Reactivation of Optic Nerve Head Astrocytes by TGF-β2 and H_2O_2 Is Accompanied by Increased Hsp32 and Hsp47 Expression

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Purpose. Histologic studies have previously demonstrated increased expression of small heat shock proteins (Hsps) in reactive optic nerve head (ONH) astrocytes of patients with glaucoma. Transforming growth factor (TGF)-β2 and hydrogen peroxide (H_2O_2) are known to induce ONH astrocyte reactivation. The goal of the present study was to determine whether new potentially involved Hsps, such as Hsp32, -47, -60, and -70, are expressed in the reactivation process of ONH astrocytes mediated by TGF-β2 and H_2O_2.

Methods. Cultured human ONH astrocytes were treated with 1.0 ng/mL TGF-β2 for up to 48 hours. In addition, the cells were exposed to 100, 200, or 400 μM H_2O_2 for 1 hour. Expression of Hsp32, -47, -60, and -70 was examined by immunohistochemistry, real-time PCR, and Western blot analyses.

Results. Treatment with TGF-β2 increased Hsp32 after 4 and 6 hours, whereas Hsp47 was upregulated after treatment with TGF-β2 for 12, 24, and 48 hours. Exposure of the cells to H_2O_2 could increase both Hsp32 and -47. No significant effects on the expression of Hsp60 and -70 were observed after treatment of the cells with TGF-β2 or H_2O_2.

Conclusions. TGF-β2 increased Hsp32 after short-term treatment and Hsp47 after longer periods in cultured human ONH astrocytes. H_2O_2 increased both Hsp32 and -47 levels. No effects on Hsp60 and -70 levels were induced by TGF-β2 and H_2O_2. These results may provide further insights into the cellular stress responses of reactive human ONH astrocytes. Further extensive studies are needed to examine the potential roles of Hsps in the ONH of glaucomatous eyes. (*Invest Ophthalmol Vis Sci.* 2009;50:1707–1717) DOI:10.1167/iovs.08-1961

From histologic studies it is known that glaucomatous changes of the optic nerve head (ONH) are associated with a reactivation of astrocytes,[1–4] which is characterized by increased expression of glial fibrillary acidic protein (GFAP) and neural cell adhesion molecule (NCAM).[4–6] Subsequent histologic experiments on glaucomatous optic neuropathy demonstrated that reactive ONH astrocytes are also associated with increased production of small heat shock proteins (Hsps) such as αB-crystallin[7] and Hsp27.[8] Hsps represent a large group of proteins, which stabilize protein folding and aggregation.[9,10] As molecular chaperones, they are known to be upregulated by various forms of stress.[11–15] In various neurodegenerative diseases, increased Hsp expression has been observed in reactive astrocytes.[11,16,17] In vitro studies with reactive human ONH astrocytes showed an induced expression of the small Hsps αB-crystallin and Hsp27 after exposure to elevated hydrostatic pressure.[5] Whether or not the expressions of new potentially involved Hsps such as Hsp32, -47, -60, or -70 are increased in reactive astrocytes of the ONH is still an open question that requires further investigation.

The pathogenesis of primary open-angle glaucoma (POAG) is not only associated with an increased intraocular pressure, but is also characterized by cellular stress factors such as increased transforming growth factor-β2 (TGF-β2)[18–21] and oxidative stress.[22–24] Both TGF-β2[2] and oxidative stress[23] have been suggested to play a role in the reactivation process of human ONH astrocytes of glaucomatous eyes. Results in several experimental studies have suggested that both TGF-β2[25–28] and oxidative stress[29–31] are potent inducers of Hsp upregulation in various cellular systems. In the ocular tissue, TGF-β2 has been shown to augment the synthesis of the small Hsp αB-crystallin in human trabecular meshwork cells[32] and in human ONH astrocytes.[29] Previous data from our laboratory have also demonstrated an upregulation of the small Hsp27 in human ONH astrocytes after TGF-β2 and hydrogen peroxide (H_2O_2) treatment.[33] Until now, it has been unclear which of the potentially involved Hsps, such as Hsp32, -47, -60, and -70, are expressed in reactive human ONH astrocytes. Furthermore, there are no existing data about the factors responsible for the increased expression of Hsps in reactive human ONH astrocytes.

In our study, we investigated the effects of TGF-β2 and H_2O_2 on the expression of Hsps in cultured human ONH astrocytes. Knowledge of the baseline and induced expression of Hsps may provide further insight into the cellular process of reactivation of ONH astrocytes. For Hsp47, we detected a possible involvement in the collagen synthesis and secretion in cultured human ONH astrocytes.

Materials and Methods

Cell Culture

Primary cell cultures of human lamina cribrosa astrocytes were prepared from donor eyes from the eye bank of Ludwig-Maximilians-University (Munich, Germany). Monolayer cultures were established from eyes of five human donors between 56 and 68 years of age. These eyes were obtained 4 to 12 hours postmortem without any history of eye diseases. Methods of securing human tissue were humane, in-

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cluded proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee. Astrocytes of the ONH were prepared, grown, and classified as described previously.[4,6,29] In brief, the eyes were cut equatorially behind the ora serrata, and the ONH was isolated from the neighboring tissues. The ONH was sagittally dissected under a microscope, and the lamina cribrosa was identified. Discs of the lamina cribrosa were prepared by dissection from the pre- and postlaminar region, subsequently cut into three to four explants and placed in 60-mm Petri dishes with 2 mL Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Invitrogen-Gibco, Karlsruhe, Germany), 5 ng/mL human basic pituitary fibroblast growth factor (bFGF; Sigma-Aldrich, Deisenhofen, Germany), 5 ng/mL human platelet-derived growth factor-A chain (PDGF AA; Sigma-Aldrich), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen-Gibco) at 37°C in a 5% CO_2 incubator. To isolate ONH astrocytes, we first plated the primary cell cultures in serum-free astrocytes growth medium (AGM; Cambrex Bio Science, Verviers, Belgium) for 24 hours and then changed to AGM containing 5% FBS.[34] Other cell populations, such as lamina cribrosa cells, failed to attach in serum-free medium and were removed by the change of medium. Subsequently, cultured ONH astrocytes were maintained in DMEM/F-12 with 10% FBS. ONH astrocytes were distinguished from adjacent cells by their morphology and immunohistochemical staining (data not shown).[4,29]

Primary human ONH astrocytes were characterized by positive immunostaining for Pax2 (Abcam, Cambridge, UK), glial fibrillary acidic protein (GFAP; Sigma-Aldrich), neural cell adhesion molecule (NCAM; Serotec, Düsseldorf, Germany), S100 (Invitrogen).[4,6,35–37] Pax2 is a transcription factor labeling the nuclei and is expressed by astrocyte lineage cells in the development of adult human retina and ONH.[4] Positive immunostaining for GFAP depict intermediate filaments of the cytoskeleton of astrocytes.[4,6,35] NCAM is a cell surface adhesion molecule expressed by ONH astrocytes shown as fine granules on the cell surface.[6] S100 represents an astroglia-derived calcium-binding protein involved in the maintenance of homeostasis in neurons and astrocytes.[36,37] Furthermore, primary human ONH astrocytes are distinguished from neighboring cells by negative immunostaining for smooth muscle actin (smA; Dako, Glostrup, Denmark) and A2B5 (Chemicon International, Hampshire, UK).[4,6] smA is a marker of vascular smooth muscle that is absent in astrocyte growth medium.[4] A2B5 is a cell surface marker that appears as patches of stain and labels precursors of oligodendrocytes in the myelinated optic nerve, which were removed from the explants under the phase microscope.[4,6] Among 13 primary cell cultures, only 9 were at least 95% positive for Pax2, GFAP, NCAM, and S100, and negative for smA and A2B5, and therefore were used in this study.[29] Morphologic characteristics distinguished ONH astrocytes, which are larger, star-shaped cells with thin and long processes, from small, flat, polygonal lamina cribrosa cells.[34,38]

To investigate the effects of TGF-β2, we grew second- to fifth-passage astrocytes to confluence in 35-mm Petri dishes in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO_2. At confluence, the cells were washed and incubated overnight in serum-free DMEM/F12. After a 24-hour incubation, this medium was replaced by fresh, serum-free DMEM/F12 supplemented with active TGF-β2 (R&D Systems, Wiesbaden, Germany) to a final concentration of 1.0 ng/mL. Under these conditions, the cells were incubated for 2, 4, 6, 12, 24, or 48 hours. In control cultures, the medium was changed at the same time points, but no TGF-β2 was added.

To test the effects of oxidative stress on astrocytes, we incubated confluent cells of passages 3 to 5 for 24 hours in serum-free DMEM/F12 at 37°C and 5% CO_2. Then, the medium was replaced by fresh serum-free DMEM/F12 medium, and the cells were exposed to 100, 200 or 400 μM hydrogen peroxide (H_2O_2) for 1 hour. After H_2O_2 treatment, the cells were placed in serum-free DMEM/F12 medium for 24 hours. In control cultures, the medium was changed at the same time points but no H_2O_2 was added. The tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) was used to test cell viability before and at the end of treatment and did not reveal any signs of increased cell death in TGF-β2 or H_2O_2-treated cells (data not shown). All experiments were performed at least in triplicate in astrocyte cultures from three different donors.

Immunohistochemistry

Cultured human ONH astrocytes, grown in four-well plastic chamber slides, were treated with TGF-β2 or H_2O_2 as described earlier. After incubation, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) for 15 minutes, and subsequently washed twice with PBS containing 0.1% Triton X-100. Primary incubation with all samples was performed with a rabbit anti-human Hsp32 antibody (Stressgen Bioreagents, Hamburg, Germany), mouse anti-human Hsp47 antibody (Stressgen Bioreagents), goat anti-human Hsp60 antibody (Stressgen Bioreagents), or rabbit anti-human Hsp70 antibody (Stressgen Bioreagents), diluted 1:200 in PBS containing 5% bovine serum albumin (BSA) for 4 hours at room temperature (RT). Control samples were incubated with PBS and 5% BSA but without the primary antibodies. Afterward, the cells were washed three times with PBS, and incubated with fluorescein-conjugated goat anti-rabbit Cy3 antibody, goat anti-mouse Cy3 antibody, or donkey anti-goat Cy3 antibody (diluted 1:500 in PBS; Dianova, Hamburg, Germany) for 1 hour at RT. The cells were then rinsed in PBS, mounted with Kaiser's glycerin jelly (Merck, Darmstadt, Germany) and analyzed by fluorescence microscope (DMR; Leica Microsystems, Wetzlar, Germany). Representative areas were documented on computer (IM 1000 software; Leica Microsystems, Heerbrugg, Switzerland). All experiments were performed at least in triplicate in astrocyte cultures from three different donors.

RNA Isolation and Real-Time PCR

Total RNA was isolated from 10-cm Petri dishes by the guanidium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). The structural integrity of the RNA samples was confirmed by electrophoresis in 1% Tris-acetate-EDTA (TAE)-agarose gels. Yield and purity were determined photometrically. After RNA isolation, mRNA was transcribed to cDNA via reverse transcriptase. This cDNA was then used for specific real-time PCR. Quantification of human Hsp32, -47, -60 and -70 mRNA was performed with specific primers (Table 1) during 40 cycles with a thermocycler (LightCycler System; Roche Diagnostics, Mannheim, Germany). Primers and probes were found with a commercial computer program (ProbeFinder, ver. 2.04; Exiqon, Woburn, MA). The standard curve was obtained from probes of three different untreated human ONH astrocyte cultures. To normalize differences of the amount of total RNA added to each reaction, we simultaneously processed 18S rRNA in the same sample as an internal control. The level of Hsp32, -47, -60, and -70 mRNA was determined as the relative ratio (RR), which was calculated by dividing the levels of mRNA of those Hsps by the level of the 18S rRNA housekeeping gene in the same samples. All experiments were performed at least in triplicate in astrocyte cultures from three donors.

Table 1. Primers Used for Real-Time PCR

Gene Target	Gene Sequence	Gene Position
Hsp32	5′-gggtgatagaagaggccaaga-3′	673–693
	5′-agctcctgcaactcctcaaa-3′	720–739
Hsp47	5′-gcgggctaagagtagaatcg-3′	122–141
	5′-atggccaggaagtggtttg-3′	213–231
Hsp60	5′-tcagtgtgccttgaactctatga-3′	1364–1386
	5′-ttatctaaatcctggagtacaacctg-3′	1430–1455
Hsp70	5′-cagcagacaccagcagaaaa-3′	1739–1758
	5′-cttggatccagcttgagagg-3′	1785–1804
18S rRNA	5′-ctcaacacgggaaacctcac-3′	1348–1367
	5′-cgctccaccaactaagaacg-3′	1438–1457

Protein Extraction and Western Blot Analysis

Cells grown on 35-mm tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in RIPA cell lysis buffer. After centrifugation (19,000*g* for 30 minutes at 4°C) in a microfuge, the supernatants were transferred to fresh tubes and stored at −70°C for future use. The protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Denatured proteins (2 μg) were separated under reducing conditions by electrophoresis on a 5% SDS-polyacrylamide stacking gel and an 8% or 10% SDS-polyacrylamide separating gel. Thereafter, the proteins were transferred onto a polyvinyl difluoride membrane (Roche) with semidry blotting (Hsp32, -47, -60, and -70) or by tank blot (Col1α1) and probed with a rabbit anti-human Hsp32 antibody (Stressgen Bioreagents), mouse anti-human Hsp47 antibody (Stressgen Bioreagents), goat anti-human Hsp60 antibody (Stressgen Bioreagents), rabbit anti-human Hsp70 antibody (Stressgen Bioreagents), or rabbit anti-human Col1α1 antibody (Rockland, Gilbertsville, PA), as described before.[32] These antibodies were used at a dilution of 1:200, respectively. Chemiluminescence was detected with the imager (LAS-1000; RayTest, Pforzheim, Germany). Exposure times ranged between 1 and 20 minutes. Quantification was performed on a computer (AIDA software; RayTest). All experiments were performed at least in triplicate in astrocyte cultures from three donors.

Transfection of siRNA

Cultured human ONH astrocytes were seeded in six-well plates at a density of 8×10^5 cells per well and grown for 24 hours in DMEM/F-12 with 10% FBS. The astrocytes were transfected with an Hsp47 specific siRNA (c = 0.1 μM; On-Target*plus* siRNA, cat. no. J-011230-05; Dharmacon, Lafayette, CO) using a transfection reagent (Dharmafect1; Dharmacon) according to the manufacturer's instructions. After 24 hours, the medium was changed to serum-free medium containing 1.0 ng/mL TGF-β2 or to regular culture medium, and the astrocytes were treated for another 12 hours. Corresponding control astrocytes were treated under the same conditions without the addition of siHsp47 for the transfection procedure. After treatment, the astrocytes were harvested for RNA isolation, and the medium of the astrocytes was collected for protein extraction. Medium samples were concentrated 40× by centrifugation (Centricon columns; Millipore, Billerica, MA). Hsp47 knockdown efficiency was tested on RNA samples by semiquantitative PCR using Hsp47 primers. The effect of Hsp47 knockdown on intracellular type I collagen accumulation was evaluated by semiquantitative PCR and Western blot experiments.

Semiquantitative PCR

Gene-specific PCRs were performed in a total volume of 25 μL containing 5 μL cDNA, 2.5 μL 10× PCR buffer (Mg^{2+} free), 0.5 μL 10 mM dNTP mix, 0.5 μL 10 μM primer (forward and reverse each), 0.75 μL 50 mM $MgCl_2$, 0.1 μL (5 U/μL) *Taq* polymerase (all from Invitrogen), and H_2O. The 25-μL PCR steps were 30 seconds of denaturation at 96°C, 30 seconds of annealing, and 45 seconds of extension at 72°C, followed by an end-extension step of 5 minutes at 72°C after the last cycle. The primers for Hsp47 were (forward, 5′-TTCTGCCTCCTGGAGGCG-3′; reverse, 5′-CGCTCAGCACTGCCTTGG-3′, position, 257-274; product size, 267; annealing temperature, 58.0°C), for Col1α1 were (forward, 5′-GATGGACTCAACGGTCTCC-3′; reverse 5′-CCTTGGGGTTCTTGCTGATG-3′; position, 3576-4034; product size, 458; annealing temperature, 57.0°C), and for GAPDH (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′; position, 6-231; product size, 225; annealing temperature, 57.0°C). The functionality of primers was tested on cDNAs obtained from different tissues before the experiments to exclude false-negative results (data not shown). The band intensity was measured in light units with an imaging workstation (Lumi-Imager; Roche, Mannheim, Germany). Quantification was performed with the accompanying software (Lumi-Analyst software, Roche). The final amount of PCR product was expressed as the ratio of the Hsp47 or the Col1α1 gene amplified to that of the GAPDH gene.

Results

Characterization of Human Astrocyte Cultures

All human ONH astrocyte cultures were characterized by negative immunohistochemical staining for smA and A2B5, which excluded potential vascular smooth muscle cells and oligodendrocytes and astrocytes of the myelinated optic nerve (Fig. 1). Furthermore, all cell cultures showed positive immunohistochemical staining for Pax2, GFAP, NCAM-1, and S100, identifiers of reactive astrocyte cultures of the ONH (Fig. 1).

Effects of TGF-β2 on Hsp32 Expression

Human ONH astrocytes were treated with 1.0 ng/mL TGF-β2 for 2, 4, 6, 12, 24, and 48 hours (Fig. 2). By immunohistochemical staining, treatment with TGF-β2 for 4 hours showed a maximum increase of Hsp32 expression (Fig. 2B) compared with untreated control cells (Fig. 2A). Since immunohistochemistry is not a reliable quantification method, we also performed real-time PCR and Western blot analysis. The signals generated by real-time PCR analysis in untreated control cells were set to 100% (Figs. 2C, 2E). Keeping in serum-free medium had no influence on Hsp expression for all investigated time periods. There was an upregulation of Hsp32 mRNA expression after TGF-β2 treatment for 4 (2.1 ± 0.7-fold) and 6 (2.6 ± 0.3-fold) hours compared with untreated control cells (Fig. 2C). However, the Hsp32 mRNA level did not

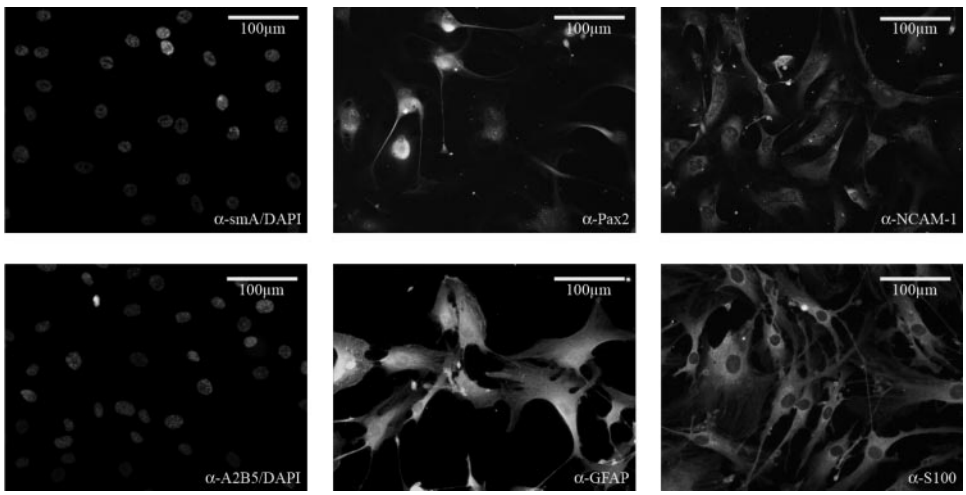

Figure 1. Immunofluorescence staining of the cultured ONH astrocytes. Cells were characterized by negative staining for smA and A2B5 (shown with DAPI nuclear counterstaining) and positive staining for Pax2, GFAP, NCAM, and S100.

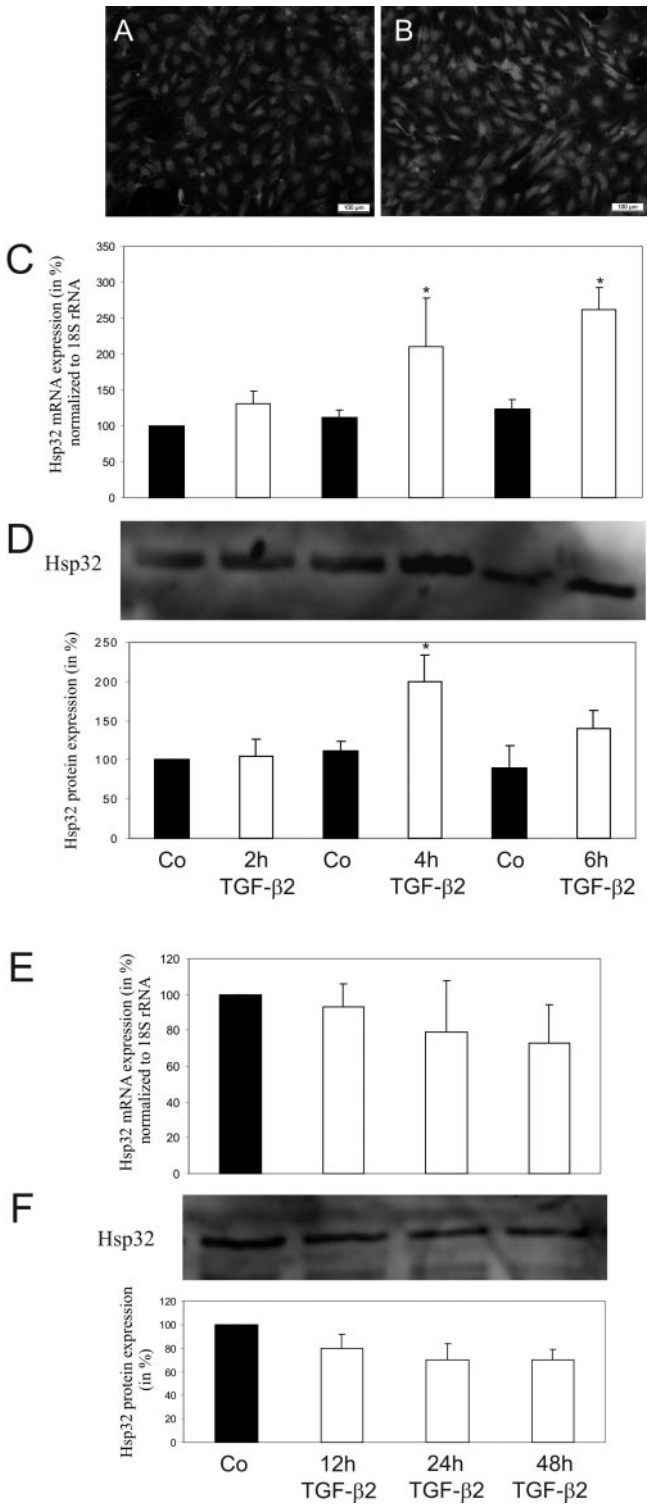

FIGURE 2. TGF-β2 increased the expression of Hsp32. (**A**) By immunohistochemistry, basal levels of Hsp32 staining were observed in untreated control ONH astrocytes. (**B**) Treatment with 1.0 ng/mL TGF-β2 for 4 hours increased the expression of Hsp32 compared with untreated control levels. (**C**, **E**) Real-time PCR analysis of TGF-β2-induced Hsp32 mRNA expression. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp32 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**, **F**) For Western blot analysis of Hsp32 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp32 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*$P < 0.05$). Co, control.

change after 12 (0.9 ± 0.1-fold), 24 (0.8 ± 0.3-fold), and 48 (0.7 ± 0.2-fold) hours of TGF-β2 treatment compared with untreated control cells (Fig. 2E).

At the protein level, Western blot analysis revealed an upregulation of Hsp32 expression after treatment with TGF-β2 for 4 (2.0 ± 0.3-fold) and 6 (1.4 ± 0.2-fold) hours compared with untreated control cells (Fig. 2D). In contrast, treatment with TGF-β2 for 2 (1.1 ± 0.2-fold), 12 (0.8 ± 0.1-fold), 24 (0.7 ± 0.1-fold), and 48 (0.7 ± 0.1-fold) hours showed no marked changes of Hsp32 protein expression (Figs. 2D, 2F).

Effects of H_2O_2 on Hsp32 Expression

Cultured human ONH astrocytes were treated with 100, 200, and 400 μM hydrogen peroxide (H_2O_2) for 1 hour (Fig. 3). Immunohistochemical staining demonstrated maximum Hsp32 increase after exposure of cells to 200 μM H_2O_2 for 1 hour (Fig. 3B) compared with untreated control cells (Fig. 3A). Real-time PCR analysis was performed 24 hours after stress exposure (Fig. 3C). The signals generated in untreated control cells were set to 100% (Fig. 3C). Exposure to 200 and 400 μM H_2O_2 for 1 hour increased the mRNA expression of Hsp32 1.9 ± 0.5- and 1.7 ± 0.7-fold compared with untreated control levels (Fig. 3C).

At the protein level, Western blot analysis showed an increase of Hsp32 protein amount after treatment with 200 μM H_2O_2 (1.8 ± 0.4-fold), and 400 μM H_2O_2 (1.6 ± 0.3-fold) compared with untreated control levels (Fig. 3D).

Effects of TGF-β2 on Hsp47 Expression

Immunohistochemical staining revealed maximum Hsp47 expression after 48 hours of TGF-β2 treatment (Fig. 4B) compared with untreated control levels (Fig. 4A). Expression of Hsp47 mRNA expression was upregulated by prolonged TGF-β2 treatment (Fig. 4E). Although the Hsp47 levels rarely changed up to 6 hours of treatment, they increased by 1.6 ± 0.2-fold after 12, 2.1 ± 0.5-fold after 24, and 2.3 ± 0.5-fold after 48 hours (Figs. 4C, 4E).

At the protein level, no significant changes of Hsp47 protein expression was observed by Western blot analysis after short-time TGF-β2 treatment for 2 (1.0 ± 0.1-fold), 4 (0.9 ± 0.1-fold), and 6 (0.8 ± 0.1-fold) hours (Fig. 4D). On the other hand, prolonged treatment with TGF-β2 for 12, 24, and 48 hours increased the Hsp47 protein levels by 1.6 ± 0.2-, 2.0 ± 0.3-, and 2.2 ± 0.1-fold compared with untreated control levels (Fig. 4F).

Effects of H_2O_2 on Hsp47 Expression

Immunohistochemical staining demonstrated maximum Hsp47 increase after exposure of cells to 200 μM H_2O_2 for 1 hour (Fig. 5B) compared with untreated control levels (Fig. 5A). Treatment with H_2O_2 increased the Hsp47 expression both at the mRNA (Fig. 5C) and protein (Fig. 5D) level. Exposure to 100, 200, and 400 μM H_2O_2 for 1 hour upregulated the Hsp47 mRNA expression by 1.5 ± 0.2, 1.6 ± 0.2, and 1.7 ± 0.3-fold compared with untreated control levels (Fig. 5C). At the protein level, Western blot analysis revealed increased Hsp47 protein expression by 1.5 ± 0.3-, 1.6 ± 0.5-, and 1.5 ± 0.3-fold after exposure of cells to 100, 200, and 400 μM H_2O_2 compared with untreated control levels (Fig. 5D).

Effects of TGF-β2 on Hsp60 Expression

Immunohistochemical staining revealed similar Hsp60 expression after 12 hours of TGF-β2 treatment (Fig. 6B) compared

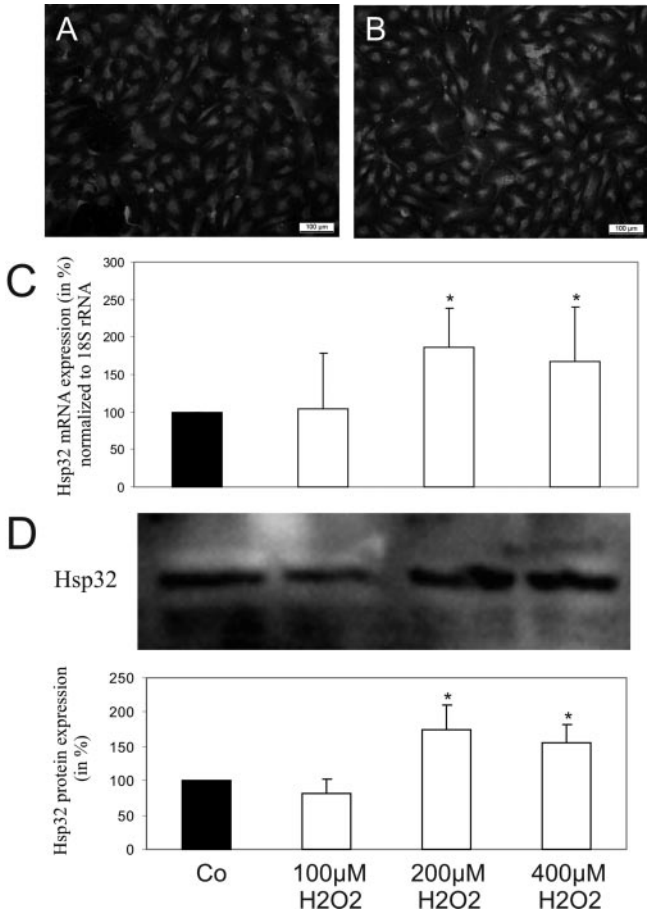

FIGURE 3. H_2O_2 increased the expression of Hsp32. (**A**) By immunohistochemistry, basal levels of Hsp32 staining were observed in untreated control ONH astrocytes. (**B**) Treatment with 200 μM H_2O_2 for 1 hour increased the expression of Hsp32 compared with untreated control levels. (**C**) Real-time PCR analysis of H_2O_2-induced Hsp32 mRNA expression. Results were normalized to 18S rRNA as a reference. The steady state mRNA level of Hsp32 in untreated control cells was considered to be100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**) For Western blot analysis of Hsp32 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp32 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*$P < 0.05$). Co, control.

with untreated control levels (Fig. 6A). There was no effects on Hsp60 mRNA expression for all investigated periods (TGF-β2 for 2 hours, 0.8 ± 0.3-fold; 4 hours, 0.9 ± 0.1-fold; 6 hours, 1.1 ± 0.3-fold; 12 hours, 1.4 ± 0.3-fold; 24 hours, 1.2 ± 0.2-fold; 48 hours, and 0.8 ± 0.4-fold; Figs. 6C, 6E).

Western blot analysis showed no significant effects of TGF-β2 treatment on Hsp60 protein expression compared with untreated control levels (TGF-β2 for 2 hours, 1.0 ± 0.1-fold; 4 hours, 0.9 ± 0.1-fold; 6 hours, 1.0 ± 0.1-fold; 12 hours, 1.1 ± 0.01-fold; 24 hours, 0.8 ± 0.04-fold; and 48 hours, 0.8 ± 0.1-fold; Figs. 6D, 6F).

Effects of H_2O_2 on Hsp60 Expression

Immunohistochemical staining revealed no Hsp60 increase after exposure of cells to 100 μM H_2O_2 for 1 hour (Fig. 7B) compared with untreated control levels (Fig. 7A). An upregulation of Hsp60 mRNA was observed after treatment with 200 μM (1.5 ± 0.2-fold) and 400 μM (1.5 ± 0.2-fold) H_2O_2 for 1 hour compared with untreated control levels (Fig. 7C). There were no significant changes of Hsp60 protein level after all investigated time periods (100 μM H_2O_2, 0.9 ± 0.1-fold; 200 μM H_2O_2, 0.8 ± 0.3-fold; and 400 μM H_2O_2, 0.8 ± 0.2-fold; Fig. 7D).

Effects of TGF-β2 on Hsp70 Expression

Immunohistochemical staining revealed similar Hsp70 expression after 12 hours of TGF-β2 treatment (Fig. 8B) compared with untreated control levels (Fig. 8A). Real-time PCR analysis revealed no significant effects on Hsp70 mRNA expression after TGF-β2 treatment (TGF-β2 for 2 hours, 1.1 ± 0.2-fold; 4 hours, 1.1 ± 0.3-fold; 6 hours, 1.0 ± 0.2-fold; 12 hours, 1.2 ± 0.4-fold; 24 hours, 1.1 ± 0.5-fold; and 48 hours, 0.8 ± 0.6-fold; Figs. 8C, 8E).

Similar results were achieved at the protein levels. Treatment with TGF-β2 for 2 (1.2 ± 0.1-fold), 4 (0.8 ± 0.1-fold), 6 (0.9 ± 0.1-fold), 12 (1.2 ± 0.1-fold), 24 (0.8 ± 0.2-fold) and 48 (0.9 ± 0.1-fold) hours showed no significant effects on Hsp70 protein expression (Figs. 8D, 8F).

Effects of H_2O_2 on Hsp70 Expression

Immunohistochemical staining demonstrated no Hsp70 increase after exposure of cells to 200 μM H_2O_2 for 1 hour (Fig. 9B) compared with untreated control levels (Fig. 9A). Exposure of cells to 100, 200, and 400 μM H_2O_2 demonstrated minor changes in Hsp70 mRNA expression compared with untreated control levels (100 μM H_2O_2, 1.0 ± 0.3-fold; 200 μM H_2O_2, 1.2 ± 0.1-fold; and 400 μM H_2O_2, 0.8 ± 0.2-fold; Fig. 9C). Western blot analysis also revealed no significant effects of H_2O_2 on Hsp70 protein levels (100 μM H_2O_2, 1.1 ± 0.1-fold; 200 μM H_2O_2, 1.2 ± 0.1-fold; and 400 μM H_2O_2, 1.0 ± 0.1-fold) compared with untreated control levels (Fig. 9D).

Involvement of Hsp47 Knockdown on TGF-β2–Induced Col1α1 Expression

The effect of siHsp47 on the TGF-β2–mediated upregulation of Col1α1 was analyzed by semiquantitative PCR and Western blot analysis. By semiquantitative PCR analysis, it can be seen that Hsp47 is effectively knocked down by the use of Hsp47 siRNA (Figs. 10A, 10B). Treatment with 1.0 ng/mL TGF-β2 for 12 hours increased Col1α1 mRNA expression by 1.5 ± 0.2-fold compared with untreated control levels (Figs. 10A, 10B). However, when cells were transfected with Hsp47 siRNA before TGF-β2 treatment, there was no marked change of Col1α1 mRNA expression (1.1 ± 0.1-fold) compared with that in untreated control cells (Figs. 10A, 10B).

To examine the effect of siHsp47 on the Col1α1 secretion in TGF-β2-treated astrocytes, we conducted Western blot analyses on the media of treated cells (Figs. 10C, 10D). Treatment with TGF-β2 for 12 hours could induce an increased extracellular Col1α1 protein secretion into the media by 1.8 ± 0.2-fold compared with untreated control levels, whereas the media of siHsp47-transfected astrocytes treated with TGF-β2 showed only a 1.2 ± 0.1-fold expression of Col1α1 protein compared with untreated control levels (Figs. 10C, 10D).

DISCUSSION

Under neuropathologic conditions, increased synthesis of heat shock proteins (Hsps) in reactive astrocytes may provide a protective cellular response to stressful events.[11,16,17] Until now, little is known about the expression of Hsps in reactive human ONH astrocytes, which may play a role in ocular diseases such as in glaucomatous neurodegeneration of the optic

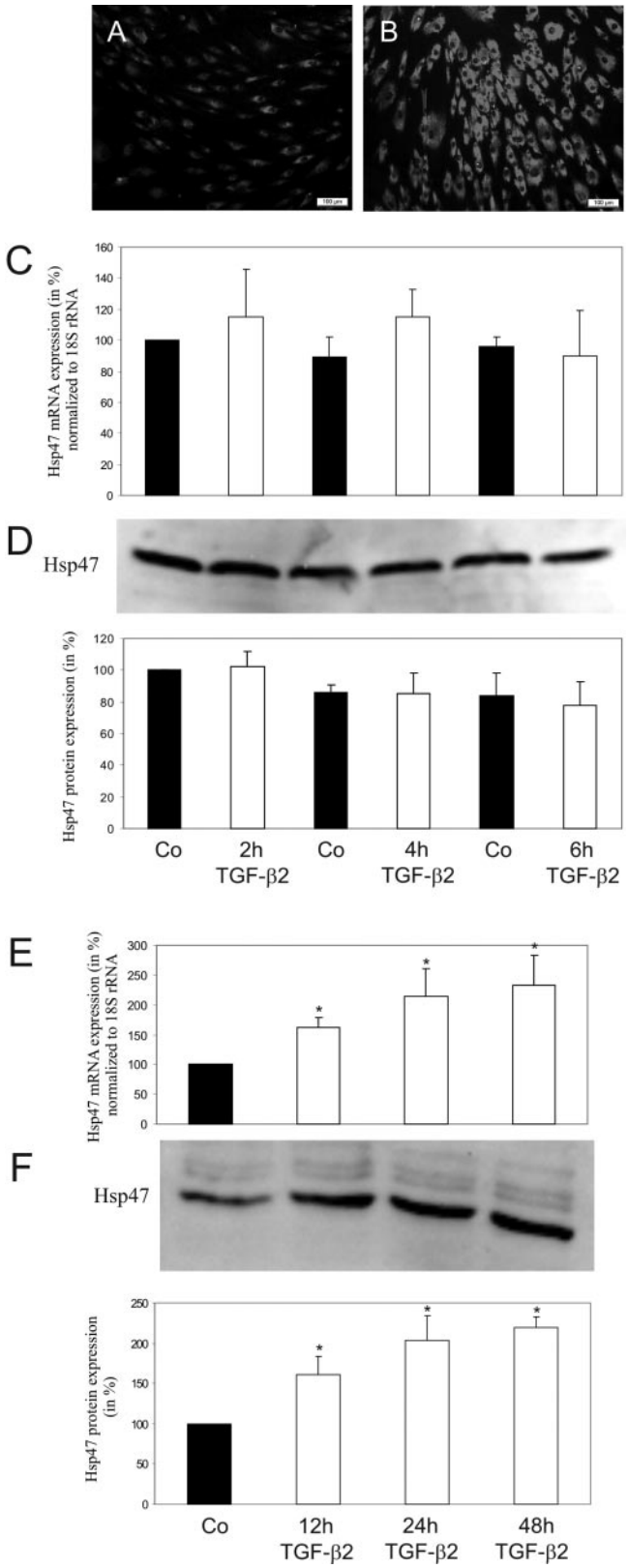

FIGURE 4. TGF-β2 increased the expression of Hsp47. (**A**) By immunohistochemistry, basal levels of Hsp47 staining were observed in untreated control ONH astrocytes. (**B**) Treatment with 1.0 ng/mL TGF-β2 for 48 hours increased the expression of Hsp47 compared with untreated control levels. (**C**, **E**) Real-time PCR analysis of TGF-β2-induced Hsp47 mRNA expression. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp47 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**, **F**) For Western blot analysis of Hsp47 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp47 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*$P < 0.05$). Co, control.

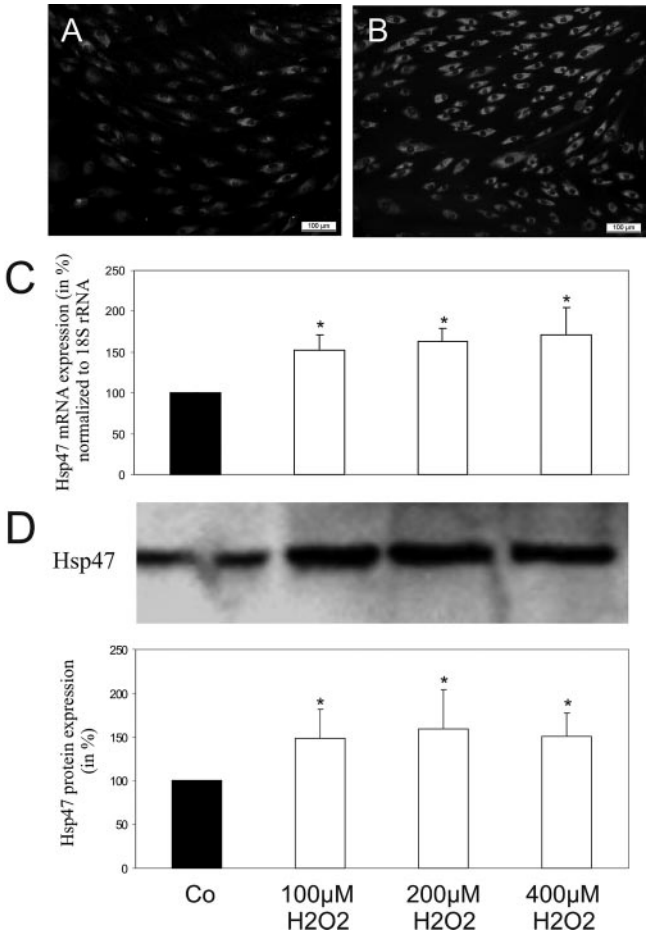

FIGURE 5. H_2O_2 increased the expression of Hsp47. (**A**) By immunohistochemistry, basal levels of Hsp47 staining were observed in untreated control ONH astrocytes. (**B**) Treatment with 200 μM H_2O_2 for 1 hour increased the expression of Hsp47 compared with untreated control levels. (**C**) Real-time PCR analysis of H_2O_2-induced Hsp47 mRNA expression. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp47 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**) For Western blot analysis of Hsp47 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp47 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*$P < 0.05$). Co, control.

nerve. TGF-β2[2,29] and H_2O_2[23] have been used to induce reactivation of astrocytes. In this study, we examined the effects of these two reactivation inducing factors on the expression of Hsp32, -47, -60, and -70 in cultured human ONH astrocytes.

Hsp32 is known as the stress-inducible form of the enzyme heme oxygenase (HO)-1, which catabolizes the pro-oxidant heme to biliverdin, free iron, and carbon monoxide.[11] The

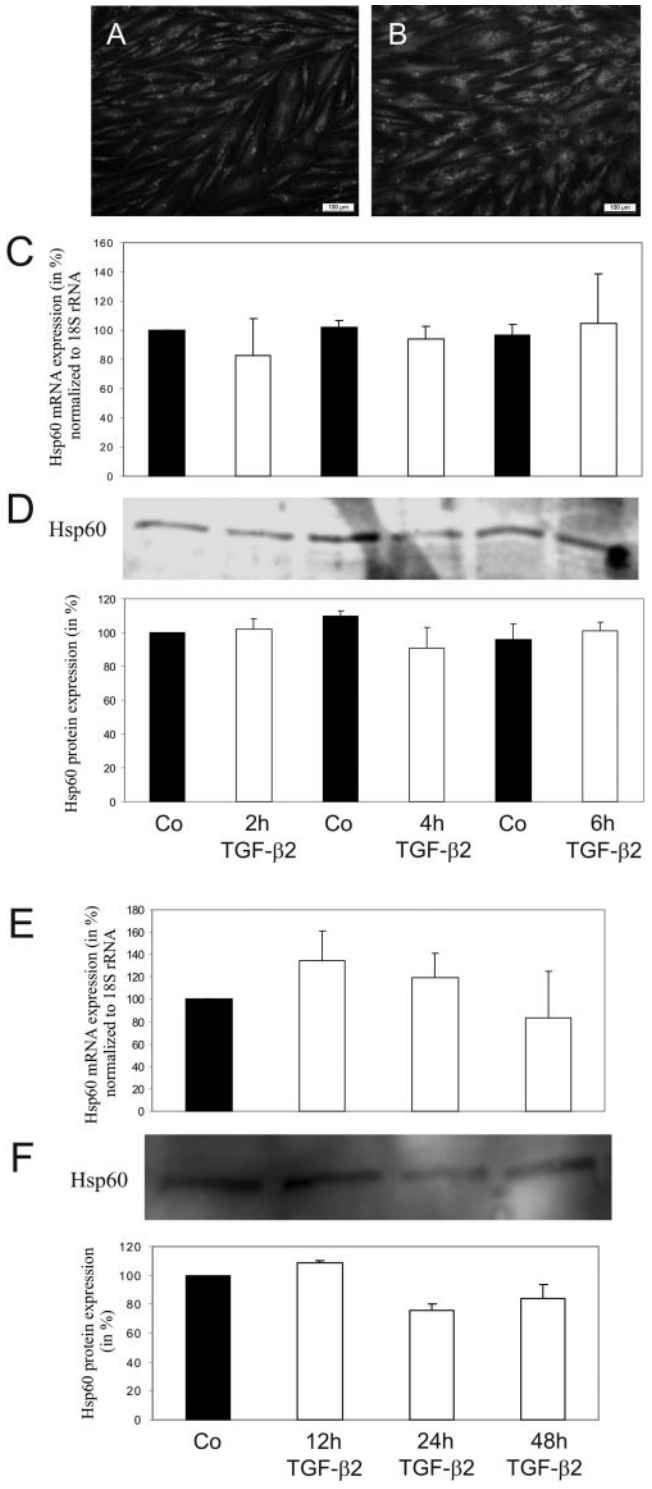

FIGURE 6. TGF-β2 had no effect on Hsp60 expression. (**A**) By immunohistochemistry, basal levels of Hsp32 staining were observed in untreated control ONH astrocytes. (**B**) TGF-β2 treatment did not induce the expression of Hsp60 compared with untreated control levels. (**C**, **E**) Real-time PCR analysis of Hsp60 mRNA expression after TGF-β2 treatment. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp60 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**, **F**) For Western blot analysis of Hsp60 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp60 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors. Co, control.

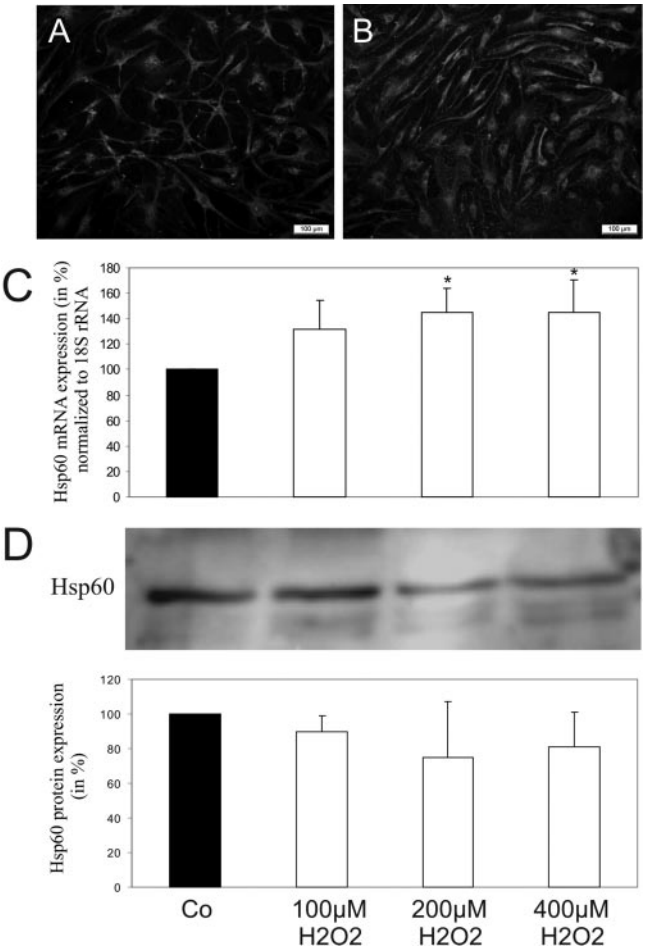

FIGURE 7. H_2O_2 had no influence on Hsp60 expression. (**A**) By immunohistochemistry, basal levels of Hsp60 staining were observed in untreated control ONH astrocytes. (**B**) H_2O_2 exposure did not induce Hsp60 expression compared with untreated control levels. (**C**) Real-time PCR analysis Hsp60 mRNA expression after H_2O_2 exposure. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp60 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**) For Western blot analysis of Hsp60 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp60 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors (*$P < 0.05$). Co, control.

Hsp32 gene is explicitly sensitive to stress stimuli including TGF-β2 and H_2O_2.[39,40] In our experiments, both TGF-β2 and H_2O_2 were able to upregulate Hsp32 mRNA and protein expression. Hsp32 expression was increased after short-time treatment with TGF-β2 for 4 and 6 hours and after exposure of cells to H_2O_2 for 1 hour. In contrast, prolonged treatment with TGF-β2 for 12, 24, and 48 hours demonstrated no significant effects on Hsp32 mRNA and protein expression. These results are consistent with in vitro studies on cerebral astrocytes, which showed maximally increased mRNA and protein Hsp32 levels within 2 to 4 hours after stress exposure and reached

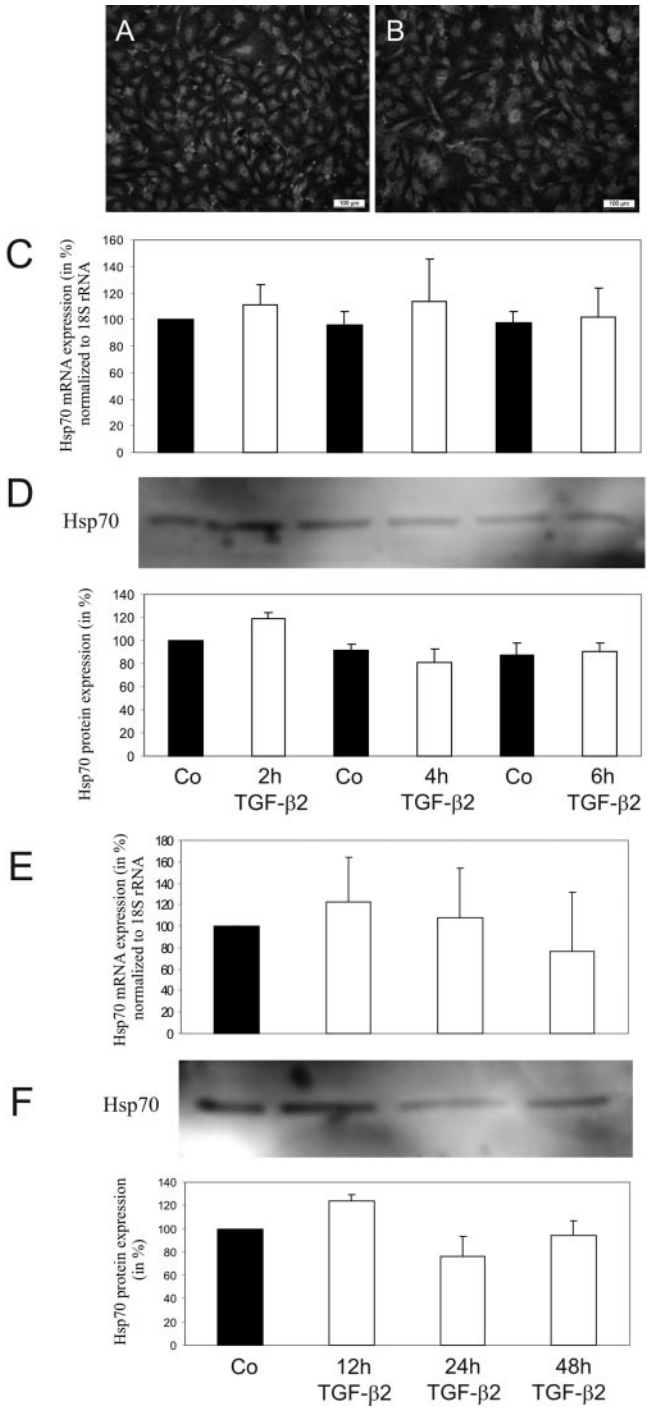

FIGURE 8. TGF-β2 had no effect on Hsp70 expression. (**A**) By immunohistochemistry, basal levels of Hsp70 staining were observed in untreated control ONH astrocytes. (**B**) TGF-β2 treatment did not induce the expression of Hsp70 compared with untreated control levels. (**C**, **E**) Real-time PCR analysis of Hsp70 mRNA expression after TGF-β2 treatment. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp70 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**, **F**) For Western blot analysis of Hsp70 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp70 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors. Co, control.

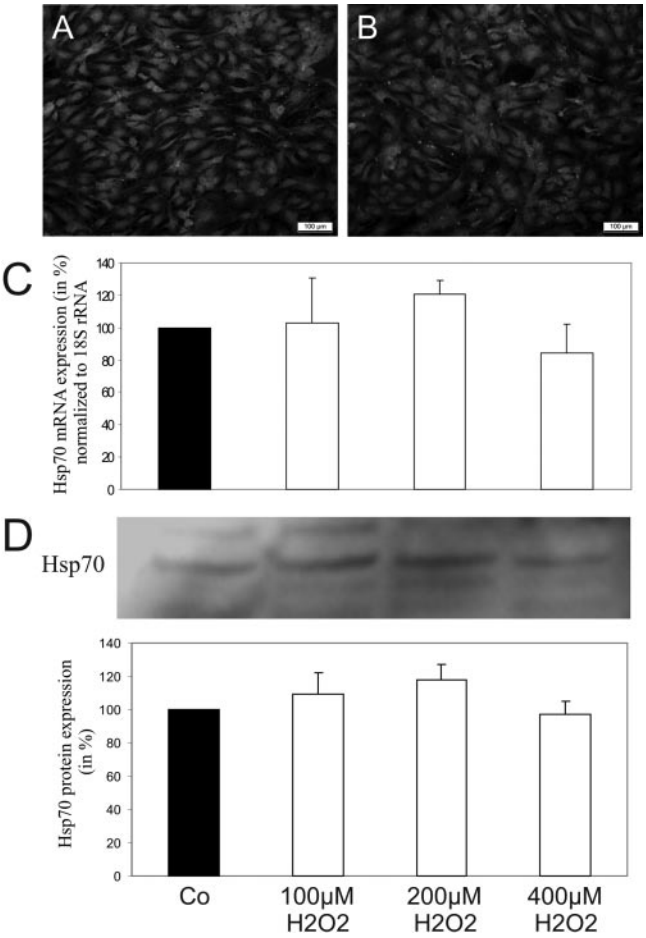

FIGURE 9. H_2O_2 had no influence on Hsp70 expression. (**A**) By immunohistochemistry, basal levels of Hsp70 staining were observed in untreated control ONH astrocytes. (**B**) H_2O_2 exposure did not induce Hsp70 expression compared with untreated control levels. (**C**) Real-time PCR analysis Hsp70 mRNA expression after H_2O_2 exposure. Results were normalized to 18S rRNA as the reference. The steady state mRNA level of Hsp70 in untreated control cells was considered to be 100%. Results are given as the mean ± SD of nine experiments with three different cell cultures from different donors. (**D**) For Western blot analysis of Hsp70 protein expression, lysates containing approximately equal amounts of protein (2 μg) were separated by SDS-PAGE and blotted for immunochemical detection of Hsp70 content. Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of nine experiments with three different cell cultures from different donors. Co, control.

basal levels within 8 hours after stress exposure.[41,42] This early increase in Hsp32 expression, which subsided after prolonged TGF-ß2 exposure up to 48 hours, may indicate that Hsp32 is explicitly a marker of early stress response. Increased production of Hsp32 has been implied in the cellular protection against oxidative stress and may play an important role in antioxidant defense responses.[42–44] It is overexpressed in several neurodegenerative conditions including Alzheimer and Parkinson disease.[39,45,46] Whether or not Hsp32 is increasingly expressed in reactive ONH astrocytes in glaucomatous optic neuropathy awaits further investigation.

TGF-β2[26,47–49] and oxidative stress[50,51] are known stress stimuli for Hsp47 induction as observed in various cellular systems. Our experiments revealed a major induction of Hsp47 expression after prolonged treatment with TGF-β2 for 12, 24, and 48 hours, whereas treatment with TGF-β2 for a shorter time did not lead to significant changes of Hsp47 protein

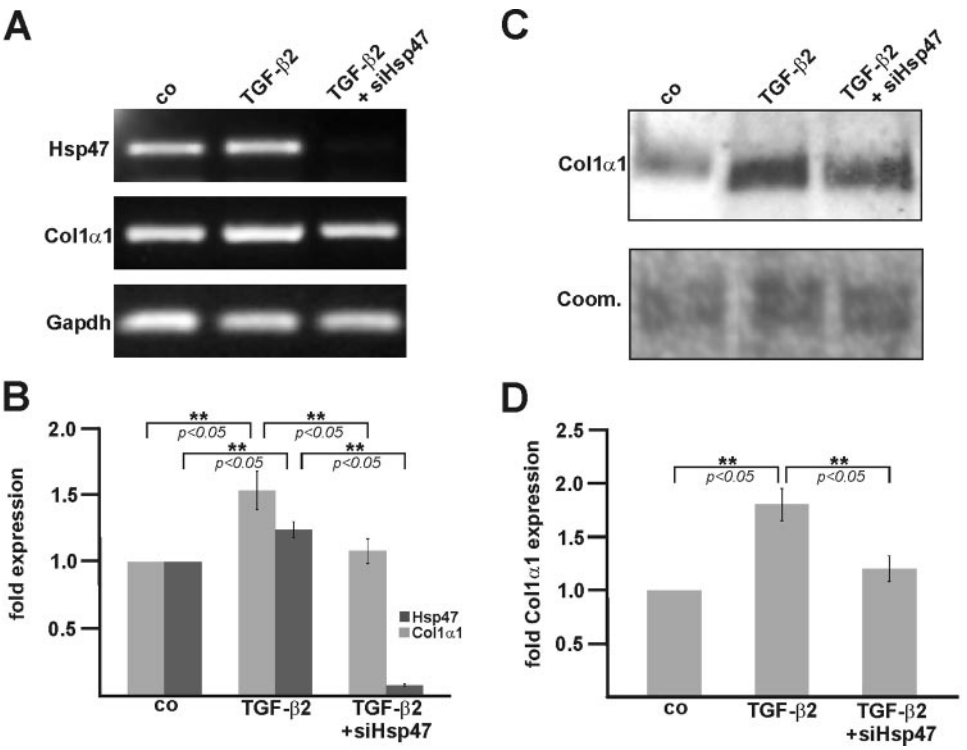

FIGURE 10. (**A**) Semiquantitative PCR analysis of Hsp47 and Col1α1 in untreated control cells, in TGF-β2-treated cells, and in cells transfected with siHsp47 before TGF-β2 treatment. Application of equal cDNA amounts in the PCR was controlled by GAPDH PCR. (**B**) Data represent the mean ratio of the optical density of the Hsp47 or Col1α1 PCR products normalized to the GAPDH amplicon of the same cDNA and are expressed as *x*-fold changes compared with untreated control levels. Results are given as the mean ± SD of six experiments with three different cell cultures from different donors (**$P < 0.05$). Co, control. (**C**) Representative Western blot analysis on concentrated medium of astrocytes treated under the same conditions as described in (**A**). Coomassie staining of gels demonstrates equal loading and protein contents of media probes. (**D**) Data are expressed as *x*-fold changes compared with untreated control levels and represent the mean ± SD of results of six experiments with three different cell cultures from different donors (**$P < 0.05$). Co, control.

expression. Similarly, previous studies on cortical reactive astrocytes of the rat brain demonstrated induced Hsp47 expression after prolonged stress exposure from 10 hours to 14 days.[11] Our experiments also showed increased Hsp47 levels after H_2O_2 exposure. The reasons for Hsp47 induction are as yet unknown. There are assumptions based on various studies that induced Hsp47 expression may participate in glial cell protection and adaptation to damage.[11] Another aspect of the role of Hsp47 in reactive ONH astrocytes may provide its function as an important collagen-specific molecular chaperone and its role in collagen biosynthesis as observed in different diseases such as in arteriosclerosis, pulmonary fibrosis, and keloid formation.[52–57] Treatment of astrocytes with TGF-β2 induced an increased Col1α1 expression, which is consistent with results of our previous studies.[58] By transfection of ONH astrocytes with Hsp47-specific siRNA before TGF-β2 treatment, we could observe a less marked increase of Col1α1 synthesis and secretion. These results confirmed that Hsp47 may play a critical role in collagen accumulation by enhancing synthesis and secretion of collagen in vitro. Whether Hsp47 is also involved in collagen synthesis in the ONH in vivo is still unknown.

Our results on Hsp60 induction revealed no significant effects of TGF-β2 and H_2O_2 on both Hsp60 mRNA and protein expression. In agreement with our observations, no enhanced expression of Hsp60 was found after TGF-β2 treatment of human cerebral astrocytes.[28] Similarly, previous investigations in vitro have shown no induction of Hsp60 after H_2O_2 exposure.[59,60] In accordance with our in vitro results, Tezel et al.[8] have observed no prominent immunohistochemical staining for Hsp60 in reactive ONH astrocytes of glaucomatous eyes.

Comparable results were obtained for Hsp70 induction in cultured human ONH astrocytes. Our experiments revealed no significant effects of both TGF-β2 and H_2O_2 treatment on Hsp70 levels. These results are in contrast to observations with cerebral astrocytes, which respond to numerous stress stimuli by rapid upregulation of Hsp70.[61–63] While extensive studies exist on stress-induced Hsp70 expression in neuronal cells,[11,64] little is known about stress-induced Hsp70 expression in astrocytes.[64] Therefore, whether Hsp70 is increased in human glaucomatous ONH astrocytes awaits further investigations.

In summary, we were able to show that both TGF-β2- and H_2O_2-induced reactivation of ONH astrocytes is accompanied by increased Hsp32 and -47 expression in vitro. These results may provide further insights into the cellular stress responses of reactive human ONH astrocytes. Further extensive in vitro and in vivo studies are needed to examine the potential roles of Hsps in the ONH of glaucomatous eyes.

Acknowledgments

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References

1. Neufeld AH, Liu B. Glaucomatous optic neuropathy: when glia misbehave. *Neuroscientist.* 2003;9:485–495.
2. Pena JD, Taylor AW, Ricard CS, Vidal I, Hernandez MR. Transforming growth factor beta isoforms in human optic nerve heads. *Br J Ophthalmol.* 1999a;83:209–218.
3. Pena JD, Varela HJ, Ricard CS, Hernandez MR. Enhanced tenascin expression associated with reactive astrocytes in human optic nerve heads with primary open angle glaucoma. *Exp Eye Res.* 1999;68:29–40.
4. Yang P, Hernandez MR. Purification of astrocytes from adult human optic nerve heads by immunopanning. *Brain Res Protoc.* 2003;12:67–76.
5. Salvador-Silva M, Ricard CS, Agapova OA, Yang P, Hernandez MR. Expression of small heat shock proteins and intermediate filaments in the human optic nerve head astrocytes exposed to elevated hydrostatic pressure in vitro. *J Neurosci Res.* 2001;66:59–73.
6. Kobayashi S, Vidal I, Pena JD, Hernandez MR. Expression of neural cell adhesion molecule (NCAM) characterizes a subpopulation of type 1 astrocytes in human optic nerve head. *Glia.* 1997;20:262–273.
7. Furuyoshi N, Furuyoshi M, May CA, Hayreh SS, Alm A, Lütjen-Drecoll E. Vascular and glial changes in the retrolaminar optic nerve in glaucomatous monkey eyes. *Ophthalmologica.* 2000;214:24–32.

8. Tezel G, Hernandez R, Wax MB. Immunostaining of heat shock proteins in the retina and optic nerve head of normal and glaucomatous eyes. *Arch Ophthalmol.* 2000;118:511–518.
9. Samali A, Orrenius S. Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress Chaperones.* 1998;3:228–236.
10. Sharp FR, Massa SM, Swanson RA. Heat-shock protein protection. *Trends Neurosci.* 1999;22:97–99.
11. Acarin L, Paris J, Gonzalez B, Castellano B. Glial expression of small heat shock proteins following an excitotoxic lesion in the immature rat brain. *Glia.* 2002;38:1–14.
12. Valentim LM, Rodnight R, Geyer AB, et al. Changes in heat shock protein 27 phosphorylation and immunocontent in response to preconditioning to oxygen and glucose deprivation in organotypic hippocampal cultures. *Neuroscience.* 2003;118:379–386.
13. Hayase T, Yamamoto Y, Yamamoto K, Muso E, Shiota K. Stressor-like effects of cocaine on heat shock protein and stress-activated protein kinase expression in the rat hippocampus: interaction with ethanol and anti-toxicity drugs. *Leg Med (Tokyo).* 2003;1:87–90.
14. Mahmoud KZ, Edens FW, Eisen EJ, Havenstein GB. The effect of dietary phosphorus on heat shock protein mRNAs during acute heat stress in male broiler chickens (Gallus gallus). *Comp Biochem Physiol C Toxicol Pharmacol.* 2004;137:11–18.
15. Calabrese V, Boyd-Kimball D, Scapagnini G, Butterfield DA. Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: the role of vitagenes. *In Vivo.* 2004;18:245–267.
16. Hwang IK, Ahn HC, Yoo KY, et al. Changes in immunoreactivity of HSP60 and its neuroprotective effects in the gerbil hippocampal CA1 region induced by transient ischemia. *Exp Neurol.* 2007;208:247–256.
17. Stahnke T, Stadelmann C, Netzler A, Brück W, Richter-Landsberg C. Differential upregulation of heme oxygenase-1 (HSP32) in glial cells after oxidative stress and in demyelinating disorders. *J Mol Neurosci.* 2007;32:25–37.
18. Lütjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGF-β2 for the pathogenesis of the disease. *Exp Eye Res.* 2005;81:1–4.
19. Picht G, Welge-Luessen U, Grehn F, Lütjen-Drecoll E. Transforming growth factor-β2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:199–207.
20. Tripathi RC, Li J, Chan WFA, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-β2. *Exp Eye Res.* 1994;58:723–727.
21. Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-β2 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2007;48:715–726.
22. Kumar DM, Agarwal N. Oxidative stress in glaucoma: a burden of evidence. *J Glaucoma.* 2007;16:334–343.
23. Malone PE, Hernandez MR. 4-Hydroxynonenal, a product of oxidative stress, leads to an antioxidant response in optic nerve head astrocytes. *Exp Eye Res.* 2007;84:444–454.
24. Sacca SC, Izzotti A, Rossi P, Traverso C. Glaucomatous outflow pathway and oxidative stress. *Exp Eye Res.* 2007;84:389–399.
25. Takenaka IM, Hightower LE. Transforming growth factor-beta 1 rapidly induces Hsp70 and Hsp90 molecular chaperones in cultured chicken embryo cells. *J Cell Physiol.* 1992;152:568–577.
26. Sasaki H, Sato T, Yamauchi N, et al. Induction of heat shock protein 47 synthesis by TGF-β and IL-1 beta via enhancement of the heat shock element binding activity of heat shock transcription factor 1. *J Immunol.* 2002;168:5178–5183.
27. Hatakeyama D, Kozawa O, Niwa M, et al. Upregulation by retinoic acid of transforming growth factor-beta-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases. *Biochem Biophys Acta.* 2002;1589:15–30.
28. Bajramović JJ, Bsibsi M, Geutskens SB, et al. Differential expression of stress proteins in human adult astrocytes in response to cytokines. *J Neuroimmunol.* 2000;106:14–22.
29. Yu AL, Fuchshofer R, Birke M, et al. Hypoxia/reoxygenation and TGF-β increase αB-crystallin expression in human optic nerve head astrocytes. *Exp Eye Res.* 2007;84:694–706.
30. Calabrese V, Lodi R, Tonon C, et al. Oxidative stress, mitochondrial dysfunction and cellular stress response in Friedreich's ataxia. *J Neurol Sci.* 2005;233:145–162.
31. Alge CS, Priglinger SG, Neubauer AS, et al. Retinal pigment epithelium is protected against apoptosis by αB-crystallin. *Invest Ophthalmol Vis Sci.* 2002;43:3575–3582.
32. Welge-Lussen U, May CA, Eichhorn M, Bloemendal H, Lütjen-Drecoll E. αB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci.* 1999;40:2235–2241.
33. Yu AL, Fuchshofer R, Birke M, Kampik A, Bloemendal H, Welge-Lussen U. Oxidative stress and TGF-β2 increase heat shock protein 27 expression in human optic nerve head astrocytes. *Invest Ophthalmol Vis Sci.* 2008;49:5403–5411.
34. Lambert W, Agarwal R, Howe W, Clark AF, Wordinger RJ. Neurotrophin and neurotrophin receptor expression by cells of the human lamina cribrosa Invest. *Ophthalmol Vis Sci.* 2001;42:2315–2323.
35. Buniatian GH. Further similarities between astrocytes and perisinusoidal stellate cells of liver (Ito cells): colocalization of desmin and glial fibrillary acidic protein in astroglial primary cultures. *Biol Cell.* 1997;89:169–177.
36. Esposito G, De Filippis D, Cirillo C, Sarnelli G, Cuomo R, Iuvone T. The astroglial-derived S100beta protein stimulates the expression of nitric oxide synthase in rodent macrophages through p38 MAP kinase activation. *Life Sci.* 2006;78:2707–2715.
37. Zimmer DB, Cornwall EH, Landar A, Song W. The S100 protein family: history, function, and expression. *Brain Res Bull.* 1995;37:417–429.
38. Hernandez MR, Igoe F, Neufeld AH. Cell culture of the human lamina cribrosa. *Invest Ophthalmol Vis Sci.* 1988;29:78–89.
39. Schipper HM. Heme oxygenase expression in human central nervous system disorders. *Free Radic Biol Med.* 2004;37:1995–2011.
40. Hartung R, Parapuram SK, Ganti R, Hunt DM, Chalam KV, Hunt RC. Vitreous induces heme oxygenase-1 expression mediated by transforming growth factor-beta and reactive oxygen species generation in human retinal pigment epithelial cells. *Mol Vis.* 2007;13:66–78.
41. Dwyer BE, Nishimura RN, Lu SY. Differential expression of heme oxygenase-1 in cultured cortical neurons and astrocytes determined by the aid of a new heme oxygenase antibody: response to oxidative stress. *Brain Res Mol Brain Res.* 1995;30:37–47.
42. Applegate LA, Luscher P, Tyrell R. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res.* 1991;51:974–978.
43. Chen K, Gunter K, Maines MD. Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. *J Neurochem.* 2000;75:304–313.
44. Liu Y, Zhu B, Luo L, Li P, Paty DW, Cynader MS. Heme oxygenase-1 plays an important protective role in experimental autoimmune encephalomyelitis. *Neuroreport.* 2001;12:1841–1845.
45. Takahashi M, Dore S, Ferris CD, et al. Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron.* 2002;28:461–473.
46. Schipper HM, Liberman A, Stopa EG. Neural heme oxygenase-1 expression in idiopathic Parkinson's disease. *Exp Neurol.* 1998;150:60–68.
47. Martelli-Junior H, Cotrim P, Graner E, Sauk JJ, Coletta RD. Effect of transforming growth factor-beta1, interleukin-6, and interferon-gamma on the expression of type I collagen, heat shock protein 47, matrix metalloproteinase (MMP)-1 and MMP-2 by fibroblasts from normal gingiva and hereditary gingival fibromatosis. *J Periodontol.* 2003;74:296–306.
48. Pan H, Halper J. Regulation of heat shock protein 47 and type I procollagen expression in avian tendon cells. *Cell Tissue Res.* 2003;311:373–382.
49. Razzaque MS, Foster CS, Ahmed AR. Role of collagen-binding heat shock protein 47 and transforming growth factor-beta1 in conjunctival scarring in ocular cicatricial pemphigoid. *Invest Ophthalmol Vis Sci.* 2003;44:1616–1621.
50. Hart DA, Reno C, Hellio Le Graverand MP, Hoffman L, Kulyk W. Expression of heat shock protein 47(Hsp47) mRNA levels in rabbit connective tissues during the response to injury and in pregnancy. *Biochem Cell Biol.* 2000;78:511–518.

51. Chander V, Singh D, Tirkey N, Chander H, Chopra K. Amelioration of cyclosporine nephrotoxicity by irbesartan: a selective AT1 receptor antagonist. *Ren Fail.* 2004;26:467–477.
52. Nagata K. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol.* 1998;16:379–386.
53. Tasab M, Batten MR, Bulleid NJ. Hsp47: a molecular chaperone that interacts with and stabilizes correctly-folded procollagen. *EMBO J.* 2000;19:2204–2211.
54. Turner PC, Bergeron M, Matz P, et al. Heme oxygenase-1 is induced in glia throughout brain by subarachnoid hemoglobin. *J Cereb Blood Flow Metab.* 1998;18:257–273.
55. Sauk JJ, Nikitakis N, Siavash H. Hsp47 a novel collagen binding serpin chaperone, autoantigen and therapeutic target. *Front Biosci.* 2005;10:107–118.
56. Nakayama S, Mukae H, Sakamoto N et al. Pirfenidone inhibits the expression of HSP47 in TGF-beta1-stimulated human lung fibroblasts. *Life Sci.* 2008;82:210–217.
57. Chen JJ, Zhao S, Cen Y, Liu XX, Yu R, Wu DM. Effect of heat shock protein 47 on collagen accumulation in keloid fibroblast cells. *Br J Dermatol.* 2007;156:1188–1195.
58. Fuchshofer R, Birke M, Welge-Lussen U, Kook D, Lütjen-Drecoll E. Transforming growth factor-beta 2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. *Invest Ophthalmol Vis Sci.* 2005;46:568–578.
59. Kemp TJ, Causton HC, Clerk A. Changes in gene expression induced by H(2)O(2) in cardiac myocytes. *Biochem Biophys Res Commun.* 2003;307:416–421.
60. Goldbaum O, Richter-Landsberg C. Stress proteins in oligodendrocytes: differential effects of heat shock and oxidative stress. *J Neurochem.* 2001;78:1233–1242.
61. Guzhova I, Kislyakova K, Moskaliova O, et al. In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. *Brain Res.* 2001;914:66–73.
62. Uehara T, Kaneko M, Tanaka S, Okuma Y, Nomura Y. Possible involvement of p38 MAP kinase in HSP70 expression induced by hypoxia in rat primary astrocytes. *Brain Res.* 1999;823:226–230.
63. Fauconneau B, Petegnief V, Sanfeliu C, Piriou A, Planas AM. Induction of heat shock proteins (HSPs) by sodium arsenite in cultured astrocytes and reduction of hydrogen peroxide-induced cell death. *J Neurochem.* 2002;83:1338–1348.
64. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Int J Hyperthermia.* 2005;21:379–392.