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Heme Oxygenase-1 Induction Attenuates Corneal Inflammation and Accelerates Wound Healing after Epithelial Injury

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Abstract

Purpose—Heme oxygenase (HO) is considered a fundamental endogenous immunomodulatory, cytoprotective, and anti-inflammatory system. This protective function is primarily ascribed to the inducible HO-1. The authors examined the effect of HO-1 induction on corneal inflammation and wound healing in mice undergoing epithelial injury.

Methods—C57BL/6 mice were treated with SnCl₂ the day before epithelial injury and once daily thereafter. The corneal epithelium was removed with the use of a corneal rust ring remover in anesthetized mice. Reepithelialization was measured by fluorescein staining. The inflammatory response was examined by histology and was quantified by the myeloperoxidase assay. Inflammatory lipid mediators were detected and quantified by LC/MS/MS-based lipidomic analysis. HO-1 expression was assessed by real-time PCR, and HO activity was determined by measuring HO-dependent carbon monoxide production.

Results—Epithelial injury caused a time-dependent transient increase in HO-1 expression and HO activity that was significantly amplified by treatment with SnCl₂, resulting in a twofold to threefold increase in mRNA levels and a similar increase in corneal HO activity. Induction of HO-1 was associated with a significant acceleration of wound healing when compared with a vehicle-treated group and with attenuation of the inflammatory response, evidenced by a significant decrease in the number of infiltrating cells and by a significant reduction in the expression and production of proinflammatory lipid mediators and cytokines.

Conclusions—Increased expression of HO-1 provides a mechanism that modulates inflammation and promotes wound closure; pharmacologic amplification of this system may constitute a novel strategy to treat corneal inflammation while accelerating wound repair after injury.

Wound healing in the cornea is unique because of the need to maintain transparency. The same is true for corneal inflammation, which is intimately linked to the reparative effort. Inflammation must be tightly controlled to avoid the consequences of an excessive inflammatory response, such as delayed wound healing and new vessel formation, both of which have adverse effects on corneal transparency. At the same time, a crucial equilibrium must be maintained between those inflammatory events that serve host defense and those that threaten the cornea. Little is known about the endogenous counterregulatory systems that

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control inflammation in the corneal epithelial surface. One innate circuit, however, that has been studied extensively in numerous tissues is the heme oxygenase (HO) system.^{1,2}

HO is the rate-limiting enzyme in heme catabolism. It cleaves heme to biliverdin, carbon monoxide (CO), and iron; biliverdin is subsequently converted by biliverdin reductase to bilirubin. Two isoforms, HO-1 and HO-2, are expressed in most tissues. HO-1 is an inducible enzyme, whereas HO-2 displays, in general, a constitutive expression that is developmentally regulated.³ The HO system has been implicated in the regulation of inflammation. This role has been assigned primarily to HO-1 based on studies showing that its overexpression provides protection against inflammation-mediated injury, including xenograft rejection,⁴ endotoxin challenge,⁵⁻⁷ and ischemia-reperfusion injury,⁸⁻¹¹ whereas deficiency in its expression is associated with a chronically inflamed state and increased leukocyte recruitment, as reported in a human¹² and in mice^{13,14} null for the HO-1 gene. Although the mechanisms involved in this cytoprotection are largely unknown, the elimination of excess cellular heme and the presence of 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. Biliverdin and bilirubin exhibit potent antioxidant properties.¹⁵ Carbon monoxide may also be cytoprotective because of its bioactions, including cGMP-mediated modulation of vascular tone, and inhibition of apoptosis, platelet aggregation, complement activation, and inflammatory cytokine production.^{16,17} The upregulation of HO-1 and the administration of bilirubin or CO have been shown to downregulate the inflammatory response by attenuating the expression of adhesion molecules, thus inhibiting leukocyte recruitment^{18,19} by repressing the induction of cytokines and chemokines,^{4,5,8,20,21} or by inhibiting proinflammatory hemoproteins such as COX-2 and cytochrome P450 (CYP).²²⁻²⁵

Our knowledge of the HO system in the corneal surface is limited. Previous studies have demonstrated the presence of HO activity and expression in human and rabbit corneas and HO-1 inducibility after oxidative stress *in vitro* and hypoxic injury *in vivo*.^{23,24,26-29} These studies led to the seminal finding that the induction of HO-1 alleviates hypoxic injury-induced ocular surface inflammation and pointed to a potential important function for the corneal HO system.^{23,24} Our postulated mechanism of action of HO-1 induction at that time was the downregulation of the expression and activity of the corneal epithelial CYP 4B1, an injury-induced system that generates powerful inflammatory eicosanoids and that participates in the inflammatory and neovascular response of the cornea.^{30,31} However, in a recent study, we showed that the protective mechanisms of the HO system in the cornea may reach far beyond the simple downregulation of inflammatory hemoproteins. This study showed that a deficiency in HO activity, as in HO-2 null mice, exacerbates ocular surface inflammation; it increased cell infiltration, expression of inflammatory genes, and production of proinflammatory lipid mediators and impaired wound healing, allowing an acute inflammation to become chronic with the stigma of chronic corneal inflammation such as neovascularization, ulceration, and perforation.³²

In the present study, the impact of increased HO activity on the inflammatory and reparative response was examined in mice using a model of epithelial injury in the eye in which inflammatory and reparative responses are well characterized.³³ We used SnCl₂, an effective metal inducer of corneal HO-1 *in vitro* and *in vivo*.^{24,28,34} We demonstrated that HO-1 induction attenuated inflammation and accelerated wound healing, further substantiating the notion that HO-1 represents a fundamental protective system in the cornea. Pharmacologic amplification of this system may constitute a novel strategy to accelerate corneal wound healing while attenuating inflammation.

Materials and Methods

Animals and Epithelial Injury

All animal experiments were performed according to an institutionally approved protocol and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice (C57Bl/6J; Jackson Laboratory, Bar Harbor, ME) were anesthetized with ketamine (50 mg/kg) and xylazine (20 mg/kg) intramuscularly, and a drop of tetracaine-HCl 0.5% was applied to the eye to deliver local corneal anesthesia before animals were subjected to injury. The corneal epithelium was removed up to the corneal/limbal border with a 0.5-mm corneal rust ring remover (Algerbrush II; Alger Equipment, Lago Vista, TX), as previously described.³² SnCl₂ has been documented as a nontoxic metal inducer of corneal HO-1 in vitro and in vivo and was used in this study at a dose that proved to be effective in previous studies.^{23,24} SnCl₂ dissolved in 0.1 M potassium phosphate buffer (pH 7.4) was administered topically (10 µg/10 µL twice a day) and intraperitoneally (1 mg/100 µL daily) starting 1 day before corneal epithelial debridement. Wound closure (reepithelialization) was monitored daily for 7 days after injury using fluorescein staining. Images of the anterior surface were taken with a dissecting microscope (Carl Zeiss, Jena, Germany) coupled to a digital camera (AxioCam HRC; Carl Zeiss) and analyzed using Zeiss software (Axiovision 4.5). Mice were humanely killed at the indicated time points, eyes were removed, and corneas free of conjunctival tissue were dissected and processed for selected analyses.

Histology

Dissected corneas were washed twice with phosphate-buffered saline (PBS; pH 7.4), and were fixed in 4% paraformaldehyde–PBS for 1 hour at 4°C. Corneas were washed five times with PBS, placed in 30% sucrose for 24 hours, and embedded in OCT compound (Sakura Finetek, Torrance, CA). Cryostat sections were cut transversely into 10-µm–thick sections, stained with hematoxylin and eosin (H&E), and mounted on microscope slides in cytosol (Cytosol XYL; Richard-Allan Scientific, Kalamazoo, MI). Sections were then visualized and analyzed (Axiovision Imager M1; Carl Zeiss).

Myeloperoxidase Activity

Measurement of myeloperoxidase (MPO) activity was used to quantify polymorphonuclear cells (PMNs) in dissected corneas, as previously described.³² 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-thawing. The particulate matter was removed by centrifugation, and MPO activity in the supernatant was measured by spectrophotometry using *o*-dianisidine dihydrochloride reduction as a colorimetric indicator. Calibration curves for the conversion of MPO activities to a PMN number were established with PMNs that were collected from zymosan A-induced peritonitis in untreated mice.

Real-Time 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 (RNeasy Protect Mini Kit; Qiagen, Valencia, CA) and quantified by spectrophotometry (Nanodrop Technologies, Wilmington, DE). Reverse transcription reaction of total RNA (5 µg) was performed using the first-strand synthesis system (Superscript III; Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Quantitative real-time PCR was performed using a PCR kit (QuantiTect SYBR Green; Qiagen) and the real-time PCR system (Mx3000; 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'; CYP4B1 sense, 5'-CTTTTGGTCATGCCCTTGAGA-3', and anti-sense, 5'-CAGCTTTAGCATAGTCAGGCTC-3'; 18S sense, 5'-TGTCTCAAAGATTAAGCCATGCAT-3', and anti-sense, 5'-AACCATAACTGATTTAA-TGAGCCATTC-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 the 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 was expressed in comparison to the reference gene 18S RNA, as described.³⁵

HO Activity/CO Measurement

Corneas were dissected from vehicle-treated and SnCl₂-treated C57bl6 mice at indicated time points. Dissected corneas were homogenized in PBS (0.01 M, pH 7.4) in the presence of a protease inhibitor cocktail (Complete Mini; Roche, Indianapolis, IN) and 100 μ M butylhydroxytoluene. Protein concentration was measured by using the Bradford assay. Aliquots of corneal homogenates were subsequently incubated for 2 hours in the dark at 37°C with 2 mM hemin in the presence of an NADPH-generating system (100 mM MgCl₂, 68.1 mM glucose-6-phosphate, 33 U/mL glucose-6-phosphate dehydrogenase, and 26 mM NADPH) in a final volume of 1 mL potassium phosphate buffer (0.1 mM, pH 7.4). To determine NADPH-independent CO production, the samples were also incubated in potassium phosphate buffer without the NADPH-generating system. CO released in the headspace gas was analyzed by gas chromatography–mass spectrometry (GC-MS, HP5989A interfaced to HP5890; Hewlett Packard, Palo Alto, CA). The amount of CO was calculated from standard curves constructed with the abundance of ions m/z 28 and m/z 29 or m/z 31, as previously described.³²

Measurements of IL-1 and MIP-2

Dissected corneas were homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL) containing protease inhibitor cocktail (Halt; Pierce). IL-1 (IL-1 α) and macrophage inflammatory protein (MIP)-2 were measured in corneal homogenates using a custom multiplex sandwich ELISA for protein analysis (SearchLight mouse microarray; Pierce Biotechnology, Woburn, MA).

LC/MS/MS-Based Lipidomics

Dissected corneas were homogenized with 0.5 mL ice-cold distilled water, to which were added 1 mL ice-cold methanol, 0.5 ng PGB₂, and 0.5 ng deuterated eicosanoids including d4-PGE₂, d8-12-HETE, and d8-8,9-epoxyeicosatrienoic acid (8,9-EET). Corneal suspensions were placed at –20°C for at least 30 minutes and were centrifuged at 10,000g for 10 minutes at 4°C, and supernatants were collected. Supernatants were diluted with 20 mL HPLC-grade water and acidified to pH 4.0 with HCl (1 N). Acidified samples were immediately loaded onto primed C18-ODS cartridges (AccuBond II; 500 mg; Agilent Technologies, Palo Alto, CA). Cartridges were washed with 10 mL water followed by 10 mL hexane, and compounds were eluted in 5 mL methanol. The methanol fraction was taken to dryness under a gentle stream of nitrogen, resuspended in methanol (100 μ L), and stored at –80°C. Eicosanoids were identified and quantified by LC/MS/MS-based lipidomic analyses. In brief, extracted samples were analyzed by a triple quadrupole linear ion trap LC/MS/MS system (3200 QTRAP; Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a mini-bore column (150 $\times$ 2 mm; LUNA C18-2; Applied Biosystems/MDS Sciex) using a mobile-phase (methanol/water/acetate, 65:35:0.03, vol/vol/vol) with a 0.35-mL flow rate. MS/MS analyses were carried out in negative ion mode, and fatty acids were quantified by multiple reaction monitoring (MRM

mode) using established transitions for each eicosanoid. Calibration curves (10–500 pg) for each compound had been established with synthetic standards.

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. $P < 0.05$ was considered significant. All data are presented as mean $\pm$ SE.

Results

Increased Induction of HO-1 after SnCl₂ Treatment

Epithelial injury leads to a rapid and transient increase of HO-1 expression.³² Treatment with SnCl₂ caused a further increase in HO-1 expression and HO activity. As seen in Figure 1A, HO-1 mRNA significantly increased by 2.0-, 1.7-, 5.0-, 3.2-, and 3.3-fold in corneas treated with SnCl₂ compared with corresponding vehicle-treated corneas at days 1, 2, 3, 4, and 7 days after injury, respectively. Induction of HO-1 mRNA expression with SnCl₂ was followed by an increase in HO activity, as seen in Figure 1B. HO activity was measured as the NADPH-dependent production of CO and was higher in corneas treated with SnCl₂ than in corresponding vehicle-treated corneas. CO production was significantly higher in SnCl₂-treated corneas 1, 2, 3, and 4 days after injury (Fig. 1B). Hence, administration of SnCl₂ resulted in a marked induction followed by increased HO function defined by the production of CO. The production level of CO at day 7 returned to pretreatment levels despite a significant increase in HO-1 mRNA expression, suggesting the possibility of a feedback control on the levels of HO activity or protein inactivation as a result of sustained induction.³⁶

Accelerated Wound Healing in SnCl₂-Treated Mice

The corneal epithelial injury model used involves the removal of the entire corneal epithelium with use of a corneal rust ring remover (Algerbrush II; Alger Equipment). It is an established epithelial-initiated injury model in which the inflammatory and reparative response has been well characterized.^{32,33} In untreated mice, the epithelial injury produced a consistent wound (6.60 ± 0.054 mm², $n = 20$) that exhibited a linear rate of reepithelialization with $8.85\% \pm 0.83\%$, $39.15\% \pm 2.44\%$, $68.98\% \pm 3.57\%$, $84.88\% \pm 3.21\%$, and $97.85\% \pm 1.36\%$ wound closure at days 1, 2, 3, 4, and 7 after injury, respectively (Figs. 2A, 2B). In contrast, reepithelialization of corneal wounds (6.04 ± 0.16 mm², $n = 16$) in SnCl₂-treated mice was significantly enhanced by 49%, 57%, 16%, 8%, and 2% compared with wild-type mice at days 1, 2, 3, 4, and 7 after injury, respectively.

Attenuated Inflammatory Response in SnCl₂-Treated Mice

Accelerated reepithelialization in corneas from SnCl₂-treated mice was associated with marked attenuation of PMN infiltration assessed by histologic examination (H&E staining) and MPO activity. H&E staining of corneal sections showed a significant difference in the magnitude of the inflammatory response to injury between vehicle-treated and SnCl₂-treated corneas and differences in the rate of reepithelialization (Fig. 3A). SnCl₂ treatment reduced ($P < 0.02$) the number of infiltrating cells by 40% to 50% (from 2852 ± 430 , 1307 ± 163 , and 651 ± 31 cells/mm² in vehicle-treated corneas to 1338 ± 183 , 727 ± 40 , and 374 ± 18 cells/mm² in SnCl₂-treated corneas at days 2, 4, and 7 after injury, respectively).

Measurement of corneal MPO activity showed a similar pattern. In response to injury, PMN infiltration as measured by MPO was rapid and transient in both groups, a pattern consistent with an acute self-resolving inflammatory response (Fig. 3B). However, this response was

significantly reduced in the SnCl₂-treated group showing twofold less PMN infiltrate in days 1 and 2 after injury and a 50% decrease in days 3, 4, and 7 after injury.

Induction of HO-1–Reduced Levels of Inflammatory Mediators

Induction of HO activity directly affects functional expression of heme-containing enzymes. In this regard, COX-2 and CYP4B1 are of special interest because these heme-containing enzymes generate well-established inflammatory and angiogenic lipid mediators and are considered markers of inflammation, especially in the cornea.^{30,37} They are localized primarily to the corneal epithelium and stromal keratinocytes and are induced in response to injury.^{38–40} The level and activity of both enzymes are regulated by HO.^{22,24} Therefore, we examined whether the attenuated inflammatory response and accelerated reepithelialization of the injured cornea in SnCl₂-treated mice corresponded with comparable reductions in inflammatory lipid mediators. Corneas were processed for lipidomic analysis using LC/MS/MS. As seen in Figure 4A, the major metabolites of COX-2 and CYP4B1, PGE₂, and 12-HETE/12-HETrE, respectively, were detected in corneas 3 days after injury. More important, corneas treated with SnCl₂ produced significantly less PGE₂, 12-HETE, and 12-HETrE when compared with control corneas treated with vehicle. In addition, the levels of 11,12-epoxyeicosatrienoic acid (11,12-EET), a putative intermediate in the synthesis of 12-HETrE,⁴¹ were lower in corneas treated with SnCl₂ than in control corneas (Figs. 4A, 4B). mRNA levels of CYP4B1, the major enzymatic source of 12-HETE and 12-HETrE in the cornea, have been shown to increase after epithelial injury.³² Figure 4C demonstrates again that injury induced a rapid twofold increase in CYP4B1 mRNA levels in the vehicle-treated corneas that returned to control levels thereafter. In contrast, CYP4B1 levels were largely suppressed in corneas treated with SnCl₂ (Fig. 4C). Hence, CYP4B1 expression in SnCl₂-treated corneas 1 day before injury and 1 day after injury were approximately 50% and 20% of that in corresponding vehicle-treated corneas, respectively. CYP4B1 levels remained suppressed during the course of reepithelialization (Fig. 4C), suggesting that HO-1 induction brings about accelerated reepithelialization by controlling, at least in part, the level of induction of one of the major proinflammatory pathways in the cornea, CYP4B1.^{31,42}

Cytokines and chemokines play important roles in the regulation of the inflammatory and repair responses of the cornea.⁴³ Among them are IL-1 and MIP-2, the expressions of which are greatly elevated on injury, and their continual accumulation leads to increased PMN infiltration and, thereby, inflammation.⁴⁴ We examined whether HO-1 induction after treatment with SnCl₂ is associated with decreased production of these proinflammatory mediators. As seen in Figure 5, injury caused a rapid and transient increase in the levels of h IL-1 and MIP-2, which peaked at day 4 after injury and declined by day 7, when reepithelialization was about completed. This transient increase was greatly attenuated by SnCl₂ treatment. The maximum increase in the levels of IL-1 and MIP-2 at day 4 was reduced by approximately 60% to 70%, suggesting that HO-1 induction contributes to accelerated healing by attenuating, at least in part, the inflammatory response.

Discussion

This study demonstrates the effectiveness of HO-1 induction in accelerating the corneal wound healing process while reducing inflammation endangered by corneal epithelial injury. These results are consistent with those of an earlier study in which we found a marked reduction in hypoxia-induced inflammation and neovascularization in the rabbit cornea by induction of HO-1 using SnCl₂-soaked contact lenses.^{23,24} Those results, together with recent findings showing that HO deficiency exacerbates injury-induced corneal inflammation and repair,³² lends substantial support to the notion that the HO system may be a key intrinsic corneal anti-

inflammatory and pro-reparative circuit in the cornea.⁴⁵ The present study further examines the contribution of HO-1 to the inflammatory and reparative response after epithelial injury.

HO-1 is an inducible enzyme whose expression is rapidly and robustly increased after injury to a given tissue. Among the most prominent inducers of HO-1 are heavy metals, which activate its transcription through a metal-responsive element. Tin is one of these metals, and SnCl₂ has been widely used as a nontoxic, effective inducer of HO-1 in many tissues, including the cornea.^{23,24} It is also an effective inducer of HO-1 mRNA and HO activity in cultured rabbit and human corneal epithelial cells.^{24,28} In the present study, SnCl₂ was administered before injury and every day thereafter. This regimen resulted in greater, sustained induction of the corneal HO-1 and was followed by increased HO-1 function, as evidenced by increased production of CO. The cellular source of the induced HO-1 was unclear. In the uninjured murine corneas, HO-1 expression was largely absent; there was some positive immunostaining in the epithelium, stromal keratinocytes, and endothelium. On injury, HO-1 expression is increased in all these layers, including infiltrating cells.³² The number of proliferating cells may explain the changes in HO-1 mRNA; however, the magnitude of the increase in mRNA (twofold), the 50% inhibition of PMN infiltration, and the 50% increase in reepithelialization in SnCl₂-treated eyes (e.g., at day 2) suggested further de novo induction, presumably in all corneal cell types participating in this response. The greater increase in HO-1 expression and activity is likely to represent the anti-inflammatory and protective property of SnCl₂ treatment.

Our ability to measure HO-derived CO equally implies the presence of biliverdin, the second byproduct of HO catalytic activity, which is rapidly reduced by biliverdin reductase to bilirubin. This is an important observation with respect to mechanisms of cytoprotection in the cornea. Although the mechanisms involved in the ability of HO-1 to attenuate corneal inflammation and to enhance the rate of repair in the injured cornea are unclear, in other tissues the elimination of excess cellular heme and the production of the enzymatic products of HO, CO, and bilirubin have been shown to protect against tissue damage by exerting antioxidant and anti-inflammatory effects, including the inhibition of adhesion molecules and leukocyte recruitment^{18,19} and the suppression of cytokine/chemokine expression.^{4,5,8,20,21} The cellular mechanisms underlying CO anti-inflammatory action (inhibition of IL-1, IL-6, TNF- α , and MIP-1) have been shown to involve the p38 MAPK and the JNK signaling pathways and the transcription factor AP-1.^{5,20,46} At physiological concentrations, bilirubin (and, to a lesser extent, biliverdin) surpasses α -tocopherol as the most potent protector against lipid peroxidation¹⁵ and other reactive oxygen species (ROS).⁴⁷ The administration of biliverdin/bilirubin decreases LPS-induced proinflammatory transcription factors (NF κ B) and cytokines (IL-6, TNF α)²¹; this may be mediated by its ROS-scavenging mechanisms because the expression of many proinflammatory genes are redox sensitive.⁴⁸ To this end, HO-1 induction after SnCl₂ treatment was associated with a significant decrease in the levels of IL-1 and MIP-2, inflammatory mediators with PMN chemotactic and activating properties in the cornea. Whether increased HO activity and the production of the cytoprotective biliverdin/bilirubin and CO contributed to the attenuated increase in IL-1 and MIP-2 must be examined. Although the lack of specific HO inhibitors impedes such examination, the recent finding that biliverdin rescues the exaggerated inflammatory response to corneal epithelial injury in HO-2-deficient mice³² suggests that HO activity plays an important role in regulating this response in the cornea.

Given the ample evidence that CO and biliverdin/bilirubin are anti-inflammatory molecules, it is reasonable to assume that injury to the cornea induces HO-1 expression, which is followed by increased HO activity. The increased production of CO and bilirubin serves to enhance cellular defense systems that counteract the detrimental effects of ROS, proinflammatory cytokines, and lipid mediators. It is also reasonable to assume that amplification of their production by the induction of HO-1, as observed in corneas treated with SnCl₂, contributes,

at least in part, to the attenuated inflammatory response, which is tightly coupled with enhanced reparative capability. Our data support these assumptions. Thus, in corneas treated with SnCl_2 , which showed increased expression of HO-1 and increased production of CO, the inflammatory response, despite a transient/resolution pattern similar to that in vehicle-treated corneas, was reduced, as evidenced by a decrease of cell infiltrates. Although cellular infiltrates are key components of the repair process,⁴⁹ exaggerated responses are detrimental in that they delay wound healing. Hence, the reduced number of cells infiltrating corneas treated with SnCl_2 may contribute to the significant accelerated reepithelialization seen in these corneas. In addition, reports suggest a role for HO-1 in cutaneous wound repair.^{50–52} Moreover, HO-1 induction and administration of CO or biliverdin has been shown to inhibit apoptosis in different cell types and tissues.^{53–56} As indicated by Wilson et al.,⁴³ keratocyte apoptosis is the earliest stromal event noted after epithelial injury and remains a likely target for modulation of the overall wound-healing response. It is possible that HO-1 induction affects the rate of reepithelialization by additional mechanisms independent of inflammation reduction.

The inflammatory response consistently involves the activation of corneal cells and the recruitment of leukocytes to produce lipid and protein molecules that mediate initiation, amplification, and resolution of inflammation. Among the lipid mediators are numerous arachidonic acid-derived eicosanoids of the cyclooxygenase, lipoxygenase, and cytochrome P450 monooxygenase (e.g., CYP4B1) pathways.^{57–61} In the cornea, cyclooxygenase (specifically, COX-2)-derived PGE_2 ⁶² and CYP4B1-derived 12-HETE/12-HETrE⁵⁹ have been implicated as proinflammatory mediators, whereas lipoxygenase-derived 15-HETE and its lipoxin derivatives have been implicated as anti-inflammatory mediators.^{33,63} In addition, 5-lipoxygenase-derived 5-HETE and LTB_4 have been implicated as proinflammatory mediators introduced largely by infiltrating leukocytes.^{64,65} Noteworthy are findings that HO-1 downregulates the proinflammatory COX-2 and CYP-derived activities^{24,25} while it acts in concert with the anti-inflammatory 15-lipoxygenase pathway.⁴⁵ In the present study, lipidomic analysis clearly showed that the induction of HO-1 brought about by treatment with SnCl_2 significantly reduced the levels of PGE_2 , 12-HETE, 12-HETrE, and 5-HETE. Treatment with SnCl_2 also reduced 5-HETE levels by 20% (data not shown). The decreased production of these metabolites, which are potent neutrophil chemoattractants and inflammatory and angiogenic factors in the cornea, may constitute part of the mechanism by which HO-1 induction attenuates inflammation in this model of injury.

In summary, many studies in numerous tissues have defined the multiple roles of the HO system: it is immunomodulatory, anti-inflammatory, and cytoprotective. Our data in the cornea are consistent with these activities. The need for such a regulatory circuit is obvious when you consider the inflammatory challenges to which the cornea must respond. The hypoxia that eyelid closure invokes during sleep causes a low-grade inflammation that lasts for hours until awakening. The shearing stress of blinking, atmospheric irritants, and sunlight are other examples of inflammatory challenges. Keratitis sicca is now recognized as an example of a chronic low-grade inflammation–based condition. The xenobiotic cyclosporine A, used in the treatment of keratitis sicca, has properties similar to those of HO-1, but the HO system has the advantage of being endogenous to the cornea, and its expression is easily induced. Moreover, the use of corticosteroids for their anti-inflammatory effect is contraindicated after corneal abrasion injury because they slow or stop corneal reepithelialization. Herein, we see accelerated reepithelialization and an anti-inflammatory effect. Therapeutic induction of HO-1 has been successful in other tissues and should be considered for the cornea, and extensive studies to understand the role and the mechanisms of action of this endogenous cytoprotective system in the cornea should be pursued.

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Figure 1.

Effect of SnCl₂ treatment on HO-1 expression and activity. (A) Corneal HO-1 mRNA levels in vehicle- and SnCl₂-treated mice 1 day before (–d1) and 0 to 7 days (d0–d7) after injury. Results are the mean ± SE; *n* = 3 to 5. **P* < 0.05 from corresponding vehicle-treated mice. (B) Corneal HO activity in vehicle- and SnCl₂-treated mice at –d1 and at d0 to d7 after injury. Results are expressed as the amount of CO produced per milligram protein per hour and are the mean ± SE; *n* = 3 to 5. **P* < 0.05 from corresponding vehicle-treated mice.

Figure 2.

Effect of SnCl₂ treatment on wound healing. (A) Fluorescein-stained corneas after injury in vehicle- and SnCl₂-treated mice (16×). (B) Wound closure as percentage change from d0. The area of the injury (d0) in vehicle- and SnCl₂-treated mice was 6.60 ± 0.05 and 6.70 ± 0.04 mm², respectively. Results are mean $\pm$ SE; $n = 14$ to 50 . * $P < 0.005$ from vehicle-treated mice. † $P < 0.05$ from vehicle-treated mice.

Figure 3.

Effect of SnCl₂ treatment on the inflammatory response. (A) Corneal MPO activity in vehicle- and SnCl₂-treated mice at -d1 and at d0 to d7 after injury. Results are expressed as the number of PMNs per cornea and are the mean $\pm$ SE; $n = 3$ to 5 . * $P < 0.05$ versus vehicle-treated mice. (B) H&E staining of corneas at d2 (a, b), d4 (c, d), and d7 (e, f) after injury.

Figure 4.

Effect of SnCl₂ treatment on inflammatory lipid mediator pathways. (A) Representative LC/MS/MS profiles of lipid mediators in injured corneas (d3) from vehicle- and SnCl₂-treated mice. *Upper*: elution profile of authentic standards. *Middle*: elution profile of lipids in vehicle-treated corneas 3 days after injury. *Lower*: elution profile of lipids in SnCl₂-treated corneas 3 days after injury. (B) Quantitative lipidomic analysis. Results are mean ± SE; *n* = 4. **P* < 0.05 from vehicle-treated mice. (C) Corneal CYP4B1 mRNA levels in vehicle- and SnCl₂-treated mice at -d1 and at d0 to d7 after injury. Results are the mean ± SE; *n* = 3 to 5. **P* < 0.05 from corresponding vehicle-treated mice.

Figure 5. Effect of SnCl₂ treatment on levels of IL-1 (A) and MIP-2 (B) after epithelial injury. Corneas were harvested at the indicated time, homogenized, and assayed for levels of IL-1 and MIP-2 by ELISA. Results are the mean $\pm$ SE; $n = 3$. * $P < 0.05$ from corresponding vehicle-treated eyes.