Oxidative Stress Is an Early Event in Hydrostatic Pressure–Induced Retinal Ganglion Cell Damage

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Purpose. To determine whether oxidative adduct formation or heme oxygenase-1 (HO-1) expression are altered in retinal ganglion cell (RGC) cultures exposed to elevated hydrostatic pressure and in a mouse model of glaucoma.

Methods. Cultured RGC-5 cells were subjected to 0, 30, 60, or 100 mm Hg hydrostatic pressure for 2 hours, and the cells were harvested. Parallel experiments examined the recovery from this stress, the effect of direct 4-hydroxy-2-nonenal (HNE) treatment, and the effect of pretreatment with resveratrol or quercetin. Mice were anesthetized and intraocular pressure was increased to 30, 60, or 100 mm Hg for 1 hour; then the retinas were harvested. HNE adduct formation and HO-1 expression were assessed by immunocytochemistry and immunoblotting.

Results. Increases of HNE-protein adducts (up to 5-fold) and HO-1 expression (up to 2.5 fold) in pressure-treated RGC-5 cells were dose dependent. During recovery experiments, HNE-protein adducts continued to increase for up to 10 hours; in contrast, HO-1 expression decreased immediately. HNE, at a concentration as low as 5 μM, led to neurotoxicity in RGC-5 cells. HNE adducts and HO-1 expression increased in the mouse retina and optic nerve after acute IOP elevation up to 5.5-fold and 2-fold, respectively. Antioxidant treatment reduced the oxidative stress level in pressure-treated RGC-5 cells.

Conclusions. This study demonstrates that oxidative stress is an early event in hydrostatic pressure/IOP-induced neuronal damage. These findings support the view that oxidative damage contributes early to glaucomatous optic neuropathy. (*Invest Ophthalmol Vis Sci.* 2007;48:4580–4589) DOI:10.1167/iovs.07-0170

Glaucoma is a blinding optic neuropathy marked by progressive and accelerated loss of retinal ganglion cells (RGCs) and their axons. It affects approximately 70 million people worldwide and is the leading cause of irreversible blindness.[1] It is generally recognized that elevated intraocular pressure (IOP) is the most significant risk factor for accelerated RGC death in glaucoma.[1,2] However, the mechanism of cellular damage caused by elevated IOP is still unknown.

Apoptosis of RGCs has been reported in rats with elevated IOP[3,4] and in glaucoma patients.[5] Hydrostatic pressure (30 or 100 mm Hg, 2 hours) induces apoptosis in cultured RGCs[6] and several other neuronal cell lines, including B35 and PC12 cells.[7] We recently showed that 30 mm Hg hydrostatic pressure for 3 days induces mitochondrial fission, dysfunction, and cellular ATP reduction in immortalized RGCs.[8] It has been proposed that oxidative stress is related and contributes to RGC loss in glaucomatous neuropathy[9–14] and that it contributes to programmed cell death.[15,16] Moreno et al.[11] have shown that chronic IOP elevation induced by weekly injections of hyaluronic acid into the anterior chambers of rat eyes reduced levels of superoxide dismutase (SOD), catalase, and glutathione. However, products of oxidative damage on proteins and other related early changes after acute IOP elevation have not been reported.

In this study, we examined the formation of 4-hydroxy-2-nonenal (HNE) adducts, a lipoxidation product,[17–19] and the expression of heme oxygenase-1 (HO-1), a common oxidative stress-induced protein,[20–22] in immortalized RGCs subjected to elevated hydrostatic pressure and in mouse eye tissues after acute IOP elevation. Our results demonstrate that oxidative damage occurs within hours of elevated hydrostatic pressure or elevated IOP.

Materials and Methods

Antibodies and Chemicals

Antibodies included: polyclonal antibody to HNE (1:1000; Axxora-USA, San Diego, CA), HO-1 polyclonal antibody (1:5000; Stressgen Bioreagents, San Diego, CA), anti-actin mouse monoclonal antibody (1:3000; Calbiochem, La Jolla, CA), goat anti-mouse IgM for actin antibody (1:2000; Calbiochem), HNE chemical (Cayman Chemical, Ann Arbor, MI), horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2000; Bio-Rad, Hercules, CA), and fluorescence labeled goat anti-rabbit IgG antibody (1:200; Molecular Probes, Eugene, OR). Also used were polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, Pittsburgh, PA), and Western blotting detection reagent (ECL Plus; Amersham Biosciences). Other chemicals were from Sigma (St. Louis, MO). Animal use adhered to the guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Pressure System

To achieve stable and adjustable hydrostatic pressure, a customized pressure chamber was designed to expose cultured RGC-5 cells to elevated hydrostatic pressure. The chamber inlet (Plexiglas; Altuglas International, Philadelphia, PA) was connected by a low-pressure two-stage regulator (Gilmont Instruments, Barnant Company, Barrington, IL) to a gas tank of 5% CO_2 and 95% air (Airgas Inc., San Diego, CA). Two levels of needle valve gas-pressure controllers provided constant hydrostatic pressure ranging from 0 to 200 mm Hg. Hydrostatic pres-

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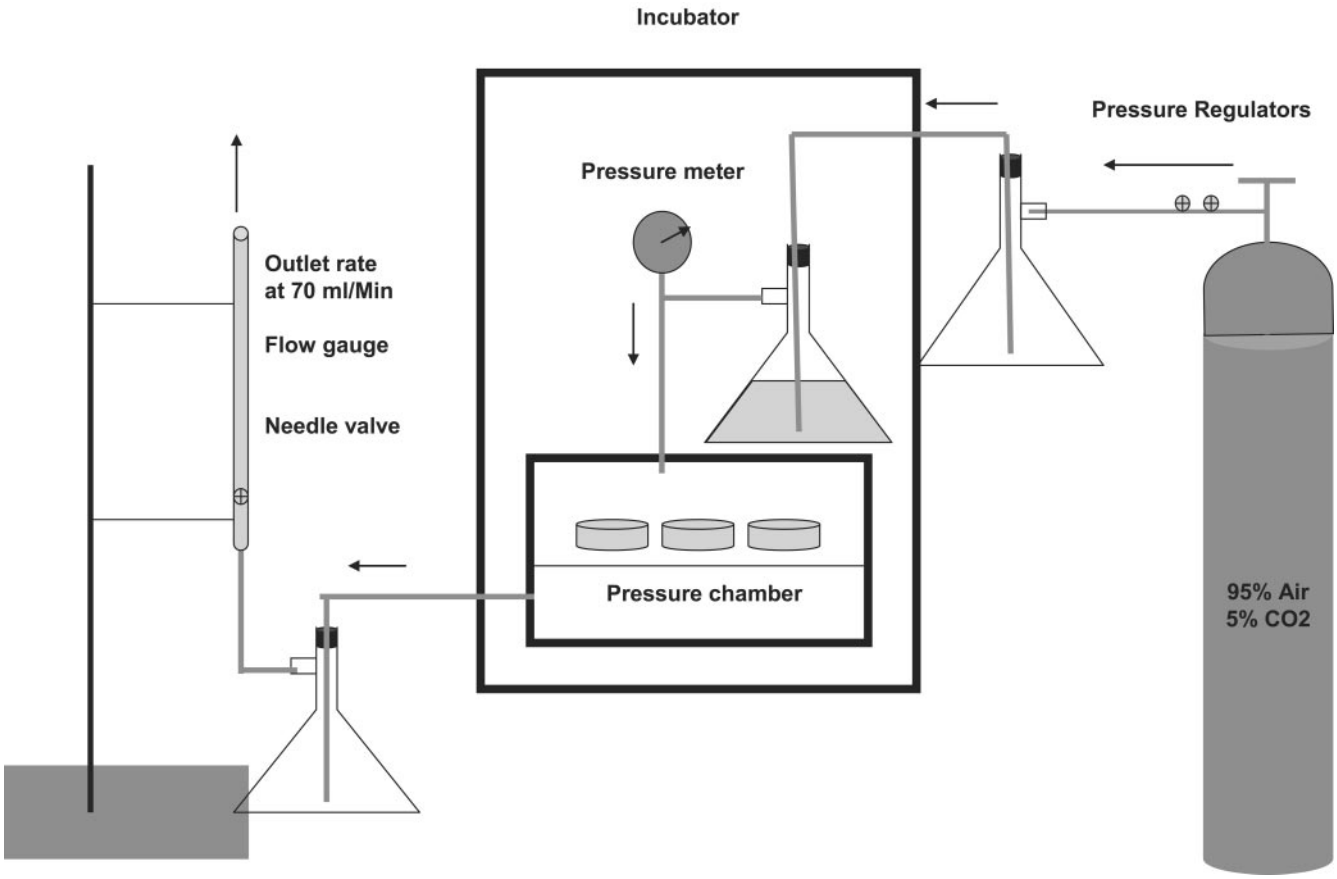

FIGURE 1. Schematic drawing of modified pressure chamber set-up.

sure in the chamber was monitored using a pressure gauge directly connected with the inlet circuit proximal to the chamber. Gas was warmed and humidified by bubbling through 2 L distilled water before flow through the chamber. Both the water flask and the pressure chamber were maintained at 37°C by placement inside an electronically controlled conventional incubator. Gas flow of 70 mL/min through the chamber was achieved using a ball-type flow gauge regulated with a needle valve in the outlet circuit (Fig. 1).

The possibility that elevated hydrostatic pressure could alter medium gas composition was assessed by analyzing different aspects in culture media in pressured and control cultures before and after pressurization. Measurements for pH, PCO_2, and PO_2 within the media were made using a portable blood gas analyzer (iSTAT Corp., East Windsor, NJ). Cultures were taken from the chamber, and the medium sample was transferred to the microchips within 1 to 2 seconds, quickly sealed with the attached cap, and read immediately.

RGC-5 Cell Culture

The RGC-5 cell line is a rat retinal ganglion cell line transformed by adenovirus carrying early region 1A (E1A). This cell line has many similarities to normal RGCs, including Thy-1 and Brn-3C expression and sensitivity to glutamate neurotoxicity or neurotrophin withdrawal.[23,24] Nevertheless, a number of differences between these cells and primary RGCs have been demonstrated, including the proliferation ability, nonneuronal appearance, and lack of repertoire of ion channels characteristic of RGCs.[23] Because it was the only available RGC cell line, we used it as a preliminary model for RGC studies in culture system. RGC-5 cells were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO) at 5% CO_2 and 37°C.[23]

To examine the pressure-induced oxidative damage, RGC-5 cells were subjected to elevated hydrostatic pressure for 2 hours, as reported elsewhere.[6] Control cells plated from an identical passage of cells were incubated simultaneously in a conventional 5% CO_2 culture incubator at 37°C. After 2-hour pressure, the cells were harvested immediately or were incubated for 1, 3, 6, or 10 hours in the conventional culture incubator for recovery experiments.

Immunocytochemistry

RGC-5 cells were plated on coverslips in 24-well plates at 10,000 cells/well. After 16 hours, the cells were exposed to elevated pressure at 30, 60, or 100 mm Hg for 2 hours. Cells were then washed with cold PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After permeabilization with 0.1% Triton X-100 in PBS for 15 minutes at room temperature, cells were washed with PBS, blocked with 1% BSA for 1 hour at room temperature, and incubated with primary antibodies for 16 hours at 4°C. After they were rinsed in PBS for 30 minutes, cells were incubated with fluorescent dye–conjugated goat anti-mouse IgG secondary antibody (Alexa Fluor 488; Molecular Probes, Invitrogen, Carlsbad, CA; 1:200) for 1 hour at room temperature. After PBS wash, the cells were counterstained with nucleic acid stain (Hoechst 33342; 1 μg/mL; Molecular Probes) in PBS for 5 minutes at room temperature and then mounted on glass slides with a water-soluble nonfluorescing compound (Fluoromount-G; Southern Biotechnology Associates, Birmingham, AL).

Images were captured under a fluorescence microscope (Eclipse E800; Nikon Instruments Inc., Melville, NY) equipped with digital camera (SPOT; Diagnostic Instrument, Sterling Heights, MI). Images were collected with appropriate software (Simple PCI, version 6.0; Compix Inc., Cranberry Township, PA) and exported as image-edited files (Adobe Photoshop; Adobe, San Jose, CA).

Western Blotting

Typically, two plates of cells were used for one sample preparation. RGC-5 cells were cultured and treated in 10-cm dishes. After treatment, the cells were washed once with cold PBS, scraped into 1.5 mL Eppendorf tubes, and centrifuged at 1000*g* for 3 minutes at 4°C. After the supernatant was removed, 100 μL cell lysis buffer (Chemicon, Pittsburgh, PA) was added to the pellets, and the samples were ultrasonicated on ice 10 times for 5 seconds each at 5-second intervals. Cell lysates were then centrifuged at 14,000*g* for 10 minutes at 4°C, and the protein concentration of the supernatant was determined using DC protein assay (Bio-Rad). The same amount of protein was loaded into 10% precast SDS-PAGE (Invitrogen, Carlsbad, CA). As HNE protein adducts formed on many of the proteins, multiple bands appeared on the blots. Thus, to facilitate the measurement of the total amount of all HNE protein adducts, we ran the gel just long enough to separate the bands. After briefly running for one third of the gel (for HNE blotting) or the full length of the gel for other blotting, samples were electrotransferred to a PVDF membrane.

Membranes were blocked in 10% nonfat dry milk in PBS with 1% Tween (PBS-T) for 1 hour with gentle shaking and rinsed in PBS-T, and the primary antibody was applied and incubated for 16 hours at 4°C with gentle shaking. After five 10-minute washes in PBS-T, secondary antibodies were applied for 1 hour at room temperature. After washings, the blot was developed by an enhanced chemiluminescence method and analyzed using a laser gel scanning system (Storm 860; Amersham).

Reblotting the membranes allowed probing with different antibodies. After the first probing, the membranes were stripped by incubation with acetonitrate for 20 minutes at room temperature, then with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 20 minutes at 50°C. Membranes were then washed with PBS-t five times for 5 minutes each before they were probed with the second primary antibody.

Calcein AM Live/Dead Viability Assay

Pressure-treated RGC-5 cells on coverslips in the same 10-cm plates were assayed by a viability/cytotoxicity assay (Live/Dead; Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. In this assay, polyanionic calcein is retained within live cells, producing an intense uniform green fluorescence (excitation/emission, 495/515 nm). Ethidium homodimer, which normally is excluded, enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence on binding to nucleic acids (excitation/emission, 495/635 nm). For positive control, RGC-5 cells were briefly treated (10 minutes) with 0.1% digitonin so that they could be permeabilized. Cell survival was calculated as the ratio of cells stained with the calcein AM to the sum of calcein AM-stained cells and ethidium homodimer-stained cells.

MTT Cell Viability Assay

Cell number and viability also were measured in RGC-5 cells cultured in 96-well plates using 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) according to the manufacturer's recommendations (Cell Proliferation Kit 1; Roche Diagnostics, Indianapolis, IN). Briefly, cells were grown in 96-well plates with a final volume of 100 μL culture medium per well. After exposure to elevated hydrostatic pressure, 10 μL MTT labeling reagent (final concentration, 0.5 mg/mL) was added to each well, and the cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 4 hours. Next, 100 μL solubilization solution was added to each well, and the plates were incubated for 16 hours in a humidified atmosphere of 5% CO_2 incubator at 37°C. Absorbance at 560 nm was then measured using a microplate reader (Spectra Max200; Molecular Devices Corp., Sunnyvale, CA). Background at 690 nm was subtracted accordingly for each sample. Data were presented as the percentage of cell viability in control wells. Each set of data was collected from multiple replicate wells of each experimental group ($n = 4$).

Antioxidant Treatments

Antioxidant treatments were performed by putting different concentrations of resveratrol or quercetin (20 or 40 μM in dimethyl sulfoxide [DMSO]) into the low-serum cell culture medium for 30 minutes before exposure to pressure. Control cells were put in the same amount of DMSO without antioxidants. Cells were subjected to 60 mm Hg pressure for 2 hours and then were harvested and immediately analyzed.

Mouse Acute IOP Elevation and Retina Sample Preparation

Three-month-old C57LB/6 mice were obtained (Harlan Labs, Inc., San Diego, CA) and were maintained in an animal facility for at least 1 week before the experiments. Mice were anesthetized by injections of ketamine (100 mg/kg) and xylazine (20 mg/kg). Because of the high mortality rate in 2-hour experimental groups, all acute IOP elevations were induced in a 1-hour period by insertion of a microneedle connected to a pressure transducer (WPI, Sarasota, FL) into the anterior chamber of the mouse eye and then by tubing to an elevated reservoir. Transducer output was connected by a bridge amplifier and an analog-to-digital signal converter (AD Instruments, Boulder, CO) to specific computer software (Chart 3.6; AD Instruments) to monitor the pressure in real time. Adjustment of hydrostatic pressure was achieved by fixing the liquid reservoir to certain heights.

For Western blotting, mice were perfused using 10 mL PBS, and the retina samples were collected immediately and frozen in −80°C. Each retina was placed in a 1.5-mL Eppendorf tube with 50 μL tissue lysis buffer (Chemicon) and was ultrasonicated and then centrifuged. The supernatants were collected and protein concentrations were measured using the DC protein assay (Bio-Rad).

For immunohistochemistry, mice were fixed by transcardial perfusion using 10 mL PBS followed by 10 mL 4% fresh prepared paraformaldehyde in PBS. Retina samples were collected immediately and fixed in 4% paraformaldehyde for 2 hours at room temperature, dehydrated through 70%, 80%, 90%, 95%, and 100% ethanol, and incubated with 50% ethanol/50% wax and 100% wax for 1 hour each. Then samples were embedded in pure wax for sectioning. Embedded samples were cut by a conventional microtome at 5-μm thickness, and the sections were mounted on glass slides. Immunohistochemistry was performed as described.

Statistical Analysis

Experiments were repeated at least three times. Data are presented as the mean ± SEM. One-way analysis of variance (ANOVA) and Tukey multiple comparison post hoc test were used for the comparison of the data. $P < 0.05$ was considered statistically significant.

Results

Effect of Hydrostatic Pressure on Culture Media

To determine whether hydrostatic pressure might alter pH, PCO_2, or PO_2 in the culture media, media samples from RGC-5 cell cultures that were exposed to hydrostatic pressure of 30 mm Hg, 60 mm Hg, or 100 mm Hg, respectively, for 2 hours were immediately analyzed using a blood gas analyzer. No significant difference in the levels of pH, PCO_2, or PO_2 in the media were observed between the pressure-treated and control media samples from RGC-5 cultures (Table 1; $P > 0.05$; $n = 3$).

Effect of Elevated Hydrostatic Pressure on HNE Adduct Formation and HO-1 Expression in Cultured RGC-5 Cells

To investigate the effect of hydrostatic pressure on oxidative stress, RGC-5 cells were exposed to 30 mm Hg, 60 mm Hg, or 100 mm Hg for 2 hours, as in other studies.[3,4] Immunocy-

TABLE 1. Measurements of pH, PCO_2, and PO_2 Levels in Cell Culture Media of Pressure-Treated and Control RCG-5 Samples

	Control	30 mm Hg	60 mm Hg	100 mm Hg
pH	7.74 ± 0.01	7.69 ± 0.03	7.73 ± 0.06	7.71 ± 0.07
PCO_2 (mm Hg)	25.60 ± 0.10	28.10 ± 1.57	26.20 ± 3.95	27.20 ± 3.86
PO_2 (mm Hg)	127.67 ± 1.53	134.00 ± 2.65	132.67 ± 9.61	130.67 ± 5.86

Statistical analysis showed no significant differences in pH, PCO_2, and PO_2 levels among the pressure-treated and control RGC-5 culture media ($P > 0.05$; $n = 3$). Data are the mean ± SD.

tostaining with anti-HNE polyclonal antibody showed that the level of HNE adducts was increased greatly in RGC-5 cells exposed to elevated pressure compared with nonpressure control cells. Interestingly, HNE immunoreactivity was greatest in the 60 mm Hg–treated cell samples (Fig. 2A). HNE immunostaining revealed diffuse immunostaining within RGC-5 cells (Fig. 2B), and different pressure treatment did not change the distribution of HNE adducts.

Consistent with these findings, Western blot analysis with the same antibody demonstrated significant increases in the level of HNE adducts in cells treated with 30 mm Hg (281% ± 60% [mean ± SEM]) and 60 mm Hg (515% ± 160) but not with 100 mm Hg (258% ±176%; $P > 0.05$) pressure treatment ($n = 4$). HNE immunoreactivity was greatest at 60 mm Hg, with more than a fivefold increase compared with control samples (Fig. 2C).

HO-1 expression was increased significantly after 2-hour treatment at 30 mm Hg (184% ± 26%) or 60 mm Hg (231% ± 37%), respectively. At 100 mm Hg (118% ± 12%) of pressure, however, the protein level was not significantly changed compared with controls. HO-1 immunoreactivity was greater in 60 mm Hg–treated samples, with more than a 2.5-fold increase, than in control samples (Fig. 2D).

Immediately after the pressure treatment, calcein AM viability/cytotoxicity assay revealed 2 to 4% of RGC-5 cells were nonviable in 100 mm Hg pressure-treated samples, which is consistent with recent findings of a pressure effect on RGC-5 cells.[6] There was no evidence of cell death in any of the other treatment groups. Actin level relative to the total protein amount was altered less than 1% ± 4% (SD; $n = 4$) by pressure treatment (data not shown). Thus, actin staining of the blots was used to confirm uniform gel loading (Figs. 2C, 2D).

Changes in HNE Adduct Formation and HO-1 Expression during RGC-5 Cell Recovery after Removal of Hydrostatic Pressure

To investigate RGC recovery from oxidative damage induced by hydrostatic pressure, RGC-5 cells were exposed to 60 mm Hg pressure for 2 hours and then were transferred to a conventional incubator for 1 to 10 hours. Western blot using anti-HNE polyclonal antibody showed that amount of HNE adducts kept increasing for 6 to 10 hours after the pressure treatment (Fig. 3A).

In contrast, immunoblot studies using anti–HO-1 monoclonal antibody showed that HO-1 expression was initially increased under pressure treatment but decreased immediately after it was discontinued (Fig. 3B).

Because of the continuing proliferation of RGC-5 cells, we did not follow the recovery time for more than 10 hours in RGC-5 cells (the proliferation cycle of RGC-5 cells is approximately 18 hours).

HNE Toxicity in RGC-5 Cells

To determine whether HNE induces RGC-5 cell death in culture, different concentrations of HNE chemical were administered for 16 hours, and the effect on the RGC-5 cells was measured.

Results showed that HNE, at concentrations as low as 5 μM, caused significant RGC-5 cell death. At 10 μM, HNE induced 30% RGC-5 cell death within 16 hours. At 50 μM, HNE killed almost all the cells in 16 hours (Figs. 4A, 4B).

Antioxidant Pretreatment Attenuates HNE Adduct Formation in RGC-5 Cells

Control experiments included both vehicle-treated cultures maintained at ambient pressure and vehicle-treated cultures exposed to 60 mm Hg pressure for 2 hours. Compared with the pressure-treated control cultures, pretreatment of RGC-5 cells with 20 μM or 40 μM resveratrol reduced HNE adduct formation caused by pressure treatment by 61% and 60%, respectively. In contrast, HNE adduct formation after pressure treatment was not altered significantly with 20 μM quercetin pretreatment and was reduced by 29% with 40 μM quercetin pretreatment (Figs. 5A, 5B).

Effect of Acute IOP Elevation on Oxidative Stress Level in Mouse Model

To investigate the effect of elevated intraocular pressure in vivo, we used a microneedle-injection model to induce transient elevation of IOP. IOP of 30, 60, and 100 mm Hg was applied for 1 hour to the mouse eyes (2-hour experimental groups experience high mortality rates).

Immunohistochemistry of retina sections with anti-HNE polyclonal antibody showed that HNE adducts were increased in a pressure-dependent manner. HNE immunoreactivity was mainly located in the ganglion cell layer, the inner nuclear layer, and the photoreceptor layer (Fig. 6A). Immunohistochemistry of optic nerve sections with anti-HNE polyclonal antibody showed that HNE adducts were also increased, especially in the optic nerve head portion (Fig. 6B).

Western blot studies using anti-HNE polyclonal antibody consistently showed that elevated IOP for 1-hour induced pressure-dependent increases in HNE adduct formation in retina samples up to 5-fold to the control samples (Fig. 6C).

In the same samples, HO-1 expression is also increased significantly with 60- and 100-mm Hg treatment but not with 30-mm Hg treatment (Fig. 6D).

DISCUSSION

We demonstrate that a single 1- to 2-hour elevation of hydrostatic pressure in vitro or of intraocular pressure in vivo significantly increased the formation of HNE adducts on many retinal proteins. In addition, it increased the expression of the inducible antioxidative protein (HO-1) in cultured RGC-5 cells and in mouse retinas. These results indicate that oxidative stress is an early response to elevated pressure or IOP. Further, short-term pressure elevation induced measurable neuronal damage. Our results provide additional evidence that oxidative damage in response to pressure elevation is an important underlying mechanism of hydrostatic pressure/IOP-induced cellular dam-

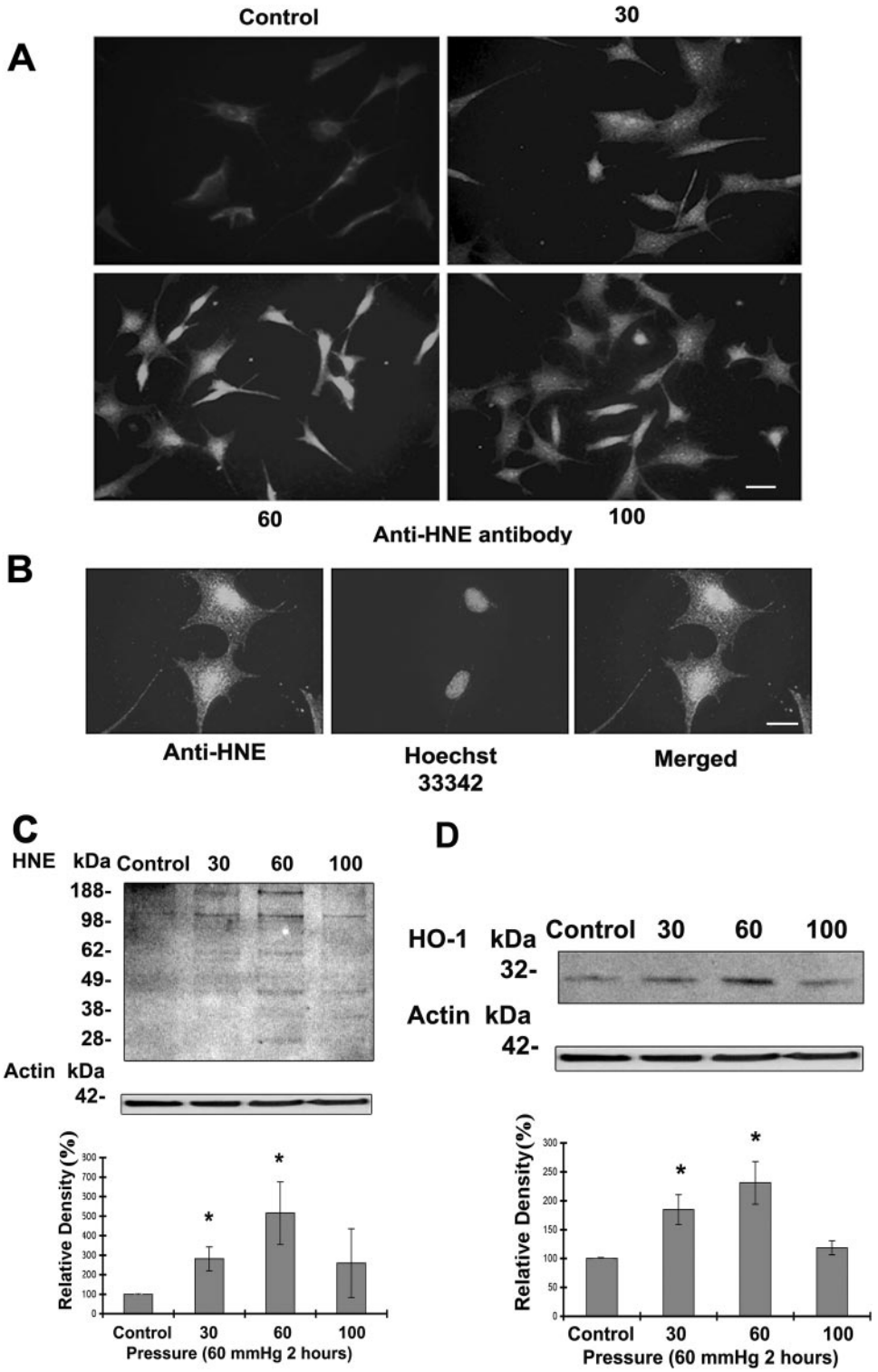

FIGURE 2. HNE adduct level and HO-1 expression are increased after different hydrostatic pressure treatment in RGC-5 cell culture. RGC-5 cells were treated with hydrostatic pressure (mm Hg), as indicated, for 2 hours. (**A**) Immunocytostaining with anti-HNE polyclonal antibody showed that HNE adduct levels were increased with elevated pressure and peaked in 60 mm Hg–treated RGC-5 samples. Scale bar, 20 μm. (**B**) Cellular distribution of HNE adducts in pressure-treated RGC-5 cells. After pressure treatment, HNE adducts showed diffuse distribution, and different pressure treatments did not alter the pattern. (Sample cells used here were from 60 mm Hg–treated samples). Scale bar, 5 μm. (**C**) Western blot showed that HNE adduct formation was increased after treatment with different hydrostatic pressure in RGC-5 cell culture. Quantitative measurement using Western blot showed that HNE adduct formation increased significantly under pressure of 30 mm Hg and 60 mm Hg, but not 100 mm Hg, for 2 hours. HNE adduct formation peaked at 60 mm Hg–treated samples. Values are mean ± SEM of four independent experiments. (**D**) Western blot showed that the heme oxygenase-1 level increased significantly under pressures of 30 mm Hg and 60 mm Hg but not 100 mm Hg. Heme oxygenase-1 level peaked at 60 mm Hg–treated samples. Values are the mean ± SEM of four independent experiments. $^*P < 0.05$.

age and neuron death. The inhibition of oxidative damage by antioxidant treatments indicates that these agents may provide preventive or therapeutic intervention for glaucoma.

As our results show, the HNE adduct formation and HO-1 expression were increased with different pressures, indicating that free radical production was induced greatly in these in vitro and in vivo experiments. These results demonstrate that cellular oxidative stress is increased within 1 to 2 hours by one-time acute pressure treatment. Given that oxidative stress induces apoptosis in neurons,[25] hydrostatic pressure induced oxidative stress could well be the mechanism responsible for the similar pressure-induced apoptosis in RGC-5 cells,[6] animal models,[3,4] and glaucoma patients with high IOP.[1,2]

HNE is the most well-studied product of endogenous lipoxidation that occurs in response to oxidative stress.[17,19,26] Most of the HNE protein adducts are considered irreversible except the lysine Michael adducts, which are only partially reversible.[27] Thus, the half-life of these adducts is largely determined by protein turnover rate and the level of accumulated HNE-protein adducts, and it provides a good indicator for oxidative

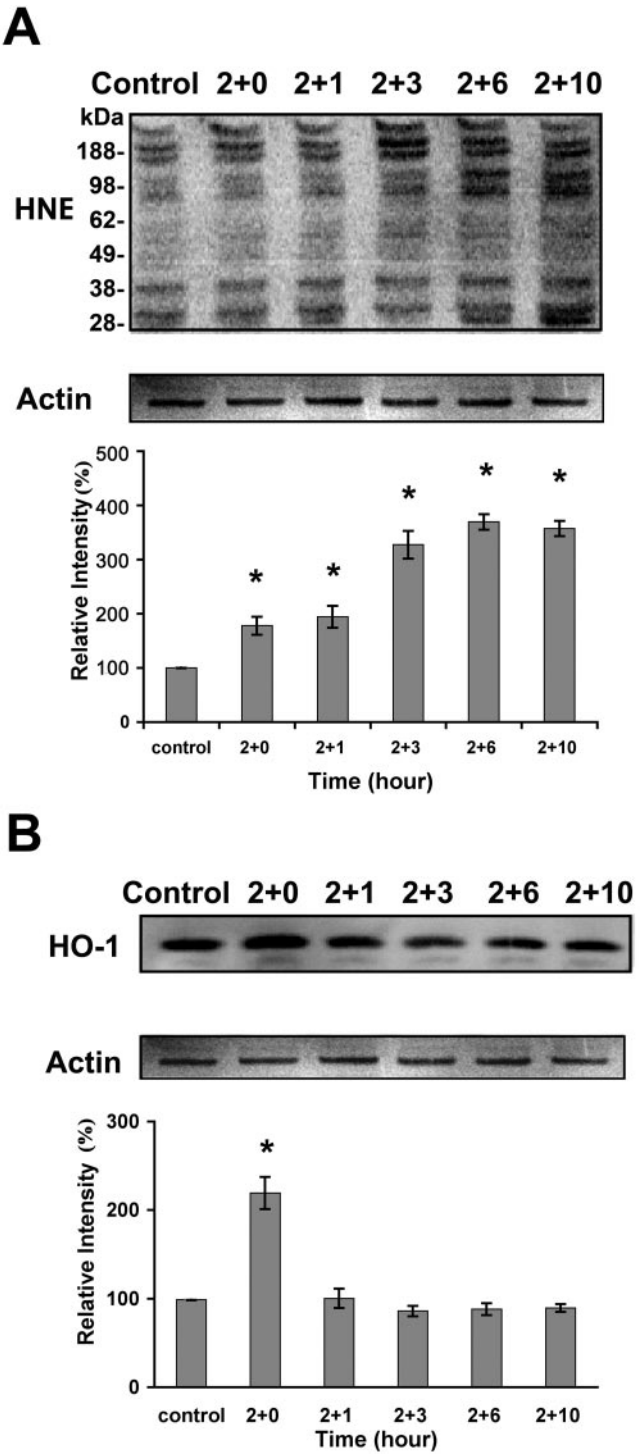

FIGURE 3. Different response levels of HNE adducts and HO-1 expression in recovery experiments of pressure-treated RGC-5 cells. After RGC-5 cells were treated with 60 mm Hg for 2 hours, pressure was discontinued. Cells were collected in different times. (**A**) Western blot showed that HNE adduct formation kept increasing within 10 hours after discontinuation of pressure (2 + 1 indicates 2 hours of pressure and 1 hour of cessation of pressure). Values are mean ± SEM of four independent experiments. $^*P < 0.05$. (**B**) Western blot showed that the heme oxygenase-1 level increased significantly under pressure of 60 mm Hg but decreased immediately after the cessation of pressure. Values are the mean ± SEM of four independent experiments. $^*P < 0.05$.

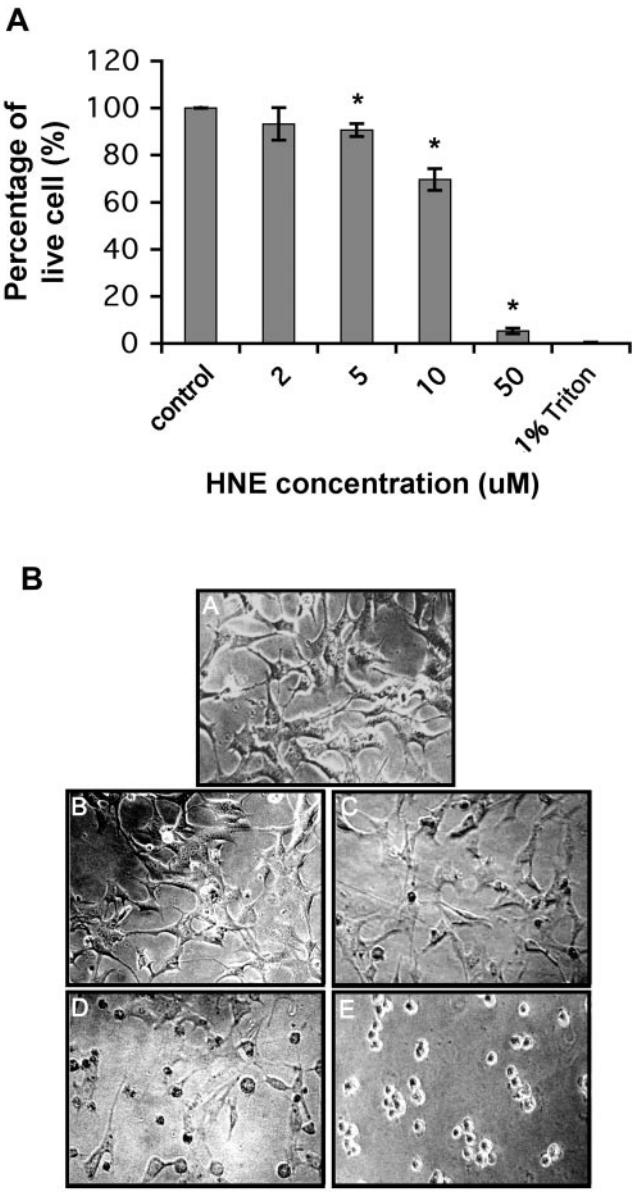

FIGURE 4. HNE toxicity in RGC-5 cell cultures. RGC-5 cells were treated with different concentrations of HNE for 16 hours. (**A**) Cell viability was assessed with MTT assay. Results showed a significant decrease in cell number in HNE-treated samples at or above 5 μM. (**B**) Light microscopy showed that RGC-5 cells were killed by HNE at concentrations greater than 5 μM. (**A**) Control. (**B**) 2 μM HNE. (**C**) 5 μM HNE. (**D**) 10 μM HNE. (**E**) 50 μM HNE. Scale bar, 20 μm. Values are the mean ± SD of four independent experiments. $^*P < 0.05$.

stress level.[5,18,19] Hence, the present observation of increased HNE adducts strongly indicates increased oxidative stress.

The level of phase 2 detoxification proteins, especially HO-1 expression, is recognized as a good biomarker for oxidative stress.[28,29] HO-1 protein also functions as an antioxidative protein that degrades the heme molecule and reduces oxidative stress in cells.[29] It has been used as a reliable marker of oxidative stress in many studies.[21,26] Its increase in the present study also indirectly demonstrates that pressure can increase oxidative stress.

RGCs are the major cell types that are substantially lost in glaucoma, but the reason is unknown.[1] To facilitate our studies, especially for quantitative analysis, RGC-5 cells were used. These cells have certain characterizations and limitations com-

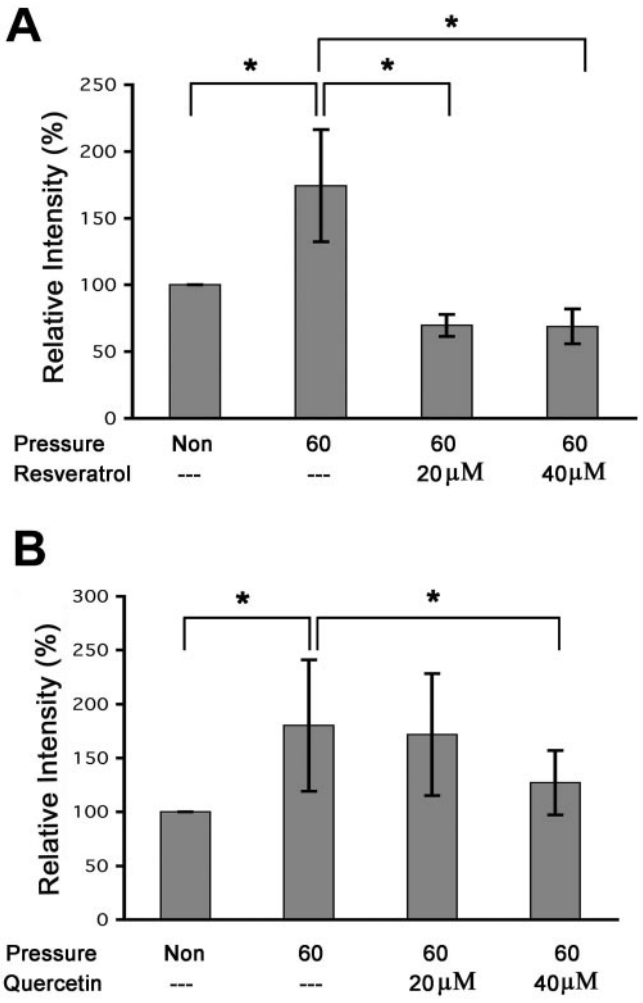

FIGURE 5. Antioxidant pretreatment reduced HNE adduct levels in RGC-5 cells subjected to 60 mm Hg pressure for 2 hours. (**A**) Resveratrol pretreatment (20 or 40 μM, 30 minutes) reduced the level of HNE adducts in RGC-5 cells subjected to 60 mm Hg pressure. (**B**) Quercetin pretreatment (20 or 40 μM; 30 minutes) reduced HNE adduct levels in RGC-5 cells subjected to 60 mm Hg pressure. $^*P < 0.05$.

pared with RGCs. RGC-5 cells have been used for different studies of hydrostatic pressure–induced apoptosis and protective interventions.[6,30,31]

Pressure levels used in this study were 30 mm Hg, 60 mm Hg, and 100 mm Hg above normal atmosphere (approximately 760 mm Hg). Pressures of 30 mm Hg and 60 mm Hg mimic the conditions seen in glaucoma patients with high IOP. Although pressure of 100 mm Hg mimics ischemia in the mouse retina, which leads to extensive and rapid neuron loss,[6] 30 mm Hg is generally considered more clinically relevant. Our recent results showed that 30 mm Hg hydrostatic pressure for 3 days triggered mitochondrial dysfunction in differentiated RGC-5 cells.[8] We did not detect any significant differences in pH, PCO_2, or PO_2 levels between media from any of the pressured samples and the controls. Thus, the changes observed in the present study appear to reflect a direct effect of pressure on the induction of oxidative stress.

When we examined the recovery of pressure-treated RGC-5 cells, the HNE adduct formation continued to increase over the next 10 hours despite a return to atmospheric pressure. This differed from HO-1 expression, which was reduced to a normal level within 1 hour. These results indicate two distinct types of responses in the recovery process. One type is the cumulative response extending the stress effect for a longer time after the stress of one-time acute pressure and presumably causing elongated cumulative damage to the cells. Another type is the tightly regulated cellular responses such as HO-1 expression, which is quickly adjusted according to stress level. Evidence supports that the cumulative responses are likely detrimental and that the tight-regulated responses are protective.[32,33] The balance of the detrimental and protective functions in neurons is critical for their function and survival under or after stress.

The effectiveness of resveratrol and quercetin has been tested in our pressure models. Results showed that resveratrol is more effective in reducing HNE adduct formation than quercetin. Further studies will be needed to demonstrate the basis for this difference.

A limitation of the RGC-5 cell line is its high proliferation rate (approximately 18 hours/generation). Therefore, we did not investigate recovery for periods longer than 10 hours. However, these can be achieved in primary retinal ganglion cell culture and animal studies. Our preliminary data from current ongoing animal studies showed that HNE adducts increased for several hours after elevated pressure, which supports these findings in the RGC-5 cell cultures. Because of intersubject variability, future studies with a large number of mice will be needed to properly investigate the issue of recovery in mice.

HNE, as one of the critical toxic products of reactive oxygen species (ROS), can induce cell death by apoptosis in multiple cell lines.[19,34] We tested HNE toxicity in RGC-5 cells. Results showed that 5 μM HNE could induce significant cell death in 16 hours. It is unclear whether cellular levels of HNE can reach equivalent levels, but the mean physiological concentration of HNE has been found to range from 0.1 to 1 μM in several types of tissue and body fluids, which can be significantly higher in pathologic conditions.[25,35] Previously, we found that HNE at a concentration of 10 μM could induce neuron death in M17 and SH-SY5Y cell cultures, even in 4 hours.[34] In addition, HNE can directly induce HO-1 expression in ERK-dependent pathways in cell cultures.[35] These data support that HNE and other ROS may be the critical players in RGCs death found in glaucoma patients.

Immunohistochemistry with anti-HNE antibody showed that the HNE adduct was primarily located in the cell layers of the retina and suggests that oxidative damage was greater near the neuronal cell bodies. This may reflect a higher level of free radical production in these locations and may locally overcome endogenous antioxidative mechanisms. Because this commonly occurs when local antioxidative functions are compromised, the present results that show one-time acute-pressure stress can cause significant free radical production must reflect that pressure induces local compromise of the cellular antioxidative functions in the retinas. The pressure difference between RGC-5 cells and the mouse model in the peak level of HNE adducts and HO-1 expression could be attributed to ischemia induced in the mouse eye with 100 mm Hg pressure that produces more oxidative damage in mice but not in RGC-5 cell cultures or because the cell cultures are more vulnerable than RGCs in retinas under pressure. These results show directly that single acute elevations of IOP in mouse eyes can cause significant increases in oxidative stress, which could play a critical role in RGC death.[36]

One issue raised by these data is that if oxidative stress appears in multiple layers of the retina, why is neuronal loss in glaucoma largely restricted in RGCs. Studies have shown that ROS can induce the apoptosis of photoreceptor cells in retina degeneration.[37] However, there is little photoreceptor cell death in glaucoma patients.[1] Perhaps RGCs are more sensitive than other retinal neurons because they have longer axons with substantial mitochondria and abundant polyunsaturated

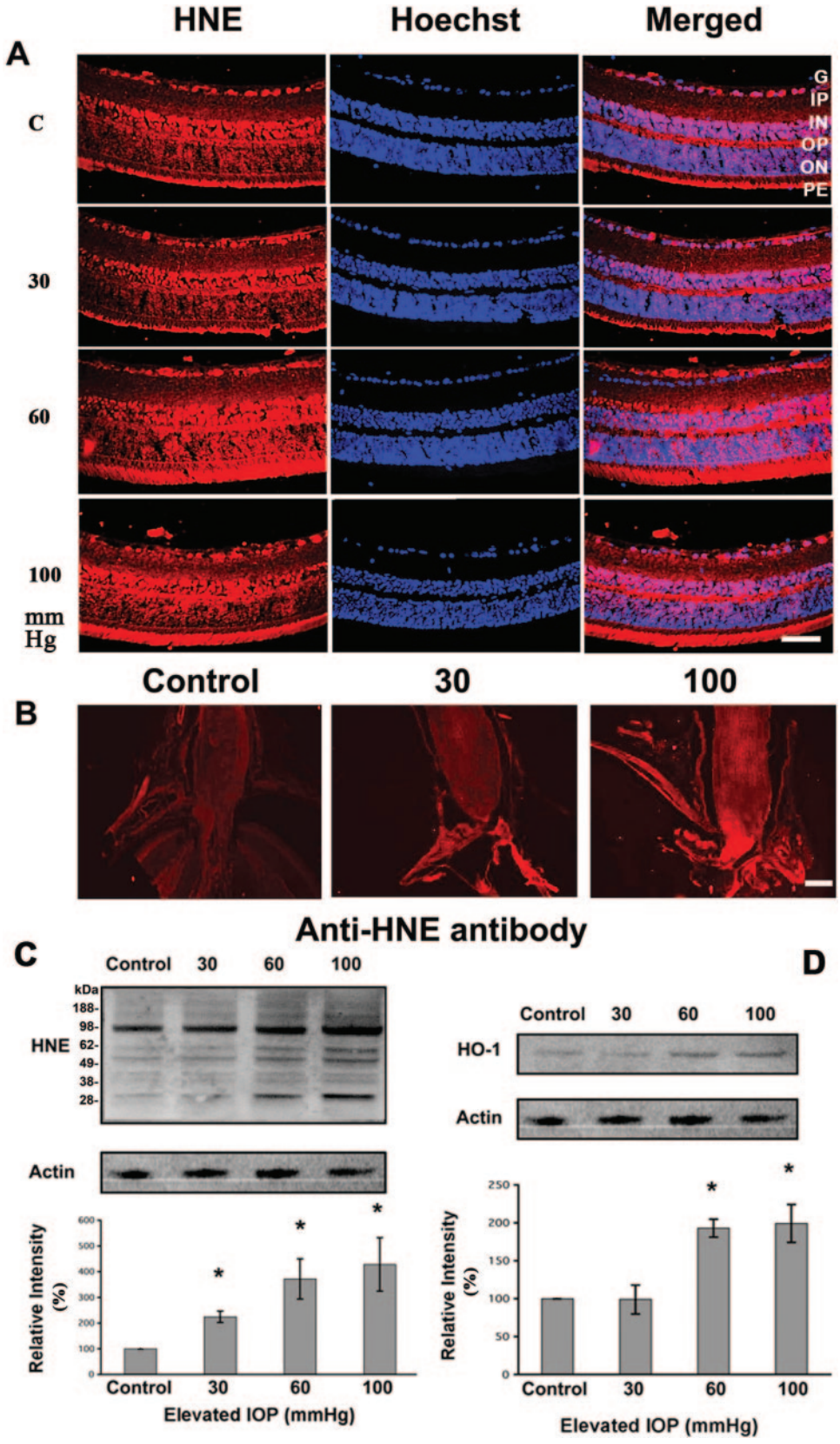

FIGURE 6. HNE adducts and HO-1 expression are increased with different hydrostatic pressure treatment in a mouse acute IOP model. (**A**) Immunohistostaining showed that HNE adducts were increased in retina samples from mouse eyes treated with different hydrostatic pressure. Scale bar, 20 μm. (**B**) Optic nerve samples from mouse eyes treated with different hydrostatic pressure showed increased level of HNE adducts. Scale bar, 0.2 mm. (**C**) Western blot showed that HNE adduct formation in retina samples increased significantly under pressure of 30 mm Hg, 60 mm Hg, and 100 mm Hg in the mouse acute IOP elevation model. Values are mean ± SD of three independent experiments. (**D**) Western blot showed that the HO-1 level in retina samples increased significantly under pressure of 30 mm Hg, 60 mm Hg, and 100 mm Hg in the mouse acute IOP elevation model. Values are mean ± SD of three independent experiments. G, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear layer; OP, outer plexiform layer; ON, outer nuclear layer; PE, pigment layer. $^*P < 0.05$.

lipids.[38] Alternatively, oxidative stress in RGCs may be accompanied by other stresses arising from axon projection through the deformed optic nerve head. Further investigations will be needed to clarify this issue.

The present study focused on early markers of oxidative stress in pressure treatments. These treatments also may induce changes in the level and activity of the cellular antioxidative components, including glutathione-*S*-transferase, SOD,

and catalase. In addition, protein carboxylation and advanced glycation end product formation may be increased. These issues will be addressed in future studies. Based on the present data, we propose that when IOP was increased, the cell membrane was altered through an unknown mechanism that enhanced cellular oxidative stress. This increased the rate of cellular damage. When the rate of damage increased beyond the ability of the RGCs to repair themselves, the resultant accumulation of damage eventually contributed to RGC death.

Pressure-induced oxidative damage occurs in many clinically relevant conditions besides glaucoma, such as intracranial pressure elevation in hydrocephalus,[39] traumatic brain injury,[40] bacterial meningitis,[41] stroke,[42] and intracranial hypertension.[43,44] Oxidative stress induced by hydrostatic pressure plays an important role in the pathophysiology of these diseases. In this study, we directly showed that single acute elevations of pressure could induce significant oxidative damage after even a brief time in RGCs in vitro and in vivo. This could be a contributing mechanism for pressure-induced neuron death in many pathologic conditions, including glaucoma. These early changes suggest the potential use of antioxidants to treat or prevent pressure-induced neuronal damage in humans.

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References

1. Weinreb RN, Khaw PT. Primary open-angle glaucoma. *Lancet*. 2004;363:1711–1720.
2. Anderson DR, Hendrickson A. Effect of intraocular pressure on rapid axoplasmic transport in monkey optic nerve. *Invest Ophthalmol*. 1974;13:771–783.
3. Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res*. 1995;61:33–44.
4. McKinnon SJ, Lehman DM, Kerrigan-Baumrind LA, et al. Caspase activation and amyloid precursor protein cleavage in rat ocular hypertension. *Invest Ophthalmol Vis Sci*. 2002;43:1077–1087.
5. Tatton NA, Tezel G, Insolia SA, Nandor SA, Edward PD, Wax MB. In situ detection of apoptosis in normal pressure glaucoma: a preliminary examination. *Surv Ophthalmol*. 2001;45(suppl 3): S268–S272.
6. Agar A, Li S, Agarwal N, Coroneo MT, Hill MA. Retinal ganglion cell line apoptosis induced by hydrostatic pressure. *Brain Res*. 2006; 1086:191–200.
7. Agar A, Yip SS, Hill MA, Coroneo MT. Pressure related apoptosis in neuronal cell lines. *J Neurosci Res*. 2000;60:495–503.
8. Ju WK, Liu Q, Kim KY, et al. Elevated hydrostatic pressure triggers mitochondrial fission and decreases cellular ATP in differentiated RGC-5 cells. *Invest Ophthalmol Vis Sci*. 2007;48:2145–2151.
9. Nickells RW. Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death. *Surv Ophthalmol*. 1999;43(suppl 1):S151–S161.
10. Osborne NN, Wood JP, Chidlow G, Bae JH, Melena J, Nash MS. Ganglion cell death in glaucoma: what do we really know? *Br J Ophthalmol*. 1999;83:980–986.
11. Moreno MC, Campanelli J, Sande P, Sanez DA, Keller Sarmiento MI, Rosenstein RE. Retinal oxidative stress induced by high intraocular pressure. *Free Radic Biol Med*. 2004;37:803–812.
12. Ferreira SM, Lerner SF, Brunzini R, Evelson PA, Llesuy SF. Oxidative stress markers in aqueous humor of glaucoma patients. *Am J Ophthalmol*. 2004;137:62–69.
13. Gherghel D, Griffiths HR, Hilton EJ, Cunliffe IA, Hosking SL. Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. *Invest Ophthalmol Vis Sci*. 2005;46: 877–883.
14. Izzotti A, Sacca SC, Cartiglia C, De Flora S. Oxidative deoxyribonucleic acid damage in the eyes of glaucoma patients. *Am J Med*. 2003;114:638–646.
15. Chong ZZ, Li F, Maiese K. Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog Neurobiol*. 2005;75:207–246.
16. Lehtinen MK, Yuan Z, Boag PR, et al. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell*. 2006;125:987–1001.
17. Liu Q, Raina AK, Smith MA, Sayre LM, Perry G. Hydroxynonenal, toxic carbonyls, and Alzheimer disease. *Mol Aspects Med*. 2003; 24:305–313.
18. Sayre LM, Zelasko DA, Harris PL, Perry G, Salomon RG, Smith MA. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J Neurochem*. 1997;68: 2092–2097.
19. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog Lipid Res*. 2003;42:318–343.
20. Elbirt KK, Bonkovsky HL. Heme oxygenase: recent advances in understanding its regulation and role. *Proc Assoc Am Physicians*. 1999;111:438–447.
21. Schipper HM. Heme oxygenase-1: transducer of pathological brain iron sequestration under oxidative stress. *Ann NY Acad Sci*. 2004; 1012:84–93.
22. Smith MA, Kutty RK, Richey PL, et al. Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. *Am J Pathol*. 1994;145:42–47.
23. Krishnamoorthy RR, Agarwal P, Prasanna G, et al. Characterization of a transformed rat retinal ganglion cell line. *Brain Res Mol Brain Res*. 2001;86:1–12.
24. Moorhouse AJ, Li S, Vickery RM, Hill MA, Morley JW. A patch-clamp investigation of membrane currents in a novel mammalian retinal ganglion cell line. *Brain Res*. 2004;1003:205–208.
25. Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol*. 2000;1:120–129.
26. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. 1991;11:81–128.
27. Nadkarni DV, Sayre LM. Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem Res Toxicol*. 1995;8:284–291.
28. Kamalvand G, Pinard G, Ali-Khan Z. Heme-oxygenase-1 response, a marker of oxidative stress, in a mouse model of AA amyloidosis. *Amyloid*. 2003;10:151–159.
29. Maines MD. The heme oxygenase system: update 2005. *Antioxid Redox Signal*. 2005;7:1761–1766.
30. Kim CI, Lee SH, Seong GJ, Kim YH, Lee MY. Nuclear translocation and overexpression of GAPDH by the hyper-pressure in retinal ganglion cell. *Biochem Biophys Res Commun*. 2006;341:1237–1243.
31. Sappington RM, Chan M, Calkins DJ. Interleukin-6 protects retinal ganglion cells from pressure-induced death. *Invest Ophthalmol Vis Sci*. 2006;47:2932–2942.
32. Chen K, Gunter K, Maines MD. Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. *J Neurochem*. 2000;75:304–313.
33. Zarkovic K. 4-Hydroxynonenal and neurodegenerative diseases. *Mol Aspects Med*. 2003;24:293–303.
34. Lin D, Lee HG, Liu Q, Perry G, Smith MA, Sayre LM. 4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal. *Chem Res Toxicol*. 2005;18:1219–1231.
35. Iles KE, Dickinson DA, Wigley AF, Welty NE, Blank V, Forman HJ. HNE increases HO-1 through activation of the ERK pathway in pulmonary epithelial cells. *Free Radic Biol Med*. 2005;39:355–364.
36. Fazio VM, Rinaldi M, Ciafre S, Barrera G, Farace MG. Control of neoplastic cell proliferation and differentiation by restoration of 4-hydroxynonenal physiological concentrations. *Mol Aspects Med*. 1993;14:217–228.
37. Harada C, Nakamura K, Namekata K, et al. Role of apoptosis signal-regulating kinase 1 in stress-induced neural cell apoptosis in vivo. *Am J Pathol*. 2006;168:261–269.

38. Sanvicens N, Gomez-Vicente V, Masip I, Messeguer A, Cotter TG. Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J Biol Chem*. 2004;279:39268–39278.
39. Farooqui AA, Ong WY, Horrocks LA. Biochemical aspects of neurodegeneration in human brain: involvement of neural membrane phospholipids and phospholipases A2. *Neurochem Res*. 2004;29:1961–1977.
40. Socci DJ, Bjugstad KB, Jones HC, Pattisapu JV, Arendash GW. Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. *Exp Neurol*. 1999;155:109–117.
41. Yang XF, Liu WG, Shen H, et al. Correlation of cell apoptosis with brain edema and elevated intracranial pressure in traumatic brain injury. *Chin J Traumatol*. 2005;8:96–100.
42. Koedel U, Pfister HW. Oxidative stress in bacterial meningitis. *Brain Pathol*. 1999;9:57–67.
43. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation*. 2004;109:2357–2362.
44. Hall SR, Wang L, Milne B, Hong M. Left ventricular dysfunction after acute intracranial hypertension is associated with increased hydroxyl free radical production, cardiac ryanodine hyperphosphorylation, and troponin I degradation. *J Heart Lung Transplant*. 2005;24:1639–1649.
45. Hall SR, Wang L, Milne B, Hong M. Mannitol but not dantrolene prevents myocardial dysfunction following intra-cranial hypertension in rats. *Am J Transplant*. 2005;5:2862–2869.