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Heme Oxygenase Induction with Attenuation of Experimentally Induced Corneal Inflammation

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ABSTRACT. Heme oxygenase (HO), by catabolizing heme to bile pigments, down-regulates cellular levels of heme and hemeproteins; certain of the latter, i.e. cytochrome P450s, generate pro-inflammatory products from endogenous substrates. Two HO isozymes, the products of distinct genes, have been described; HO-1 is the inducible one, whereas HO-2 is believed to be constitutively expressed. We studied the inducing effects of several metal compounds [$CoCl_2$, $SnCl_2$, $ZnCl_2$, heme, and cobalt protoporphyrin (CoPP)] on HO-1 mRNA content and enzyme activity in cultures of rabbit corneal epithelial (RCE) cells; these metal compounds are known to induce HO in other tissues. Additionally, we studied HO-1 expression in an experimental model of ocular inflammation produced in rabbit corneas by extended contact lens wear, and the relation of HO expression to the induced inflammatory process. $SnCl_2$ added to RCE cells *in vitro* produced marked time- and concentration-dependent increases in HO-1 mRNA and HO-1 enzyme activity; $CoCl_2$, $ZnCl_2$, and CoPP were inducers of HO as well, though to a lesser degree than $SnCl_2$. Corneas treated for 6 days with contact lenses impregnated with $SnCl_2$ displayed substantially less corneal inflammation, swelling, and new vessel invasion than did controls; attenuation of ocular inflammation was paralleled by $SnCl_2$-induced increases in HO mRNA and HO activity in corneal epithelial cells from treated eyes. It is suggested that amelioration of the inflammatory response produced by extended contact lens wear is due, in part, to the induction of high levels of HO-1 activity by $SnCl_2$, which results in diminished production of pro-inflammatory mediators generated through heme-dependent metabolic processes. Regulation of HO activity in this manner may have clinical applications. BIOCHEM PHARMACOL 53;8:1069–1075, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. heme oxygenase; metal induction; inflammation suppression; cornea; neovascularization

HO‖ controls the initial and rate-limiting step in heme catabolism. The enzyme cleaves heme to biliverdin, which is converted subsequently to bilirubin by biliverdin reductase. Iron is released when the heme ring is opened while carbon monoxide is liberated. The heme molecule plays a central role in biological processes as the prosthetic moiety of hemeproteins involved in cell respiration, energy generation, oxidative biotransformation, growth differentiation processes, and the generation of inflammatory mediators such as eicosanoids and nitric oxide. Two HO isozymes, the products of distinct genes, have been described [1]. HO-1, which is distributed ubiquitously in mammalian tissues, is induced strongly and rapidly by many compounds that elicit cell injury; the natural substrate of HO, heme, is itself a potent inducer of the enzyme [2]. HO-2, which is believed to be constitutively expressed, is present in high concentrations in such tissues as brain and testis and is thought to be non-inducible [3]. HO-1 activity is increased in whole animal tissues and in cultured cells following treatment with heme, metals, and inflammatory cytokines as well as in hypoxic and oxidative conditions [4–7]. HO-1 is also induced by heat shock, and the enzyme belongs to a class of macromolecules known as stress proteins, which are responsive to various types of acute cellular injuries [8, 9].

Induction of HO-1 is thought to be of considerable importance in the initiation of cellular protective mechanisms following exposure to various forms of cell stressful stimuli [6, 10, 11]. This idea derives, in part, from the fact that increased HO activity enables the removal of heme, a lipid-soluble, transmissible form of the potent prooxidant iron, and results in the generation of bilirubin and biliverdin, heme metabolites with significant antioxidant and anti-complement properties [12–14]. Indeed, a study by Nath *et al.* [15] provides evidence that induction of HO-1 coupled

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‖ *Abbreviations:* HO, heme oxygenase; HO-1, HO-2, heme oxygenase isoforms; RCE, rabbit corneal epithelial; CoPP, cobalt protoporphyrin; and GADPH, glyceraldehyde-3-phosphate dehydrogenase.

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to ferritin synthesis is a rapid, protective antioxidant response *in vivo* in rhabdomyolysis-induced kidney injury in the rat. Other studies have also shown that induction of HO-1 in skin fibroblasts is of value in protection against UV light-induced oxidative stress [16–18], and we recently demonstrated [19] that HO-1 gene overexpression via adenovirus-mediated HO-1 cDNA transfer protects coronary endothelial cells from oxidative injury produced by exposure to free heme/hemoglobin.

The corneal epithelium is uniquely threatened by processes or agents that produce hypoxic as well as oxidative tissue injury and inflammation such as exposure to the most energetic wavelengths of sunlight, atmospheric oxygen, and a variety of agents that generate reactive oxygen species. A common property of many of these agents is their ability to enhance cellular hemeprotein prooxidant systems. The absence of blood vessels in the normal cornea poses a further risk to this tissue since this denies the corneal epithelium access to circulatory plasma-based antioxidant systems. HO-1 in the corneal epithelium may thus participate in protective mechanisms against ocular injury and inflammation.

We recently characterized a model of contact lens-induced corneal inflammation in the rabbit eye and demonstrated that treatment of inflamed eyes with $SnCl_2$, a potent inducer of HO-1, markedly reduces the inflammatory response [20, 21]. However, the link between $SnCl_2$ treatment and HO-1 expression in this model was not established. The present study was undertaken to examine the presence and inducibility of HO-1 in corneal epithelial cells and to assess the relation of this enzyme activity to the development of experimentally induced inflammation in the corneal surface of the rabbit eye. The results of this study demonstrate that corneal epithelium expresses basal and inducible HO-1 mRNA and enzyme activity and that an increase in HO-1 expression is associated with marked alleviation of ocular inflammation produced by an experimental model of contact lens-induced injury of the eye.

MATERIALS AND METHODS

Rabbit Corneal Epithelial Cell Line

RCE cells immortalized via SV40-adenovirus vector transfection were a gift from Dr. Kaoru Araki, Department of Ophthalmology, Osaka University Medical School. Cells were maintained in 75 cm^2 culture flasks (Falcon Labware, Oxnard, CA) according to the method of Araki *et al.* [22]. Briefly, cells were grown in a supplemented hormone epithelial medium (SHEM) consisting of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM-F12; Gibco, Grand Island, NY) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (Gibco), 5 μg/mL insulin (Collaborative Biomedical Products, Bedford, MA), 0.1 μg/mL cholera toxin (Calbiochem, La Jolla, CA), 10 ng/mL human epidermal growth factor, 0.5% DMSO (Sigma, St. Louis, MO) and 50 μg/mL gentamycin (Gibco). The cultures were maintained in a humidified atmosphere (95% air and 5% CO_2) at 37°. The culture medium was changed every 3 days. RCE cells were serially passaged at a 1:4 split ratio using a 0.05% trypsin/0.053 mM EDTA solution (Gibco). Subconfluent cells (80% confluent) were treated with various inducers for 1–24 hr after which RNA was extracted or microsomes were prepared for measurements of HO-1 mRNA levels or HO enzyme activity, respectively. Anhydrous $SnCl_2$, $ZnCl_2$, and $CoCl_2$ (Aldrich, Milwaukee, WI) were dissolved in potassium phosphate buffer (0.1 M, pH 7.4). CoPP (Porphyrin Products Inc., Ogden, UT) was prepared in Tris buffer, pH 8.0.

Contact Lens-Induced Inflammation Model

The model of contact lens-induced inflammation of the cornea has been described previously in detail [20, 21]. Briefly, male New Zealand albino rabbits (2.0 to 2.5 kg body weight) were anesthetized with ketamine HCl (50 mg/kg) and xylazine (20 mg/kg) intramuscularly. Seven days prior to contact lens placement, the right nictitating membrane was removed surgically in anesthetized rabbits after topical proparacaine-HCl (0.5%), and the remaining tissue was cauterized. Extended wear contact lenses (Vistakon; Acuvue: +4.0 diopters, 9.1 mm base curve, 14.0 mm diameter, 58% water) were dehydrated for 30 min and then rehydrated in either phosphate buffer (0.1 M, pH 7.4) or $SnCl_2$ in phosphate buffer (100 μg/mL) for 30 min. In this fashion, the hydrogels functioned as both a drug depot as well as an inflammatory stimulus. Lenses were then placed onto the proposed right eye in stacked fashion (2 lenses, one on top of the other) and the eye was gently reposited. To keep the lenses in place and to enhance hypoxic conditions, the lids were sutured closed. At the indicated time, sutures were removed, the test eyes exposed and the lenses removed. After topical proparacaine, the eyes were examined with a slit lamp and photographed. The degree of *in situ* inflammation was scored subjectively as described previously [20]. Animals were killed with an overdose of sodium pentobarbital (90 mg/kg). Corneal epithelium was scraped and processed immediately for RNA extraction.

HO-1 mRNA and Activity Analysis

For measurement of mRNA levels, total RNA was isolated by lysis of cultured cells or freshly scraped corneal epithelium in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% *N*-laurylsarcosine, 0.1 M 2-mercaptoethanol, and northern blot analysis using the rat HO-1 cDNA as a probe was performed as previously described [23]. Filters were exposed to Kodak XAR film at −70°. All filters were reprobed with cDNA encoding GADPH to ensure that equal amounts of RNA were loaded onto each lane. For measurement of enzyme activity, RCE cells (6 × T175 flasks) were treated with $SnCl_2$ (500 μM, the concentration used to soak the lenses or control vehicle) for 24 hr. The cells were then harvested, and microsomes were prepared following cell homogenization and centrifugation at

100,000 g for 90 min. The microsomal pellet was resuspended in potassium phosphate buffer (pH 7.4) and used for determination of enzyme activity as described [24].

RESULTS

Detection of HO-1 mRNA in a Corneal Epithelial Cell Line

Due to the limited availability of primary cell cultures from rabbit corneal epithelium, we used the RCE cell line as a model to characterize *HO-1* gene expression. Upon reaching confluence, RCE cells were treated with agents known to cause induction of HO-1 mRNA in various cell lines, such as $SnCl_2$, $CoCl_2$, $ZnCl_2$, and CoPP, at concentrations of 10–150 μM. Control experiments consisted of RCE cells treated with the appropriate vehicle. The effects of these agents on HO-1 mRNA levels were assessed, and the results are depicted in Fig. 1. A basal level of HO-1 mRNA was evident albeit to a much lesser extent than that of the rabbit liver. Treatment with all agents used resulted in an accumulation of HO-1 mRNA. A quantitative evaluation of the mRNA changes by scanning densitometry relative to GAPDH mRNA levels indicated a 50-fold increase in HO-1 mRNA levels in RCE cells treated with $SnCl_2$ (10 μM). CoPP (10 μM) showed similar potency, increasing HO-1 mRNA levels by 30-fold. On the other hand, 150 μM $CoCl_2$ was needed to achieve the same induction; $ZnCl_2$ at 100 μM produced a 10-fold increase of HO-1 mRNA over control. $CoCl_2$ and $ZnCl_2$ at 10 μM did not produce an increase of HO-1 mRNA (data not shown). These results indicate that the RCE cell line responds to HO-1 inducers as do many other tissues. Since the potency of $SnCl_2$ in inducing RCE HO-1 mRNA surpassed that of the other agents studied, we used $SnCl_2$ in the subsequent experiments.

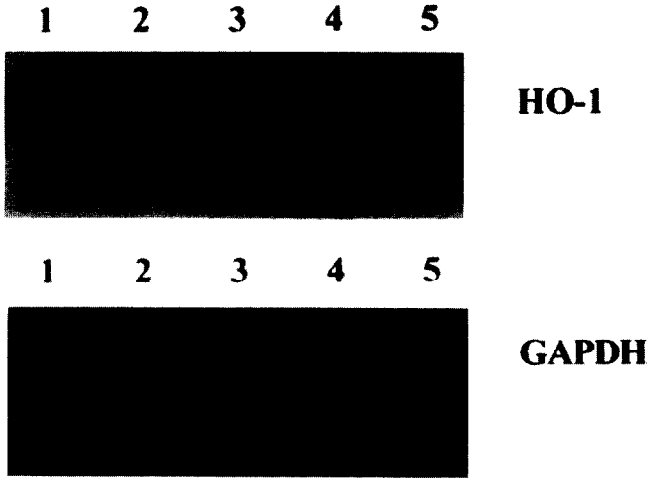

FIG. 1. Effects of metals and metalloporphyrins on HO-1 mRNA expression. RCE cells were cultured as indicated in Materials and Methods and treated for 1 hr with the following: lane 1, control; vehicle-treated cells; lane 2, $SnCl_2$ (10 μM); lane 3, $CoCl_2$ (150 μM); lane 4, CoPP (10 μM); and lane 5, $ZnCl_2$ (100 μM); the lower panel shows that equal amounts of RNA were transferred among samples as indicated by the GADPH probe. Total RNA was extracted and hybridized with the ^{32}P-labeled rat HO-1 cDNA as described in Materials and Methods.

Time- and Concentration-Dependent Induction of HO-1 mRNA by $SnCl_2$

RCE cells were grown and maintained in 75 cm^2 flasks; upon reaching confluence, they were treated with $SnCl_2$ and RNA was extracted as described in Materials and Methods. The time-dependent response to $SnCl_2$ is shown in Fig. 2. The vehicle used for solubilization of $SnCl_2$ did not change HO-1 mRNA (lane 2) levels as compared with the control (lane 1). Optimal induction of HO-1 in RCE was at 16 hr ($SnCl_2$ 100 μM) (lane 4), with a subsequent return to control levels at 24 hr (lane 5). Additional experiments were conducted to demonstrate the long-term effect of $SnCl_2$ in cells grown in a medium containing $SnCl_2$ (100 μM) for up to 6 days. Results showed that in the continuous presence of $SnCl_2$ cells maintained high levels of HO-1 mRNA while no toxicity (loss of cell viability) was detected (data not shown). The concentration–response to $SnCl_2$, at 16 hr, is shown in Fig. 3. Cells were incubated with $SnCl_2$ at concentrations of 0.1 to 100 μM for 16 hr, after which the RNA was extracted and analyzed. Compared with untreated cells, HO-1 mRNA levels were not increased over controls (lanes 1 and 2) after the addition of $SnCl_2$ at 0.1 μM (lane 3). Induction of HO-1 mRNA by $SnCl_2$ (5–100 μM) was concentration-related (lanes 4–6). Hybridization of the filters with radiolabeled GAPDH and ethidium bromide staining of RNA confirmed that similar amounts of total RNA were transferred to the filters in each lane of the paired experiments (data not shown).

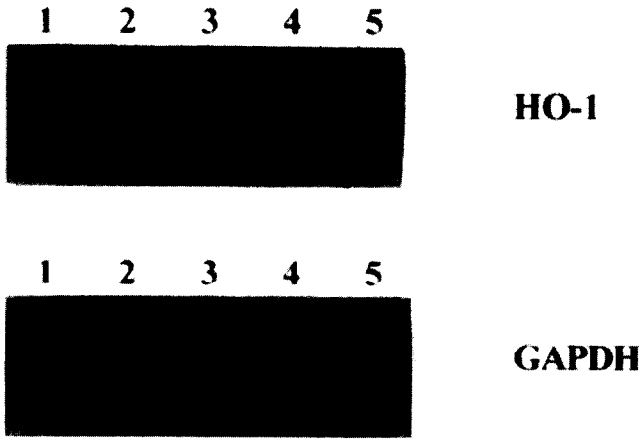

FIG. 2. Time-dependent effect of $SnCl_2$ on the induction of HO-1 mRNA in RCE cells. Cells were treated with $SnCl_2$ for various lengths of time. Control cells, lane 1; cells treated with vehicle, lane 2; $SnCl_2$, 8 hr, lane 3; $SnCl_2$, 16 hr, lane 4; and $SnCl_2$, 24 hr, lane 5; the lower panel shows that equal amounts of RNA were transferred among samples as indicated by the GADPH probe. Total RNA was extracted and northern blot analysis was performed with rat HO-1 cDNA as described in Materials and Methods.

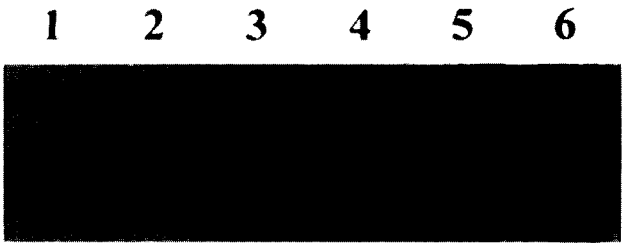

FIG. 3. Concentration-dependent effect of $SnCl_2$ on the induction of HO-1 mRNA in RCE cells. Cells were treated with various concentrations of $SnCl_2$ for 16 hr and compared with untreated cells, lane 1; cells treated with vehicle, lane 2; $SnCl_2$ (0.1 μM), lane 3; $SnCl_2$ (5 μM), lane 4; $SnCl_2$ (10 μM), lane 5; and $SnCl_2$ (100 μM), lane 6. Total RNA was extracted and northern blot analysis was performed with the rat HO-1 cDNA probe as described in Materials and Methods.

Effect of mRNA-Inducing Agents on HO Activity

Translation of HO-1 mRNA into active HO-1 enzyme was verified in the RCE cells. RCE cells grown in 175 cm^2 flasks were treated with either vehicle control or $SnCl_2$ for 24 hr. After combining 6 flasks, microsomes were prepared and HO activity was assessed as described in Materials and Methods. As seen in Fig. 4, HO activity was detectable in untreated (control) cells [16 ± 3 pmol/mg/hr, mean ± SEM (two experiments, triplicate assays, variation within assays <5%)]; with $SnCl_2$ treatment, HO activity significantly increased (6-fold) over the controls (103 ± 21 pmol/mg/hr, mean ± SEM). These results indicate that transcriptional activation of the HO-1 gene by $SnCl_2$ is followed by translation into functional HO enzyme in the cultures.

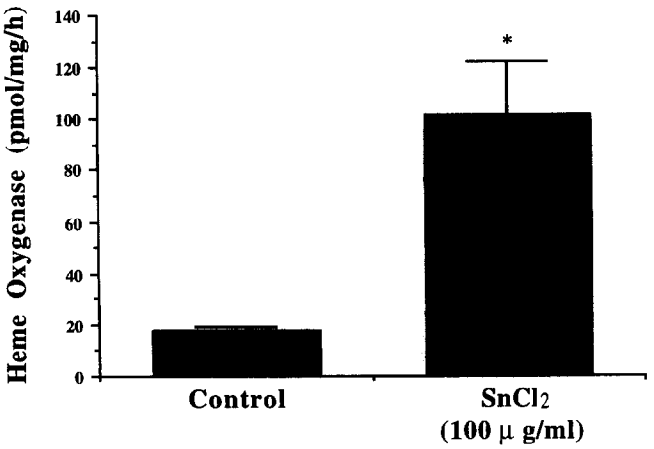

FIG. 4. Effect of $SnCl_2$ on HO activity in RCE cells. Confluent cultures were treated with $SnCl_2$ (100 μg/mL; 500 μM) or the vehicle control (0.1 M phosphate buffer, pH 7.4) for 24 hr. Microsomes were prepared and the HO activity was measured as described in Materials and Methods. Data are expressed as specific activity in pmol/mg/hr of two separate experiments measured in triplicate. The variation between triplicate assays in each treatment was within 5%. Key: (*) $P < 0.05$ versus the vehicle treatment.

Effect of $SnCl_2$ on Corneal Epithelial HO-1 mRNA In Vivo

To determine whether induction of HO-1 would be associated with moderation or suppression of the inflammatory response to contact lens wear, total RNA was extracted from control and closed eye-hydrogel contact lens-treated corneal epithelium ±$SnCl_2$ (500 μM) after 6 days of wear. As shown in Fig. 5, Northern blot analysis of HO mRNA showed detectable levels of HO-1 mRNA for the untreated control eyes (lanes 1–4). In corneal epithelium from vehicle-treated lenses, HO-1 mRNA was elevated slightly over the control (lane 7). Corneal epithelium from $SnCl_2$-treated contact lenses, in contrast, demonstrated a marked increase in HO mRNA levels (lanes 5 and 6). Ethidium bromide staining indicated that similar amounts of RNA were loaded in each lane. The *in vivo* induction of HO mRNA at 6 days was associated with a substantial increase in HO enzyme activity (data not shown) and was further correlated with the severity of the *in situ* inflammatory response. Figure 6 depicts representative slit lamp photographs of the ocular surfaces (N = 3–6) after 6 days of closed eye-hydrogel contact lens wear. In the vehicle-treated eyes, there was a progressive *in situ* inflammatory response becoming prominent at day 3 [21] and more severe at day 6 (Fig. 6A). The inflammatory response consisted of limbal vasodilation, conjunctional swelling, dilation of the iridial vessels, decreased corneal transparency (increased thickness, cloudiness, and opacity) and neovascularization. One-

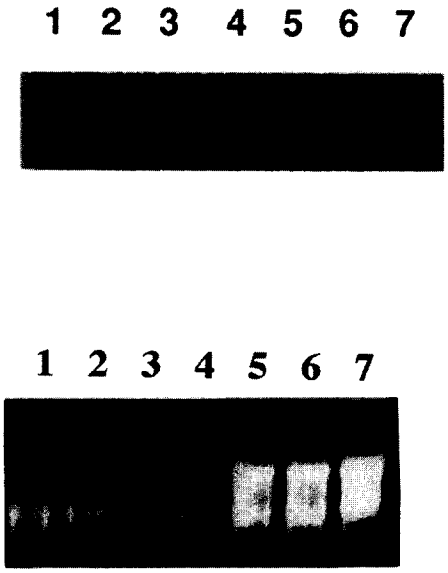

FIG. 5. Effect of one-time $SnCl_2$ treatment on HO-1 mRNA in corneal epithelium from 6-day closed eye-hydrogel contact lens-treated eyes. Total RNA was extracted from corneal epithelial scrapings from control, vehicle, and $SnCl_2$ (100 μg/mL) treated eyes as described above. RNA was hybridized with the rat HO-1 cDNA probe as described in Materials and Methods. Lanes 1–4, control, untreated eyes; lanes 5 and 6, eyes treated with $SnCl_2$-treated lenses; lane 7, corneal epithelium from vehicle-treated lenses. Lower panel, ethidium bromide staining of the gel.

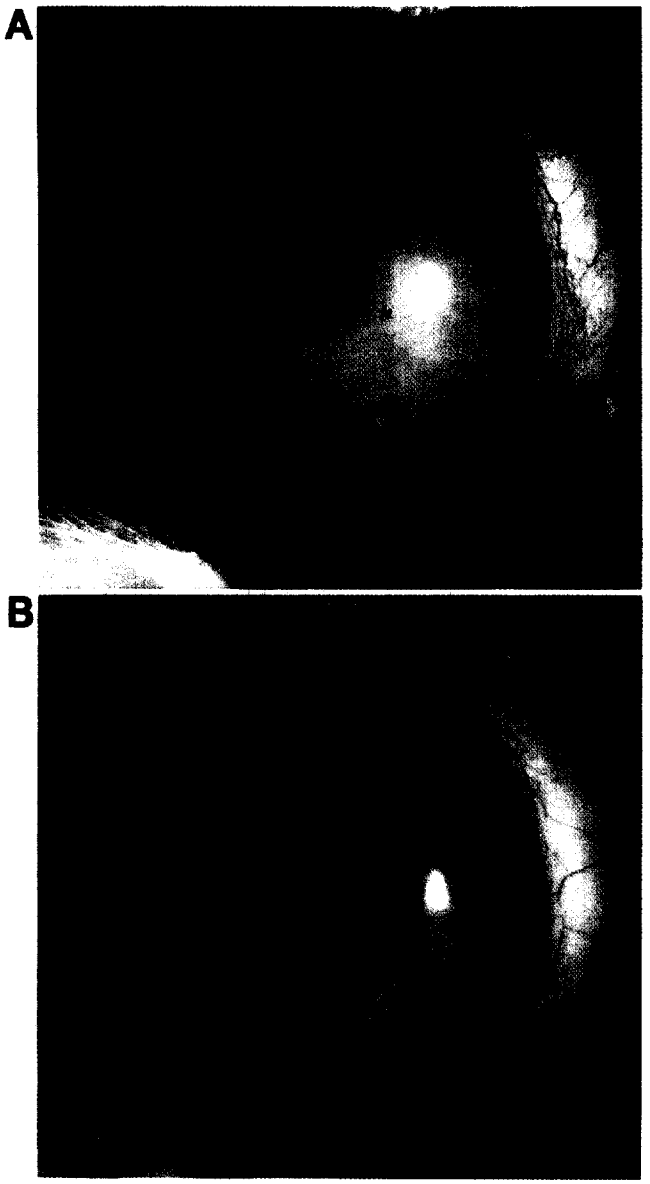

FIG. 6. Effect of one-time treatment with $SnCl_2$ on the *in situ* ocular inflammatory response at 6 days of closed eye-hydrogel contact lens wear. Lenses were treated with either vehicle (0.1 M phosphate buffer, pH 7.4) or $SnCl_2$ (100 μg/mL) for 30 min prior to lens placement and tarsorrhaphy. At 6 days, sutures were removed and eyes were examined. Biomicroscopic photographs of the ocular surface were taken with a photographic attachment to the slit lamp. Photographs are representative of N = 5 from each group. (A) Eye placed with vehicle-treated lenses (day 6, vehicle); (B) eye placed with $SnCl_2$-treated lenses. In (A), arrow indicates area of neovascular ingrowth and represents region of corneal edema, both of which were attenuated significantly in (B).

time treatment of the hydrogel lenses with $SnCl_2$ (100 μg/mL) resulted in marked attenuation of the inflammatory response (Fig. 6B). $SnCl_2$ was effective in suppressing both limbal and iridial vasodilation as well as the extent of epithelial defects and neovascularization of the cornea. Quantitative analysis by subjective (blinded) inflammatory scoring showed a significant correlation between the treatment with $SnCl_2$, which attenuated the increase in inflammatory response beginning at day 3 and reaching significance at day 7 (alleviation by ≈60%). Figure 7 summarizes the effect of $SnCl_2$ treatment on the *in situ* inflammatory response; corneal thickness, indicative of corneal edema, was decreased by 60% as compared with eyes with untreated contact lenses (Fig. 7A), while the subjective inflammatory score was reduced by 75% (Fig. 7B).

DISCUSSION

The results of the present study indicate that metal compounds, which are known to induce HO-1 expression in liver and other mammalian cells, act similarly in corneal

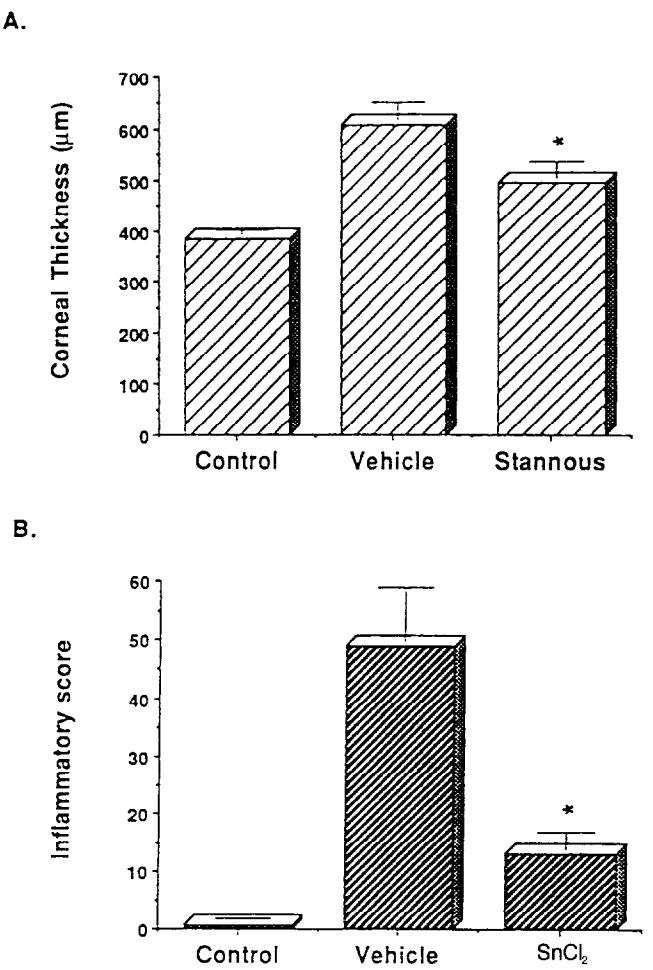

FIG. 7. Effect of one-time treatment with $SnCl_2$ on corneal thickness and inflammatory score in eyes after 6 days of exposure to lenses treated with either vehicle (0.1 M phosphate buffer, pH 7.4) or $SnCl_2$ (100 μg/mL) in phosphate buffer for 30 min prior to lens placement and tarsorrhaphy. At 6 days, sutures were removed and eyes were examined. Corneal thickness was assessed by ultrasonic pachymetry as described in Materials and Methods. Biomicroscopic photographs of the ocular surface were taken with a photographic attachment to the slit lamp. Photographs were subjectively scored as described in Materials and Methods. Data are presented as means ± SEM (N = 5). Key: (*) $P < 0.05$ versus control.

epithelium. $SnCl_2$ was the most potent of the several compounds studied, with small concentrations of the metal producing substantial increases in HO-1 mRNA content and in HO enzymic activity in the treated RCE cells. The effect of the metal was concentration- and time-dependent; exposure of the cells continuously over 6 days to $SnCl_2$ resulted in a sustained increase in *HO-1* expression.

Study of the effect of increased HO activity on the severity of experimentally induced inflammation in the cornea was an important aspect of these experiments. As noted above, we have shown in earlier work that transfection of the *HO-1* gene and its selective overexpression into rabbit coronary endothelial cells substantially moderated cell damage resulting from exposure to free heme/hemoglobin [19]. Several studies in *in vivo* animal models clearly affirm the role of HO-1 as a tissue-protective response to injury and inflammation. Nath *et al.* [15] showed that HO-1 induction coupled to ferritin synthesis provided significant protection against rhabdomyolysis-induced injury to the kidney, and inhibition of HO activity deteriorated renal function in this model system. Similarly, Agarwal *et al.* [25] demonstrated that induction of HO-1 occurred rapidly in oxidant-induced nephrotoxicity and pretreatment of rats with an HO inhibitor worsened renal function following administration of cisplatin or gentamycin. Willis *et al.* [26] in a recent report also implicated the direct involvement of HO-1 in the resolution of complement-dependent acute inflammation.

The results of this study demonstrate that $SnCl_2$ induction of *HO-1* expression in corneal epithelium is associated with a substantial attenuation of the corneal inflammatory response elicited by extended contact lens wear; this ameliorative effect is graphically depicted in Fig. 6. Its proximate mechanism likely involves a number of factors including enhanced catabolism of the prooxidant heme to the antioxidant metabolites biliverdin and bilirubin; diminution in the cellular content and activity of hemeprotein species, such as cytochrome P450 isozymes involved in the production of pro-inflammatory mediators as shown earlier; and possibly the local generation of carbon monoxide, which could inactivate other heme-containing proteins that contribute to the inflammatory response. Furthermore, Nakagami *et al.* [13] have shown that biliverdin and bilirubin are potent, anti-complement factors, thus providing an additional dimension to the anti-inflammatory effects of overexpression of the *HO-1* gene.

The exact mechanism by which $SnCl_2$ attenuates the corneal inflammatory response in the closed eye-hydrogel contact lens model of injury is unknown and may include some or all of the mechanisms indicated above. In characterizing this model of injury, we have established significant correlations between the synthesis of cytochrome P450-derived eicosanoids, the subjective inflammatory score (r = 0.963), and the progressive increase in corneal thickness (r = 0.971), thus implicating these eicosanoids as mediators of the inflammatory response; furthermore, administration of $SnCl_2$ significantly inhibited corneal epithelial synthesis of the cytochrome P450-derived eicosanoids (82%) [20, 21], suggesting that inhibition of cytochrome P450 activity constitutes part of the mechanism of the anti-inflammatory effect of HO-1 induction.

The potency of $SnCl_2$ in eliciting local induction of HO-1 in corneal epithelium and the attenuation of tissue inflammation associated with enhanced *HO-1* expression in the treated cells provide further evidence of a significant relationship between HO activity and the genesis of tissue inflammation. Our findings also indicate that this relationship can be regulated by simple pharmacological means and raise the possibility that regulation of the HO activity–inflammatory response relationship in this manner may be applicable to the clinical setting.

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