Oxidative Stress Induces Heme Oxygenase-1 Immunoreactivity in Müller Cells of Mouse Retina in Organ Culture

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Purpose. Heme oxygenase (HO)-1 immunoreactivity (IR) was examined in normal untreated retina and in retinal explants after in vitro treatment with stress agents.

Methods. Enucleated eyes from young adult C3H mice were immediately fixed and cryosectioned and the retina sections processed for immunocytochemistry with antibodies against HO-1 and glial fibrillary acidic protein (GFAP). From other eyes retinas were isolated and maintained in organ culture, either untreated for 4 days maximum or for 21 hours during which the explants were treated the first 3 hours with selected doses of sodium arsenate or hydrogen peroxide. Thereafter, the explants were processed identically with the normal tissue.

Results. In the normal retina, HO-1 and GFAP IR was very low. The culturing itself resulted in an increase in both HO-1 and GFAP immunolabeling in Müller cells of explanted retinas. Both sodium arsenate and hydrogen peroxide further induced strong HO-1 IR in Müller cells but not in other retinal cells. In contrast to HO-1, GFAP staining in Müller cells was not altered as a result of treatment, either by sodium arsenate or hydrogen peroxide at any concentration used.

Conclusions. The results show for the first time that HO-1 can be induced in the retina in vitro by conditions of oxidative stress and that enzyme expression is confined exclusively to Müller cells. (*Invest Ophthalmol Vis Sci.* 2001;42:1370–1374)

Based on a wealth of available data describing structural and functional interactions, the retina is viewed as a structure in which information primarily flows from the photoreceptor cells to the inner retina with less dominant feedback mechanisms. This generally accepted idea regarding the route for information processing within the retinal circuitry is changing, largely because of explorations of the pathways through which agents affect the retinal tissue. There is emerging evidence that some neurotrophic factors rescue differentiated photoreceptor cells from (inherited) retinal degeneration.[1,2] However, a search for their effector pathways has indicated that these neurotrophic factors bind to specific receptors on the cell surface of Müller cells, whose somata are located in the retinal inner nuclear layer.[3] It follows that photoreceptor cell rescue by neurotrophic factors is channeled by a circuit running from the inner retina to the photoreceptor cells.

To get a fuller appreciation of the cellular systems used by different categories of agents affecting the retina, we investigated the retinal response to oxidative stress as expressed by induction of heme oxygenase (HO). Interest in retinal oxidative mechanisms is partly clinically oriented, relating to the potential contribution of oxidative stress to disorders such as diabetic retinopathy and retinopathy of prematurity.

HO (EC 1.14.99.3) is an enzyme that, together with biliverdin reductase, converts heme into the bile pigment bilirubin.[4–6] Two isoforms of HO (HO-1 and HO-2) have been isolated and characterized. Despite their close functional similarity, these proteins are the products of different genes,[6,7] and they exhibit differences in their sizes, biochemical characteristics, antigenicity, and tissue distribution.[8,9] HO-1 can be induced by a variety of conditions that stress cells, including exposure to heavy metals, oxidative stress, inflammatory cytokines, and heat stress.[5,9,10] The induction of HO-1 has been demonstrated both in vitro and in vivo in various mammalian cells and organs.[11–13] Oxidative insult, in particular, appears to be a major factor in HO-1 induction under pathologic conditions.[14–17]

Recently, it has been demonstrated that both the neural retina and retinal pigment epithelium (RPE) contain the HO-1 isoenzyme protein and mRNA.[18,19] In human retinoblastoma and RPE culture model systems, HO-1 mRNA and protein levels can both be elevated by known stress agents.[18,19] In the normal retina, however, the cell type(s) in which HO-1 is induced has not been established. In the present study, we used a well-characterized retinal organ culture system to demonstrate the modulation of HO-1 expression in mouse neural retina by sodium arsenate and hydrogen peroxide, known inducers of oxidative stress. We present evidence that HO-1 immunoreactivity (IR) is exclusively increased in retinal Müller cells. For comparison, the expression of glial fibrillary acidic protein (GFAP), the marker for activated Müller cells, was also studied in this experimental paradigm. Preliminary reports of this study have been presented in abstract form.[20]

Materials and Methods

Retinal Organ Culture

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Normal adult (4–8 weeks of age) C3H mice were killed by cervical dislocation and the eyes enucleated. For normal analysis of the retina, anterior segments were removed and the eye cup put in fixative. For explant analysis, eyes were incubated in phosphate-buffered saline (PBS), con-

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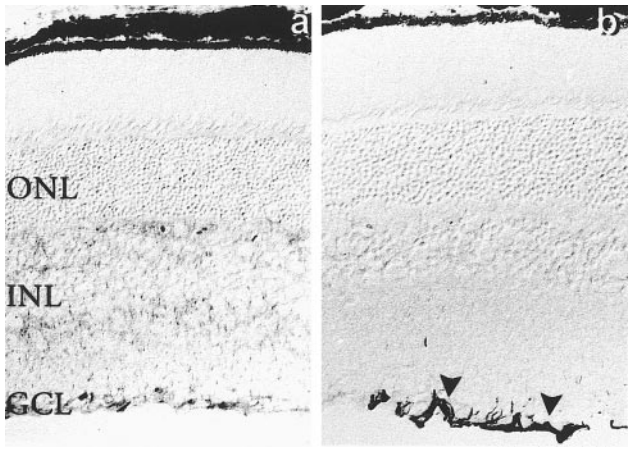

FIGURE 1. Normal mouse retina. Anti-HO-1 (**a**) and anti-GFAP (**b**) IR in 10-μm cryosections. HO-1 IR was weak and scattered in the inner retina, and GFAP IR was strong in the ILM (*arrowheads*). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Magnification, ×150.

taining proteinase K (0,5 mg/ml; Sigma, St. Louis, MO), and 5× antibiotic–antimycotic solution (Gibco, Paisley, UK). To stop the proteinase K action, eyes were placed in R16 medium (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco) for 20 minutes at 37°C. Under sterile conditions, muscles and connective tissue were peeled off the eyes, anterior segments were removed, and neural retinas without RPE attached were isolated. Retinal explants were maintained as organ cultures in R16 medium, supplemented with 10% FCS and antibiotic–antimycotic solution (culture medium) at 37°C in a humid atmosphere supplemented with 5% CO_2, essentially as described earlier.[21]

HO-1 Induction

Untreated explants were cultured for 4 days maximum in culture medium with evaluations at regular time intervals. To induce HO-1, fresh retinal explants were rinsed in PBS and treated for 3 hours in either sodium arsenate (10, 50, or 250 μM; Sigma) or hydrogen peroxide (10 or 1000 μM; Merck, Darmstadt, Germany) in a solution of PBS. Explants treated with PBS alone served as control samples. After induction, explants were rinsed in PBS (three times, 5 minutes each) and incubated for an additional 18 hours in the wash medium. Each parameter was studied in at least three separate experiments. More than three explants were analyzed per experiment.

Immunohistochemistry

Tissues were fixed in 4% buffered paraformaldehyde (pH 7.3) for 1 hour at room temperature, rinsed in Sörensen's phosphate buffer (pH 7.3), immersed in 25% sucrose in PBS, embedded in optimal cutting temperature compound (Tissue-Tek; Miles, Elkhart, IN), and cryosectioned at 10 μm. Nonspecific binding was blocked with normal goat serum (1:200; Vector, Burlingame, CA) for 30 minutes. The primary antibodies, rabbit anti-HO-1 (Stressgen, Victoria, British Columbia, Canada) and rabbit anti-GFAP (Milab, Malmö, Sweden), were diluted 1:10,000 and 1:4,000, respectively. The sections were incubated overnight in a humid chamber at room temperature. Binding was visualized through incubations with biotinylated goat anti-rabbit antibody (1:200; Vector), followed by avidin-peroxidase conjugates (Vector). Color reaction was developed with diaminobenzidine in the presence of hydrogen peroxide. Control samples were incubated with normal rabbit serum instead of the primary antibodies or without any antibodies.

RESULTS

HO-1 and GFAP Immunoreactivity in Normal Mouse Retina and in Untreated Retinal Explants

In normal mouse retina, only scattered and weak immunoreaction was seen for HO-1 in the inner plexiform layer (IPL). Strong GFAP immunolabeling was observed only in the inner limiting membrane (ILM; Figs. 1a, 1b). Immediately after isolation, no changes were detected in HO-1 or GFAP IR in retinal explants compared with IR in normal in situ mouse retinas (Figs. 2a, 2c). After 4 days of culturing, HO-1 IR increased slowly in the ILM and expanded to Müller cell processes in the IPL (Fig. 2b). In contrast, strong GFAP IR appeared in Müller cell processes as early as 2 days into culture, and the label was retained through the 4-day culture period (Fig. 2d).

HO-1 and GFAP IR after Inducing Oxidative Stress in Retinal Explants

In PBS-treated explants, weak HO-1 IR was observed in the ILM and a few Müller cell processes (Fig. 3a). By 18 hours after treatment with sodium arsenate, however, the staining pattern of HO-1 changed dramatically. A 10-μM dose of this chemical caused a strong immunoreaction in descending Müller cell processes and a few Müller cell bodies as well (Fig. 3b). The intensity and frequency of the stained elements increased with higher (50 or 250 μM) arsenate concentrations (Figs. 3c, 3d, 3g). Similar changes in staining pattern were detected in retinal explants 18 hours after hydrogen peroxide treatment. Heavy labeling was observed in the outer limiting membrane (OLM), the ILM, and the Müller cell bodies and processes (Figs. 3e, 3f). Strong IR was already present at the lowest dose tested (10 μM; Fig. 3e). Of particular interest was the difference in staining pattern that was observed after application of sodium arsenate and hydrogen peroxide. Specifically, IR in the OLM was stron-

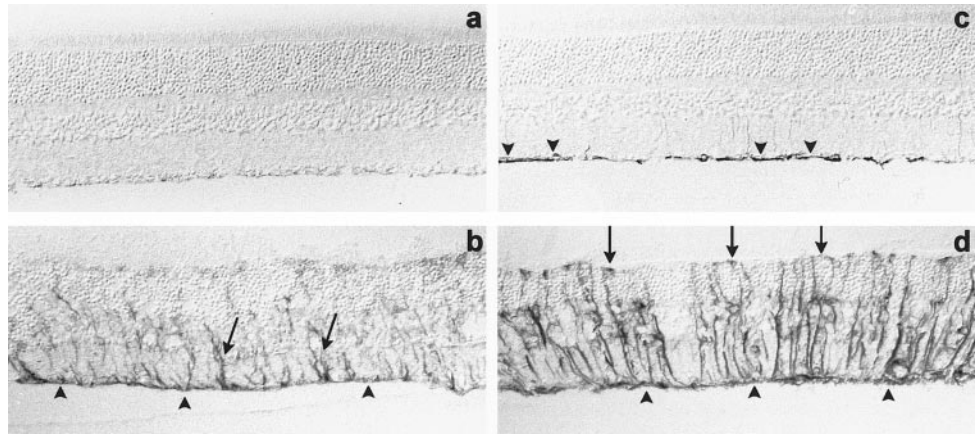

FIGURE 2. HO-1 (**a**, **b**) and GFAP (**c**, **d**) immunoreactivity in retinal explants just after tissue isolation (**a**, **c**) and 4 days' culture (**b**, **d**). After 4 days in culture, weak HO-1 IR was seen mostly in basal Müller cell processes (**b**; *arrows*) and in the ILM (**b**, **c**, and **d**; *arrowheads*). In contrast, GFAP IR was stronger and was confined to Müller cell processes, expanding from OLM (**d**; *small arrows*) to ILM. Magnification, ×200.

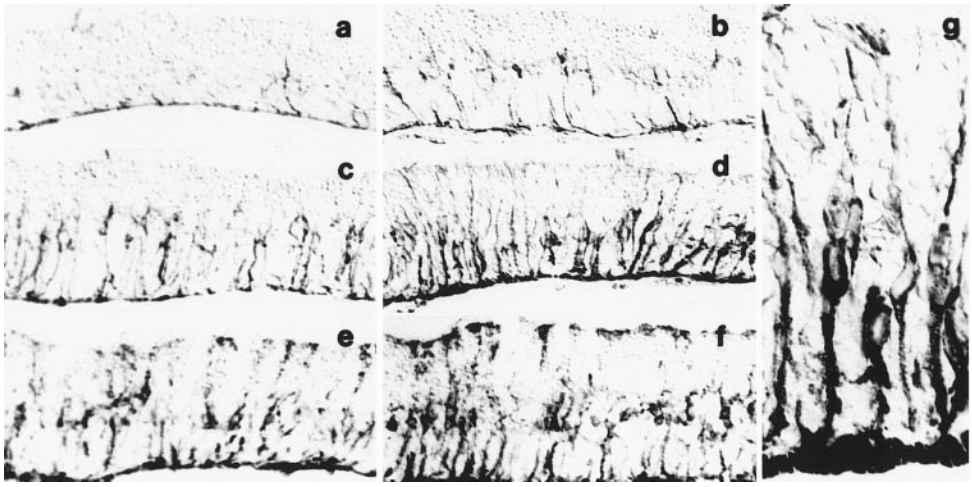

FIGURE 3. Cryosections of retinal explants displaying HO-1 IR 18 hours after treatment with sodium arsenate: (**b**) 10 μM, (**c**) 50 μM, and (**d**) 250 μM; or hydrogen peroxide: (**e**) 10 μM and (**f**) 1000 μM. (**a**) Untreated control; (**g**) high magnification of explant treated with 250 μM arsenate. Magnification, (**a** through **f**) ×200; (**g**) ×800.

ger after the treatment with hydrogen peroxide (Figs. 3e, 3f) than after application of arsenate (Figs. 3a through 3d).

In contrast to HO-1, GFAP IR was not altered as a result of treatment, either by sodium arsenate or hydrogen peroxide (Figs. 4a through f). Müller cell processes and the ILM were always heavily stained, but neither the intensity of the staining nor its distribution pattern changed after these chemicals were applied.

DISCUSSION

Under conditions of oxidative stress, HO-1 manifests itself as an anti-oxidative defense factor.[14,22] It is suggested that the anti-oxidative effect of HO-1 is associated with the production of bilirubin, an effective free radical scavenger,[16] and with the reduction of heme, a powerful pro-oxidant.[23] Oxidative stress has been implicated in retinal degeneration under severe pathologic conditions, including retinal inflammation,[24] diabetes-associated retinopathy,[25] retinal aging,[26] and light-induced injury.[27,28] Therefore, the question arises of whether HO-1 participates in the general defense mechanisms of the retina against oxidative stress and which cells are responsible for the enzyme expression.

In the normal mouse, only scattered and weak HO-1 labeling was detected in the inner retina. This staining appeared to be slightly more pronounced in both plexiform layers and the ILM. In these retinal locations horizontal fiberlike protrusions of the Müller cells occurred and may represent basal enzyme expression by Müller cells. In agreement with previous studies,[29,30] GFAP was found in normal mouse retina only in the ILM. Two types of inducers were used in our model system: sodium arsenate, which causes oxidative stress by affecting glutathione levels in the cells,[31] and hydrogen peroxide, which is an active oxygen intermediate and participates in production of oxygen free radicals.[32] Under the present experimental conditions, we did not detect changes in GFAP expression after either sodium arsenate or hydrogen peroxide treatment. GFAP is a marker for activated Müller cells and is expressed in a number of pathologic conditions, including retinal degeneration[29] and retinal detachment.[33] The refractory response of GFAP to the chemicals tested indicates that the mechanisms activating the HO-1 and GFAP genes in retinal Müller cells are different and respond to separate traumatic events.

Glial cells, including retinal Müller cells, possess cellular mechanisms providing maintenance of high intracellular glutathione concentration.[34] The high level of glutathione in Müller cells makes them susceptible to glutathione-depleting agents. Glutathione depletion and the subsequent HO-1 induction[35] is the most likely mechanism triggered by sodium arsenate in the Müller cells from the treated retinal explants. In contrast, neurons were found to contain much less glutathione than did glia.[36] This may explain why retinal photoreceptors are unresponsive to this glutathione-depleting agent and do not show an induction of HO-1 expression.

In the retina, photoreceptors are the most vulnerable to oxidative damage because of their oxygen-rich environment and the high content of polyunsaturated fatty acids in their membranes.[37] Because hydrogen peroxide generates active

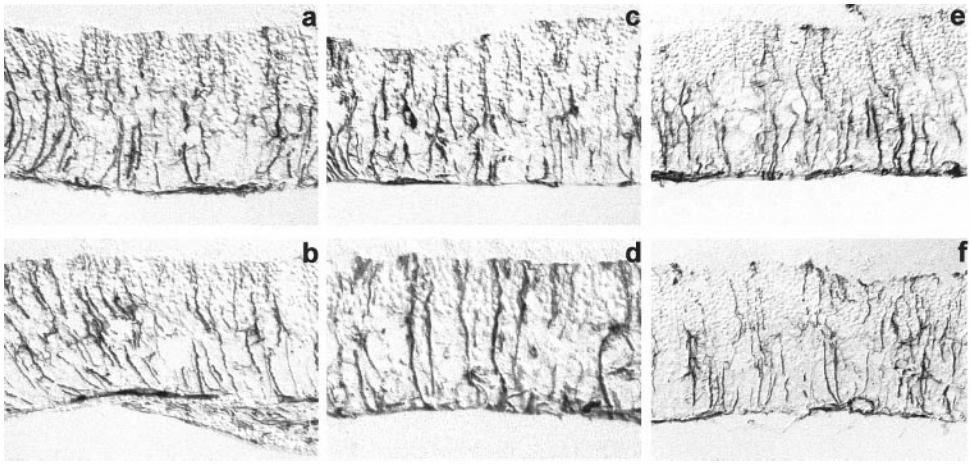

FIGURE 4. GFAP IR of retinal explants after treatment with HO-1 inducers. (**a**) Untreated control; (**b**) 10 μM sodium arsenate; (**c**) 50 μM sodium arsenate; (**d**) 250 μM sodium arsenate; (**e**) 10 μM hydrogen peroxide; and (**f**) 1000 μM hydrogen peroxide. There was no difference in intensity and location of IR between control- and inducer-treated explants. Magnification, ×200.

oxygen species and causes lipid peroxidation chain reactions,[32] we anticipated that photoreceptor cells subjected to hydrogen peroxide would express HO-1 as part of a defense mechanism against this form of oxidative stress. Support came from prior observations on the retinoblastoma cell system.[18] However, this expression did not occur. In a surprising finding, hydrogen peroxide, similar to sodium arsenate, induced HO-1 expression exclusively in Müller cells. One reason for this may be that Müller cells provide metabolic support to adjacent neural cells. For example, they contribute to spatial buffering currents and are the exclusive site for glutamine synthesis that removes excess and potentially toxic amounts of glutamate from the retina.[38] This protective mechanism is obviously absent from the retinoblastoma cell model system.

The present study shows that HO-1 is induced in neural retina as a result of oxidative stress and that this response is primarily limited to Müller cells. Further investigation is needed to understand the functional role of HO-1 in the retina. But, it appears that HO-1 may play an important role in protecting this tissue from oxidative damage. Overexpression of HO-1 has been clearly shown to protect cells from oxidative stress in other systems.[39–41] Also, HO-1-deficiency is reported to have an adverse effect on cellular resistance to oxidative stress.[42,43] The cytoprotective role of HO stems from its ability to catalyze the degradation of the pro-oxidant heme into biliverdin, the precursor of the potent antioxidant bilirubin.[14,44] HO reaction also generates Fe^{2+}, an agent that is deleterious to the cell. It is thought, however, that this in turn leads to the induction of ferritin, which could sequester free Fe^{2+}.[45] The expression of HO-1 in brain in response to injury is primarily seen in glial cells.[46] The increased HO activity could enhance the ability of these cells to protect neighboring neurons from oxidative stress. In comparison, HO-1 could play an important role in the ability of Müller cells, which are also glial cells, to protect photoreceptor cells from oxidative damage.

HO may also contribute to the neurotransmitter activity in the retina. Of particular interest may be the link with carbon monoxide (CO) and nitric oxide (NO). CO, a product of HO reaction, is a gaseous neural messenger that can mimic the action of NO.[47,48] CO can regulate the cyclic guanosine monophosphate (cGMP) concentration in cells by its ability to modulate the activity of the soluble form of guanylate cyclase. Under normal physiological conditions HO-2, the constitutive form of HO, is thought to be responsible for this action. The localization of HO-2 is similar to that of constitutive forms of NO synthase (NOS). In neural retina, HO-2 IR is detected mainly in neuronal cells.[49] A recent study shows that HO-2 IR is present in the inner retina, ganglion cells, and amacrine cells, and that CO, similar to NO, can increase cGMP production in the retina.[50] Under stressful conditions HO-1 and HO-2 may collaborate when NO concentration in the cell reaches a toxic level because of the activity of the inducible form of NO synthase (iNOS).[51] The CO- and NO-generating systems are interdependent; NO could induce HO-1 and CO could regulate NOS activity.[52]

The consistent induction of HO-1 in Müller cells permits speculation that these glial elements may, in addition, play an important role in protection of photoreceptors and other retinal neural cells against oxidative damage.

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