivation of MnSOD initiated by ONOO<sup>-</sup> can occur independent of tyrosine nitration of residues located in the enzyme's active site. Complete inactivation requires not only nitration of critical tyrosine residues, but also tyrosine oxidation and subsequent formation of dityrosine.<sup>87</sup> Since that pivotal study, both prostacyclin synthase of rat mesangial cells stimulated with IL-1 $\beta$  and Ca-ATPase of aged rat skeletal muscle have been shown to be both nitrated and inactivated.<sup>88,89</sup> In the mouse model of Parkinson's disease, tyrosine hydrolase was found to be modified by nitration as well.<sup>90</sup>

**Tyrosine Nitration in EIU:** Using antibodies against nitrated proteins, tyrosine nitration has been observed in the experimental model of autoimmune uveitis. Wu et al, have demonstrated ONOO<sup>-</sup> formation in photoreceptors of the retina, and to a lesser extent ganglion cells and blood vessels of the retina. Nitration appeared to correlate with the area of pathologic oxidation.<sup>91</sup> Nitrotyrosine immunoreactivity has also been detected in the rabbit model of EIU where an increase in nitrated proteins was seen in inflamed rabbit eyes versus controls.<sup>92</sup>

**Ebselen:** The discovery that ONOO<sup>-</sup> could elicit tissue damage has led to the search for therapeutic agents which could scavenge ONOO<sup>-</sup> directly or reduce the amount of O<sub>2</sub><sup>-</sup> available, thereby decreasing ONOO<sup>-</sup> formation. The ideal scavenger would have a higher affinity of ONOO<sup>-</sup> than that of ONOO<sup>-</sup> with CO<sub>2</sub>. Ebselen, with a 1000 fold higher reaction rate has been determined to be that ideal scavenger.<sup>76</sup> This compound has been

extensively studied since the early 1980s as a glutathione-peroxidase (GPx) mimic, an anti-inflammatory agent, and most recently, a ONOO<sup>-</sup> scavenger.<sup>93,94</sup> Ebselen via a selenium atom within the molecule acts as a ONOO<sup>-</sup> scavenger in the presence of thiols, reducing ONOO<sup>-</sup> to NO<sub>2</sub><sup>-</sup> and generating its by-product selenoxide. A secondary reaction includes the reduction of selenoxide back to ebselen.<sup>95</sup> Thus, through this catalytic cycle ebselen provides a sustained line of defense against ONOO<sup>-</sup> induced toxicity. Mechanistically, ebselen has been shown in vitro in a dose-dependent manner, to preferentially inhibit the activity of iNOS.<sup>96</sup> In rat Kupffer cells stimulated with LPS, ebselen suppressed TNF- $\alpha$  production and also inhibited the nuclear translocation of NF- $\kappa$ B.<sup>97</sup> Inhibition of nuclear translocation was suggested to occur by interrupting upstream signaling. i.e phosphorylation of IKK. The multiple actions of this compound and its extremely low toxicity in humans has led to the development of ebselen for clinical use.<sup>94</sup> As a result, clinical studies have been undertaken to explore the beneficial effects of the antioxidant in disease conditions with an underlying inflammatory component.<sup>94</sup>

**Summary:** Anterior uveitis is a painful and debilitating ocular condition whose pathogenesis has yet to be defined making it an important condition to investigate in order that effective therapies be developed. Through utilization of the ocular model EIU, it has been determined that iNOS and its product NO are important mediators in this condition, however, the manner in which these mediators act is not completely understood. Positive and negative regulation of iNOS occurs via the transcription factor NF- $\kappa$ B and the immunosuppressive cytokine, TGF $\beta$ , respectively. Although studies

discussing the role of NF- $\kappa$ B in EIU are sparse, TGF $\beta$ 2, a major regulator in the ocular immune environment of the eye, has been extensively studied and levels have been determined to decrease during uveitis as levels of NO increase. ONOO<sup>-</sup>, a cytotoxic component of NO metabolism, has been suggested to be a part of the pathogenicity as well, however, direct involvement of the species has yet to be determined.

Here, the role iNOS and NO in EIU has been more thoroughly explored. It has been determined that TGF $\beta$ 2 can negatively modulate transcriptional and translational expression of iNOS through a NF- $\kappa$ B independent mechanism. Furthermore, ONOO<sup>-</sup> is involved in the pathogenesis of EIU, and by scavenging ONOO<sup>-</sup>, via the antioxidant, ebselen, EIU could be suppressed. Moreover, ebselen acts by inhibiting NF- $\kappa$ B activation.

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## Chapter 1

**TGF $\beta$ 2 suppresses inducible nitric oxide synthase (iNOS) in rat iris/ciliary body (ICB) cells stimulated with IL-1 $\beta$  and in endotoxin-induced uveitis (EIU)**

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## ABSTRACT

**Purpose:** TGF $\beta$ 2 and iNOS have been extensively studied in anterior uveitis, TGF $\beta$ 2 as an anti-inflammatory/immunosuppressive factor and iNOS as a pro-inflammatory factor. However, their relationship within the condition has never been explored. The purpose of this study was to define the relationship between TGF $\beta$ 2 and iNOS *in vitro* in rat cultured iris-ciliary body epithelial cells and *in vivo* in the endotoxin-induced uveitis (EIU) rat model and, furthermore to evaluate the therapeutic ability of TGF $\beta$ 2 in EIU.

**Methods:** Rat ICB were exposed to TGF $\beta$ 2 overnight and then stimulated for various times with IL-1 $\beta$  for *in vitro* studies. Intraperitoneal TGF $\beta$ 2 and footpad endotoxin injections were co-administered to rats for *in vivo* studies. iNOS expression was examined through RT-PCR and immunoblotting. Transcriptional regulation was determined by investigating I $\kappa$ B/ NF- $\kappa$ B activation. Eyes from *in vivo* studies were also examined histologically.

**Results:** TGF $\beta$ 2 suppressed iNOS mRNA and protein expression *in vitro* and *in vivo*. This suppression, however, did not involve inhibition of the I $\kappa$ B/NF- $\kappa$ B signaling pathway. Furthermore, TGF $\beta$ 2 could not suppress EIU *in vivo*.

**Conclusion:** Understanding the manner in which TGF $\beta$ 2 suppresses iNOS expression may provide insight into the pathogenesis of anterior uveitis.

## INTRODUCTION

TGF $\beta$  belongs to a superfamily of cytokines whose mammalian isoforms include TGF $\beta$ 1, 2, 3. This family of cytokines influences cellular, physiological, and immunological processes. TGF $\beta$  functions in immune processes by modulating the expression of adhesion molecules, providing a chemotactic gradient for leukocytes and other cells participating in an inflammatory response, and inhibiting them once they have become activated. For example, TGF $\beta$  can recruit mononuclear cells to the site of inflammation and can upregulate pro-inflammatory cytokines IL-1 $\alpha$  and TNF- $\alpha$ , indirectly impacting immune processes.<sup>1,2,3</sup> On the contrary, TGF $\beta$  can deactivate macrophages at the site of inflammation by increasing the expression of IL-1 receptor antagonist or by modulating the profile of cytokines important in activation.<sup>4,5,6</sup> These activities indicate a role of TGF $\beta$  in disease progression and resolution.

Recently, TGF $\beta$  has been found to control antimicrobial and tumoricidal pathways and immune responses via regulation of the enzyme inducible nitric oxide synthase (iNOS).<sup>7</sup> Regulation of iNOS by TGF $\beta$  has been demonstrated in a plethora of cell types such as renal mesangial cells, smooth muscle cells, retinal pigment epithelial cells, endothelial cells, and keratinocytes.<sup>8,9,10,11,12</sup> The manner in which TGF $\beta$  suppresses the production of NO, in these cell types, has been evaluated. For instance, in macrophages, TGF $\beta$  was shown to reduce the stability and rate of translation of iNOS mRNA, and increase the rate of degradation of iNOS protein.<sup>13</sup> Studies in smooth muscle cells suggested that TGF $\beta$  could reduce transcription, as well as affect posttranscriptional and translational levels of iNOS.<sup>14</sup> The transcriptional effects of

TGF $\beta$  on iNOS expression may include alterations in binding or downregulation of transcription factors which regulate its expression, in particular nuclear factor-kappa B (NF- $\kappa$ B). NF- $\kappa$ B has been reported to be the principal mediator of LPS-induced iNOS gene transcription.<sup>15</sup> TGF- $\beta$ 1 has been shown to increase the expression of I $\kappa$ B $\alpha$ , which in turn, decreases NF- $\kappa$ B transcriptional activity.<sup>16</sup> Other possible transcription factor targets which might be downregulated are interferon regulatory factor 1 (IRF-1) and activating protein 1 (AP-1) which both contain promoter sites on the iNOS gene.<sup>17,18</sup>

Both TGF $\beta$  and iNOS have been found to be important anti-inflammatory and inflammatory mediators, respectively in the pathogenesis of the ocular inflammatory condition, anterior uveitis. TGF $\beta$  behaves to suppress the inflammatory response whereas iNOS encourages its progression. In the eye, TGF $\beta$ 1 and TGF $\beta$ 2, by suppressing T cell proliferation, play a major role in maintaining ocular immune privilege environment<sup>19</sup>. Loss of TGF $\beta$ 2 activity during uveitis has been correlated with susceptibility to the inflammatory condition.<sup>20</sup> Administration of TGF $\beta$  post-induction of uveitis has been demonstrated to ameliorate the condition. A study by Peng et al showed a decrease in leukocyte count, protein concentration, and IL-6 levels in EIU mice injected intraperitoneally with TGF- $\beta$ 1 eight hours post-endotoxin injection.<sup>21</sup> In addition, a 50% reduction in cellular infiltrate was noted when TGF- $\beta$ 2 was injected intravitreally in LPS-injected rabbit eyes.<sup>22</sup> The involvement of iNOS and NO in the pathogenesis of uveitis was emphasized through studies utilizing NOS inhibitors in endotoxin-induced uveitis (EIU). A reduction in iNOS activity and clinical and histopathological signs was seen with the NOS inhibitor, NG-nitro-L-arginine.<sup>23</sup> Allen

et al further underscored the importance of iNOS in EIU when it was determined that EIU could be abrogated by utilizing a selective iNOS inhibitor, aminoguanidine.<sup>24</sup> These data in accordance with the finding that as TGF $\beta$  levels decrease, NO production increases<sup>25</sup> makes the case that susceptibility to anterior uveitis is linked to dysregulation of iNOS gene expression due to a reduction in TGF $\beta$  activity.

Therefore, the purpose of this study was to determine if TGF $\beta$ 2 negatively modulates iNOS mRNA and protein expression in *in vitro* and *in vivo* models of anterior uveitis. Furthermore, because NF- $\kappa$ B has been recently identified as an important mediator in anterior uveitis,<sup>26</sup> determine if modulation was mediated by its inactivation and lastly, to investigate if administration of TGF $\beta$ 2 exogenously could confer inhibition of the inflammatory condition. *In vitro* studies were conducted using iris/ciliary body (ICB) cells stimulated with IL-1 $\beta$ . The ICB is a major source of TGF $\beta$ 2 and iNOS in the anterior segment. *In vivo* studies were performed using dissected ICB from rats injected with endotoxin (endotoxin-induced uveitis). We determined that TGF $\beta$ 2 can modulate iNOS protein expression at the level of the gene; however, this is through an I $\kappa$ B $\alpha$  independent mechanism. Furthermore, co-administration of TGF $\beta$ 2 with endotoxin did not suppress EIU, emphasizing complexity in which this cytokine behaves physiologically.

## **MATERIALS AND METHODS**

**Isolation and Cytokine stimulation of Iris/Ciliary Body (ICB) Cells:** Normal male Spague Dawley rats were euthanized by exposure to a saturated atmosphere of carbon dioxide. Both eyes were removed and iris/ciliary body micro-dissected. Excised pieces of ICB were placed in collagenase (Worthington Biochemical, Lakewood, NJ) and dispase (Boehringer Mannheim, Indianapolis, IN) at 1mg/ml on ice. Tissues were then incubated at 37°C for 45 minutes to an hour (depending on number of ICB dissected). After incubation cells were spun at 1500 rpm for 5 minutes. Single cell suspensions of ICB were made by pushing the enzyme-treated tissue through a 21-gauge needle followed by a push through a 23-gauge needle. Cells were washed with Dulbecco's modified essential media (DMEM, Mediatech, Herndon, VA) containing 15% fetal bovine serum (FBS, Mediatech) and plated on 100 mm plates in 15% FBS/DMEM. Monolayers grown in parallel were examined for cell type diversity and exhibited characteristics consistent with smooth muscle and epithelial cells (the predominant cell types of ICB). Confluent cells were trypsinized and replated in 100 mm plates and grown to 80-90% confluency. Cells were pretreated with doses of TGFβ2 (1ng/ml, 10 ng/ml, 30 ng/ml) in serum-free media overnight. The following day, cells were rinsed and treated in serum-free media with either fresh doses of TGFβ2 alone, IL-1β at 300U/ml, TGFβ2 and IL-1β for 4 hours for detection of iNOS mRNA. iNOS protein was examined in parallel cultures after 6 hours. For IκBα studies, the conditions were analogous, however, cells were stimulated for 30 minutes. All concentration and time points were derived after dose response and kinetic studies were performed. Cells were always examined morphologically prior to termination of experiment for viability. After

treatment, cells were either harvested with Tri-Reagent for RNA or with hypotonic buffer for cytoplasmic protein extraction.

**Induction of Uveitis and TGF $\beta$ 2 administration:** Adult male Sprague Dawley rats (150-175 grams) were purchased (Charles River, Raleigh, NC) and given unrestricted access to food and water. The animals were handled according to the Association for Research in Vision and Ophthalmology Resolution for the Use of Animals in Ophthalmic and Vision Research and the North Carolina State University Institutional Animal Care and Use Committee. This study consisted of three treatment groups: 1) animals injected with lipopolysaccharide (LPS, 150  $\mu$ g; Sigma, St. Louis, MO) in sterile, pyrogen free saline, in one hind footpad and then 1 ml of sterile saline, intraperitoneal; 2) animals injected with LPS hind footpad and 12  $\mu$ g TGF $\beta$ 2 dissolved in 1 ml sterile saline intraperitoneal, and 3) animals injected with saline hind footpad and 1 ml saline intraperitoneal. At six hours, rats were euthanized similar to in vitro rats, ICB micro-dissected, and snap frozen in protease inhibitor cocktail (Sigma). To evaluate TGF $\beta$ 2 as a therapeutic agent, rats were treated using the protocol stated above, and euthanized at 24 hours. Eyes were placed in 10% neutral buffered formalin and processed for routine histology.

**RNA extraction and Reverse Transcriptase-PCR:** Total cellular RNA was extracted and reverse transcribed as previously described.<sup>27</sup> Briefly, 1  $\mu$ g of RNA was incubated with Moloney–murine leukemia virus (MMLV), MMLV reverse transcriptase buffer, 20 mM dNTP, 0.5  $\mu$ g/ $\mu$ l random hexamers, 20u/ $\mu$ l of RNAase inhibitor at 37°C in 20  $\mu$ l.

The reaction was terminated at 90°C for 5 minutes. Amplification of rat iNOS was performed in a 50 µl reaction by incubating 1 µg of cDNA in 20mM Tris/HCl containing 50mM KCl, 1.5 mM of MgCl<sub>2</sub>, 10 mM dNTP, and 0.5U Taq DNA polymerase with 15 pmoles of the following oligonucleotide primers: GCCCTCACCTACTTCCTGGAC (sense) and CGCACAAAGCAGGGCACTGG (anti-sense). Rat β-actin (sequences ACCACAGCTGAGAGGGAAATCG and AGAGGTCTTTACGGATGTCAACG) was amplified as an internal control under identical conditions. PCR was performed with the protocol, 45s at 94°C, 45s at 60°C, 1.5 min at 72°C for 35 cycles. PCR products( 619 bp for iNOS and 281 bp β-actin) were visualized on a 1.5% agarose gel using ethidium bromide.

**Preparation of Cytoplasmic extracts:** Cytoplasmic extracts were prepared as previously described.<sup>28</sup> Briefly, cells were harvested in 100 µl of hypotonic buffer (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1X Protease Inhibitor Cocktail). Extracts were placed in centrifuge tubes, and incubated on ice for 5 minutes. Samples were centrifuged for 5 minutes at 3,000 rpm at 4°C, supernatants (cytoplasmic extract) collected, and put in new centrifuge tubes. The concentration of cytoplasmic proteins was determined by BCA assay according the manufacturer's instruction (Pierce, Rockford, IL) by using bovine serum albumin (BSA) as a standard.

**Protein Extraction:** Total protein was extracted from in vivo samples as previously described.<sup>27</sup>

**Western Blotting :** For detection of iNOS, 50 µg of cytoplasmic extracts or total protein from in vivo tissue were placed in Laemmli sample buffer, heated at 95°C, and resolved on a 7.5% SDS polyacrylamide gel. Protein bands were transferred electrophoretically onto PVDF. Blots were placed in 5% nonfat drymilk/TBS-T and subsequently incubated with a polyclonal anti-iNOS antibody (1:4000, Transduction Laboratories, Lexington, KY) at room temperature overnight. Blots were rinsed three times in TBS-T and incubated with a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature, rinsed in TBS-T and immunoreactive proteins were visualized by enhanced chemiluminescence. IκBα protein was detected similarly with exceptions being 30 µg of protein from cytoplasmic extracts and protein from tissue extracts were used and blots were incubated with a polyclonal antibody to IκBα (1:3000, Santa Cruz Biotechnology) at room temperature for one hour.

## RESULTS:

***TGF $\beta$ 2 suppresses mRNA and protein expression of iNOS in vitro:*** The ability of TGF $\beta$ 2 to suppress iNOS transcription in ICB stimulated with IL-1 $\beta$  (Fig 1A) was examined at various doses, *in vitro*. Cells were treated overnight with either 1 ng/ml, 10 ng/ml or 30 ng/ml of TGF $\beta$ 2 in serum-free media. The following day, cells were rinsed and fresh doses of TGF $\beta$ 2 were added to plates along with IL-1 $\beta$  for 4 hours. Other treatment groups consisted of control cells, cells treated with TGF $\beta$ 2 alone, or cells stimulated with IL-1 $\beta$  alone. Our results demonstrated a decrease in iNOS transcription in all treatment groups, however this effect was dose-independent. Cells treated with 10ng/ml (lane 4) of TGF $\beta$ 2 exhibited expression similar to the controls of media and TGF $\beta$ 2 alone (lanes 1 and 2, respectively). Minimal iNOS expression was seen in IL-1 $\beta$  stimulated cells treated with 30 ng/ml (lane 5) and 1 ng/ml which proved to be least effective (lane 3) in suppressing iNOS transcription. Examination of protein expression, *in vitro* (Fig 1B) determined that TGF $\beta$ 2 suppressed iNOS protein expression in the presence of IL-1 $\beta$  (lane 4) compared to IL-1 $\beta$  alone (lane 3). Suppression, however, was at the level of the controls, media alone (lane 1) and TGF $\beta$ 2 alone (lane 2).

***TGF $\beta$ 2 suppresses iNOS protein expression in EIU.*** The ICB has been shown to be primary producers of iNOS in the anterior segment during EIU <sup>29</sup>. Kinetic studies of iNOS protein expression *in vivo* have detected (data not shown) protein expression as early as 3 hours, at 6 and 24 hours, with maximal expression at 6 hours. Therefore, a reduction of iNOS protein expression by TGF $\beta$ 2 was investigated in ICB at 6 hours.

iNOS protein expression was examined in rats co-treated with 12 µg of TGFβ2 intraperitoneally and a footpad injection of endotoxin. The results were similar to those seen *in vitro*. iNOS expression in EIU rats injected with TGFβ2 was reduced as compared to those endotoxin alone (Fig 2, lanes 3, 4, respectively). Control levels, *in vivo*, were undetectable (lanes 1 and 2).

***TGFβ2 does not inhibit IκBα degradation in ICB stimulated with IL-1β or in EIU.*** NF-κB has been shown to be a regulator of iNOS transcription as evident by the κB site on the promoter. Expression of iNOS mediated by NF-κB has been shown to occur in the presence of LPS. Moreover, the inhibitory element, IκBα, has been shown to be modulated by TGFβ. NF-κB has been identified as an inflammatory mediator in EIU.<sup>26</sup> Therefore, in this study, degradation of IκBα was used as indicator of NF-κB nuclear translocation *in vitro* and *in vivo*. It has been determined previously that maximal IκBα degradation occurs at 30 minutes *in vitro* in ICB stimulated with IL-1β (data not shown). Thus, IκBα degradation was evaluated in our treatment groups at 30 minutes. Our results demonstrated at 30 ng/ml (Fig 3A, lane 2), as compared to control (lane 1), TGFβ2 could increase IκBα protein. This finding is consistent with the literature. Surprisingly, in the presence of IL-1β, this effect was remarkably overcome as compared to cells stimulated with IL-1β alone (lane 3).

Activation of NF-κB has been shown to occur between 3 and 6 hours *in vivo* in the model of EIU.<sup>26</sup> Therefore we examined degradation of IκBα *in vivo* at 3 and 6 hours (Fig 3B). TGFβ2, similar to our *in vitro* results, was unable to inhibit activation of

NF- $\kappa$ B, in the presence of LPS. At 3 hours, I $\kappa$ B $\alpha$  degradation occurred at the same level in both EIU rats (lane 3) and EIU rats treated with TGF $\beta$ 2 (lane 2). The 6 hour time point represented a partial recovery of protein in both EIU (lane 5) and TGF $\beta$ 2/EIU rats (lane 4) as indicated by expression in ICB of control eyes (lane 1).

***TGF $\beta$ 2 administration does not suppress EIU in vivo.*** Because iNOS has been shown to be a key mediator in various inflammatory conditions and the specific suppression of its production of NO has led to the reduction of various inflammatory conditions. We assessed the ability of exogenous administration of TGF $\beta$ 2 to act therapeutically. Rats co-injected with TGF $\beta$ 2 and endotoxin or co-injected with endotoxin and saline were euthanized at 24 hours and eyes removed for histology. As Fig 4 shows, relative to ICB of EIU eyes (panel C), TGF $\beta$ 2 co-administered did not ameliorate the condition (panel B). An influx of inflammatory cells was seen in the ICB of both treatment groups as compared to control ICB (panel A).

## DISCUSSION

The immunosuppressive cytokine TGF $\beta$  and the pro-inflammatory mediator iNOS have been extensively studied in anterior uveitis. However, their relationship within the condition has never been explored. In this report, we demonstrated that TGF $\beta$ 2 decreased expression of iNOS mRNA in ICB stimulated with IL-1 $\beta$  and in ICB dissected from EIU eyes. This effect was translated at the protein level where iNOS expression, *in vitro* and *in vivo*, was also decreased. We explored the mechanism of transcriptional regulation of iNOS by determining if nuclear translocation of the pro-inflammatory transcription factor, NF- $\kappa$ B was inhibited. This was completed by investigating whether degradation of the inhibitory element, I $\kappa$ B $\alpha$  occurred in the presence of IL-1 $\beta$ . TGF $\beta$  has been identified as a regulator of NF- $\kappa$ B activation as assessed by an increase in I $\kappa$ B $\alpha$  transcription<sup>16</sup>. Furthermore, transcriptional activation by I $\kappa$ B /NF- $\kappa$ B has been shown to be important in EIU. Results from our *in vitro* study demonstrated that TGF $\beta$ 2 could suppress I $\kappa$ B $\alpha$  transcription relative to control, however not in the presence IL-1 $\beta$ . *In vivo* results were similar. TGF $\beta$ 2 inability to inhibit I $\kappa$ B $\alpha$  degradation explains why a total suppression of iNOS did not occur. This conclusion is derived from a study by Perrella et al who showed in vascular smooth muscle cells stimulated with LPS that TGF $\beta$ 1 could only partially inhibit iNOS transcription.<sup>30</sup> The authors concluded that partial suppression was linked to the magnitude of induction of iNOS by NF- $\kappa$ B. Accordingly, we speculate that TGF $\beta$ 2 reduction of iNOS gene transcription is not a direct result of decreased NF- $\kappa$ B activity, and that suppression may involve downregulation of other transcription factors important in iNOS gene regulation.

IRF-1 has been shown to be a critical modulator of iNOS expression. This transcription factor is induced by pro-inflammatory cytokines, IFN- $\beta$ , and IFN- $\gamma$ . Additionally, IFN- $\gamma$  has been identified as a potent stimulator of iNOS in macrophages. IRF-1 significance in iNOS gene regulation is best noted in studies that demonstrated that macrophages from IRF -/- mice produced barely detectable levels of iNOS mRNA which correlated with minimal NO production.<sup>31</sup> Furthermore, synergistic activation of IFN- $\gamma$  and LPS is only seen when IRF-1 binds to its response element on the iNOS promoter.<sup>18</sup> The role of IRF-1 has not been studied in anterior uveitis. This perhaps may be a new mediator to explore, primarily, because IRF-1 regulates, through IFN- $\gamma$ , the T<sub>H</sub>1 response. The immunopathogenesis of anterior uveitis has been shown to be T-cell mediated. Therefore, IRF-1 and the cytokines important in its regulation may play a significant role in the pathogenesis of anterior uveitis.

A response element to AP-1 exists on the iNOS promoter as well. AP-1, similar to NF- $\kappa$ B, can increase levels of iNOS in response to LPS. However, Tran-thi et al demonstrated in Kupffer cells stimulated with LPS, that DNA binding activity of AP-1 could not be inhibited by TGF $\beta$ .<sup>32</sup>

The most promising insight, pertinent to our study, is the finding that Smad3, a downstream transcription factor in TGF $\beta$  signaling, is a key player in the transcriptional regulation of iNOS by TGF $\beta$ .<sup>33</sup> Smad3, a member of the Smad family of transcription factors, has been termed a “R-Smad” or receptor-mediated Smad. During TGF $\beta$  signaling, it associates with the other R-Smad, Smad2, and is phosphorylated by TGF $\beta$  Type I receptor. The Smad2/Smad3 complex heterooligomerizes with Smad4 and

translocates to the nucleus to alter transcription of genes regulated by TGF $\beta$ . In the study by Werner et al., in macrophages treated with LPS, transient transfection of Smad3 construct was able to suppress iNOS, NF- $\kappa$ B, AP-1, activity similar to that of TGF $\beta$ . Addition of TGF $\beta$  enhanced suppression. Smad3 was shown to be the only Smad capable of this response. Transactivation was hypothesized to occur through association of Smad3 with the p300/CREB transcriptional complex. This complex is essential for transcriptional activation of genes regulated by NF- $\kappa$ B and AP-1 alike. Thus, TGF $\beta$  may not directly impact or alter transcription factors important in iNOS regulation, it may, through Smad3, reduce iNOS transcription by competing for transcriptional activators essential to its activation.

Although studies have strongly suggested a role for transcriptional regulation, posttranscriptional and posttranslational modifications remain a strong possibility. In murine macrophages stimulated with IFN- $\gamma$ , the authors reported that treatment of cells with TGF $\beta$ 1 reduced mRNA levels but not at the level of the gene. The reduction in mRNA levels was attributed to a decrease in mRNA stability.<sup>13</sup> Conversely, Perrella et al demonstrated in IL-1 $\beta$  stimulated rat smooth muscle cells, that downregulation of iNOS mRNA was not linked to a reduction of mRNA stability but was due to a 65% drop in the transcriptional rate.<sup>14</sup> Collectively, these data highlight the intricate factors involved in iNOS gene regulation. Studies will be undertaken to elucidate relevant pathways critical to understanding the mechanisms involved in our system.

Our final study evaluated the therapeutic potential of TGF $\beta$ 2 in the model of EIU. TGF $\beta$  has been explored as a therapeutic agent in the model of arthritis where systemic administration of TGF $\beta$ 1 profoundly antagonized the development of polyarthritis.<sup>34</sup>

Systemic injection of TGF $\beta$ 2, in our study, as noted histologically did not render rats injected with endotoxin protection. This is in contrast to other ocular studies which have evaluated the therapeutic potential of this cytokine. Rabbit eyes injected intraocularly with 100 ng of TGF $\beta$ 2 and LPS exhibited a decrease in inflammatory cell infiltrate in the anterior uvea and in the aqueous humor.<sup>22</sup> An intraperitoneal injection of TGF $\beta$ 1 eight hours after LPS injection reduced inflammation and IL-6 levels in the mouse model of EIU.<sup>21</sup> Therefore, these studies suggest that the disparity in response in our system versus responses noted in others may be correlated to species differences and these differences are also associated with routes of administration.

In conclusion, this study has further extended the role of TGF $\beta$  as an immunosuppressive agent, i.e. regulatory element in iNOS gene transcription. Understanding the mechanism in which this cytokine suppresses iNOS and regulates leukocyte function, may provide the necessary insight for use of this cytokine in the treatment of anterior uveitis and other debilitating inflammatory conditions.

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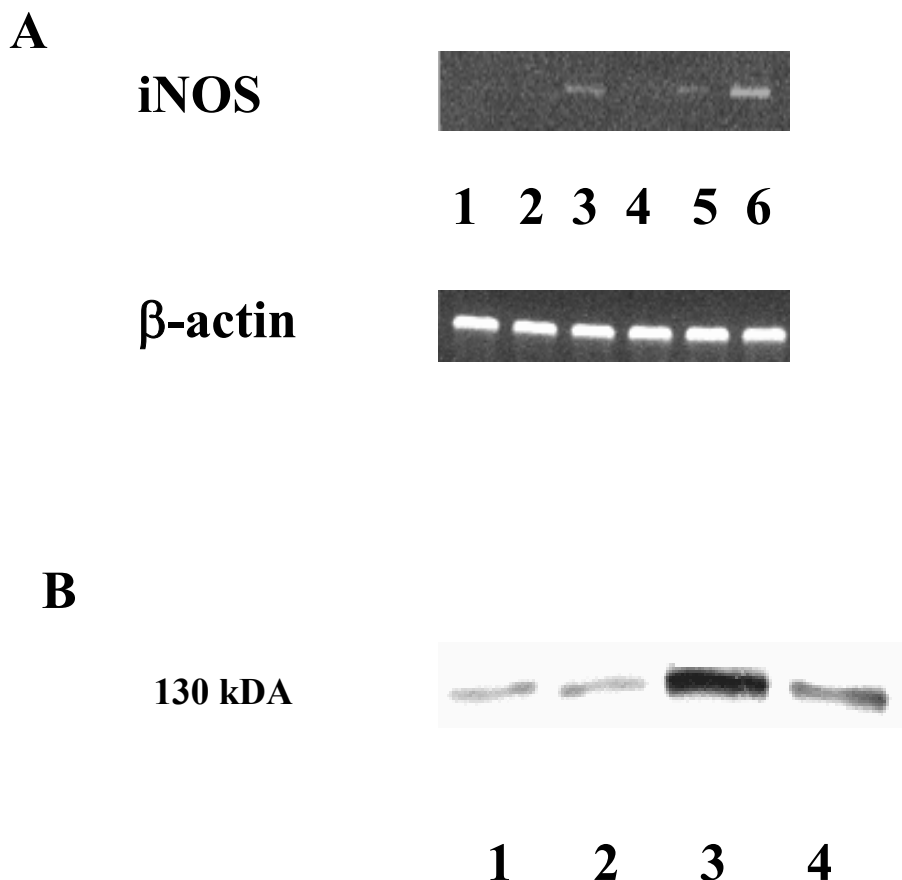


**Figure 1. TGFβ2 suppresses iNOS mRNA and protein expression *in vitro*.** (A) PCR for iNOS was performed from cDNA of ICB cells treated with 1 ng/ml (lane 3), 10 ng/ml (lane 4) or 30 ng/ml (lane 5) of TGFβ2 overnight and then stimulated with 300 U/ml of IL-1β for 4 hours (lanes 3- 6). iNOS mRNA expression was decreased in all treatment groups as compared to ICB cells stimulated with IL-1β for 4 hours. Expression of iNOS at 10 ng/ml TGFβ2 (lane 4) was similar to control cells, cells in serum-free media (lane 1) and cells treated with TGFβ2 alone (lane 2). (B) Protein expression of iNOS was examined at 6 hours under similar treatment conditions. Results demonstrate that as compared to IL-1β stimulated cells (lane 3), TGFβ2 suppresses iNOS expression (lane 4). Control levels demonstrate some basal level of iNOS expression (lane 1, media alone; lane 2, TGFβ2 alone).

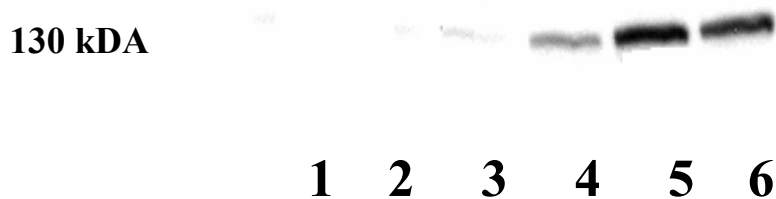
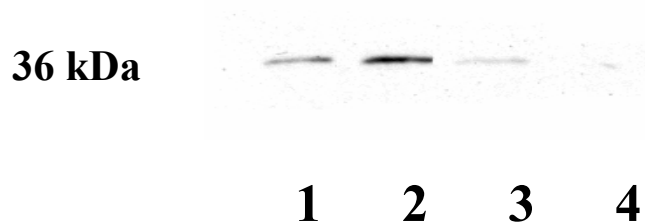


**Figure 2. TGF $\beta$ 2 suppresses iNOS protein expression *in vivo*.** Rats were co-injected with a footpad injection of endotoxin and an intraperitoneal injection of 1 TGF $\beta$ 2. At 6 hours, animals were euthanized and ICB dissected. A representative blot demonstrates that TGF $\beta$ 2 at 30 ng/ml decreases iNOS expression (lanes 3, 4) in EIU rats as compared to rats injected with endotoxin alone (lanes 5, 6). Expression of iNOS in control animals was undetectable (lanes 1, 2).

A



B

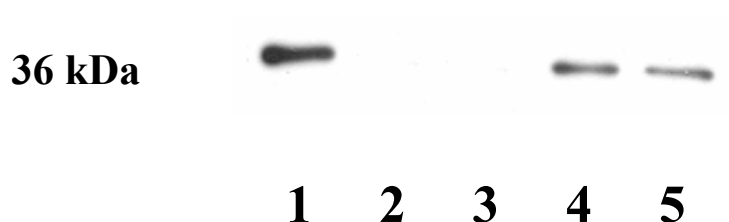


**Figure 3. TGF $\beta$ 2 does not inhibit IkB $\alpha$  degradation in ICB stimulated with IL-1 $\beta$  *in vitro* or *in vivo*.** (A) ICB were treated and stimulated as described in iNOS protein studies. The data indicate that TGF $\beta$ 2 alone represses degradation of IkB $\alpha$  (lane 2) as compared to control lane (lane 1). However, in the presence of IL-1 $\beta$  this effect is overcome. Degradation of IkB $\alpha$  occurs in TGF $\beta$ 2 treated ICB cultures stimulated with IL-1 $\beta$  (lane 4) and those treated with IL-1 $\beta$  alone (lane 3). (B) Degradation of IkB $\alpha$  was examined at 3 and 6 hours in rats co-injected with TGF $\beta$ 2 and endotoxin and compared to those injected with endotoxin alone. Results at 3 hours demonstrated that TGF $\beta$ 2 does not block degradation of IkB $\alpha$  (lane 2) as compared to rats injected with endotoxin alone (lane 3). At six hours, protein levels of both TGF $\beta$ 2 treated and EIU (lane 4 and 5, respectively) rats resemble control (lane 1).

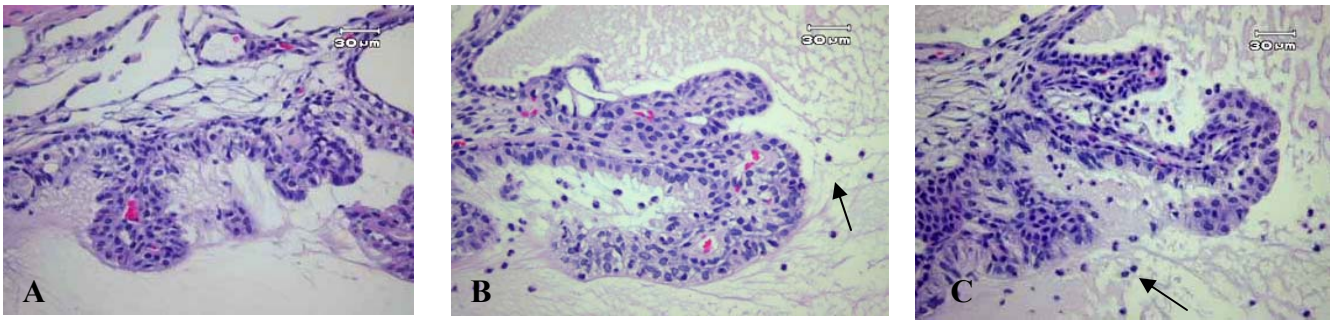


**Figure 4. TGFβ2 administration does not suppress EIU.** Animals were co-injected either with TGFβ2 alone and saline (panel A), TGFβ2 and endotoxin (panel B) or endotoxin alone and saline as reported in Materials and Methods. Animals were euthanized at 24 hours, eyes removed and placed in formalin for routine histology. Hematoxylin and eosin staining shows that compared to animals injected with endotoxin (panel C), TGFβ2 administration does not markedly diminish inflammatory cells infiltrating the ICB (panel B). Arrows indicate presence of leukocytic infiltrate.

## Chapter 2

### Nitration of Manganese Superoxide Dismutase during Ocular Inflammation

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## Summary

Reactive nitrogen species, in particular, peroxynitrite ( $\text{ONOO}^-$ ) have been proposed to play an important role in the pathogenesis of endotoxin-induced uveitis (EIU). Tyrosine nitration by  $\text{ONOO}^-$  has been shown in other model systems to inhibit the activity of the superoxide anion quenching enzyme, manganese superoxide dismutase (MnSOD), perhaps contributing to progression of disease. In this study, we confirm through immunoanalysis that nitrated proteins are produced during EIU, and furthermore, that MnSOD is a target of nitration during the inflammatory response. In addition, through microsequencing analyses, nitrated albumin-apparent in both control and EIU eyes- was identified. Positive immunostaining of nitrated proteins was seen in the ciliary epithelium, inflammatory cells, and protein exudate of eyes from rats injected with endotoxin. Incubation of nitrotyrosine immunoprecipitates from the iris and ciliary body (ICB) with a polyclonal antibody against MnSOD revealed that nitrated MnSOD was present only in the ICB of EIU rats. When total activity of the enzyme was examined, it was observed that despite the presence of nitrated MnSOD, activity was increased relative to control. Analysis of MnSOD mRNA and protein from the ICB of both groups demonstrated an increase in mRNA expression and consequently a 3-5 fold increase in MnSOD protein in EIU rats as compared to control rats. Further examination of MnSOD protein expression through immunohistochemistry noted enhanced immunostaining in the ciliary epithelium of eyes of EIU rats. Additional investigation of a 70kDa band apparent in nitrotyrosine immunoprecipitates from the ICB of control and EIU rats revealed that the plasma protein albumin is nitrated as well. This protein is present as a result of the breakdown of the blood aqueous barrier during inflammation. In summary, two

endogenous nitration targets, albumin and MnSOD, were identified. Nitrated MnSOD appears to be specifically targeted to the ICB during inflammation, underscoring the importance of the interface in EIU. Furthermore, the expression and activity of the enzyme is increased in the ICB during EIU, perhaps regulating reactive nitrogen species produced within the cells. This study implicates ONOO<sup>-</sup> in the pathogenesis of EIU and imparts the putative role MnSOD plays in disease resolution.

***Key Words***

Uveitis

Nitric Oxide

Peroxynitrite

Nitration

MnSOD

Albumin

Reactive Nitrogen Species

## 1. INTRODUCTION

Nitric oxide has emerged as a key molecule in the pathology of disease. Pathophysiological effects are related to generation of its toxic metabolites including nitrogen dioxide ( $\text{NO}_2$ ), nitrite ( $\text{NO}_2^-$ ), nitryl ion ( $\text{NO}_2^+$ ) and peroxynitrite ( $\text{ONOO}^-$ ), collectively termed 'reactive nitrogen species' (RNS) (Patel et al., 1999). Studies from animal models of uveitis have implicated reactive oxygen/nitrogen species in the pathogenesis of the disease. Experimental models of uveitis in rats, rabbits, or mice have demonstrated that inducible nitric oxide (NO) is an important uveitic mediator (Bellot et al., 1996, Parks et al., 1994, Wu, Zhang and Rao, 1997). Inhibition of inducible nitric oxide synthase (iNOS), the enzyme responsible for the production of NO, suppresses the inflammatory response (Allen et al., 1996, Goureau et al., 1995, Tilton et al., 1994). Researchers have proposed that this effect may be due to the inhibition of  $\text{ONOO}^-$  (Allen, Keng and Privalle, 1998)

$\text{ONOO}^-$ -the best understood and most reactive species—was initially viewed as excellent scavenger and neutralizer of  $\text{O}_2^-$ , however it is now thought of as a potent mediator of cytotoxicity (Patel et al., 1999).  $\text{ONOO}^-$  reacts with biomolecules through oxidation and nitration reactions. Oxidative processes are the most efficient reactions and include oxidation of protein-metal centers of biomolecules, such as the iron center of hemoglobin, to form methemoglobin, the zinc-thiolate centers of DNA binding transcription factors, and the seleno-cysteine residues of the antioxidant, glutathione (Squadrito and Pryor, 1998). Nitration of phenolic residues occurs less frequently, although under pathological conditions, the rate of formation can increase 100 fold (Crow and Beckman, 1996).

Recent studies have identified specific proteins modified by nitration in human, animal, and cellular models of disease (MacMillan-Crow et al., 2000, Souza et al., 1999, Viner et al., 1996, Zou, Martin and Ullrich, 1997)]. Manganese superoxide dismutase (MnSOD), the mitochondrial enzyme critical in regulating  $O_2^-$  levels within the cell, was the first nitrated protein to be unambiguously identified (MacMillan-Crow et al., 1996). MnSOD was found to be tyrosine nitrated and inactivated during human chronic renal allograft rejection. Additional studies demonstrated that complete inactivation of MnSOD initiated by  $ONOO^-$  can occur independent of tyrosine nitration of residues located in the enzyme's active site. Complete inactivation requires not only nitration of critical tyrosine residues, but also tyrosine oxidation and subsequent formation of dityrosine (MacMillan-Crow, Crow and Thompson, 1998, MacMillan-Crow and Thompson, 1999). Earlier studies which demonstrated embryolethality of homozygous MnSOD knockout mice have provided unequivocal evidence that MnSOD is essential for life (Lebovitz et al., 1996, Li et al., 1995), thus, nitration of MnSOD may underlie many pathologic situations.

Here, we are the first to report that MnSOD is nitrated during an acute inflammatory response, specifically, endotoxin-induced uveitis (EIU). The enzyme was only found to be nitrated in the iris and ciliary body of rats injected with endotoxin as compared to those injected with saline. In addition, we also report that MnSOD mRNA and protein are upregulated during the uveitic response. These results establish  $ONOO^-$  as a participant in the pathogenesis of this eye disease and suggest that upregulation of MnSOD may play a role in disease resolution.

## **2. MATERIALS AND METHODS**

### ***Endotoxin-Induced Uveitis (EIU)***

To induce uveitis, one footpad of female Lewis rats (150 g, Charles River farms, Clayton, NC) was injected with lipopolysaccharide (LPS, 250 µg; Sigma, St. Louis, MO) in 100 µl of 0.9% sterile, pyrogen-free saline. Control rats were injected with saline alone. Twenty-four hours later, animals were euthanized via overexposure to halothane (Halocarbon Laboratories, River Edge, NJ) and cervical dislocation. Eyes from control and EIU rats were either removed and placed in 10% neutral buffered formalin for immunohistochemistry or the iris ciliary body (ICB) dissected. Dissected ICBs were placed in either Tri-Reagent (Sigma) for RNA extraction or snap frozen on dry ice for protein extraction.

### ***Immunohistochemistry of nitrated proteins***

Formalin-fixed tissue was paraffin-embedded and 5 µm sections cut and immunostained. Sections were deparaffinized in xylene, rehydrated in a series of alcohols, and rinsed in PBS. Peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide in methanol for 20 minutes. Slides were rinsed with 3 changes of PBS (pH 7.2) and incubated with 1% bovine serum albumin (BSA, Sigma) for 20 minutes at room temperature. Sections of whole eye were immediately incubated with rabbit polyclonal anti-nitrotyrosine antibody (1:200 in 1% BSA, Upstate Biotechnology Inc) overnight at 4°C. Nitrotyrosine immunoreactivity was determined using a Quick Staining Kit (Dako, Inc, Carpinteria, CA). Positive immunostaining was detected using diaminobenzidine (DAB). Sections were counterstained with hematoxylin and mounted with permount.

For MnSOD staining, after deparaffinization and rehydration, slides were rinsed in PBS and placed in 2 changes of 50 mM glycine (pH 3.5) with 0.01% EDTA at 95°C for antigen retrieval. Slides were then rinsed with deionized water (3X) and peroxidase activity quenched. After three washes in PBS, sections were blocked with 0.25% casein (Dako Inc) for 1 hour and immediately incubated with rabbit polyclonal anti-MnSOD (1:50 in 1% BSA; Upstate Biotechnology Inc, Lake Placid, NY) overnight. Slides were rinsed in three changes of PBS and immunoreactivity to MnSOD was detected using an LSAB2 kit (Dako, Inc) per manufacturer's instruction. Positive immunostaining was detected using DAB.

### ***Protein extraction***

Total protein from the ICB of control and EIU rats was extracted from samples snap frozen on dry ice. Tissue was then homogenized with a pestle in 150 µl of protease inhibitor cocktail (Sigma, St. Louis, MO) in PBS. Samples were spun at 12,000 rpm for 5 minutes at 4°C. Supernatants were collected and protein concentrations measured by Bradford Assay (BioRad, Hercules, CA).

### ***Nitrotyrosine immunoprecipitation***

Extracted protein (200 µg) from above was pre-cleared with 10 µl of Protein G (Life Technologies, Rockville, MD) and placed on a nutator for 20 minutes at 4°C. Beads were pelleted, supernatant collected and incubated with 10 µg of anti-nitrotyrosine agarose conjugate (Upstate Biotechnology Inc, Lake Placid, NY) overnight at 4°C. Twenty-four hours later beads were pelleted and prepared for immunoblotting.

### ***Western blot analysis***

For detection of both nitrated MnSOD (immunoprecipitated extracts, see above) and MnSOD protein (40 µg of total protein) samples were placed in Laemmli sample buffer (0.5M Tris, 10% glycerol, 2% SDS, 700 mM 2-β-Mercaptoethanol, 0.12% bromophenol blue), boiled for 10 minutes and resolved on a 15% SDS polyacrylamide gel. Protein bands were transferred electrophoretically onto PVDF membrane. Blots were placed in 10% nonfat drymilk/TBS-T (10 mM Tris, 140 mM NaCl, 0.15% Tween) and subsequently incubated with a rabbit polyclonal anti-MnSOD antibody (1:1000, Upstate Biotechnology Inc, Lake Placid, NY) at room temperature for one hour. Blots were rinsed three times in TBS-T and incubated with a horseradish peroxidase- conjugated anti-rabbit secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature. Blots were rinsed in TBS-T (3X-10 minutes) and immunoreactivity to MnSOD (24 kDa) was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ)

### ***MnSOD activity assays***

Activity of MnSOD in tissue extracts was measured using the cytochrome c reduction method (McCord and Fridovich, 1969) in the presence of 2mM potassium to inhibit copper zinc SOD (Cu Zn SOD) and extracellular SOD (EcSOD).

### ***RNA extraction and RT-PCR***

Total RNA from the ICB of control and EIU rats was isolated using Tri-Reagent (Sigma) per manufacturer's instruction (Sigma). RNA was reverse transcribed in a 20 µl reaction at 42°C. Briefly, 1 µg of RNA was incubated with 200 units/ul of Moloney–murine leukemia virus (MMLV), MMLV reverse transcriptase buffer (1X), 20 mM

dNTP, 0.5 µg/µl random hexamers, 20u/µl of RNAase inhibitor [Promega Corp, Madison,WI]). The reaction was terminated by heating to 90°C for 5 minutes. Amplification of rat MnSOD (Sugino et al., 1998) was performed in a 50 µl reaction by incubating 1 µg of cDNA in 20mM Tris/HCl containing 50mM KCl, 1.5 mM of MgCl<sub>2</sub>, 10 mM dNTP, and 0.5 U Taq DNA polymerase (Perkin Elmer, Roche Diagnostics) with 15 pmoles of the following oligonucleotide primers: 5'-ATTAACGCGCAGATCATGCAG-3'(sense) and 5'-TTTCAGATAGTCAGGTCTGACGTT-3'(anti-sense). Rat β-actin -sequences 5'-ACCACAGCTGAGAGGGAAATCG-3' and 5'-AGAGGTCTTTACGGATGTCAACG-3' was amplified as an internal control under identical conditions. PCR was performed under the following conditions 45 secs at 94°C, 45 secs at 60°C, 1.5 min at 72°C for 30 cycles. PCR products (483 bp MnSOD and 281 bp β-actin) were visualized at 25 and 30 cycles on a 1.5% agarose gel.

### ***Statistics***

Statistical differences in MnSOD activity from control and EIU samples were determined using a one-tailed paired Student's t test.

### 3. RESULTS

#### *Nitrated proteins in EIU*

Increased ONOO<sup>-</sup> production has been implicated in the pathogenesis of several diseases, therefore we were interested in determining if it was involved in the progression of EIU. ONOO<sup>-</sup> can modify proteins through the addition of NO<sub>2</sub> moiety and this modification can be detected immunologically through the use of an antibody against nitrated tyrosine residues (van der Vliet et al., 1996). Protein tyrosine nitration was investigated through immunohistochemistry. Paraffin sections from control and EIU eyes were probed with an antibody against nitrotyrosine and immunoreactivity was detected using DAB. Positive immunostaining was seen in the ciliary epithelium in sections from control (Fig. 1[A]) and EIU eyes (Fig. 1[C]). In addition, inflammatory cells and protein exudate from EIU eyes immunostained positive (arrows; Fig. 1[C]). Specificity of staining was demonstrated by probing serial sections from control and EIU rats with primary antibody incubated with 10mM 3-nitrotyrosine (Sigma), (Fig. 1[B] and Fig. 1[D], respectively).

#### *Tyrosine nitration of MnSOD and albumin in EIU*

Because the ICB is known to be a source and target of RNS, specific nitration targets were investigated through nitrotyrosine immunoprecipitation of protein extracts from ICB of control and EIU rats (Fig. 2, lanes 1 and 2, respectively). Following immunoprecipitation, samples were separated on 15% SDS-PAGE and stained with Coomassie Blue. Results showed that although the protein profile of nitrated products appeared similar in both treatment groups, the amount of nitrated proteins was increased in ICB from EIU rats.

Since MnSOD has been demonstrated to be a sensitive target of tyrosine nitration *in vivo* (MacMillan-Crow et al., 1996), anti-MnSOD Western analysis of nitrotyrosine immunoprecipitates from ICB of control and EIU rats was performed. Results showed that MnSOD is nitrated only in extracts from EIU rats (Fig. 3, lanes 3, 4) and not in extracts from control eyes (Fig. 3, lanes 1, 2). Since earlier reports have documented that tyrosine nitration and oxidation (through the formation of dityrosine) of MnSOD parallels inactivation of the enzyme (MacMillan-Crow et al., 1998, MacMillan-Crow and Thompson, 1999), analysis of MnSOD was performed using a standard cytochrome c reduction method. As illustrated in Fig 4, a statistically significant increase in the activity of MnSOD in extracts from control (lane 1) and EIU eyes (lane 2) was observed.

A Coomassie-stained band of about 70 kDa- apparent in the nitrotyrosine immunoprecipitation of both control and EIU rats- was excised and analyzed through microsequencing (arrow, Fig. 2). The band was identified as albumin. This plasma protein, known to be a part of the protein exudate present in the anterior chamber (Allen et al., 1996, Fleisher, Ferrell and McGahan, 1990), enters the anterior chamber as a result of the breakdown of blood aqueous barrier. To our knowledge this is the first report of its nitrated presence in the eye *in vivo*.

### ***Upregulation of MnSOD in EIU***

It has been documented in inflammatory models involving kidney, lung, and intestine that LPS as well as cytokines such as IL-1 and TNF- $\alpha$  increase MnSOD expression (Gwinner, Tisher and Nick, 1995, Tannahill et al., 1997, Visner et al., 1990). The manner in which these mediators affect the expression of MnSOD in uveitis has yet to be reported. Therefore, the expression of MnSOD was investigated using RT-PCR,

Western analysis, and immunohistochemistry. Through RT-PCR, it was determined that MnSOD mRNA is upregulated during EIU. PCR product was removed at 25 and 30 cycles from control and EIU eyes and compared. At 25 cycles mRNA expression of MnSOD (483 bp fragment) was found to be increased in eyes from EIU rats (Fig. 5A, lanes 4-6) whereas in control eyes negligible expression was detectable (Fig. 5A, lanes 1-3). However, at 30 cycles, expression is seen in both control and EIU eyes with a greater intensity of expression in the inflamed tissue (Fig. 5B, lanes 1-3; lanes 4-6, respectively).

The expression of MnSOD protein was examined by Western blot. Extracts of ICB from EIU and control eyes were immunoblotted and probed with a polyclonal antibody against MnSOD. An immunoreactive band at 24 kDa corresponding to monomeric MnSOD was seen in both groups of animals (Fig 6A), nonetheless, densitometric analysis revealed a 3-5 fold increase in expression of MnSOD in ICB from EIU eyes (Fig. 6B, lanes 3, 4) versus that of controls (Fig. 6B, lanes 1, 2).

Immunohistochemistry was utilized to determine the localization of MnSOD protein expression using a polyclonal antibody against MnSOD. The iris and ciliary epithelium from both control and EIU eyes immunostained positive for MnSOD (Fig. 7A, 7B, respectively). However, corresponding to RT-PCR and Western analysis, an elevated expression of MnSOD was seen in EIU eyes as compared to control. Immunoreactivity was not observed in the inflammatory cells infiltrating the anterior chamber (arrows), indicating the probable source of nitrated MnSOD at 24 hours to be the ciliary epithelium.

#### 4. DISCUSSION

Previous studies have highlighted the importance of  $\text{ONOO}^-$  in models of ocular inflammation. Wu et al implicated  $\text{ONOO}^-$  in the etiology of experimental autoimmune uveitis via the detection of nitrated proteins in the photoreceptors and nerve fiber cell layers of the retina (Wu et al., 1997). Studies from our lab, using the rabbit model of EIU, noted positive staining of nitrated protein in extravasated inflammatory cells in the anterior chamber of the eye as well as in the epithelium of the iris and ciliary body, suggesting that  $\text{ONOO}^-$  might be involved in development of the disease (Allen et al., 1998). Our current study, using the rat model for EIU, further supports the importance of RNS in the pathogenesis of EIU. In accordance with our previous studies, positive staining was seen in the inflammatory cells and ciliary epithelium. Nitrotyrosine immunoreactivity in inflammatory cells (mainly neutrophils and macrophages) reflects the high concentration of  $\text{ONOO}^-$  produced in these cells following an immune challenge.

The nitrating source in these cells has recently become the subject of much debate. Nitration of tyrosine was primarily thought to be unique to  $\text{ONOO}^-$  and thus a marker for its formation, however, recent literature has introduced the theory that myeloperoxidase, the most abundant protein in neutrophils, may, under inflammatory conditions, induce tyrosine nitration (Eiserich et al., 1998). In activated neutrophils, the microbicidal enzyme can oxidize  $\text{NO}_2^-$  to  $\text{NO}_2^-$  or  $\text{NO}_2^+$  resulting in two species capable of nitrating phenolic residues (Burner et al., 2000). These findings introduce a new perspective as to the origin of nitration seen during a uveitic response, and potentially a new target for intervention.

Interestingly, we did not see a dramatic difference in the nitration in epithelium of control eyes compared to EIU eyes. In fact, in some areas of the ciliary epithelium, staining was more intense in control eyes as compared to EIU eyes. This may possibly be due to the release of soluble nitrated proteins into the anterior chamber of inflamed eyes during inflammation. To elaborate, it is evident from the immunohistochemistry of control eyes that nitration is a constitutive process occurring as a result of the high metabolic activity in the ciliary epithelium. These cells are responsible for maintaining the clarity of the aqueous humor by controlling the amount of protein present in the fluid (Krause and Raunio, 1969, Krause and Raunio, 1969). During inflammation, the intercellular junctions of these cells are compromised and they essentially become a semi-porous bilayer allowing various constituents to become a part of the aqueous humor and subsequently enter the posterior chamber (Dernouchamps, 1982). The release of these proteins from the ciliary epithelium into the posterior chamber during inflammation may account for the differences in nitrotyrosine immunoreactivity seen in the ciliary epithelium relative to protein exudate. More importantly, it could account for the differences in nitration seen in the ciliary epithelium of control eyes compared to EIU eyes.

Tyrosine nitration, once thought to be a ubiquitous event, has now been shown to be a selective process—not all tyrosine residues of proteins are nitrated nor are all proteins nitrated in vivo (Patel et al., 1999). Nitration selectivity was first recognized in rejected human renal allografts via the detection of nitrated and inactivated MnSOD (MacMillan-Crow et al., 1996). Since that pivotal study, several endogenous targets have been identified (Crow et al., 1997, MacMillan-Crow et al., 2000, Souza et al., 1999,

Viner et al., 1996, Zhang et al., 2000, Zou et al., 1997). Results from our study indicate that nitration is also a discriminatory process in EIU. Immunoprecipitation results from protein extracts from the ICB of control eyes revealed that there is a constitutive level of nitration *in vivo*, however, as identified after immunoblotting, only tissue extracts from the ICB of EIU eyes contained nitrated MnSOD. The nitration of MnSOD did not result in a loss of total activity as demonstrated by the cytochrome c reduction method. It seems that both tyrosine nitration and oxidation (through dityrosine formation) of MnSOD are required for complete inactivation of the enzyme (MacMillan-Crow et al., 1996). No detectable levels of dityrosine could be measured (data not shown) and furthermore, no apparent higher molecular weight aggregates of MnSOD were observed following SDS-PAGE (Fig. 6A).

The increase in enzymatic activity in the ICB of EIU eyes may provide another explanation as to differences seen in nitrotyrosine immunostaining of ciliary epithelium of EIU eyes compared to control. An increase in the specific activity of MnSOD may reduce the level of circulating nitrating species within the cells of the ciliary epithelium and thus reduce the amount of nitration detected. Proteins released into the posterior chamber as a result of the compromised integrity of the ciliary epithelium are not privy to the increase in enzymatic activity and therefore are more susceptible to RNS released by the cells of the inflammatory infiltrate and surrounding epithelium.

It was suggested that an increase in cellular expression might be a compensatory response to nitration (MacMillan-Crow et al., 1996). Thus, we explored the expression of MnSOD during EIU by investigating its cellular expression through RT-PCR, immunoblotting, and immunohistochemistry.

It has been well documented *in vitro* that LPS and other inflammatory mediators can induce expression of MnSOD. Visner et al demonstrated in rat pulmonary epithelial cells (PEC) that in response to LPS, IL-1, and TNF- $\alpha$  mRNA levels of MnSOD increase (Visner et al., 1990). In glomerular epithelial cells (GEC), mRNA and protein levels of MnSOD increase in cells stimulated with IL-1 $\alpha$  or LPS. A 40- fold induction in MnSOD mRNA was seen in GEC stimulated with IL-1 $\alpha$  alone (Gwinner et al., 1995). We report similar results *in vivo* for the eye. An increase in mRNA corresponded to a 3-5 fold induction of MnSOD protein in tissue extracts of EIU eyes as compared to control. This 3-5 fold induction may be responsible for the dramatic increase in activity seen in these cells versus control. Similar results were seen in control and EIU eyes through immunohistochemistry. Through immunohistochemistry, we also determined that the predominant source of MnSOD at the peak of inflammation is the epithelia of the ciliary body not the inflammatory cells infiltrating the interface. These findings were not surprising considering the abundance of mitochondria in these cells (specifically, the nonpigmented epithelium of the ciliary body), a reflection of their high metabolic activity.

Thus, we speculate based upon our results and the aforementioned studies that the increase in cellular expression and activity of MnSOD is a result of inflammatory mediators (IL-1, TNF- $\alpha$ ) acting upon the ICB, conceivably regulating the production of RNS produced in these cells during inflammation. Thus, nitrated MnSOD produced by the ICB (24 hours post-injection) manifests “as a consequence” of the increased NO activity generated in these cells in response to mediators. Moreover, the increase in activity is not only a result of the unobserved oxidation of tyrosine residues but also a

result of the increase in cellular expression of the enzyme. Perhaps, if the level of nitrated MnSOD exceeded the level of enzyme expression, tyrosine oxidation would have been detected, and a loss of activity resulted.

It is likely that the mechanism of nitration of MnSOD is ONOO<sup>-</sup> dependent rather than myeloperoxidase driven. This is supported by studies which demonstrated NO or ONOO<sup>-</sup> can irreversibly inhibit mitochondrial enzymes important in respiration and the likelihood of this inhibition was related to the propensity of NO and O<sub>2</sub><sup>-</sup> to interact within the mitochondrial matrix (Brown and Borutaite, 1999). In addition, results from studies using human kidney extract show that ONOO<sup>-</sup> can nitrate tyrosine residues of MnSOD *in vitro* (MacMillan-Crow et al., 1996).

We identified through microsequencing another nitration target, albumin. This nitrated protein has been previously identified *in vivo* (Greenacre et al., 1999). The band was apparent in nitrotyrosine immunoprecipitates from both control and EIU ICB. The presence of the plasma protein in both groups results as a consequence of the filtration process occurring in the ICB and the impairment of this process during inflammation.

In conclusion, this is the first study to demonstrate that nitration may occur via ONOO<sup>-</sup> or a myeloperoxidase dependent mechanism, and that specific proteins are targeted for nitration during an inflammatory response in the eye. We also identified a ONOO<sup>-</sup> mediated nitration target, MnSOD. Nitrated MnSOD appears to be targeted to the iris and ciliary epithelium during inflammation, underscoring the importance of the interface in ocular inflammation. Additionally, through the identification of another target, nitrated albumin, we introduce the possibility that there may be other biomolecules targeted (and possibly inactivated) that may be critical to the regulation of the

inflammatory response. Future studies will focus on intervention of EIU using scavengers of RNS and employing nitrated MnSOD as a biomarker of efficacy.

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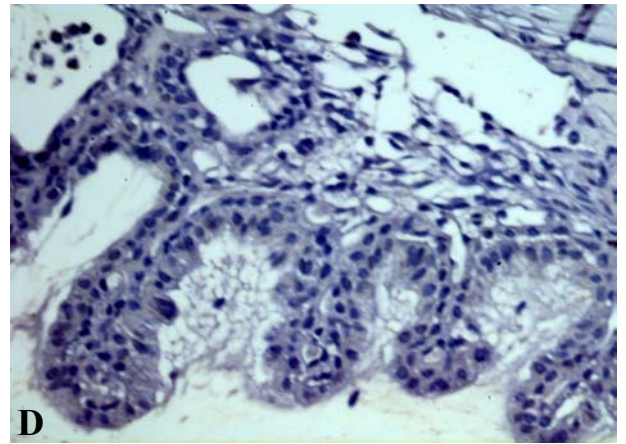
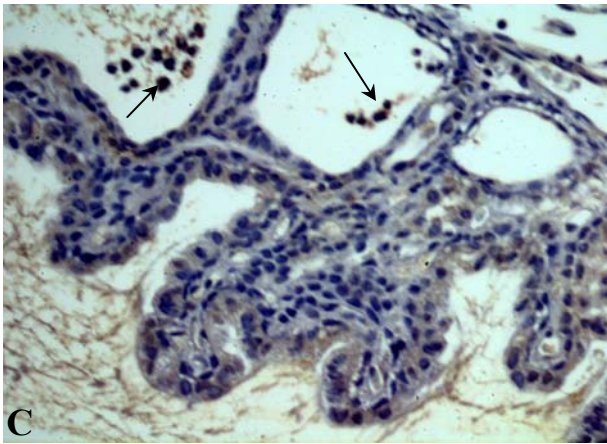
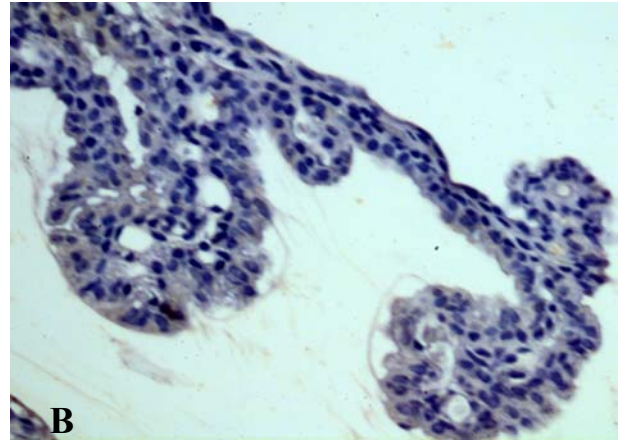
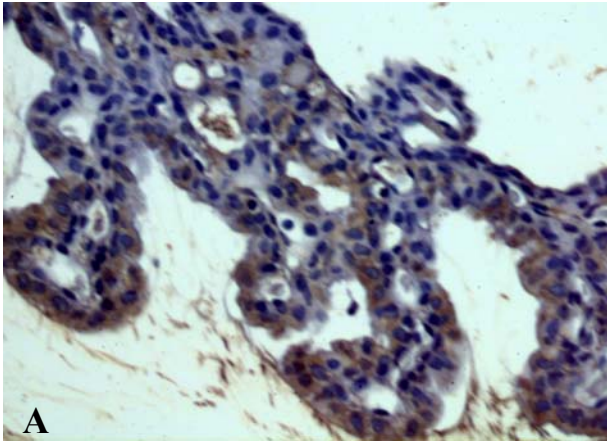


**Figure 1. Immunohistochemistry of nitrated proteins.** Immunohistochemistry of sections from control and EIU eyes demonstrates that nitration plays a role in normal ocular physiology as well as inflammation induced by endotoxin. The ciliary epithelium from control eyes immunostained positive for nitrotyrosine (A). In EIU eyes, ciliary epithelium, inflammatory cells (arrows), and protein exudate immunostained positive (C). Incubating sections with antibody in 10mM nitrotyrosine diminished staining in sections from control and EIU eyes (B, D, respectively). (magnification 40X)

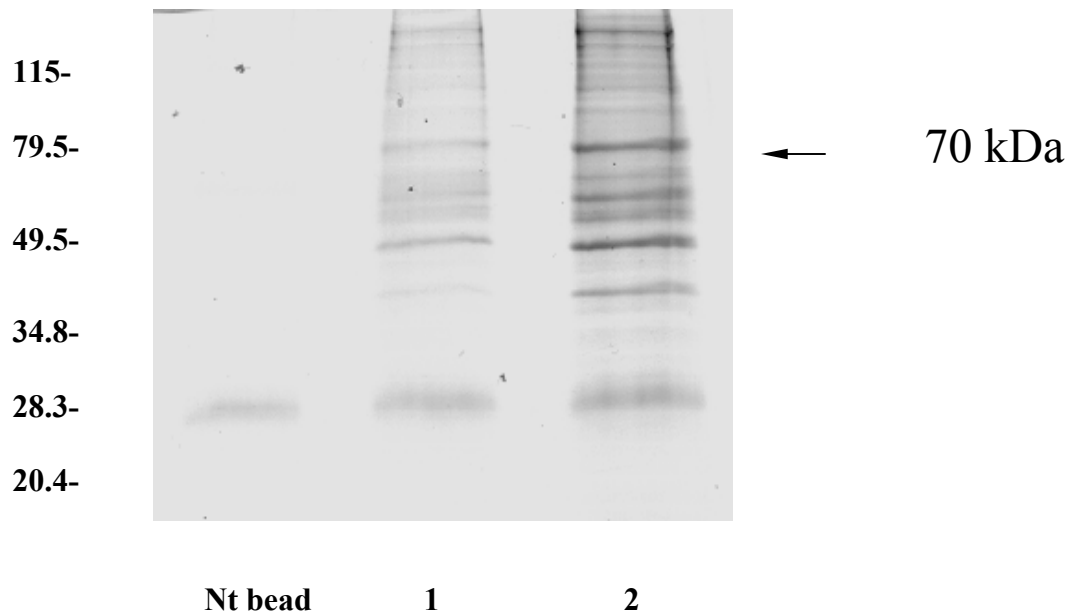


**Figure 2. Coomassie stain of immunoprecipitated proteins.** 200  $\mu$ g of protein from tissue extracts from control and EIU eyes was immunoprecipitated with anti-nitrotyrosine conjugated beads. Nitrated proteins present in control eyes indicate constitutive nitration occurring *in vivo* (lane 1). Protein extracts from the ICB of EIU rats demonstrated enhanced nitration as a result of inflammation (lane 2). Fractionation of 10  $\mu$ g of anti-nitrotyrosine conjugate beads (nt beads) serve as control.

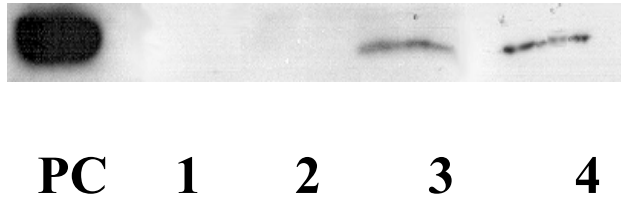


**Figure 3. Identification of nitrated MnSOD.** Immunoblotting of nitrated protein from ICB protein extracts and subsequent incubation with anti-MnSOD antibody revealed that nitrated MnSOD is present in EIU eyes (lanes 3,4) and absent in control (lanes 1, 2) indicating the discriminate nitration of MnSOD *in vivo* during inflammation. Recombinant human MnSOD was run as a positive control (PC).

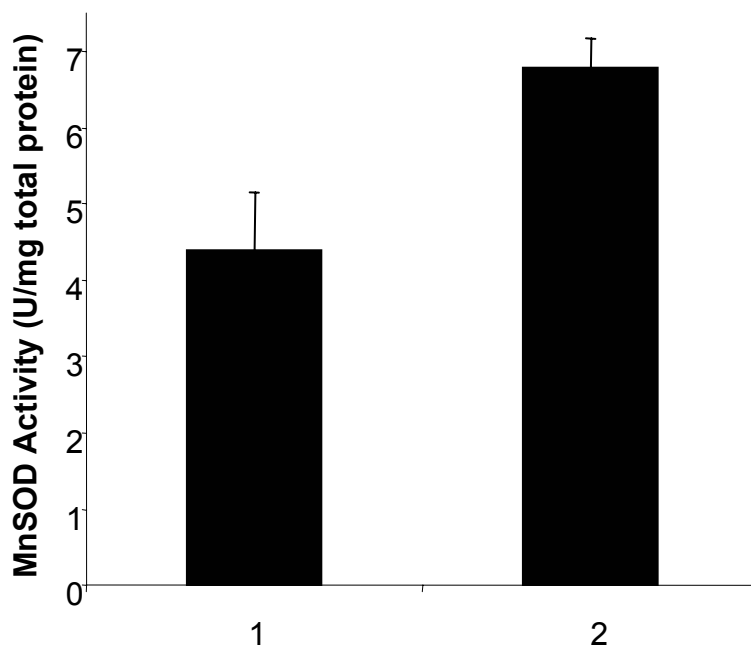


**Figure 4. Total activity of MnSOD.** Cytochrome c reduction method was performed on protein extracts from ICB of control and EIU eyes to determine total activity of MnSOD. Samples were run in triplicate. Specific activity of MnSOD (U/mg) was significantly increased in EIU eyes (lane 2) as compared to control (lane 1), 6.8 and 3.2, respectively, as determined by Student's t test ( $p < 0.05$ ).

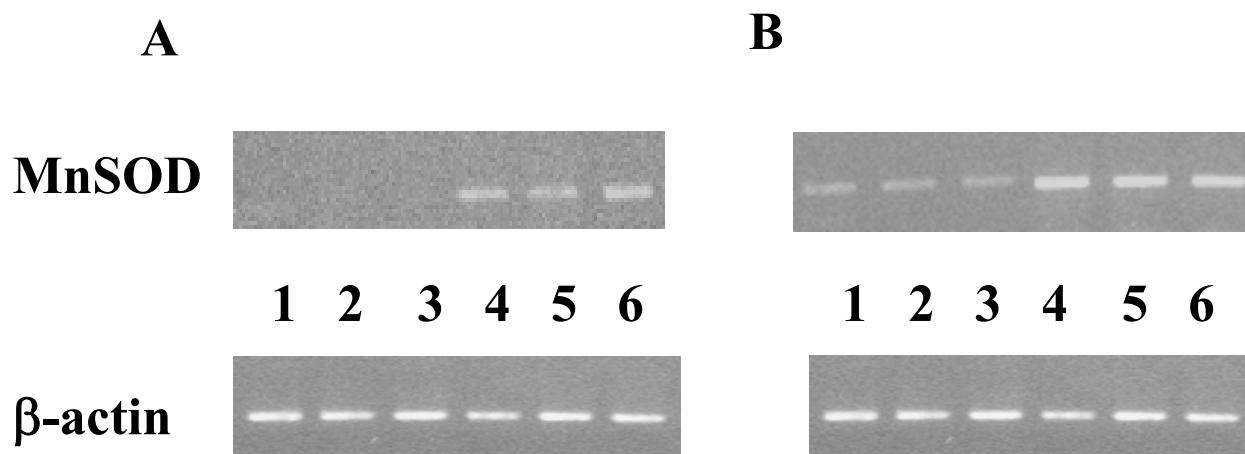
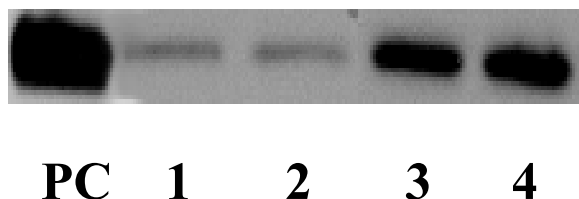


**Figure 5. mRNA expression of MnSOD in EIU.** mRNA expression of MnSOD in the ICB of control and EIU eyes was examined at 25 cycles (A) and 30 cycles (B) of PCR. An increase in MnSOD expression is seen in EIU eyes (lanes 4-6) as compared to control (lanes 1-3) at 25 and 30 cycles, however, a more dramatic difference is seen at 25 cycles.  $\beta$ -actin represents internal standard.

**A**



**B**

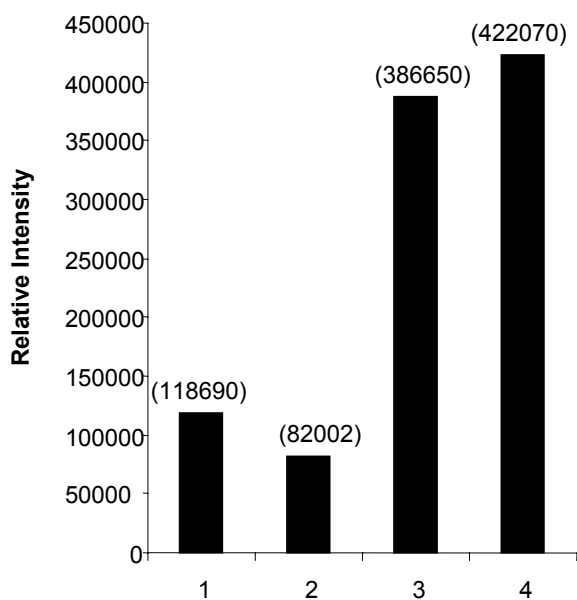


**Figure 6. Protein expression of MnSOD.** Extracts from control (lanes 1, 2) and EIU eyes (lanes 3, 4) were subjected to standard Western analysis and subsequently incubated with an anti-MnSOD polyclonal antibody. Recombinant human MnSOD served as a standard control (PC). Immunoreactivity was noted at 24 kDa as approximated by molecular weight standards. MnSOD protein expression is elevated in EIU eyes (A, lanes 3, 4) as compared to control (A, lanes 1, 2). Densitometric analysis revealed a 3-5 fold increase in MnSOD expression of EIU eyes (B, lanes 3, 4) compared to control (B, lanes 1, 2).

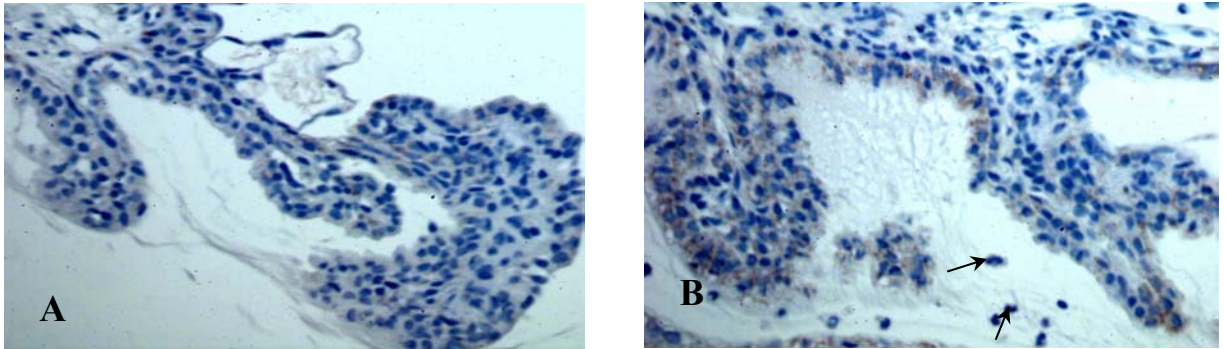


**Figure 7. Immunohistochemistry of MnSOD.** Sections from control (A) and EIU eyes (B) were incubated with a polyclonal antibody against MnSOD. Complementary to RT-PCR and Western Analysis, elevated expression of MnSOD was seen in sections from EIU eyes (B). Slight staining was seen in the ciliary epithelium from control eyes (A). (magnification 40X)

## Chapter 3

**Peroxynitrite scavenger, ebselen, inhibits endotoxin-induced uveitis (EIU) via suppression of iNOS and inhibition of I $\kappa$ B $\alpha$  degradation**

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## ABSTRACT

We determined previously via identification of nitrated MnSOD, that peroxynitrite (ONOO<sup>-</sup>) -induced tyrosine nitration was a component of endotoxin-induced uveitis (EIU). We now propose that scavenging ONOO<sup>-</sup> may prevent nitration of MnSOD and other proteins essential to cellular homeostasis; consequently, EIU may be inhibited. The anti-inflammatory component of the seleno-molecule ebselen includes the ability to scavenge ONOO<sup>-</sup>. The purpose of this study was to determine if ebselen could protect against EIU, and in using the absence or presence of nitrated MnSOD, determine if ONOO<sup>-</sup> was scavenged. In addition, by exploring mediators upregulated during inflammation we examined the mechanisms involved in protection *in vivo* using the model of EIU and *in vitro* using iris/ciliary body cells (ICB) stimulated with IL-1 $\beta$ . It was determined histologically, in rats co-injected with ebselen and endotoxin, that ebselen could in a dose-dependent manner decrease inflammatory cells infiltrating the ICB and anterior chamber compared to those injected with endotoxin alone. Furthermore, in the ICB of ebselen treated EIU rats, nitration of MnSOD was inhibited compared to untreated EIU rats. Ebselen, however, could not suppress inflammatory levels of MnSOD protein expression but did decrease levels of iNOS, although this effect did not manifest at the level of transcription. Inhibition of I $\kappa$ B $\alpha$  degradation of EIU rats treated with ebselen suggests that ebselen acts by preventing activation of NF- $\kappa$ B. Collectively, this study suggests that ebselen suppresses EIU by decreasing both ONOO<sup>-</sup> and NF- $\kappa$ B activity. In conclusion, ebselen may be an excellent therapeutic candidate for treatment of anterior uveitis.

## INTRODUCTION

Aberrant levels of nitric oxide during inflammatory disease states have pathological consequences. This is because nitric oxide (NO), as a radical species, is capable of producing reactive intermediates which can interact with various constituents of the cell and elicit damage. The enzyme inducible nitric oxide synthase (iNOS) produces high levels of NO during the inflammatory response and thus has been implicated and investigated as a critical mediator of several pathological conditions (Iadecola et al., 1997, Koprowski et al., 1993, Merrill et al., 1993). The role of iNOS in anterior uveitis remains somewhat controversial. Initial studies in the model of EIU collectively demonstrated that iNOS activity increased during EIU and activity was reduced in response to inhibitors of nitric oxide synthases (NOS) (Goureau et al., 1995, Mandai et al., 1994). Additional studies using the selective iNOS inhibitor aminoguanidine reported a reduction in EIU and vascular changes associated with the condition (Allen et al., 1996, Tilton et al., 1994). Conversely, further investigations evaluating the role of NOS isoforms in EIU suggested that the constitutive isoform, presumably endothelial NOS, plays the essential role in the onset and development of EIU (Mandai et al., 1996). Furthermore, Smith et al, demonstrated that iNOS deficient mice remain susceptible to EIU (Smith et al., 1998). The difficulty in definitively establishing the key isoform essential to NO cytotoxicity during EIU has led us to focus on the cytotoxic metabolite of NO metabolism,  $\text{ONOO}^-$ , in hopes of elucidating the pathogenic mechanism of NO in anterior uveitis.

$\text{ONOO}^-$  is formed by the interaction of NO and  $\text{O}_2^-$ . Once formed  $\text{ONOO}^-$  can undergo direct reaction with thiols or react rapidly within the vasculature with carbon

dioxide ( $\text{CO}_2$ ). Its reaction with  $\text{CO}_2$  is one of the fastest *in vivo* and the intermediates formed as a result of the  $\text{ONOO}^-/\text{CO}_2$  interaction are believed to be partly responsible for one of the key ways NO cytotoxicity manifest--protein tyrosine nitration (Squadrito and Pryor, 1998). Tyrosine nitration induced by  $\text{ONOO}^-$  can potentially 1) tag a protein for proteolysis, 2) affect cell signaling by mimicking phosphorylation or preventing dephosphorylation, or 3) alter protein conformation and as a result function (Crow and Beckman, 1996). The first protein to be unambiguously identified, *in vivo*, as tyrosine nitrated by  $\text{ONOO}^-$  was manganese superoxide dismutase (MnSOD) (MacMillan-Crow et al., 1996). MnSOD, the mitochondrial enzyme which regulates  $\text{O}_2^-$  levels in the cell, was found to be tyrosine nitrated and inactivated in human chronic renal allografts. We recently reported, using the model of EIU, that MnSOD nitration is increased in iris/ciliary body (ICB) of inflamed eyes at 24 hours (Pittman et al., 2002). However, this did not result in a decrease in enzyme activity. Nevertheless, we determined that  $\text{ONOO}^-$  was a component of the inflammatory response and also identified a biomarker of its activity *in vivo*.

Because of its high reactivity, scavenging of  $\text{ONOO}^-$  has been viewed as difficult. Recently, ebselen, a seleno-organic compound, has been identified as a  $\text{ONOO}^-$  scavenger. Ebselen reacts with  $\text{ONOO}^-$  nearly 30 times faster than  $\text{ONOO}^-$  and  $\text{CO}_2$  and scavenges  $\text{ONOO}^-$  by reducing it to nitrite ( $\text{NO}_2^-$ ) (Masumoto and Sies, 1996, Squadrito and Pryor, 1998). Ebselen has also been recognized as a glutathione peroxidase (GPx) mimic and anti-inflammatory agent. Its multiple functions and low toxicity have been pivotal in ebselen's success in protecting against inflammation in numerous experimental models (Sies and Arteel, 2000). As a result, ebselen has been developed for clinical trials

and used in conditions with an underlying inflammatory component (Ogawa et al., 1999, Parnham and Sies, 2000, Saito et al., 1998, Yamaguchi et al., 1998).

Mechanistic investigations have determined that at certain doses ebselen acts by suppressing iNOS activity and eNOS activity (Hattori et al., 1994). In addition, the molecule has also been shown to decrease activation of nuclear factor kappa B (NF- $\kappa$ B). NF- $\kappa$ B is a transcription factor which is activated during inflammation via degradation of its inhibitory protein, I $\kappa$ B $\alpha$ . Activation induces transcription of pro-inflammatory genes such as TNF- $\alpha$ , iNOS, cyclooxygenase 2 (COX 2), and IL-1 $\beta$ . Ebselen has been reported to inhibit lipopolysaccharide (LPS)-induced activation of NF- $\kappa$ B in rat Kupffer cells. Inhibition of NF- $\kappa$ B by ebselen led to a decrease in TNF- $\alpha$  transcription and COX-2 expression (Shimohashi et al., 2000).

In the model of EIU, it was determined that ebselen is able to penetrate the blood aqueous and blood retina barrier of the eye and arrive unmodified (Bosch-Morell et al., 1999). Thus, as a whole, the purpose of this study was to determine if ebselen could protect against EIU, and if protection was conferred, explore the mechanisms involved. Our results demonstrate that ebselen can suppress EIU in a dose-dependent manner and that suppression involves reducing ONOO<sup>-</sup> levels as determined by a reduction in nitrated MnSOD. Mechanistic studies revealed that ebselen acts by decreasing both iNOS protein expression and activation of NF- $\kappa$ B.

## **MATERIALS AND METHODS:**

### **Endotoxin-Induced Uveitis (EIU)**

Uveitis was induced via an injection of lipopolysaccharide (LPS, 150 µg; Sigma, St. Louis, MO) in 100 µl of 0.9% sterile, pyrogen saline in the hind footpad of male Sprague Dawley rats (200 g, Charles River Farms, Clayton, NC). Ebselen (Cayman Chemicals, Ann Arbor, MI) was reconstituted in dimethyl sulfoxide (DMSO, Sigma) and was co-administered intraperitoneally, immediately after endotoxin injection, at 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, or 40 mg/kg in DMSO (total volume 0.5 ml). Twenty-five minutes later, a second dose was administered for a total dose of 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, and 80 mg/kg, respectively. Positive and negative vehicle control animals were injected in the footpad with endotoxin or saline, and subjected to same volumes of DMSO and dosing times as ebselen-treated animals. Experimental animals were euthanized via overexposure to halothane (Halocarbon Laboratories, River Edge, NJ) and cervical dislocation. For protein studies, animals were euthanized at six hours, eyes were removed, and iris ciliary body (ICB) dissected. Dissected ICB were placed in protease inhibitor cocktail (Sigma) and snap frozen on dry ice for protein extraction. To assess protection from EIU with ebselen treatment, animals were euthanized at 24 hours, eyes removed, and placed in 10% neutral buffered formalin and processed for routine histology.

**Protein Extraction:** Total protein was extracted from in vivo ICB samples as previously described (Pittman et al., 2002).

### **Identification of nitrated MnSOD**

Nitrated MnSOD was detected as previously described (Pittman et al., 2002). Briefly, extracted protein (130 µg) from above was pre-cleared with 10 µl of Protein G (Life Technologies, Rockville, MD) and placed on a nutator for 20 minutes at 4°C. Beads were pelleted, supernatant collected and incubated with 10 µg of anti-nitrotyrosine agarose conjugate (Upstate Biotechnology Inc, Lake Placid, NY) overnight at 4°C. The following day, beads were pelleted and nitrated MnSOD detected via immunoblotting.

### **Isolation, Ebselen Treatment, and Cytokine stimulation of Iris/Ciliary Body (ICB)**

**Cells, *in vitro*:** Normal male Sprague Dawley rats were euthanized by exposure to saturated atmosphere of CO<sub>2</sub>. Both eyes were removed and iris/ciliary body micro-dissected. Excised pieces of ICB were placed in collagenase (Worthington Biochemical, Lakewood, NJ) and dispase (Boehringer Mannheim, Indianapolis, IN) at 1mg/ml on ice. Tissues were then incubated at 37°C for 45 minutes to an hour (depending on number of ICB dissected). After incubation cells were spun at 1500 rpm for 5 minutes. Single cell suspensions of ICB were made by pushing the enzyme treated tissue through a 21-gauge needle followed by a push through a 23-gauge needle. Cells were washed with Dulbecco's modified essential media (DMEM, Mediatech, Herndon, VA) containing 15% fetal bovine serum (FBS, Mediatech) and plated on 100 mm plates in 15% FBS/DMEM. Monolayers grown in parallel were examined for cell type diversity and exhibited characteristics consistent with smooth muscle and epithelial cells (the predominant cell type of ICB). Confluent cells were trypsinized and replated in 100 mm

plates and grown to 80-90% confluency. For in vitro studies, cells were treated either with DMSO in serum-free media (DMSO at < 0.005% of total volume), ebselen (2.5  $\mu$ M) in DMSO alone, IL-1 $\beta$  in DMSO (150 U/ml, Roche Diagnostics, Rockville, MD), or ebselen (2.5  $\mu$ M) and IL-1 $\beta$  (150 U/ml). iNOS mRNA and protein were examined after 6 hours of treatment/stimulation. For I $\kappa$ B $\alpha$  studies, the conditions were analogous, however, cells were stimulated for 30 minutes. All concentrations and time points were derived after dose response and kinetic studies were performed. Cells were always examined morphologically for viability. After treatment cells were either harvested with Tri-Reagent for RNA or with hypotonic buffer for cytoplasmic protein extraction.

**Preparation of Cytoplasmic extracts:** Cytoplasmic extracts were prepared as previously described (Wang et al., 1999). Briefly, cells were harvested in 100  $\mu$ l of hypotonic buffer (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1X Protease Inhibitor Cocktail). Extracts were placed in centrifuge tubes, and incubated on ice for 5 minutes. Samples were centrifuged for 5 minutes at 3,000 rpm at 4°C, supernatants (cytoplasmic extract) collected, and put in new centrifuge tubes. The concentration of cytoplasmic proteins was determined by the BCA assay according to manufacturer's instruction (Pierce, Rockford, IL) by using bovine serum albumin (BSA) as a standard.

### **Western blot analysis**

iNOS protein expression was detected using 50  $\mu$ g of cytoplasmic extracts or total protein from in vivo dissected ICB. Samples were placed in Laemmli sample buffer,

heated at 95°C, and resolved on a 7.5% SDS polyacrylamide gel. Protein bands were transferred electrophoretically onto PVDF. Blots were placed in 5% nonfat drymilk/TBS-T and subsequently incubated with a polyclonal anti-iNOS antibody (1:4000, Transduction Laboratories, Lexington, KY) at room temperature for overnight. Blots were rinsed three times in TBS-T and incubated with a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature, rinsed in TBS-T and immunoreactive proteins were visualized by enhanced chemiluminescence. IκBα protein was detected similarly with exceptions being 30 µg of protein from cytoplasmic extracts and protein from ICB extracts were used and blots were incubated with a polyclonal antibody to IκBα (1:3000, Santa Cruz Biotechnology) at room temperature for one hour. MnSOD protein expression was examined as previously described using 40 µg of protein extracted from dissected ICB. (Pittman et al., 2002)

**Reverse Transcriptase-PCR of iNOS:** Total cellular RNA was extracted and reverse transcribed as previously described (Pittman et al., 2002). Briefly, 1 µg of RNA was incubated with Moloney–murine leukemia virus (MMLV), MMLV reverse transcriptase buffer, 20 mM dNTP, 0.5 µg/µl random hexamers, 20u/µl of RNase inhibitor at 37°C. The reaction was terminated at 90°C for 5 minutes. Amplification of rat iNOS was performed in a 50 µl reaction by incubating 1 µg of cDNA in 20mM Tris/HCl containing 50mM KCl, 1.5 mM of MgCl<sub>2</sub>, 10 mM dNTP, and 0.5U Taq DNA polymerase with 15 pmoles of the following oligonucleotide primers: GCCCTCACCTACTTCCTGGAC (sense) and CGCACAAAGCAGGGCACTGG (anti-sense). Rat β-actin (sequences

ACCACAGCTGAGAGGGGAAATCG and AGAGGTCTTTACGGATGTCAACG) was amplified as an internal control under identical conditions. PCR was performed using the protocol of 45s at 94°C, 45s at 60°C (annealing temperature), 1.5 min at 72°C for 35 cycles. PCR products (619 bp for iNOS and 281 bp  $\beta$ -actin) were visualized at 30 and 35 cycles on a 1.5% agarose gel using ethidium bromide.

## RESULTS:

***Ebselen administration inhibits EIU:*** To investigate whether ebselen could inhibit EIU, rats were given intraperitoneal injections of ebselen at 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg in DMSO vehicle and a simultaneous footpad injection of endotoxin (150 µg). After 25 minutes, animals were dosed again with ebselen alone at corresponding concentrations. Parallel to ebselen/EIU rat injections, control animals were injected either with endotoxin hind footpad and a corresponding amount of vehicle (vehicle/EIU rats) or vehicle and saline (vehicle control). As histology represents in Fig 1, as the dose of ebselen increases the amount of inflammatory infiltrate seen in the ciliary epithelium (panels C, D, E) decreases. This is relative to infiltrate seen in the ciliary epithelium of vehicle/EIU animals (panel F). Sections from ebselen/EIU rats were similar to the negative vehicle control (panel A). At lower doses, 20 mg/kg or less (panel B), ebselen did not protect against EIU (10 mg/kg data not shown).

***Ebselen decreases nitrated MnSOD.*** Nitrated MnSOD, a biomarker of ONOO<sup>-</sup> induced nitration, was investigated in ICB dissected from EIU rats injected with ebselen and compared to vehicle/EIU and vehicle control rats. Nitrotyrosine immunoprecipitates of protein extracts from ICB were immunoblotted and probed for MnSOD. The data indicate that ebselen can prevent nitration of MnSOD *in vivo*. Fig 2 demonstrates that ebselen reduced levels of nitrated MnSOD in ICB of EIU rats treated ebselen (lanes 3,4) to a level similar to that of vehicle control (lanes 1,2) compared to vehicle/EIU rats (lanes 5, 6).

***Ebselen administration does not decrease MnSOD expression in vivo.*** MnSOD

expression has been shown to increase in ICB in response to LPS as early as 6 hours in vivo (data not shown). In addition, ebselen has been reported to inhibit  $O_2^-$  production in immune cells (Ichikawa et al., 1987, Leurs, Timmerman and Bast, 1989). To further elucidate the manner in which ebselen reduces nitration, we examined MnSOD expression in ICB of experimental animals 6 hours after injection of endotoxin. Our results demonstrate that ebselen was unable to overcome LPS-induced expression of MnSOD. Expression of MnSOD was increased at a similar magnitude in both ebselen/EIU rats and vehicle/EIU rats (Fig 3, lanes 3-4 and lanes 5, 6 respectively) compared to vehicle control (lanes 1 and 2).

***Ebselen decreases iNOS expression in vivo and in vitro.*** Ebselen has been reported to prevent the formation of NO (Wang et al., 1992). To ascertain whether the reduction in nitration was a result of a reduction in iNOS-induced NO production, we explored expression of iNOS in ICB of experimental animals. ICB has been shown to be primary producers of iNOS in the anterior segment during EIU (Jacquemin et al., 1996).

Preliminary studies from our laboratory detected maximal expression of iNOS 6 hours post-endotoxin injection, thus, iNOS expression was examined in experimental animals at 6 hours. Results demonstrated (Fig 4A) that ebselen decreased expression of iNOS in ICB of rats injected with endotoxin (lanes 3 and 4) compared to ICB of rats injected with DMSO and endotoxin (lanes 5 and 6). iNOS expression was not detected in ICB of vehicle control animals (lanes 1 and 2).

To determine if the decrease in expression seen *in vivo* was mediated by ebselen acting on the ICB, *in vitro* ICB cultures were treated with either ebselen in the presence of IL-1 $\beta$ , IL-1 $\beta$  in DMSO, ebselen alone, or vehicle alone for 6 hours. Western analysis indicated that as compared to IL-1 $\beta$  stimulated cells (Fig 4B, lane 3), ebselen decreased iNOS expression (Fig 4B, lane 4) to the level of control cells i.e. vehicle control and ebselen control (Fig 4B, lanes 1 and 2, respectively).

***Ebselen does not suppress iNOS transcription.*** To assess if the decrease in iNOS protein expression by ebselen was transcriptionally mediated, RT-PCR was performed on RNA extracted from ICB cultures treated as previously mentioned. As demonstrated in Fig 5, the decrease in iNOS protein expression by ebselen is not mediated at the level of transcription. Similar to IL-1 $\beta$  stimulated cells at 30 (A) and 35 cycles (B) of PCR (lane 3), transcription of iNOS did not change in IL-1 $\beta$  stimulated cells treated with ebselen (lane 4). Interestingly, in ICB cells treated with ebselen alone (lane 2), basal levels of iNOS transcription was suppressed as compared to control (lane 1).

***Ebselen blocks degradation of I $\kappa$ B $\alpha$  in vivo and in vitro.*** To further elucidate the anti-inflammatory effect of ebselen on the ICB, we examined the ability of ebselen to inhibit nuclear translocation of the inflammatory transcription factor, NF- $\kappa$ B. Degradation of I $\kappa$ B $\alpha$  dictates nuclear translocation of NF- $\kappa$ B. Therefore, in this study, degradation of I $\kappa$ B $\alpha$  was used as indicator of NF- $\kappa$ B nuclear translocation *in vivo* and *in vitro*.

Activation of NF- $\kappa$ B has been shown to occur between 3 and 6 hours post-endotoxin injection, with maximal activation at 3 hours (Ohta et al., 2002). We examined

degradation of I $\kappa$ B $\alpha$  at 3 hours in ebselen/EIU rats, vehicle/EIU rats, and vehicle control rats via Western analysis (Fig 6A). As indicated by increased expression in the ICB of ebselen/EIU rats, ebselen repressed degradation of I $\kappa$ B $\alpha$  (lanes 3,4) compared to vehicle/EIU rats (lanes 5, 6). Levels of I $\kappa$ B $\alpha$  protein were equivalent to vehicle control levels (lanes 1, 2).

Time kinetic studies from our laboratory (data not shown) demonstrated that maximal degradation of I $\kappa$ B $\alpha$  occurs at 30 minutes *in vitro* in ICB stimulated with IL-1 $\beta$  (data not shown). Thus, I $\kappa$ B $\alpha$  degradation was evaluated in our treatment groups at 30 minutes. Our data demonstrate (Fig 6B) that in ebselen treated cells stimulated with IL-1 $\beta$  (lane 3), degradation of I $\kappa$ B $\alpha$  is blocked compared to vehicle/IL-1 $\beta$  cells (lane 4), however not to the level of the controls, media alone (lane 1) and ebselen alone (lane 2).

## DISCUSSION

Tyrosine nitration can modify and alter the function of critical cellular proteins. This effect is related to the amount of  $\text{ONOO}^-$  produced and the concentration and proximity of the protein target. We determined previously that  $\text{ONOO}^-$ -induced tyrosine nitration was a component of EIU. Thus, acknowledging that  $\text{ONOO}^-$  is involved in the pathogenesis of EIU, we conjectured that scavenging it might prevent nitration of MnSOD and other proteins essential to cellular homeostasis; consequently, EIU may be inhibited. The anti-inflammatory component of the seleno-molecule, ebselen includes the ability to scavenge  $\text{ONOO}^-$ . The data, presented herein, demonstrated that ebselen can inhibit EIU and this outcome is dose-dependent. Furthermore, ebselen scavenged  $\text{ONOO}^-$  as indicated by decreased iNOS protein expression and an unobservable change in MnSOD expression as compared to EIU rats. Decreased  $\text{I}\kappa\text{B}\alpha$  degradation suggested that ebselen acted by suppressing NF- $\kappa\text{B}$  activity.

Protection against EIU was achieved at a total dose of 30 mg/kg, 40 mg/kg, and 80 mg/kg, i.p., with equal protection noted at total doses of 40mg/kg and 80 mg/kg. Doses and administration times of ebselen were experimentally determined after experimental trials using doses and times noted in other inflammatory systems proved ineffective (Ishii et al., 2000, Kobayashi, Ohta and Yoshino, 2001). Dose times and concentrations were selected based upon the character of ebselen molecule. For instance, ebselen has been determined to bind to thiols with a great affinity (Ullrich et al., 1996). Physiologically, the affinity manifests through the covalent binding of ebselen to albumin. Ebselen's preferential binding to albumin is related to the highly reactive thiol group present in the albumin and the high concentration of the protein in plasma.

Ebselen binds albumin with a  $t_{1/2}$  of 1.5 hrs (Ullrich et al., 1996). This association reduces the amount of “free” ebselen in the blood and diminishes the amount available to enter target sites, effectively altering the efficacy of the compound. Therefore, we speculate that the dose times and concentrations chosen in our system increased the amount of “free” ebselen in the plasma. An increase in the amount of “free” ebselen in the blood allowed ebselen to interact with target cells such as polymorphonuclear (PMN) cells and tissues, and of primary interest, the ICB of the eye, and henceforth protect against EIU.

The action of ebselen on PMN is to inhibit their extravasation to the site of injury. Inhibition is achieved through the chemotactic molecule and lipoxygenase product, leukotriene B<sub>4</sub> (LTB<sub>4</sub>). Ebselen prohibits the formation of LTB<sub>4</sub> while increasing the production of the biologically inactive trans isomer of LTB<sub>4</sub> (Kuhl et al., 1986, Patrick, Peters and Issekutz, 1993). Moreover, as demonstrated by Issekutz et al, ebselen inhibits adhesion and transmigration of PMNs in response to IL-1 $\alpha$ , TNF $\alpha$ , and C5a (Issekutz and Lopes, 1992). From this, we conjecture that inhibition of EIU was in part due to anti-chemotactic properties of ebselen.

Once ebselen enters the ICB its activity ranges from a free radical scavenger i.e. antioxidant to an anti-inflammatory agent. Ebselen scavenges free radicals including O<sub>2</sub><sup>-</sup>, lipid hydroperoxides (GPx like activity) and ONOO<sup>-</sup>. Bosch-Morell and colleagues demonstrated, in the model of EIU, that ebselen crosses the blood aqueous barrier unmodified where it compensates for the drop in glutathione peroxidase activity during inflammation (Bosch-Morell et al., 1999). In this study, we determined that ebselen could reduce nitration of MnSOD, a ONOO<sup>-</sup> target. We also determined that nitration of

MnSOD was reduced similar to vehicle control levels in rats treated with ebselen and LPS. Masumoto and colleagues determined *in vitro* that ebselen, via a redox reaction, reduces ONOO<sup>-</sup> to NO<sub>2</sub><sup>-</sup> and is oxidized to a selenoxide (Masumoto et al., 1996). The selenoxide can revert to ebselen by reducing equivalents such as glutathione. However, this mechanism may not predominate, *in vivo*. Ebselen has also been shown to decrease NO and O<sub>2</sub><sup>-</sup> (Wang et al., 1992) production meaning that it could also prevent the formation of ONOO<sup>-</sup> and in that manner reduce *in vivo* nitration levels. We explored this possibility by looking at both the levels of MnSOD and iNOS expression in experimental animals.

Expression of MnSOD has been determined to be increased in ICB of EIU rats at 24 hours as compared to control animals (Pittman et al., 2002). In this study, expression at 6 hours was found to be similar. Ebselen was unable to suppress LPS-induced expression of MnSOD. This finding was surprising. Ebselen has been demonstrated to inhibit O<sub>2</sub><sup>-</sup> production of guinea pig PMN and alveolar macrophages (Ichikawa et al., 1987, Leurs et al., 1989). Furthermore, ebselen decreased MnSOD activity in lung epithelial cells exposed to reactive oxygen species (Li, Wright and Jackson, 2002). We, therefore, expected a decrease in expression.

In contrast to MnSOD expression, iNOS protein expression was decreased in ICB at the 40mg/kg dose. This result, as indicated by *in vitro* data, was a direct effect of ebselen acting on the ICB. The decrease, however, is not a consequence of a decrease in transcription. RT-PCR performed on cells treated *in vitro* revealed that there is no difference in iNOS mRNA expression in cells treated with IL-1 $\beta$  as to those treated with ebselen and IL-1 $\beta$ . This finding is consistent with Ishii et al, who determined that a

reduction in pulmonary inflammation occurred *in vivo* with ebselen treatment, although at 18 hours, iNOS mRNA levels were elevated (Ishii et al., 2000). On a similar note, de Mello and colleagues demonstrated that iNOS mRNA was decreased in rat insulinoma (RIN) cells pre-treated with ebselen and stimulated with IL-1 $\beta$  for 6 hours, but at 24 hours, iNOS mRNA expression was similar to IL-1 $\beta$  (de-Mello, Flodstrom and Eizirik, 1996). None of the studies investigated protein expression of the enzyme. Nevertheless, these studies suggest that the ability of the compound to affect iNOS transcription may be time and cell type dependent. Its effect on iNOS protein expression may manifest as a decrease in translation or by increasing degradation of the protein. The anti-inflammatory cytokine, TGF $\beta$  has been shown to utilize these mechanisms to suppress iNOS protein (Vodovotz et al., 1993). Studies are being undertaken to elucidate this mechanism.

From the data collected, we hypothesize that ebselen decreases nitration of MnSOD by decreasing formation of the reactants and the products. To further explain, it has been reported that NO can outcompete MnSOD for O<sub>2</sub><sup>-</sup>, (Crow and Beckman, 1996) therefore decreasing NO production via iNOS suppression would increase the likelihood that MnSOD and O<sub>2</sub><sup>-</sup> would interact, and result in decreased nitration. On the other hand, ebselen was only able to partially suppress iNOS production of NO and has been shown to inhibit eNOS activity by only 30%, (Hattori et al., 1994) henceforth NO production still occurs and as a result ONOO<sup>-</sup> formation. So, at some level, ebselen must scavenge ONOO<sup>-</sup> as well. Taken together, these data suggests that ebselen may act two fold *in vivo*, to prevent formation of ONOO<sup>-</sup> and to scavenge it.

Interestingly, a total dose of less than 20mg/kg did not afford protection and this is seen most dramatically with 20 mg/kg. In fact, 20 mg/kg dose exacerbated the condition as compared to our positive control. The reason remains unclear. However, we hypothesize that this may be associated with an increase in iNOS activity. This is supported by an *in vitro* study in peritoneal macrophages stimulated with LPS, which showed that iNOS activity was enhanced over 100% in treatments up to 1 $\mu$ M and at 2 $\mu$ M or greater, inhibited 90% (Hattori et al., 1994). Consequently, at lower doses, ebselen may be pro-inflammatory.

The I $\kappa$ B/NF- $\kappa$ B family of transcription factors is important as regulators in the inflammatory response. These transcription factors regulate the response of such pro-inflammatory mediators as TNF- $\alpha$ , IL-1 $\beta$ , and iNOS. By this reason, the relationship between ebselen, as an anti-inflammatory agent, and I $\kappa$ B/NF- $\kappa$ B has been investigated. Ebselen in rat Kupffer cells, as determined by immunofluorescence, could inhibit nuclear translocation of NF- $\kappa$ B (Shimohashi et al., 2000). Mechanistic evaluation determined that ebselen inactivates NF- $\kappa$ B by inhibiting phosphorylation of JNK, a downstream signal protein in the MAPK pathway. In contrast, in IL-1 $\beta$  stimulated RIN, ebselen could not prevent the binding activity of NF- $\kappa$ B (de-Mello et al., 1996). We explored activation of NF- $\kappa$ B in ICB by looking at degradation of I $\kappa$ B $\alpha$ , the inhibitory component of NF- $\kappa$ B nuclear translocation. *In vivo*, ebselen suppressed I $\kappa$ B $\alpha$  similar to control levels. These results were confirmed *in vitro*, demonstrating that ebselen acts on the ICB to suppress I $\kappa$ B $\alpha$ . Studies to determine if suppression manifested in a reduction of NF- $\kappa$ B dependent genes were inconclusive (data not shown). As previously discussed, transcription of iNOS was not changed as a result of ebselen treatment, however the

transcription of this enzyme is not only mediated by NF- $\kappa$ B but several other transcription factors as well. Future studies will investigate the effect of ebselen on DNA binding activity of NF- $\kappa$ B in ICB *in vivo*.

In summary, we demonstrated that the ONOO<sup>-</sup> scavenger, ebselen, in a dose-dependent manner, could inhibit EIU. These data suggest that the effect is achieved by inhibiting chemotaxis of neutrophils, reducing formation of and scavenging of ONOO<sup>-</sup>, and by inhibiting NF- $\kappa$ B activation. This study uncovers a potential candidate for the treatment of anterior uveitis. Further investigations into the anti-inflammatory activities of ebselen in EIU are warranted.

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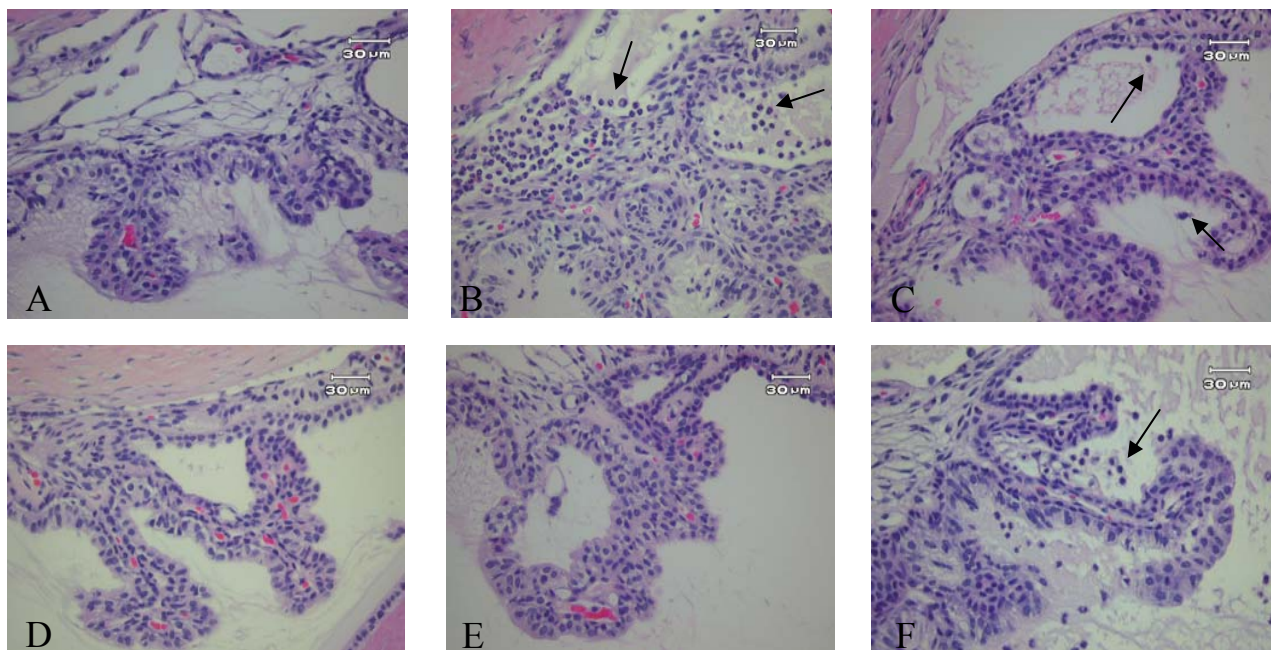


**Figure 1: Ebselen protects against EIU.** Sections of ICB from EIU rats injected intraperitoneally with ebselen at total doses of 20 mg/kg (panel B), 30 mg/kg (panel C), 40 mg/kg (panel D), or 80 mg/kg (panel E) were stained with hematoxylin and eosin and examined for the presence of inflammatory cells. Results demonstrate a dose-dependent decrease in inflammation in sections compared to vehicle (DMSO)/EIU (panel F). Sections were similar to the negative vehicle control (panel A). Arrows indicate presence of leukocytic infiltrate.

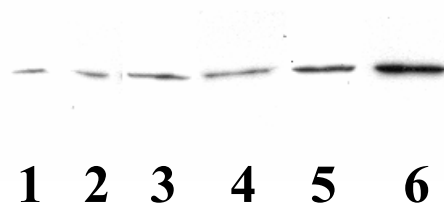


**Figure 2: Ebselen decreases nitration of MnSOD.** Nitrotyrosine immunoprecipitates from ICB of EIU rats injected with a total dose of 40 mg/kg ebselen, ICB of vehicle/EIU rats and vehicle negative control animals were immunoblotted and probed for nitrated MnSOD. Data indicate a decrease in nitration in ebselen animals (lanes 3 and 4) compared to vehicle/EIU rats (lanes 5 and 6). Nitrated MnSOD levels are similar to that of vehicle negative control (lanes 1 and 2).



**Figure 3. Ebselen administration does not decrease MnSOD expression in vivo.** MnSOD expression was examined from ICB of EIU rats injected with a total dose of 40 mg/kg ebselen and compared to MnSOD expression from vehicle/EIU rats and vehicle negative control animals. Results show that MnSOD expression in both ebselen/EIU rats (lanes 3 and 4) and vehicle/EIU rats (lanes 5 and 6) is similar. Expression is greater than that seen in the vehicle negative control animals (lanes 1 and 2).

**A**



**B**

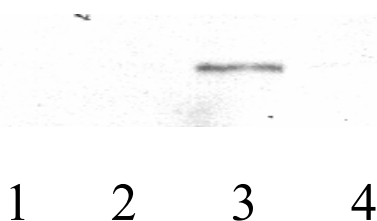


**Figure 4: Ebselen decreases iNOS expression in vivo and in vitro.** iNOS expression was examined *in vivo* (**A**) and *in vitro* (**B**). (**A**) *In vivo* animals consisted of EIU rats injected with 40 mg/kg ebselen, vehicle/EIU rats, or vehicle negative control rats. Animals were euthanized after 6 hours. Data demonstrates a decrease in iNOS expression in EIU rats treated with ebselen relative to vehicle/EIU rats. No detectable levels of iNOS expression are seen in vehicle negative control animals. (**B**) *In vivo* results were confirmed *in vitro*. iNOS expression was examined in ICB cell cultures stimulated with IL-1 $\beta$  (150 U/ml) and co-treated with 2.5  $\mu$ M ebselen in vehicle (DMSO). Results were compared with expression of iNOS from cells stimulated with IL-1 $\beta$  in vehicle, 2.5  $\mu$ M ebselen in vehicle, or media in vehicle. iNOS expression was only seen in cells treated with IL-1 $\beta$  and vehicle (lane 3). No expression was detectable in ebselen treated cells stimulated with IL-1 $\beta$  (lane 4) or the controls ebselen alone and media alone (lanes 1 and 2, respectively).

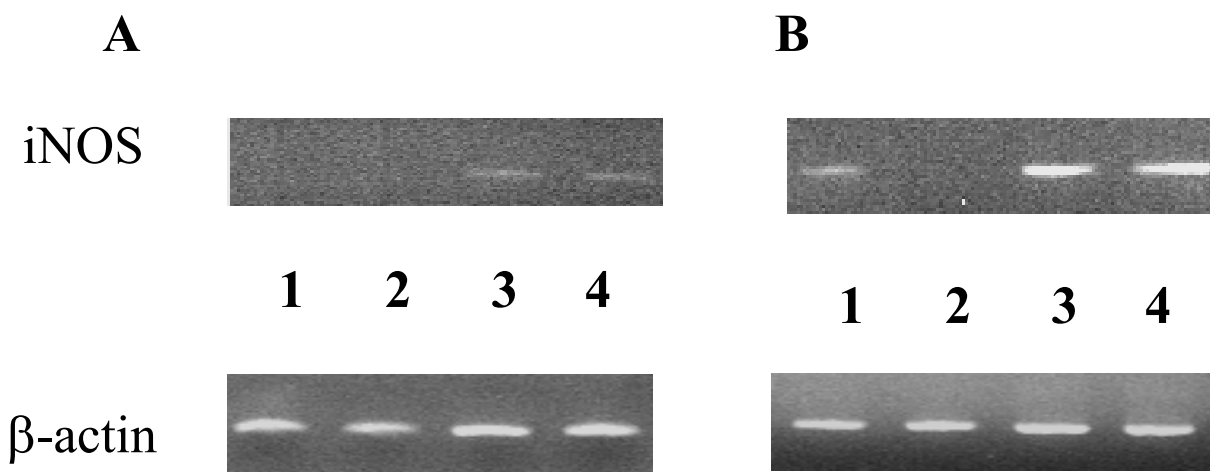
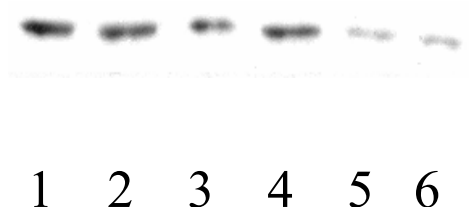


**Figure 5. Ebselen does not suppress iNOS transcription.** mRNA expression of iNOS was examined at 30 (A) and 35 (B) cycles of RT-PCR in ICB cells stimulated with IL-1 $\beta$  (150 U/ml) and co-treated with 2.5  $\mu$ M ebselen in vehicle (DMSO). Results were compared with PCR performed on cDNA from cells stimulated with IL-1 $\beta$  in vehicle, 2.5  $\mu$ M ebselen in vehicle, or media in vehicle. Findings indicate that mRNA expression of iNOS in ebselen treated ICB cultures stimulated with IL-1 $\beta$  (lane 3) is similar to expression in cells stimulated with IL-1 $\beta$  and vehicle (lane 4). Ebselen affects basal expression of iNOS as indicated by cells treated with ebselen and vehicle (lane 2) compared to vehicle and media (lane 1).  $\beta$ -actin represents an internal standard.

A



B

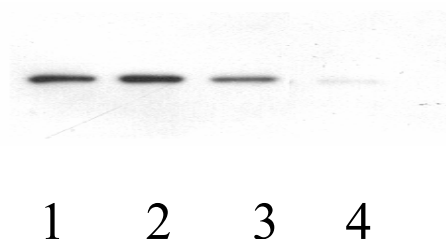


**Figure 6. Ebselen blocks degradation of IκBα in vivo and in vitro.** IκBα degradation was evaluated *in vivo* (A) and *in vitro* (B). (A) IκBα expression was examined from ICB of EIU rats injected with a total dose of 40 mg/kg ebselen and compared to IκBα expression from vehicle/EIU rats and vehicle negative control rats. Results demonstrate that IκBα degradation is repressed in ICB from ebselen treated rats (lanes 3 4), similar to vehicle negative control (lanes 1, 2). Degradation-- indicated by decreased IκBα expression-- is seen in vehicle/EIU rats (lanes 5, 6). (B) *In vitro* data demonstrate similar results. IκBα degradation is blocked in ICB cells stimulated with IL-1β and co-treated with 2.5 μM ebselen (lane 3) compared to cells stimulated with IL-1β alone and vehicle (lane 4). Results were similar to the controls, ebselen alone and media alone (lanes 1 and 2, respectively).

## Summary and Future Studies:

In this study, we examined the relationship among cellular and inflammatory mediators identified as critical to the onset and development of the inflammatory condition, anterior uveitis. The pathogenesis of the condition is linked to the increase in NO production and the concomitant decrease in activity of TGF $\beta$ 2. Progression of the inflammatory response directed by NO occurs through the high levels of NO produced by iNOS. iNOS transcription is regulated, in part, by the transcription factor, NF- $\kappa$ B. NF- $\kappa$ B activation occurs when its bound cytoplasmic inhibitory element, I $\kappa$ B $\alpha$ , is degraded. Degradation of I $\kappa$ B $\alpha$  allows NF- $\kappa$ B to enter the nucleus and activate transcription of pro-inflammatory genes. As a result of increased iNOS transcription, high levels of NO are produced and lead to cytotoxicity via its toxic metabolite ONOO $^-$ . ONOO $^-$  elicits toxicity by many mechanisms but the one best understood is that of tyrosine nitration. Tyrosine nitration can modify and alter the function of proteins and therefore, may alter function of proteins critical to the regulation of the inflammatory response. In contrast to iNOS, TGF $\beta$ 2 is anti-inflammatory in the eye. TGF $\beta$  maintains the immune privilege environment of the eye by downregulating expression of inflammatory mediators. Several studies from the literature have demonstrated that TGF $\beta$ 2 can decrease expression of iNOS during inflammation. In addition, studies have reported that TGF $\beta$  decreases NF- $\kappa$ B activation by increasing I $\kappa$ B $\alpha$  transcription. From this data, we hypothesized that when TGF $\beta$ 2 activity decreases during uveitis, NF- $\kappa$ B activity increases, as does the expression of pro-inflammatory genes, in particular iNOS. Increase in iNOS expression leads to an increase in NO activity and production of ONOO $^-$ . ONOO $^-$  can then nitrate and inactivate proteins critical to the regulation of the

inflammatory response. We further proposed that exogenous administration of TGF $\beta$ 2 i.e. increasing TGF $\beta$ 2 activity or scavenging ONOO $^-$  may protect against EIU.

Scavenging of ONOO $^-$  was examined by using the ONOO $^-$  scavenger, ebselen. All studies were undertaken using either the *in vivo* model of anterior uveitis, endotoxin-induced uveitis (EIU) or in the *in vitro* system of iris ciliary body (ICB) cells stimulated with IL-1 $\beta$ .

The purpose of the first study was to determine whether or not TGF $\beta$ 2 could negatively regulate iNOS mRNA and protein expression during EIU as reported in other inflammatory models. Moreover, if suppression occurred, determine the mechanisms involved and if exogenous administration could protect against EIU. We determined in the *in vitro* model, that TGF $\beta$ 2 decreased iNOS mRNA and protein expression in ICB stimulated with IL-1 $\beta$ . *In vivo* results were similar. TGF $\beta$ 2 decreased expression of iNOS in ICB of EIU rats injected with the cytokine. We next examined whether the downregulation of iNOS transcription by TGF $\beta$ 2 was a result decreased NF- $\kappa$ B. This was investigated by examining degradation of I $\kappa$ B $\alpha$  in vitro and in vivo. TGF $\beta$ 2 did not suppress IL-1 $\beta$  induced activation *in vitro* or LPS-induced activation *in vivo* of NF- $\kappa$ B. We hypothesized that perhaps other transcription factors were involved including IRF-1 and AP-1 or that, as demonstrated in murine macrophages, binding sites critical to its expression are blocked by activation of the receptor-mediated Smad, Smad3. Although TGF $\beta$ 2 suppressed iNOS mRNA and protein expression, it could not protect against EIU. Animals were co-injected with TGF $\beta$ 2 and endotoxin and euthanized at 24 hours. We speculated the lack of protection was associated with species utilized in our study and administration route of the cytokine. In summary, TGF $\beta$ 2 does regulate iNOS

gene expression during EIU, and regulation manifests as decreased mRNA transcription and protein expression of iNOS. However, suppression of iNOS is not mediated through NF- $\kappa$ B, nor does suppression denote protection against EIU.

The purpose of the second study was to determine whether or not the NO metabolite, ONOO<sup>-</sup>, was involved in the pathogenesis of EIU and if involved, does it nitrate proteins important in the regulation of the inflammatory response. We determined through the identification of nitrated MnSOD that ONOO<sup>-</sup> was involved in the pathogenesis of EIU. Protein extracts from ICB were examined 24 hours post-endotoxin injection for the nitrated product. MnSOD scavenges the reactive species O<sub>2</sub><sup>-</sup> and thus is critical in controlling cellular levels during inflammation. Studies in the chronic renal allograft model had determined that the enzyme was not only nitrated but also inactivated. Inactivation of MnSOD was proposed to be an integral part of allograft rejection. Although MnSOD was nitrated in our system, it was not inactivated. Activity of MnSOD was increased. Increased activity was linked to the increase in expression of MnSOD detected during the inflammatory response. In our study, the level of nitration did not exceed the level of expression; therefore we speculated that if nitration had exceeded expression, than a decrease in activity might have been noted. In summary, as determined by the presence of nitrated MnSOD, ONOO<sup>-</sup> is a component of EIU. Although MnSOD was not inactivated as a result of nitration, we speculated that nitration by ONOO<sup>-</sup> might inactivate other proteins essential to inflammatory response regulation.

The purpose of the final study was to determine whether or not ONOO<sup>-</sup> is a key cytotoxic mediator in the pathogenesis of anterior uveitis through the use of the ONOO<sup>-</sup> scavenger, ebselen. Furthermore, if protection was conferred, to explore molecular

mechanisms involved. We determined that ebselen can protect against EIU. We proposed that protection was, in part, due to the anti-chemotactic activity of ebselen on PMNs. Additional studies determined that ebselen reduced  $\text{ONOO}^-$  induced nitration, as observed by a reduction in nitrated MnSOD. We surmised that ebselen reduced nitration by preventing the formation of and scavenging of  $\text{ONOO}^-$ . This conclusion was formulated through observations that 1) *in vitro* and *in vivo* that ebselen decreased iNOS protein expression, however not at the level of the gene and 2) that *in vivo* ebselen, although recognized as a scavenger of  $\text{O}_2^-$ , could not overcome LPS-induced expression of MnSOD. Further examination into the molecular mechanisms revealed that ebselen inhibited activation of NF- $\kappa$ B. Activation was explored by examining repression of I $\kappa$ B $\alpha$  degradation. These results were confirmed *in vitro*. In summary, mechanisms involved in suppression of EIU by ebselen include preventing chemotaxis of PMNs, preventing the formation of and scavenging of  $\text{ONOO}^-$ , and suppressing NF- $\kappa$ B activity.

Collectively, these studies demonstrated that TGF $\beta$ 2 negatively regulates iNOS expression during EIU and that iNOS, NO and its metabolite,  $\text{ONOO}^-$  are key components in the pathogenesis of EIU, however, management of uveitis occurs when suppression of these mediators is coupled to PMN suppression and decreased activity of NF- $\kappa$ B.

Future studies will focus on determining the anti-inflammatory activities of TGF $\beta$ 2 during ocular inflammation. Primary studies will determine if suppression of iNOS modulated by TGF $\beta$ 2 results in a reduction in nitration. If a reduction manifests, this will tell us if  $\text{ONOO}^-$  is essential to the pathogenesis or if the pathogenesis is primarily related to the flux of inflammatory cells. Secondary studies will focus on the

role of the Smad family of transcription factors, in particular Smad 3. These transcription factors are critical in transducing signals initiated by TGF $\beta$  binding to its cognate receptors and are, therefore essential for expression of genes modulated by TGF $\beta$ . Final studies will assess kinetics of TGF $\beta$ 2 administration. We speculated that the lack of protection seen was related to time of injection. Perhaps dosing once immune cells have extravasated to the site of injury, i.e. during a developing or established inflammatory response might confer protection.

Overall, we hope this study and our future studies provide further insight into the pathogenesis of anterior uveitis and introduce new avenues and modalities for treatment of anterior uveitis.