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Exacerbated corneal inflammation and neovascularization in the HO-2 null mice is ameliorated by biliverdin

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Abstract

Heme oxygenase (HO-1 and HO-2) represents an intrinsic cytoprotective and anti-inflammatory system based on its ability to modulate leukocyte migration and to inhibit expression of inflammatory cytokines and proteins. HO-2 deletion leads to unresolved corneal inflammation and chronic inflammatory complications including ulceration, perforation and neovascularization. We examined the consequences of HO-2 deletion on hemangiogenesis and lymphangiogenesis in the model of suture-induced inflammatory neovascularization. A 8.0 silk suture was placed at the corneal apex of wild type and HO-2 null mice. Neovascularization was assessed by vital microscopy and quantified by image analysis. Hemangiogenesis and lymphangiogenesis were determined by immunofluorescence staining using anti-CD31 and anti-LYVE-1 antibodies, respectively. Inflammation was quantified by histology and myeloperoxidase activity. The levels of HO-1 expression and inflammatory cytokines were determined by real time PCR and ELISA, respectively. Corneal sutures produced a consistent inflammatory response and a time-dependent neovascularization. The response in HO-2 null mice was associated with a greater increase compared to the wild type in the number of leukocytes (827600 ± 129000 vs. 294500 ± 57510 ; $p < 0.05$), neovessels measured by vital microscopy (21.91 ± 1.05 vs. 12.77 ± 1.55 mm; $p < 0.001$) 4 days after suture placement. Hemangiogenesis but not lymphangiogenesis was more pronounced in HO-2 null mice compared to wild type mice. Induction of HO-1 in sutured corneas was greatly attenuated in HO-2 null corneas and treatment with biliverdin diminished the exaggerated inflammatory and neovascular response in HO-2 null mice. The demonstration that the inflammatory responses, including expression of proinflammatory proteins, inflammatory cell influx and hemangiogenesis are exaggerated in HO-2 knockout mice strongly supports the notion that the HO system is critical for controlling the inflammatory and neovascular response in the cornea. Hence, pharmacological amplification of this system may constitute a novel therapeutic strategy for the treatment of corneal disorders associated with excessive inflammation and neovascularization.

Keywords

hemangiogenesis; lymphangiogenesis; inflammation; heme oxygenase; biliverdin

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INTRODUCTION

Neovascularization is regarded as a component of the inflammatory reparative response of a given tissue to injury. While neovascularization has some positive effects such as facilitating healing by enabling transport of immunologic humoral and cellular factors for repairing tissues, persistence of blood vessels within the cornea is detrimental. Neovascularization of the normally avascular cornea is seen in many pathological conditions, which include infection, mechanical and chemical injury, chronic exposure to hypoxia and following corneal transplantation. Corneal neovascularization leads to decreased vision, graft rejection and incompetent barrier function, thus, presenting a serious clinical problem for which treatment is often lacking.

The molecular mechanisms that control corneal neovascularization are not fully understood. Since inflammation is closely linked with neovascularization, many studies have concentrated on the role of humoral and corneal-derived inflammatory mediators in the regulation of this process and on angiogenic factors that drive the neovascularization process. Less attention has been focused on the identification of intrinsic corneal anti-inflammatory mediators and cellular pathways. In recent years the heme oxygenase (HO) system has emerged as a key cytoprotective signal. HO is the rate-limiting enzyme in heme catabolism. It cleaves heme into iron, sequestered by ferritin, carbon monoxide (CO), and biliverdin which is reduced to bilirubin by biliverdin reductase (Abraham and Kappas, 2008). Two isoforms, HO-1 and HO-2, are the major source of HO activity in most tissues. HO-1 is an inducible enzyme, while HO-2 displays a constitutive expression in all cell types. HO-1 and HO-2 are alike in terms of mechanisms of heme oxidation, cofactor and substrate specificity, and susceptibility to inhibition by porphyrins (Abraham and Kappas, 2008; Maines, 1998). They differ in their postulated function; whereas, HO-2 is believed to function as the constitutive HO activity contributing to cell homeostasis, HO-1 expression is relatively low in most normal tissues. Following injury, however, HO-1 expression is greatly enhanced to play a significant role in cytoprotection (Abraham and Kappas, 2008).

The realization that HO-1 is strongly induced by oxidative stress and other injurious stimuli in conjunction with the robust ability of HO-1 to guard against oxidative insult (Abraham and Kappas, 2008; Foresti et al., 2004; Otterbein and Choi, 2000) and the demonstrations of its protective role in models of inflammation-mediated injury including xenograft rejection (Song et al., 2003), endotoxin challenge (Ohta et al., 2003; Otterbein et al., 2000; Zampetaki et al., 2003), ischemia–reperfusion injury (Arai-Gaun et al., 2004; Coito et al., 2002; Hegazy et al., 2000; Minamino et al., 2001; Yet et al., 2001), and contact lens-induced hypoxic injury (Connors et al., 1995; Laniado Schwartzman et al., 1997), together with the fact that HO-1 deficiency is associated with a chronically inflamed state and increased leukocyte recruitment as reported in a human (Yachie et al., 1999) and in mice (Kapturczak et al., 2004; Poss and Tonegawa, 1997) null for the HO-1 gene, have all led to an examination of the cytoprotective and anti-inflammatory nature of the HO system. Although the mechanisms involved in this cytoprotection are largely unknown, the elimination of excess cellular heme as well as the enzymatic products of the HO system, carbon monoxide and bilirubin, have been shown to protect against tissue damage by exerting antioxidant and anti-inflammatory effects (Stocker et al., 1987) (Otterbein, 2002; Ryter et al., 2004). Upregulation of HO-1 as well as administration of biliverdin/bilirubin or CO have been shown to downregulate the inflammatory response by either attenuating the expression of adhesion molecules and, thus, inhibiting leukocyte recruitment (Wagener et al., 1999; Wagener et al., 2003), by repressing the induction of cytokines and chemokines (Minamino et al., 2001; Morse et al., 2003; Otterbein et al., 2000; Sarady-Andrews et al., 2005; Song et al., 2003), or by inhibiting proinflammatory hemoproteins such as cyclooxygenase-2 and cytochrome P450 4B1 (Connors et al., 1995; Haider et al., 2002; Laniado Schwartzman et al., 1997; Li Volti et al., 2003).

Though many of the postulated mechanisms underlying this protection are assigned to HO enzymatic activity and its products, CO and bilirubin, the contribution of HO-2, the primary source of bilirubin and CO in uninjured tissues, to the regulation of inflammation and reparative response has been largely unexplored.

We have previously documented the presence of HO activity and expression in human and rabbit corneas as well as HO-1 inducibility following oxidative stress *in vitro* and hypoxic injury *in vivo* (Abraham et al., 1995; Abraham et al., 1987; Bonazzi et al., 2000; Connors et al., 1995; Laniado Schwartzman et al., 1997; Neil et al., 1995). In a recent study, we showed that HO-2 displays a prominent constitutive expression in the cornea and that deletion of HO-2 gene markedly impairs the inflammatory and reparative response of the cornea to epithelial injury (Seta et al., 2006). Hence, HO-2 deficiency led to unresolved corneal inflammation and chronic inflammatory complications including ulceration, perforation and neovascularization. In the present study, we examined the consequences of HO-2 deletion on the corneal inflammatory response including hemangiogenesis and lymphangiogenesis in the model of suture-induced inflammatory neovascularization and examined whether supplementation of the HO-derived product, biliverdin, corrects for HO-2 deficiency.

MATERIALS AND METHODS

Animals

All animal experiments were performed following an institutionally approved protocol in accordance with the National Institute's of Health Guide for the Care and Use of Laboratory Animals. The HO-2-null mice are direct descendants of the HO-2 mutants produced by Poss and colleagues (Poss et al., 1995). These well-characterized HO-2-null mice have a C57BL/6 $\times$ 129/Sv genetic background (Rogers et al., 2003), which was used on age- and gender-matched controls (Jackson Laboratory, Bar Harbor, ME).

Suture-Induced Inflammatory Corneal Neovascularization

Mice were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (20 mg/kg). A drop of tetracaine-HCl 0.5% was applied to the eye to deliver local corneal anesthesia before subjecting animals to injury. One 8-0 silk suture was placed intrastromally with two stromal incursions extending over the corneal apex, the points of the suture placement being about 1.5 mm from the limbus, in order to obtain standardized angiogenic responses. In some experiments, eyes were treated with biliverdin (100 μ M, 10 μ l eye drop, tid). The corneal angiogenic response was documented on day 2, 4 and 7 after suture placement using a Zeiss dissecting microscope coupled to an AxioCam Hrc digital camera and Axiovision 4.5 software (Zeiss). Digital images were analyzed by image processing software (ImagePro, Media Cybernetics, Inc., Silver Spring, MD). Corneal vascularization was measured as the total length (mm) of vessels in the cornea. Mice were euthanized after 2, 4, and 7 days and the corneas were dissected and subjected to selected analyses.

Corneal Whole mounts and Morphologic Determination of Hem- and Lymphangiogenesis

The corneas were excised, rinsed in phosphate buffered saline (PBS 0.01 M, pH 7.4) and fixed in acetone for 20–30 minutes, as described previously (Cursiefen et al., 2004). After three additional washing steps in PBS and blocking with 3% BSA in PBS for 3 h, the corneas were stained over night at 4°C with FITC-conjugated rat anti-mouse CD31 (PECAM-1) (1:300, eBioscience, San Diego, CA). Thereafter the tissue was washed, blocked and stained with rabbit anti-mouse LYVE-1 (1:500, Abcam Inc., Cambridge, MA) antibody over night at 4°C. After a last washing and blocking step, LYVE-1 was detected with a Cy3-conjugated goat anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA). Corneal hemangiogenesis and lymphangiogenesis were quantified using the digital image analysis software Axiovision

(Zeiss) as described (Samolov et al., 2005). The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the ratio between the area of the vascularized (CD31⁺ for hemangiogenesis and LYVE-1⁺ for lymphangiogenesis) cornea and the total corneal area was calculated.

Histology and immunostaining

Dissected corneas were washed twice with PBS and fixed in 4% paraformaldehyde-PBS for 1 h at 4°C. Corneas were washed five times with PBS, placed in 30% sucrose for 24 h and embedded in OCT compound (Sakura Finetek, Torrence, CA). Cryostat sections were cut transversely into 7 µm thick sections, stained with Hematoxylin-Eosin and mounted on microscopic slides in Cytosol XYL (Richard-Allan Scientific, Kalamazoo, MI). Immunofluorescence of HO-2 was determined using a rabbit anti-rat HO-2 antibody and a FITC-conjugated goat anti-rabbit antibody. Immunofluorescence was detected by a confocal laser-scanning microscope (Olympus Fluoview FV300). For double Immunofluorescence studies, corneal sections (5 µm) were blocked in 5% goat serum in PBS-Triton X100 (0.3%) overnight. Then, sections were incubated with rabbit anti-mouse HO-1 antibody (1:400, Assay Designs, Inc., Ann Arbor, MI) overnight at 4°C, washed and further incubated with a Cy3-conjugated goat anti-rabbit antibody (1:1000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Thereafter, the sections were washed and stained with rat anti-mouse CD-68 (1:100, AbD Serotec, Raleigh, NC) antibody overnight at 4°C. After washing and blocking, sections were further incubated with a Alexa Fluor® 488-conjugated goat anti-rat antibody (1:1500, Invitrogen Molecular Probes, Eugene, OR) for 1 h at room temperature. To further verify cellular entity, sections were washed and counterstained for nuclei with 4',6-diamino-2-phenylindole (DAPI) for 5 minutes. Immunofluorescence was visualized using a Zeiss Axioplan-2 fluorescent microscope. Images were captured and analyzed using AxioVision 2 multi channel image processing software (Zeiss, Gottingum, Germany).

Myeloperoxidase (MPO) Activity

Measurement of MPO activity was used to quantify polymorphonuclear cells (PMNs) in dissected corneas as previously described (Seta et al., 2006). In brief, tissues were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of sonication and freeze-thaw. The particulate matter was removed by centrifugation at 18300 g for 15 min, and then MPO activity in the supernatant was measured by spectrophotometry using *o*-dianisidine dihydrochloride reduction as a colorimetric indicator. Calibration curves for conversion of MPO activities to PMN number were established with PMN that were collected from zymosan A-induced peritonitis in untreated mice.

Real-Time Polymerase Chain Reaction (PCR)

Corneas were aseptically dissected from eyes and cleaned in sterile PBS (4°C) under a dissecting microscope to remove all noncorneal tissue. Total RNA was isolated using RNeasy Protect Mini Kit (Qiagen, Germantown, MD) and RNA was quantitated using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription reaction of total RNA (5 µg) was performed using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Quantitative real-time PCR was performed using QuantiTect™ SYBR® Green PCR Kit (Qiagen, Germantown, MD) and the Mx3000 real-time PCR system (Stratagene, La Jolla, CA). Specific primers were designed based on published sequences (GenBank) and were as follows: HO-1 sense, 5'-TCCAGACACCGCTCCTCCAG-3' and anti-sense, 5'-GGATTTGGGGCTGCTGGTTTC-3'; HO-2 sense, 5'-TACTTCACATACTCAGCCCT-3' and anti-sense, 5'-ATGGGCCACCAGCAGCTCTG-3'; CAGCTTTAGCATAGTCAGGCTC-3'; 18S sense, 5'-

TGTCTCAAAGATTAAGCCATGCAT-3' and anti-sense, 5'-AACCATAACTGATTTAATGAGCCATTC-3'. PCR efficiency for each primer pair was determined by quantitating amplification with increasing concentrations of template cDNA, and specific amplification was verified by subsequent analysis of melt curve profiles for each amplification. A nontemplate control served as negative control to exclude the formation of primer dimers or any other nonspecific PCR products. RNA expression of target genes was calculated based on the real-time PCR efficiency (E) and the threshold crossing point (CP) and is expressed in comparison to the reference gene 18S as described (Pfaffl, 2001).

Chemokine analysis

Dissected corneas were homogenized in T-PER® Tissue Protein Extraction Reagent containing Halt™ protease inhibitor cocktail and 1mM phenylmethanesulfonyl fluoride. KC (IL-8 homologue), IL-6, macrophage inflammatory protein-2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) were measured in corneal homogenates using a custom multiplex sandwich ELISA for protein analysis (SearchLight mouse microarray, Pierce Biotechnology, Woburn, MA).

Statistical Analysis

Student's t-test was used to evaluate the significance of differences between groups and multiple comparisons were performed by regression analysis and one-way analysis of variance. P values less than 0.05 were considered significant. All data are presented as mean $\pm$ SE.

RESULTS

Expression of HO-1 and HO-2 in mouse cornea

Figure 1 shows immunofluorescence and immunohistochemical analyses of HO-1 and HO-2 in corneas of HO-2 null mice and congenic wild type mice. Both analyses clearly show that the wild type mouse cornea exhibited a robust expression of HO-2 protein which is primarily localized to the corneal epithelium and to a lesser extent to the stroma. In contrast, immunohistochemistry (Figure 1B) indicates that HO-1 immunoreactivity in the wild type uninjured cornea is relatively low furthering the definition of HO-1 as an inducible protein. In HO-2 null mice, both analyses demonstrated no HO-2 immunoreactivity (Figure 1A and B) while some HO-1 positive staining (Figure 1B) was observed in the stroma and the endothelium, suggesting that in the absence of HO-2, increased HO-1 expression is seen as a compensatory mechanism (Ding et al., 2006).

Amplified neovascular response in corneas of HO-2 null mice

Insertion of a suture into the stroma produced a time-dependent neovascular response that was greater in HO-2 null mice. Significant neovascularization of the cornea in wild type and HO-2 null mice was evident as early as 2 days after suture placement. At day 7 after suture placement more than 50% of the cornea was covered with blood vessels in the HO-2 null mice (Figure 2A). The time frame of up to 7 days was chosen to assess differences in the rate of neovascularization between wild type and HO-2 null mice; at longer time periods, 10 and 14 days after suture, differences were less clear. Quantitative analysis indicated that neovascularization of the avascular cornea at days 4 and 7 after suture placement was 0.8 and 2.2 fold greater in HO-2 null mice than in the corresponding wild type mice (Figure 2 B).

The accelerated rate of corneal neovascularization in HO-2 null mice was further confirmed by immunofluorescence using FITC conjugated anti-CD31 antibodies. As seen in Figure 3A, new blood vessels (hemangiogenesis) were readily labeled with CD31, a vascular endothelial cell specific marker. Quantitative analysis of CD-31 positive blood vessels within the cornea

corroborated the results obtained with the direct microscopic image analysis of a greater rate of neovasacularization in corneas from HO-2 null mice at days 4 and 7 after suture placement (Figure 3B). Hemangiogenesis as measured by CD-31 fluorescence was 3-fold higher in corneas from HO-2 null mice as compared to wild type corneas at day 4 after suture placement, whereas, at day 7 hemangiogenesis was only 50–80% greater further suggesting that the initial inflammatory and neovascular response is exaggerated in the absence of the HO-2 gene.

The inflammatory insult brought about by the insertion of a silk suture into the stroma triggered not only the growth of new blood vessels (hemangiogenesis) into the cornea but also the growth of lymph vessels (lymphangiogenesis) (Cursiefen et al., 2006). Several studies have demonstrated that these two processes occur in parallel and are driven by the inflammatory response and more specifically by activated macrophages (Maruyama et al., 2005). To gain insight into the role of HO in the growth of lymphatic vessels, we measured the degree of lymphangiogenesis (LYVE-positive vessels) in the HO-2 null corneas. As seen in Figure 3C, LYVE-positive vessels were evident in corneas of wild type and HO-2 null mice. Quantitative analysis demonstrated that lymphangiogenesis was not significantly different between wild type and HO-2 null mice (Figure 3D), suggesting that HO-2 deletion impinges on these two processes differently.

Exaggerated inflammatory response in HO-2 null mice

The intensified neovascular response was accompanied by an exaggerated inflammatory response that was clearly seen by histology and quantified by MPO measurement. As seen in Figure 4A, H&E staining of corneal sections indicated a marked influx of inflammatory cells in corneas of HO-2 null mice as compared to corresponding wild type corneas. The qualitative histological analysis was further substantiated by biochemical measurements of the number of infiltrating PMN using the MPO assay. Thus, the number of PMN in corneas of HO-2 null mice was 2–3 fold greater than in wild type corneas at all time points tested (Figure 4B). In fact, in the wild type corneas the number of PMNs reached its maximum at day 2 and stayed constant at around 250,000 cells/cornea, thereafter. In contrast, the number of PMNs in HO-2 null corneas reached its maximum at day 4 and stayed at around 750,000 cells/cornea (3-fold greater than in wild type), afterwards.

Together with the increase in cellular infiltrate, there was an increase in the levels of inflammatory cytokines including KC, IL-6, MIP-2 and MCP-1. As seen in Figure 5, suture placement induced an inflammatory response exemplified by a robust increase in the levels of these cytokines 2 days after suture placement in both wild type and HO-2 null corneas. However, these increases at days 2 and 7 were more pronounced in the HO-2 null corneas amounting to 2–3 fold higher than their corresponding wild type corneas.

Disregulation of HO-1 in HO-2 null corneas

The uninjured wild type corneas exhibited low levels of HO-1 mRNA. Insertion of a foreign body (suture) into the stroma elicited a rapid induction of HO-1 in corneas of wild type mouse (Figure 6A). Levels of HO-1 mRNA increased by 10- and 40-fold at days 2 and 4 after suture placement, respectively, and decreased by day 7 to a level that was still 8-fold higher than the control level in unsutured cornea (Figure 6A and B). On the other hand, in the HO-2 null corneas the basal (unsutured) levels of HO-1 mRNA were higher than those of wild type corneas (Figure 6A), which is in agreement with the observed positive HO-1 immunostaining in HO-2 null corneas (Figure 1B) and with previous reportings of a compensatory increase in HO-1 expression in the HO-2 null mice (Ding et al., 2006; Seta et al., 2006). Interestingly, as clearly seen in Figure 6B, suture insertion did not significantly induce a further increase in HO-1 mRNA levels. These results further indicate that induction of HO-1 in response to insult (suture insertion) is impaired in the HO-2 null mice.

We have previously documented that infiltrating macrophages are also a source for the increased HO-1 expression in the injured cornea (Seta et al., 2006). To this end, induction of HO-1 expression in macrophages has been implicated as an important step for the resolution phase of inflammation (Willis et al., 1996). Indeed, a double immunofluorescence analysis with anti-HO-1 and anti-CD68 antibodies revealed that infiltrating macrophages were a source of stromal HO-1 in wild type corneas 4 days after suture insertion (Figure 6C). In contrast, while significantly more CD-68 positive cells were detected in the stroma of HO-2 null cornea (288 ± 33 vs 72 ± 7 CD68-positive cells/mm², $n=6-9$, $p<0.001$), few of CD-68 positive cells showed HO-1 immunofluorescence in HO-2 null cornea. In fact 89.8% of CD68 cells in wild type stromas stained positive for HO-1, whereas only 26.2 % of CD68 cells in HO-2 null stromas stained positive for HO-1, suggesting that HO-1 dysregulation in HO-2 null mice is also reflected by the reduced HO-1 expression in these activated macrophages.

Treatment with biliverdin attenuates the exaggerated inflammatory and neovascular response in HO null corneas

Since upregulation of HO-1 in response to suture placement was impaired in HO-2 null mice, it is reasonable to assume that the HO-2 null mice display lower levels of HO activity as was previously demonstrated (Seta et al., 2006). Hence, replenishing the corneas with HO-derived products may rescue the exaggerated inflammatory and neovascular phenotype seen in the HO-2 null corneas. Indeed, topical application of biliverdin onto sutured HO-2 null corneas, significantly reduced neovascularization at day 2 (Figure 7A) and day 4 (Figure 7B) after suture insertion by 50 and 60 %, respectively. It also reduced the inflammatory response as evidenced by a significant 60% decrease in MPO activity, i.e., in the number of infiltrating neutrophils, 4 days after suture insertion (Figure 7C).

DISCUSSION

The present study furthers the concept that HO-2 serves a cytoprotective function in the cornea. Its deletion leads to an exaggerated inflammatory response and accelerated hemangiogenesis but lymphangiogenesis does not appear to be altered by the deletion. Moreover, lack of HO-2 expression was associated with impaired HO-1 induction leading to a deficiency in HO activity; this deficiency was corrected with the addition of biliverdin, a product of the HO system. Biliverdin supplementation attenuated the exaggerated inflammatory and neovascular response seen in the cornea following placement of an intrastromal suture.

This study makes an important observation of the robust constitutive expression of HO-2 in the cornea, which, in the intact uninjured cornea of wild type mouse, is largely localized to the corneal epithelium. This robust expression begs the question of its function in corneal homeostasis. Even though HO-1 and HO-2 catalyze the same reaction, notable differences between the two exist (Shibahara, 2003). Human HO-1 and HO-2 share 43% amino acid sequence identity and about 80% to their correspondent rat and mice proteins. HO-2 (316 amino acids; 36 kDa) contains 3 cysteine residues while HO-1 (288 amino acids; 32 kDa) contains no cysteine residues. Unlike HO-1, HO-2 contains 2 copies of the heme binding site which are not involved in the heme breakdown reaction and are conserved at equivalent positions of human, mouse and rat HO-2 (McCoubrey et al., 1997). It is therefore likely that HO-2 may serve not only to degrade heme but also to sequester heme to maintain intracellular levels or reduce heme-mediated oxidative stress. HO-2 expresses at relatively constant levels but its activity is regulated by phosphorylation under the control of different protein kinases (Boehning et al., 2003; Dore et al., 1999b). HO-2 deficient mice are fertile and survive normally for at least one year (Poss et al., 1995; Poss and Tonegawa, 1997) and, thus, show a milder phenotype than those of HO-1 null mice such as infertility and prenatal death (Poss and Tonegawa, 1997). HO-2 null mice show no noticeable morphological changes except that

cerebral HO activity is reduced (Poss et al., 1995). HO-2 null mice have increased susceptibility to hyperoxic injury in the lung (Dennery et al., 1998) and to oxidative and ischemic injury in the brain (Chang et al., 2003; Dore et al., 1999a) suggesting a cytoprotective role for HO-2 not necessarily compensated for by HO-1. Moreover, studies using HO-2 gene-deleted or pharmacologically-inhibited cerebrovascular endothelial cells concluded that HO-2 is critical for protection of the cerebrovascular endothelium against apoptotic changes induced by oxidative stress and cytokine-mediated inflammation (Basuroy et al., 2006; Parfenova et al., 2006). Interestingly, HO-1 expression is increased in lungs (Dennery et al., 1998) but not in the brain (Poss et al., 1995) of HO-2 null mice while heme levels showed no changes in these tissues (Dennery et al., 1998; Zakhary et al., 1997). The distinct properties of HO-2 including constitutive expression, activation by phosphorylation and an additional binding site for heme set it apart from HO-1. Moreover, the demonstration that HO-2 is cytoprotective without degrading heme (Kim and Dore, 2005; Kim et al., 2005) suggests that HO-2 participates not only in maintaining heme homeostasis but also in cellular defense mechanisms against injury. With regard to the cornea, this notion was first introduced in a previous report by Seta et al., and is re-enforced by the current study.

The suture-induced inflammatory neovascularization is a model in which inflammation is the underlying driving force of the neovascular response and it is likely to better resemble clinical situations where corneal neovascularization is caused by inflammatory stimuli such as a retained foreign body. In a previous study, we demonstrated that epithelial injury in HO-2-null corneas induces a massive neovascular response that is absent in normal corneas (Seta et al., 2006). The current study utilized this subtle injury to further examine the impact of HO-2 deletion on corneal neovascularization. Here we show that both the inflammatory and neovascular response are exacerbated in the absence of HO-2. The inflammatory response to the intrastromal suture was significantly higher in HO-2 null corneas as evidenced by a greater increase in cellular infiltrates and cytokine production as compared to wild type corneas. In the wild type mice, the increase in cytokine production was the highest at days 2 and 4 after suture placement; at day 7 these levels are dramatically decreased reaching the control levels prior to suture placement. In contrast, cytokine levels in HO-2 null mice at day 7 were several-fold higher than those in control corneas, despite a significant decrease at day 4. The latter may be explained by a transient, albeit small, increase in HO-1 expression at day 2 and 4, which in turn may contribute to reduced cytokine production (*vide infra*). The biphasic pattern of cytokine production in the HO-2 null mice may also represent a possible shift in the cellular composition contributing to the production of these cytokines (neutrophils, macrophages, lymphocytes and activated epithelial and stromal cells), and additional studies extending the time course and evaluating inflammatory profiles are warranted.

It is likely that the increased rate of neovascularization seen in HO-2 null corneas is the result of an accelerated inflammatory response. The mechanisms underlying the increased inflammatory response in the absence of HO-2 are largely unclear. However, the finding that the known injury-triggered HO-1 induction in HO-2 null cornea was largely absent suggests that the cytoprotective property of the HO system is lacking in this model. HO-1 induction is recognized as one of the rapid defense responses of a tissue to injury that brings into the site of injury powerful anti-inflammatory signals to initiate resolution and repair. In the HO-2 null corneas, HO-1 induction following suture insertion was attenuated suggesting that these corneas are deficient in HO-derived anti-inflammatory signals, including CO and biliverdin. CO and biliverdin/bilirubin have been shown to exert anti-inflammatory effects by inhibiting adhesion molecules and leukocyte recruitment (Wagener et al., 1999; Wagener et al., 2003), and by suppressing cytokine/chemokine expression (Minamino et al., 2001; Morse et al., 2003; Otterbein et al., 2000; Sarady-Andrews et al., 2005; Song et al., 2003). The cellular mechanisms underlie the anti-inflammatory action of CO that lead to inhibition of IL-1, IL-6, TNF α and MIP-1 expression involve the p38 MAPK as well as the JNK signaling pathway and

the transcription factor AP-1 (Morse et al., 2003; Otterbein et al., 2000; Ryter and Otterbein, 2004). Biliverdin has been shown to inhibit the activation of pro-inflammatory transcription factors including NF κ B in vitro and in vivo and to decrease inflammatory mediators such as IL-6, TNF α and leukocyte infiltration (Foresti et al., 2004; Nakao et al., 2004; Sarady-Andrews et al., 2005) (Gibbs and Maines, 2007). Induction of HO-1 as well as administration of either CO or biliverdin or both have been shown to lessened tissue damage in various models of injury. Consequently, it is reasonable to assume that deficiency in HO-1 induction and HO activity (production of CO and biliverdin) would contribute, at least in part, to increased inflammation. Equally reasonable is that induction of HO-1 expression increases production of CO and bilirubin, which in turn serve to enhance cellular defense systems that modulate the inflammatory response. Failure to increase the levels of the HO system may greatly compromise the inflammatory response of the cornea. Importantly, the demonstration that topical application of biliverdin lessened inflammation-associated corneal neovascularization in HO-2 null mice is consistent with the aforementioned arguments. To the extent that CO shares similar protective properties (Abraham and Kappas, 2008), its administration should also be effective in lessening inflammatory neovascularization. In a separate set of experiments, using the epithelial injury model in which corneal neovascularization occurs only in the HO-2 null mice, administration of a CO donor diminished neovascularization (unpublished data). While CO has been shown to increase the angiogenic response of endothelial cells in vitro (Li Volti et al., 2005), its effect in vivo may reflect its broad range of anti-inflammatory actions, specifically with regard to the complex cellular milieu of corneal neovascularization.

The finding that biliverdin effectively reduced the accelerated rate of inflammation and neovascularization in HO-2 null corneas may have important consequences for corneal transplantation. Numerous studies have shown that biliverdin/bilirubin benefit ischemia-reperfusion injury and solid-organ transplantation, including chronic rejection, and islet transplantation (Fondevila et al., 2004; Nakao et al., 2004; Yamashita et al., 2004) and suggested it as a novel therapeutic approach to maximize the function and thus the availability of donor organs (Ollinger et al., 2007). In view of the cataractogenic and pressure elevation potential of the currently used glucocorticoids to prevent or treat graft rejection, biliverdin/bilirubin might constitute an alternative treatment of choice.

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Figure 1. HO-2 and HO-1 expression in the cornea of wild type (HO-2^{+/+}) and HO-2 null mice (HO-2^{-/-})

A) Immunofluorescence of HO-2 in corneas of HO-2^{+/+} (upper panel) and HO-2^{-/-} (lower panel) mice. B) immunohistochemistry of HO-1 (a and b) and HO-2 (c and d) in corneas from HO-2^{+/+} (upper panels) and HO-2^{-/-} mice (lower panels).

Figure 2. Increased neovascular response in HO-2 null mice

A) Representative photographs of eyes from wild type (HO-2^{+/+}) and HO-2 null (HO-2^{-/-}) mice at days 2, 4 and 7 after suture placement. **B)** Neovascularization expressed as total length of penetrating blood vessels (n=4–23; *p<0.05; ***p<0.001 from wild type).

Figure 3. Analysis of hemangiogenesis and lymphangiogenesis in HO-2 null mice
A) FITC-CD31 immunofluorescent staining of corneal flat mounts of sutured eyes from wild type (HO-2^{+/+}) and HO-2 null (HO-2^{-/-}) mice on days 2, 4 and 7 after suture placement. **B)** Quantitative analysis of FITC-CD31 stained blood vessels on days 2, 4 and 7 days after suture placement. Results are expressed as % vascularized area of the cornea (n=3–8; *p<0.05; **p<0.01). **C)** Cy3-LYVE-1 immunofluorescent staining of corneal flat mounts of sutured eyes from HO-2^{+/+} and HO-2^{-/-} mice on days 2, 4 and 7 after suture placement. **D)** Quantitative analysis of Cy3-LYVE-1-stained lymph vessels on days 2, 4 and 7 days after suture placement. Results are expressed as % vascularized area of the cornea (n=3–8).

Figure 4. Amplified inflammatory response in HO-2 null corneas

A) Representative histological sections stained with H&E of corneas from wild type (HO-2^{+/+}) and HO-2 null (HO-2^{-/-}) mice 2, 4 and 7 days after suture placements. **B)** Corneal influx of inflammatory cells determined by MPO activity in HO-2^{+/+} and HO-2^{-/-} mice (n=2–14; *p<0.05; **p<0.01; ***p<0.001 from WT).

Figure 5. Increased inflammatory mediators in HO-2 null mice

A) Inflammatory cytokine profile in corneas from wild type (HO-2^{+/+}) and HO-2 null (HO-2^{-/-}) mice before and 2, 4, and 7 days after suture placement (n=4, *p<0.05; **p<0.01; ***p<0.001 from wild type).

Figure 6. Corneal HO-1 expression in wild type (HO-2^{+/+}) and HO-2 null (HO-2^{-/-}) mice
 Real time PCR analysis is given as A) relative expression of heme oxygenase 1 (HO-1) mRNA in corneas before, and on days 2, 4 and 7 after suture placement and B) as fold change from control unsutured corneas (n=10, ***p<0.001). C) Immunofluorescence analysis of CD68 (Cy3, red) and HO-1 (Alexa 488, green) in HO-2^{-/-} and HO-2^{+/+} corneas 4 days after suture insertion.

Figure 7. Effect of biliverdin treatment on neovascularization in HO-2 null mice at days 2 (A) and 4 (B) after suture placement

Biliverdin was applied topically and neovascularization was measured as described in methods (n=3–10, *p<0.05; **p<0.01 from control, untreated eyes). C) Biliverdin effect on MPO activity in HO-2 null corneas (n=3–10, *p<0.05).