Heme Oxygenase-2 Is a Critical Determinant for Execution of an Acute Inflammatory and Reparative Response

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Heme oxygenase (HO) represents an intrinsic anti-inflammatory system based on its ability to regulate leukocyte function and inhibit expression of proinflammatory cytokines. This anti-inflammatory function is linked to the inducible isoform HO-1; the role of the constitutive isoform HO-2 is unknown. The current study was undertaken to investigate the role of HO-2 in the regulation of the acute inflammatory and reparative response by using HO-2-null mice and well-established animal models of epithelial injury and antigen-induced peritonitis. Here we show that *in vivo* deletion of HO-2 disables execution of the acute inflammatory and reparative response after epithelial injury and leads to an exaggerated inflammatory response in antigen-induced peritonitis. HO-2 deletion was associated with impaired HO-1 induction, indicating that HO-2 is critical for HO-1 expression and that the subsequent failure to up-regulate the HO system may contribute to unresolved inflammation and the development of chronic inflammatory conditions. Indeed, supplementation with the HO bioactive product, biliverdin, rescued the acute inflammatory and reparative response in HO-2-null mice. Thus, HO-2 sets in place a basal tone of anti-inflammatory signals that may be a prerequisite for the ordered execution of an inflammatory and reparative response. (Am J Pathol 2006, 169:1612–1623; DOI: 10.2353/ajpath.2006.060555)

Tightly controlled inflammation contributes to normal wound healing. It enables and crucially drives tissue repair by stimulating resident cells to migrate and proliferate into the wound site, but when prolonged, it severely disrupts wound healing. Hence, a self-resolving inflammatory-reparative process must include pro- and anti-inflammatory circuits that work in concert to initiate, mediate, and resolve inflammation in a controlled manner so as to allow for the repair process to proceed toward complete restoration of structure and function.¹ The heme oxygenase (HO) system has been implicated in cellular cytoprotection, and evidence suggests that it may constitute an endogenous anti-inflammatory circuit.

HO is the rate-limiting enzyme in heme catabolism. It cleaves heme into biliverdin, carbon monoxide (CO), and iron.² Biliverdin is subsequently converted by biliverdin reductase to bilirubin, a potent endogenous antioxidant.³ 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 regulated⁴ and is altered in human pathological conditions.^{5,6} HO-1 and HO-2 are alike in terms of mechanisms of heme oxidation, co-factor and substrate specificity, and susceptibility to inhibition by porphyrins.^{2,7} 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 tissues, and its significance comes to light in response to injury.²

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 overexpres-

Supported by the National Institutes of Health (grants EY06513 to M.L.-S., EY016136 to K.G., HL34300 to M.L.-S. and N.G.A., DK56601 to N.G.A., and NS42273 to R.F.R.).

Accepted for publication July 20, 2006.

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sion down-regulates leukocyte migration and inflammatory cytokine production^{8,9}; however, lack of its expression in humans¹⁰ and mice¹¹ produces a phenotype of a severe chronic inflammatory state. In models of nonimmune and immune-driven inflammation, HO-1 levels in monocytes are enhanced during the resolution phase of acute inflammation; inhibition of HO activity converts acute inflammation to a chronic one.^{12,13} In models of cutaneous and oral mucosa wounding, the spatial and temporal increase in HO-1 correlates with infiltration of leukocytes and proliferating keratinocytes, resolution, and wound closure.^{14–16} The role of HO-2 in inflammation is primarily unexplored. However, given that the bioactions of CO and bilirubin underlie the postulated mechanisms of the HO-1 cytoprotective function, it is reasonable to assume that HO-2, which has an equal ability to produce CO and bilirubin, shares this function. CO and bilirubin have been shown to protect against tissue damage by exerting antioxidant and anti-inflammatory effects including inhibition of adhesion molecules and leukocyte re-cruitment^{17–19} and suppression of cytokine/chemokine expression.^{20–23}

In this study, the impact of impaired HO activity on the inflammatory and reparative response was examined in HO-2-null mice using a model of epithelial injury in the eye in which inflammatory and reparative responses are well characterized.²⁴ We demonstrated that epithelial injury in HO-2-null mice caused an acute inflammatory response that failed to resolve, evolving into exaggerated and uncontrolled inflammation with impaired wound closure and neovascularization. A phenotype of magnified inflammation and aberrant neutrophil function in HO-2-null mice was also confirmed in a model of antigen-induced peritonitis. Our findings point to a novel and critical role for HO-2 in providing the necessary threshold for the execution of an ordered inflammatory and reparative response.

Materials and Methods

Animal Experimentation

All animal experiments were performed following an institutionally approved protocol in accordance with the National Institutes 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.²⁵ These well-characterized HO-2-null mice have a C57BL/6 × 129/Sv genetic background,²⁶ which was used on age- and gender-matched controls. Mice 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 subjecting animals to injury. The corneal epithelium up to the corneal/limbal border was removed using an Algerbrush II with a 0.5-mm corneal rust ring remover (Alber Equipment Co., Lago Vista, TX) as previously described.²⁴ In some experiments, biliverdin was admin-

istered topically. Biliverdin hydrochloride (Frontier Scientific, Inc., Logan, UT) was dissolved in 50 mmol/L Na₂CO₃ (pH 10.6) to 1 mmol/L, further diluted 1:10 in phosphate-buffered saline (PBS) (pH 7.4) to a final concentration of 100 μ mol/L, and a 10- μ l drop was applied three times a day. Wound closure, re-epithelialization, and corneal neovascularization were monitored daily for 14 days after injury. To measure the wound area, corneas were stained with fluorescein, and images of the anterior surface were taken with a charge-coupled device camera attached to a slit lamp biomicroscope. Digital images were analyzed by imaging processing software (ImagePro; Media Cybernetics, Inc., Silver Spring, MD). Corneal neovascularization was measured as the total length (mm) of vessels penetrating the corneal surface using the ImagePro software. Mice were euthanized at the indicated time points, eyes were removed, and corneas, free of conjunctival tissue, were dissected and processed for selected analyses.

Zymosan A-Induced Peritonitis

Mice were injected intraperitoneally with 1 mg of zymosan A (Sigma, St. Louis, MO) in 0.5 ml of sterile saline. Mice were sacrificed 4 and 14 hours after zymosan A injection and inflammatory cells were collected by peritoneal lavage (5 ml of sterile PBS, pH 7.4). Total and differential cell counts were performed by microscopy using a hemocytometer and Wright-Giemsa staining. The peritoneal exudates were centrifuged at 450 × g for 10 minutes and cells were either resuspended in PBS to 5 × 10⁶ cells/ml for NADPH oxidase assays or lysed in PBS buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethyl sulfonyl fluoride, and one tablet of a complete mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for Western blot analyses.

NADPH Oxidase Assay

Superoxide (O₂⁻) production by cell exudates was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm. The assay was performed in a total volume of 1 ml of PBS containing 0.5 × 10⁶ cells, 60 μ mol/L ferricytochrome c with or without 30 μ g/ml superoxide dismutase (Sigma). Samples were equilibrated for 3 minutes at 37°C and the reaction initiated by adding formyl-methionyl-leucyl phenylalanine (10 μ mol/L) or vehicle control. The reaction was stopped after 10 minutes by placing tubes in ice-cold water, the particulate matter was removed by centrifugation, and superoxide production was immediately determined by measuring the absorbance at 550 nm. The O₂⁻ generation was defined as the superoxide dismutase-inhibitable reduction, and concentration of O₂⁻ was calculated by using the extinction coefficient of 21,100 mol/L⁻¹ cm⁻¹.

Myeloperoxidase (MPO) Activity

Measurement of MPO activity was used to quantify polymorphonuclear cells (PMNs) in dissected corneas as previously described.²⁴ 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, and 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 wild-type (WT) 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 TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA integrity was verified by agarose gel electrophoresis and quantitated by spectrophotometry. Reverse transcription reaction of total RNA (5 µg) was performed using the Superscript III first-strand synthesis system (Invitrogen) according to the manufacturer's instruction. Quantitative real-time PCR was performed using Brilliant SYBR Green QPCR Master Mix 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'-GGATTTG-GGGCTGCTGGTTTC-3'; HO-2 sense, 5'-TACTTCACATACTCAGCCCT-3' and anti-sense, 5'-ATGGGCCACCAGCAGCTCTG-3'; CYP4B1 sense 5'-TGATGTGC-TGAAGCCCTATG-3' and anti-sense 5'-CGCTCCTGAAGCTTTTCTG-3'; β -actin sense, 5'-AGCCATGTACGTAGCCATCC-3' and anti-sense, 5'-TTTGATGTC-ACGCACGATTT-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 β -actin as described.²⁷

Histology, Immunostaining, and Immunoblotting

Corneas were washed twice with PBS and fixed in 4% paraformaldehyde-PBS, pH 7.4, for 24 hours at 4°C. For histology, corneas were embedded in Paraplast (BDH, Poole, Dorset, UK), sectioned at 5-µm thickness and stained with hematoxylin and eosin. For immuno-

histochemical analysis, fixed corneas were washed with PBS and mounted in OCT compound. Cryostat sections were cut transversely into 5-µm-thick sections, mounted on microscopic slides, fixed in ice-cold acetone, and treated with 3% hydrogen peroxide in methanol for 10 minutes at room temperature to block endogenous peroxidase activity. Sections were then treated with 0.1% Triton in Tris-buffered saline, pH 7.6, and incubated with normal serum (1:5 in Tris-buffered saline) for 60 minutes, then serially treated overnight at 4°C with the primary antibodies at 1:100 dilution [rat monoclonal anti-mouse CD68 antibody (Serotec Inc., Raleigh, NC); mouse anti-mouse HO-1 and rabbit anti-mouse HO-2 antibodies (Stressgen, San Diego, CA)]. The sections were washed in Tris-buffered saline buffer, incubated with the secondary biotinylated antibody (1:50; Dakopatts, Älvsjö, Sweden), and then washed and incubated for 1 hour at room temperature using an avidin-biotin horseradish peroxidase kit (Dakopatts) according to the manufacturer's instructions. Peroxidase activity was detected using 0.33% H₂O₂ and 0.05% 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with hematoxylin to visualize cell nuclei, dehydrated, and mounted. Control reactions were performed in the absence of the primary antibodies and with isotype-matched irrelevant mouse IgGs.

For Western blot analysis, dissected corneas were homogenized in T-PER tissue protein extraction reagent containing Halt protease inhibitor cocktail (Pierce Biotechnology, Inc., Rockford, IL). Proteins were separated by gel electrophoresis, and immunoblotting was performed using the following primary antibodies: 1:1000 mouse anti-mouse HO-1 and 1:2000 rabbit anti-mouse HO-2 antibodies (Stressgen) and 1:2500 mouse anti-mouse β -actin (Sigma).

Chemokine Analysis

Dissected corneas were homogenized in T-PER tissue protein extraction reagent containing Halt protease inhibitor cocktail. KC (IL-8 homologue), macrophage inflammatory protein-2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) were measured in corneal homogenates and peritoneal exudates using a custom multiplex sandwich enzyme-linked immunosorbent assay for protein analysis (SearchLight mouse microarray; Pierce Biotechnology, Woburn, MA).

HO Activity/CO Measurement

Corneas were dissected from WT and HO-2-null mice at indicated time points. On dissection, corneas were homogenized in PBS (0.01 mol/L, pH 7.4) in the presence of protease inhibitor cocktail (Complete Mini; Roche) and 100 µmol/L butylhydroxytoluene. Aliquots (50 µl) of corneal homogenates were subsequently incubated for 2 hours in the dark at 37°C with 2 mmol/L hemin in the presence of NADPH-generating system (100 mmol/L MgCl₂, 68.1 mmol/L glucose-6-phos-

Figure 1. Neovascularization and delayed wound healing in HO-2-null mice. **A:** Fluorescein-stained corneas before and after injury in HO-2-null (HO-2^{-/-}) and WT mice. **B:** Wound closure as percent change from day 0 ($n = 16$ to 20 ; $*P < 0.05$ from day 0; $^{\dagger}P < 0.05$ from WT). **C:** Photographs of WT and HO-2-null eyes 14 days after injury. **D:** Neovascularization expressed as total length of penetrating vessels ($n = 12$; $*P < 0.001$ from WT). Original magnifications, $\times 16$ (**A**).

phate, 33 U/ml glucose-6-phosphate dehydrogenase, and 26 mmol/L NADPH) in a final volume of 1 ml of potassium phosphate buffer (0.1 mmol/L). 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 an abundance of ions m/z 28 and m/z 29 or m/z 31, as previously described.²⁸

12-HETrE Analysis

Corneas were gently homogenized in 60% ice-cold methanol. Corneal homogenates were placed at -20°C to precipitate proteins and centrifuged, and supernatants were collected. Supernatants were di-

luted with 10 vol of high performance liquid chromatography-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 of high-performance liquid chromatography-grade water followed by hexane and compounds eluted in methyl formate followed by a final elution in methanol. Methyl formate fractions were dried under nitrogen and resuspended in methanol (100 μl). For 12-HETrE determination, extracts were subjected to reverse-phase high performance liquid chromatography, and fractions corresponding to 12-HETrE were collected and derivatized to the pentafluorobenzyl ester and trimethylsilyl ether. 12-HETrE levels were quantified by negative chemical ionization-gas GC-MS using [12-²H₃]-12(R)-HETrE as the internal standard, which was added (0.1 ng) to the corneal homogenates before acidification and column extraction.²⁹

Figure 2. Exaggerated inflammation in HO-2-null mice. **A:** Corneal MPO activity in WT and HO-2-null (HO-2^{-/-}) mice ($n = 5$ to 8 ; * $P < 0.05$ from WT). **B:** H&E staining of uninjured corneas (**a, d**), 2 days (**b, e**), and 7 days (**c, f**) after injury. **C:** CD68 immunostaining in corneal epithelium (Ep) and stroma (St). **D:** KC, MIP-2, and MCP-1 levels in uninjured corneas and corneas after injury. Results are the average value from four corneas ($n = 2$).

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

Aberrant Wound Healing in HO-2-Null Mice

The injury model used involved the removal of the entire corneal epithelium. It is an established epithelial-initiated injury model in which the inflammatory and

reparative response has been well characterized.²⁴ The advantage of using the cornea is underscored by the fact that it expresses the HO system,³⁰ and an increase in corneal HO-1 expression is associated with marked alleviation of injury-induced inflammation.^{31,32} In WT mice, epithelial injury produced a consistent wound (5.53 ± 0.14 mm², $n = 20$) that exhibited a linear rate of re-epithelialization with $34.9 \pm 3.1\%$, $79.4 \pm 10\%$, and $98.7 \pm 12\%$ wound closure at days 2, 4, and 7 after injury, respectively (Figure 1, A and B). In contrast, re-epithelialization of corneal wounds (6.04 ± 0.16 mm², $n = 16$) in HO-2-null mice was inhibited by $320 \pm 28\%$, $37 \pm 4\%$, and $69 \pm 8\%$ as compared with WT mice at days 2, 4, and 7 after injury, respectively. Impaired re-epithelialization in the HO-2-null mice was

Figure 3. Impaired functional expression of HO-1 in HO-2-null mice. **A:** Real-time PCR analysis of corneal HO-1 mRNA expression in WT and HO-2-null (HO-2^{-/-}) mice ($n = 3$, * $P < 0.05$ versus uninjured, ‡ $P < 0.01$ versus HO-2-null). **B:** HO activity ($n = 3$, * $P < 0.05$ versus uninjured, ‡ $P < 0.05$ versus HO-2-null). **C:** HO-1 immunostaining in the stroma of WT (**a, b**) and HO-2^{-/-} (**c, d**) mice 4 days after injury. Immunopositive macrophages (arrows), fibroblasts (arrowheads), and granulocytes (circles). **D:** HO-2 immunostaining in WT 4 days after injury (**a**); immunopositive macrophages (arrows), fibroblasts (arrowheads), and granulocytes (circles). No positive immunostaining in HO-2-null mice (**b**). Original magnifications, $\times 100$ (**C**).

associated with ulceration and perforation of the corneal surface. Moreover, slit lamp microscopy 7 and 14 days after injury revealed consistent and massive corneal neovascularization in HO-2-null mice that was completely absent in WT mice (Figure 1, C and D). Neovascularization in this model reflects the severity of corneal injury and the extent to which re-epithelialization is impaired.

Exaggerated Inflammatory Response in HO-2-Null Mice

The impaired re-epithelialization in HO-2-null mice was associated with a persistent PMN infiltrate, whereas in WT mice PMN infiltration was transient, peaking at day 4 and beginning resolution by day 7, a pattern consistent with an acute self-resolving inflammatory response (Figure 2A). In contrast, PMN infiltration into the cornea of HO-2-null mice persisted and was threefold and

twofold higher than in WT corneas at days 7 and 14 after injury, respectively. H&E staining of corneal sections confirmed a persistent PMN infiltrate in the stroma and delayed re-epithelialization in HO-2-null mice compared with WT mice (Figure 2B). Consistent with exaggerated inflammation, immunostaining of CD68-positive cells, a marker of tissue macrophages, indicated that 7 days after injury the number of macrophages were markedly increased in stroma of HO-2-null mice when compared with WT mice (Figure 2C). The levels of the chemokines, KC, MIP-2, and MCP-1, which have been shown to correlate with PMN influx in various models of inflammation, were also measured. In WT mice, levels of these proinflammatory chemokines showed a rapid and transient increase that peaked at day 2 after injury and subsided by day 7 (Figure 2D). These changes closely correlated with re-epithelialization of the injured cornea of WT mice. By contrast, in HO-2-null mice, this response was aberrant: chemokine

Figure 4. Amplification of inflammatory lipid mediator pathways in HO-2-null mice. **A:** COX-2 immunostaining in stroma of WT and HO-2-null (HO-2^{-/-}) mice before (**a, d**), 2 days (**b, e**), and 4 days (**c, f**) after injury. Immunopositive macrophages (**arrows**) and fibroblasts (**arrowheads**). **B:** Real-time PCR analysis of CYP4B1 mRNA expression ($n = 3$, * $P < 0.05$ versus uninjured, ‡ $P < 0.05$ versus WT). **C:** CYP4B1 activity measured as 12-HETE production ($n = 4$, * $P < 0.05$ versus uninjured, ‡ $P < 0.05$ versus WT).

levels reached a maximum at day 4 and were 2- to 20-fold higher than those in WT mice. Moreover, despite a gradual decrease, levels remained elevated 14 days after injury in HO-2-null mice and were sixfold to eightfold higher than in WT mice (Figure 2D), a response consistent with a persistent and chronic inflammation.

Impaired Functional Expression of HO-1 in HO-2-Null Mice

Epithelial injury in WT mice resulted in a rapid and transient induction of HO-1 expression (Figure 3A). In contrast, injury-induced HO-1 mRNA expression in HO-2-null mice was abrogated (Figure 3A). Corneas of WT mice exhibited constitutive HO-2 mRNA levels that were not significantly altered in response to injury; HO-2 mRNA was not detected in HO-2-null mice (data not shown). Uninjured corneas of WT mice exhibited a basal level of HO activity, measured by NADPH-dependent CO production from heme, which was increased by threefold 3 days after injury, stayed elevated (twofold greater than control) at day 4 and decreased thereafter (Figure 3B). In contrast, HO-2-

null mice failed to induce HO enzyme activity in response to injury (Figure 3B), consistent with the impaired HO-1 mRNA induction. WT corneas 4 days after injury showed positive HO-1 immunostaining in inflammatory infiltrates as well as in stromal keratinocytes (Figure 3C). In the HO-2-null mice, HO-1 immunostaining, although present in these cells, was markedly attenuated (Figure 3C). HO-2 immunostaining in WT corneas 4 days after injury was present in stromal cells and inflammatory infiltrates, whereas no positive HO-2 immunostaining was observed in the cornea of HO-2-null mice (Figure 3D).

Impaired Expression of the HO System Correlates with Amplification of Inflammatory Lipid Mediator Pathways

Induction of HO activity directly impacts functional expression of heme-containing enzymes. In this regard, cyclooxygenase (COX)-2 and cytochrome P450 (CYP) 4B1 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

Figure 5. Amplified leukocyte function in HO-2-null mice. **A:** Leukocyte number in peritoneal exudates 4 and 14 hours after zymosan A injection in WT and HO-2-null (HO-2^{-/-}) mice ($n = 6$ to 9 , $*P < 0.05$ from WT). **B:** NADPH oxidase activity in peritoneal leukocytes ($n = 7$, $*P < 0.05$ from WT). **C:** KC levels in peritoneal exudates at 14 hours ($n = 3$, $*P < 0.05$ from WT). **D:** HO-1 protein expression in peritoneal leukocytes; levels are normalized to β -actin (AU, arbitrary units; $n = 4$; $*P < 0.05$ from WT).

cornea.^{33,34} The activity of both enzymes is regulated by HO.^{32,35}

Analyses of corneas by immunohistochemistry indicated exaggerated COX-2 protein expression in macrophages and stromal keratinocytes of HO-2-null mice, 2 and 4 days after injury, when directly compared with WT mice (Figure 4A). COX-2 immunostaining was not detected in uninjured corneas of WT or HO-2-null mice (Figure 4A). CYP4B1 mRNA expression in WT mice was maximally induced 2 days after injury and returned to basal levels by day 4, whereas in HO-2-null mice, CYP4B1 mRNA expression was rapidly induced 24 hours after injury and remained significantly elevated thereafter (Figure 4B). The levels of 12-hydroxyeicosatrienoic acid (12-HETRe), the CYP4B1-derived arachidonic acid metabolite, in WT mice demonstrated a rapid and transient increase of 7-, 20-, and 16-fold greater than control at 1, 3, and 4 days after injury, respectively, and returned to control levels by day 7 (Figure 4C). In contrast, 12-HETRe levels were significantly elevated in uninjured corneas of HO-2-null mice, gradually increased by twofold at day 7 after injury, and returned to levels that were observed in uninjured corneas by day 14. It is important to note that 12-HETRe content in corneas of HO-2-null mice 14 days after injury were four times higher than in corresponding corneas of WT mice (Figure 4C).

HO-2-Null Mice Exhibit Amplified Inflammation and Leukocyte Function

To assess whether the exacerbated inflammatory response in HO-2-null mice was associated specifically with epithelial injury in the cornea, we used zymosan A-induced peritonitis as a model of innate immune response that produces a well-defined acute and self-resolving inflammation.³⁶ Consistent with the observed exaggerated inflammation in the eye, the number of inflammatory cells in peritoneal exudates taken at 4 and 14 hours after zymosan A administration was 1.5- to 2-fold higher in HO-2-null mice than in WT mice (Figure 5A). More importantly, peritoneal leukocytes from HO-2-null mice displayed significantly higher levels of formyl-methionyl-leucyl phenylalanine-stimulated NADPH oxidase activity, a primary leukocyte functional response (Figure 5B). KC levels were also significantly higher in exudates from HO-2-null than WT mice (Figure 5C) consistent with a phenotype of amplified inflammation in HO-2-null mice. Moreover, similar to leukocytes in the cornea, HO-1 protein expression in peritoneal leukocytes of HO-2-null mice was significantly impaired (Figure 5D).

Biliverdin Restores the Acute Inflammatory and Reparative Response in HO-2-Null Mice

To the extent that deficiency in HO activity contributes to the exaggerated inflammation in HO-2-null mice, can this phenotype be rescued by adding back the bioactive products of the HO system? To address this question, experiments were performed in which injured eyes were treated with a daily topical application of biliverdin. It is widely accepted that bilirubin, the product of biliverdin reductase, is the more powerful bioactive metabolite of the HO pathway. However, bilirubin is highly lipophilic whereas biliverdin is water-soluble and readily penetrates tissue. Studies have shown that administration of biliverdin is as effective as administration of bilirubin in conferring antioxidant and cytoprotective actions in part because of its rapid conversion to bilirubin by biliverdin reductase, which is ubiquitously expressed,^{37,38} including in the cornea (data not shown). As seen in Figure 6A, wound size of HO-2-null mice treated with biliverdin was significantly smaller than the corresponding untreated eyes 2 days after injury. Indeed, biliverdin treatment increased the rate of re-epithelialization by twofold, reaching that of the WT mice (Figure 6B). Likewise, treatment of injured eyes with biliverdin brought about a marked 50% decrease in PMN number in HO-2-null corneas (Figure 6C) and a 50% reduction in corneal neovascularization (Figure 6D) 7 days after injury.

Discussion

The current study substantiates the notion that the HO system is a critical endogenous cytoprotective and anti-inflammatory circuit and, more importantly, it points to a

Figure 6. Biliverdin administration rescues impaired inflammatory and reparative responses in HO-2-null mice. **A:** Photographs of eyes treated with biliverdin or the vehicle (PBS, pH 7.4) 2 days after injury. **B:** Wound closure as percent re-epithelialization ($n = 4$, * $P < 0.05$ versus WT, ‡ $P < 0.05$ versus vehicle). **C:** PMN content measured as MPO activity ($n = 3$, * $P < 0.05$ versus WT, ‡ $P < 0.05$ versus vehicle) 7 days after injury. **D:** Corneal neovascularization in HO-2-null 7 days after injury ($n = 4$, * $P < 0.05$ versus vehicle). Original magnifications, $\times 16$.

yet unidentified novel role for HO-2. This is the first demonstration that HO-2 is crucial for an ordered execution and resolution of inflammation and wound healing; in the absence of HO-2, resolution of inflammation was impaired, resulting in a continuous influx of inflammatory cells and, subsequently, in an exacerbation of cellular injury. It has been proposed that HO-1 functions as a signal to terminate acute inflammation;^{12,39} our study suggests that the HO-1 function critically depends on the activity of HO-2. It should be noted that physical interactions between HO-1 and HO-2 have been documented.⁴⁰ However, the significance of such interactions *in vivo* and the regulatory mechanisms controlling these interactions need to be further explored.

Epithelial regeneration was greatly impaired in mice lacking the HO-2 gene. Moreover, wounds were not healed within the experimental time frame; in fact, ulceration, perforation, and neovascularization, hallmarks of chronic inflammation, ensued. This was in marked contrast to the WT mice in which an ordered and resolved inflammatory response led to complete wound closure and healing. The HO system has been

implicated in wound healing processes through its documented anti-inflammatory bioactions. Studies in models of epithelial injury^{14–16} demonstrated spatial and temporal increases in HO-1, which correlated with leukocyte infiltration, keratinocyte proliferation, resolution, and wound healing. These reports have suggested that HO-1 induction at the site of injury promotes healing by limiting inflammation via the production of biliverdin and CO. Our study indicates that, in the absence of HO-2, functional HO-1 induction is impaired and HO activity is abrogated. It is likely that deficiency of significant levels of biliverdin and/or CO as a consequence of negated HO activity in the HO-2-null mice contributed to amplified inflammation and impaired wound healing. This is further enforced by the demonstration that biliverdin administration rescued the HO-2 phenotype, reversing the impaired wound closure and suggesting a cause and effect relationship between HO activity and wound healing.

Kinetics of the inflammatory and reparative response in the cornea resembled that observed in other tissues. It consists of a rapid and transient leukocyte influx

followed by tissue regeneration and repair. HO-1 induction, as a fundamental cytoprotective response, was apparent in resident corneal cells and infiltrating PMNs and macrophages. Moreover, maximal HO activity was associated with the resolution phase, suggesting that timely and robust HO-1 expression is critical to the resolution of the inflammatory response. Indeed, in pleurisy models of inflammation, HO-1 expression and activity are maximal during the resolution phase; induction of HO-1 expression promotes resolution whereas inhibition of HO activity is proinflammatory.^{12,13,39} In our study, injury-induced HO-1 expression and activity was completely absent in HO-2-null mice despite a similar initial kinetic pattern of leukocyte recruitment to the wounded area. Furthermore, resolution was absent in the HO-2-null corneas. Macrophages were present in HO-2-null mice as early as 48 hours after injury and remained at high levels 1 week later. More importantly, these inflammatory cells showed a weak HO-1 immunoreactivity. To the extent that the HO catalytic products, CO and biliverdin, are stop signals to control leukocyte migration and activation by attenuating adhesion molecule expression^{8,17} and cytokine and chemokine induction,^{20,22,41–43} the lack of sufficient HO-1 levels in HO-2-null mice may increase migration, lower the threshold of leukocyte activation (ie, O₂[•] generation) and contribute to leukocyte-mediated tissue injury. This notion is substantiated by results obtained with zymosan A-induced peritonitis in HO-2-null mice that demonstrated pronounced increases in leukocyte influx, NADPH oxidase activity, and KC levels, all associated with impaired induction of PMNs and macrophage HO-1.

The exaggerated inflammation in HO-2-null mice was manifested by amplified and lasting production of proinflammatory chemokines including KC, MIP-2, and MCP-1. Sustained increases in their levels have been associated with persistent inflammation, neovascularization, and decreased re-epithelialization and tissue repair.^{44–47} The fact that these same chemokines are subjected to down-regulation by either HO-1 induction or supplementation of CO or biliverdin,^{21,43,48,49} further underscores the notion that the absence of considerable HO activity promoted the uncontrolled massive production of proinflammatory signals and, consequently, chronic inflammation and neovascularization. This is strongly supported by the demonstration that biliverdin administration rescued the impaired inflammatory and reparative response of the HO-2-null mice; it accelerated wound repair, promoted resolution, and attenuated neovascularization. It is noteworthy that HO-2 expression levels have been shown to decrease significantly in human pathologies and conditions that are associated with chronic inflammation such as pre-eclamptic pregnancies^{5,50} and prolonged cigarette smoking.⁶ Hence, it is not unreasonable to speculate that there is a causal relationship between the levels of HO-2 expression and activity and unresolved chronic inflammation.

Like cytokines and chemokines, lipid mediators produced by the wounded tissue and inflammatory cells

play an important role in orchestrating and amplifying the inflammatory-reparative response. The CYP4B1-derived 12-HETrE is one such prominent corneal-derived lipid signal whose production levels closely correlate to inflammation and neovascularization of the cornea in response to injury.^{51,52} Depletion of CYP4B1 by induction of corneal HO-1 ameliorates,^{31,32} whereas increased corneal expression of CYP4B1, aggravates inflammation and neovascularization.³³ Notably, the HO-2-null mice in which the HO system is greatly compromised displayed higher levels of CYP4B1 mRNA expression and increased production of 12-HETrE, a potent corneal-derived chemotactic^{24,53} and angiogenic eicosanoid^{53,54} *in vitro* and *in vivo*. It is likely that 12-HETrE contributes to the unresolved inflammation and massive neovascularization seen in the corneas of HO-2-null mice. Its higher levels in uninjured corneas of HO-2-null mice suggest that deletion of HO-2 removes an important control mechanism in one of the key proinflammatory circuits in the cornea, namely the CYP4B1-12-HETrE pathway. These initial high levels may play a role in the amplified PMN response in the HO-2-null mice.

In summary, this study strongly implicates a novel role for HO-2 in the regulation of the inflammatory and reparative response to injury, a cytoprotective mechanism that, hitherto, was typically associated with HO-1 induction. HO-2 may constitute an essential protective circuit that sets in place a basal tone of anti-inflammatory signals critical to the execution of self-resolving inflammatory-reparative processes.

Acknowledgments

We thank Dr. Claudia Colombrita for assisting in real-time PCR analysis and Ms. Rowena Kemp for performing GC/MS analysis.

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