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TGF-β_2- and H_2O_2-Induced Biological Changes in Optic Nerve Head Astrocytes Are Reduced by the Antioxidant Alpha-Lipoic Acid

Alice L. Yu[a] Jerome Moriniere[a] Ulrich Welge-Lussen[b]

[a]Department of Ophthalmology, Ludwig-Maximilian University, Munich, and [b]Department of Ophthalmology, Friedrich-Alexander University, Erlangen, Germany

Key Words
Optic nerve head · Antioxidant · Heat shock protein

Abstract
Background/Aims: The goal of the present study was to determine whether transforming growth factor-β_2 (TGF-β_2)- and oxidative stress-induced cellular changes in cultured human optic nerve head (ONH) astrocytes could be reduced by pretreatment with the antioxidant α-lipoic acid (LA). ***Methods:*** Cultured ONH astrocytes were treated with 1.0 ng/ml TGF-β_2 for 24 h or 200 μM hydrogen peroxide (H_2O_2) for 1 h. Lipid peroxidation was measured by a decrease in *cis*-parinaric acid fluorescence. Additionally, cells were pretreated with different concentrations of LA before TGF-β_2 or H_2O_2 exposure. Expressions of the heat shock protein (Hsp) αB-crystallin and Hsp27, the extracellular matrix (ECM) component fibronectin and the ECM-modulating protein connective tissue growth factor (CTGF) were examined with immunohistochemistry and real-time PCR analysis. ***Results:*** Both TGF-β_2 and H_2O_2 increased lipid peroxidation. Treatment of astrocytes with TGF-β_2 and H_2O_2 upregulated the expression of αB-crystallin, Hsp27, fibronectin and CTGF. Pretreatment with different concentrations of LA reduced the TGF-β_2- and H_2O_2-stimulated gene expressions. ***Conclusion:*** We showed that TGF-β_2- and H_2O_2-stimulated gene expressions could be prevented by pretreatment with the antioxidant LA in cultured human ONH astrocytes. Therefore, it is tempting to speculate that the use of antioxidants could have protective effects in glaucomatous optic neuropathy.

Introduction

Primary open-angle glaucoma is an optic neuropathy, which clinically presents with progressive excavation of the optic disk and corresponding visual field defects. Histologically, glaucomatous optic neuropathy is characterized by distinct compositional and structural changes in the optic nerve head (ONH), which are largely attributed to cellular responses of reactive ONH astrocytes [1, 2]. Characteristic responses of reactive astrocytes in the glaucomatous ONH are increased expression of so-called heat shock proteins (Hsps) [3, 4], and increased extracellular matrix (ECM) production [5–7]. Previously, it has been reported that the process of astrocytic reactivation in glaucomatous disease is accompanied by elevated expression of the small Hsps αB-crystallin [3] and Hsp27 [4]. Hsps are molecular chaperones, which stabilize pro-

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Alice Yu, MD
Department of Ophthalmology, Ludwig-Maximilian University
Mathildenstrasse 8
DE–80336 Munich (Germany)
Tel. +49 89 51 60 38 11, E-Mail alice.yu@med.uni-muenchen.de

tein folding and suppress protein aggregation [8, 9]. Besides these changes, other findings could be observed in human ONH astrocytes such as an increased expression of the ECM component fibronectin [10, 11] and the ECM-modulating protein connective tissue growth factor (CTGF) [12, 13].

In general, it is known that astrocytic reactivation takes place during stressful events [14, 15]. In the pathogenesis of primary open-angle glaucoma, various stress factors such as transforming growth factor-β_2 (TGF-β_2) [16–19] and oxidative stress [20–22] may play an important role. One common characteristic of both TGF-β_2 and oxidative stress is the generation of reactive oxygen species (ROS) [23, 24]. Increased levels of ROS are able to injure tissues by interacting with lipids, proteins, or DNA [25]. There is a growing body of evidence suggesting that ROS production is involved in the progression of glaucomatous neurodegeneration [22, 24, 26, 27]. In particular, the optic nerve as part of the human brain is very sensitive to stress from ROS compared to other tissues [28].

Based on these findings, the goal of this study was to examine in vitro whether or not TGF-β_2 and H_2O_2, two known pathogenetic factors for glaucoma, induce characteristic glaucomatous changes in cultured human ONH astrocytes via ROS production. To provide a proof-of-principle of ROS-mediated gene expressions, we used the antioxidant α-lipoic acid (LA) to block these stress-induced changes. LA has already been tested for the adjuvant therapy of other oxidative stress-associated diseases such as diabetic neuropathy, multiple sclerosis and Alzheimer's diseases [29–31]. An antioxidant effect of LA has been detected both by animal experiments and cell culture studies [32, 33]. Currently, it is discussed that antioxidants could have neuroprotective effects on the glaucomatous ONH [34, 35]. In this study, we investigated, in particular, whether or not TGF-β_2- and H_2O_2-induced synthesis of the small Hsps αB-crystallin and Hsp27, the ECM component fibronectin and the ECM-modulating protein CTGF could be reduced by pretreatment with the antioxidant LA in cultured human ONH astrocytes.

Materials and Methods

Cell Culture

Primary cell cultures of human lamina cribrosa astrocytes were obtained from the eye bank of the Ludwig-Maximilian University, Munich, Germany. Monolayer cultures were established from eyes of 5 human donors between 52 and 69 years of age. These eyes were obtained 4–12 h postmortem without any history of eye diseases. Methods of securing human tissue were humane, included 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 [36–38]. In brief, eyes were cut equatorially behind the ora serrata and the ONH was isolated from the neighboring tissues. The ONH was sagitally dissected under a microscope and the lamina cribrosa was identified. Disks of lamina cribrosa were prepared by dissection from the pre- and postlaminar region, subsequently cut into 3–4 explants and placed in Petri dishes with 2 ml Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Gibco-Life Science Technology, Karlsruhe, Germany), 5 ng/ml human basic pituitary fibroblast growth factor (Sigma, Deisenhofen, Germany), 5 ng/ml human platelet-derived growth factor-A chain (PDGF AA; Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco-Life Science Technology) at 37°C in a 5% CO_2 incubator. To isolate ONH astrocytes, the primary cell cultures were first plated in serum-free astrocyte growth medium (Cambrex Bio Science, Verviers, Belgium) for 24 h and then changed to astrocyte growth medium containing 5% FBS [39]. Other cell populations such as lamina cribrosa cells failed to attach in serum-free medium and were removed with medium change. 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) [36, 38]. Primary human ONH astrocytes were characterized by positive immunostaining for glial fibrillary acidic protein (Sigma), neural cell adhesion molecule (Serotec, Düsseldorf, Germany), vimentin (Sigma), desmin (Abcam, Cambridge, UK), S100 (Invitrogen, Karlsruhe, Germany), and Pax2 (Abcam), and negative immunostaining for A2B5 (Chemicon International, Hampshire, UK) and smooth muscle actin (Dako, Glostrup, Denmark) [36, 37, 40–42]. Only cell cultures which were at least 95% positive for glial fibrillary acidic protein, neural cell adhesion molecule, vimentin, desmin, S100, Pax2, and negative for A2B5 and smooth muscle actin were used in this study [38].

Second- to fifth-passage astrocytes were grown to confluence in 35-mm Petri dishes in DMEM/F-12 supplemented with 10% FBS at 37°C and 5% CO_2. At confluence, cells were washed and incubated overnight in serum-free DMEM/F-12 medium for 24 h. LA (Sigma) was dissolved in 95% (v/v) ethanol. Cells were pretreated with 50, 100, 150 and 200 μM LA or the corresponding ethanol volume for 24 h in fresh serum-free medium. Afterwards, cells were either treated with TGF-β_2 or oxidative stress. Cells were kept with 1.0 ng/ml active TGF-β_2 (R&D Systems, Wiesbaden, Germany) for 24 h. Oxidative stress exposure was performed with 200 μM H_2O_2 for 1 h with subsequent incubation under serum-free conditions for 24 h. In control cultures, the medium was changed at the same time points. The tetrazolium dye reduction assay [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] 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-β_2- or H_2O_2-treated cells (data not shown). All experiments were performed at least in triplicate in astrocyte cultures from 4 different donors.

Assessment of Lipid Peroxidation

Oxidative stress can be assessed by markers of lipid peroxidation. A sensitive and specific assay for lipid peroxidation is based on metabolic incorporation of the fluorescent oxidation-sensitive fatty acid, *cis*-parinaric acid (PNA), a natural 18-carbon fatty acid with 4 conjugated double bonds, into membrane phospholipids of cells [43, 44]. Oxidation of PNA results in disruption of the conjugated double bond system that cannot be resynthesized in mammalian cells. Therefore, lipid peroxidation was estimated by measuring loss of PNA fluorescence. Briefly, treated cells were incubated with 10 μM PNA (Molecular Probes, Invitrogen, UK) at 37°C for 30 min in the dark. The media was then removed and cells washed three times with PBS. Afterwards, cells were scraped into 2 ml PBS using a rubber policeman. The suspension was then added to a fluorescence cuvette and measured at 312-nm excitation and 455-nm emission. A blank (unlabeled cells) was measured and subtracted from all readings. All experiments were performed at least in triplicate in astrocyte cultures from 4 different donors.

Immunohistochemistry

Cultured human ONH astrocytes, grown in 4-well plastic chamber slides, were treated as described above. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min and subsequently washed twice with PBS containing 0.1% Triton X-100. Primary incubation with all samples was performed with a rabbit anti-human αB-crystallin antibody (Stressgen Bioreagents, Hamburg, Germany) and a rabbit anti-human Hsp27 antibody (Stressgen Bioreagents) diluted 1:200 in PBS containing 5% bovine serum albumin for 4 h at room temperature. Control samples were incubated with PBS and 5% bovine serum albumin but without the primary antibodies. Afterwards, cells were washed three times with PBS, and incubated with fluorescein-conjugated goat anti-rabbit Cy3 antibody (diluted 1:500 in PBS; Dianova, Hamburg, Germany) for 1 h at room temperature. Cells were then rinsed in PBS, mounted with Kaiser's glycerin jelly (Merck, Darmstadt, Germany) and analyzed under a fluorescence microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany). Representative areas were documented with Leica IM 1000 software (Leica Microsystems, Heerbrugg, Switzerland). All experiments were performed at least in triplicate in astrocyte cultures from 4 different donors.

RNA Isolation and Real-Time PCR

Total RNA was isolated from 10-mm Petri dishes by the guanidinium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). Structural integrity of the RNA samples was confirmed by electrophoresis in 1% Tris-acetate-EDTA agarose gels. Yield and purity were determined photometrically. After RNA isolation, mRNA was transcribed to cDNA by reverse transcriptase. This cDNA was then used for specific real-time PCR. Quantification of human mRNA was performed during 40 cycles with a LightCycler Instrument (LightCycler System, Roche Diagnostics, Mannheim, Germany). The primers selected were αB-crystallin forward primer 5′-cagctggtttgacactggac-3′ (positions, 220–239) and reverse primer 5′-gcttcacatccaggttgaca-3′ (positions, 274–293); Hsp27 forward primer 5′-tgacggtcaagaccaagga-3′ (positions, 433–451) and reverse primer 5′-tgtagccatgctcgtcctg-3′ (positions, 489–507); fibronectin forward primer 5′-ctggccgaaaatacattgtaaa-3′ (positions, 2611–2632) and reverse primer 5′-ccacagtcgggtcaggag-3′ (positions, 2707–2724); CTGF forward primer 5′-ctgcaggctagagaagcagag-3′ (positions, 826–846) and reverse primer 5′-gatgcacttttgcccttct-3′ (positions, 897–916), and 18S rRNA forward primer 5′-ctcaacacgggaaacctcac-3′ (positions, 1348–1367) and reverse primer 5′-cgctccaccaactaagaacg-3′ (positions, 1438–1457). Primers and probes were found with the program ProbeFinder Version 2.04. The standard curve was obtained from probes of 4 different untreated human ONH astrocyte cultures. To normalize differences of the amount of total RNA added to each reaction, 18S rRNA was simultaneously processed in the same sample as an internal control. The level of αB-crystallin, Hsp27, fibronectin or CTGF mRNA was determined as the relative ratio, which was calculated by dividing the level of αB-crystallin, Hsp27, fibronectin or CTGF mRNA by the level of the 18S rRNA gene in the same samples. All experiments were performed at least in triplicate in astrocyte cultures from 4 different donors.

Statistical Analysis

Results are expressed as mean ± SD. For comparison of means between two groups, an unpaired t test was employed. Statistical significance was defined as $p < 0.05$.

Results

TGF-β_2 and H_2O_2 Increased Lipid Peroxidation

In our experiments, lipid peroxidation of the cytoplasm membrane of cultured human ONH astrocytes was assessed by increased loss of PNA fluorescence. We could observe a decrease in PNA fluorescence after 24 h of TGF-β_2 treatment to 75.1 ± 5.5% and after exposure to 200 μM H_2O_2 to 71.2 ± 12.7% as compared to untreated control cells (fig. 1). Pretreatment of astrocytes with 200 μM LA could block the TGF-β_2- and H_2O_2-mediated decrease in PNA fluorescence (fig. 1).

TGF-β_2- and H_2O_2-Induced αB-Crystallin Expression Could Be Reduced by Pretreatment with LA

Immunohistochemical stainings showed a marked increase in αB-crystallin expression after TGF-β_2 treatment for 24 h (fig. 2b) compared to untreated control cells (fig. 2a). Since immunohistochemistry is not a valid quantification method, we additionally performed real-time PCR analyses to examine the mRNA expression. Human ONH astrocytes were treated with 1.0 ng/ml TGF-β_2 for 24 h or 200 μM H_2O_2 for 1 h (fig. 3). The signals generated by real-time PCR analysis in untreated control cells were set to 100% (fig. 3). There was a marked upregulation of αB-crystallin mRNA expression both after 24 h of TGF-β_2 treatment (2.7 ± 0.8-fold) (fig. 3a) and after exposure of cells to 200 μM H_2O_2 for 1 h (1.9 ± 0.5-fold) (fig. 3b) compared to untreated control cells. Expo-

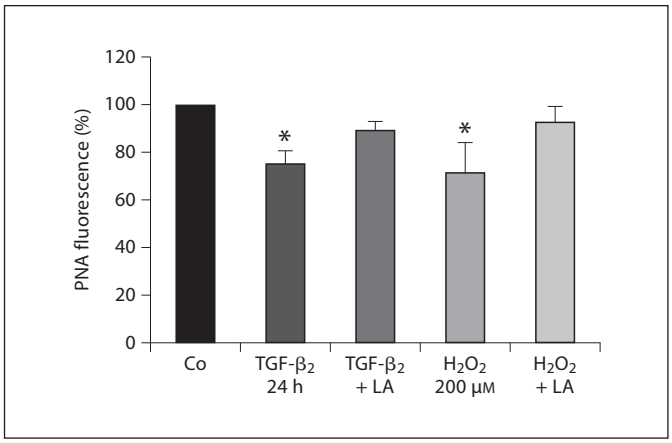

Fig. 1. Measurements of ROS by loss of PNA fluorescence in cultured human ONH astrocytes. Cells were either treated with 1.0 ng/ml TGF-β_2 for 24 h or exposed to 200 μM H_2O_2 for 1 h with subsequent incubation under serum-free conditions for 24 h. Pretreatment was conducted with 200 μM LA for 24 h before stress exposure. Data are expressed as percentage compared to untreated control cells kept for the same time periods and represent the mean ± SD of results of 12 experiments with 4 different cell cultures from different donors (* $p < 0.05$). Co = Control.

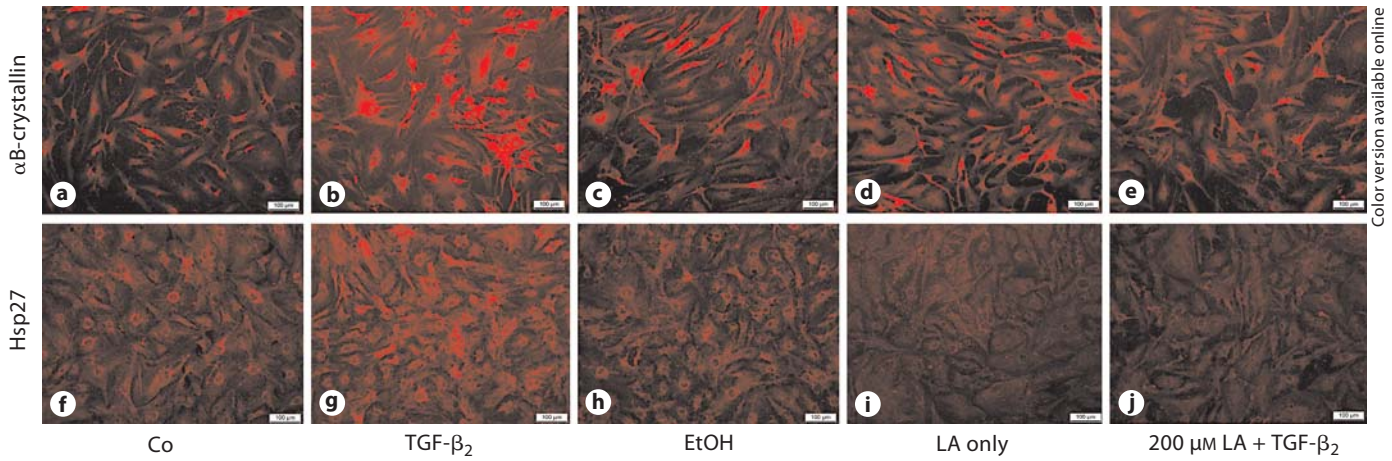

Fig. 2. Immunohistochemical stainings of cultured human ONH astrocytes for the small Hsp αB-crystallin (**a–e**) and Hsp27 (**f–j**). **a** Basal levels of αB-crystallin staining were observed in untreated ONH astrocytes incubated in serum-free medium for 24 h. **b** Treatment with 1.0 ng/ml TGF-β_2 for 24 h increased αB-crystallin expression. **c** Corresponding ethanol volume of 200 μM LA concentration (**d**) had no additional effects on αB-crystallin expression compared to untreated control cells. **e** TGF-β_2-induced αB-crystallin expression could be blocked by pretreatment with 200 μM LA. **f–j** Immunohistochemical stainings for Hsp27 expression were conducted under the same conditions as described for **a–e** and showed comparable results. Scale bar = 100 μm.

sure of cells to the maximal LA concentration (200 μM) and its corresponding ethanol volume had no effects on αB-crystallin expression (fig. 3a, b). In contrast, pretreatment of astrocytes with 50, 100, 150, 200 μM LA could reduce the TGF-β_2- and H_2O_2-induced αB-crystallin expression (fig. 3a, b). The most prominent reduction of TGF-β_2-induced αB-crystallin expression was observed with pretreatment with 150 and 200 μM LA (1.2 ± 0.2- and 1.2 ± 0.5-fold) (fig. 3a). H_2O_2-induced αB-crystallin expression was most effectively blocked by pretreatment of cells with 200 μM LA (1.4 ± 0.2-fold) (fig. 3b). These results could be confirmed by immunohistochemical stainings (fig. 2c–e).

TGF-β_2- and H_2O_2-Induced Hsp27 Expression Could Be Reduced by Pretreatment with LA

Similar to the small Hsp αB-crystallin, immunohistochemical stainings also demonstrated a marked increase in Hsp27 expression after 24 h of TGF-β_2 treatment (fig. 2g) compared to untreated control cells (fig. 2f). To perform a quantitative measurement, real-time PCR analyses were conducted. Human ONH astrocytes were treated with 1.0 ng/ml TGF-β_2 for 24 h or 200 μM H_2O_2 for 1 h (fig. 4). The signals generated by real-time PCR analysis in untreated control cells were set to 100% (fig. 4). There was a marked increase in Hsp27 mRNA expression both after 24 h of TGF-β_2 treatment (3.2 ± 0.6-fold) (fig. 4a)

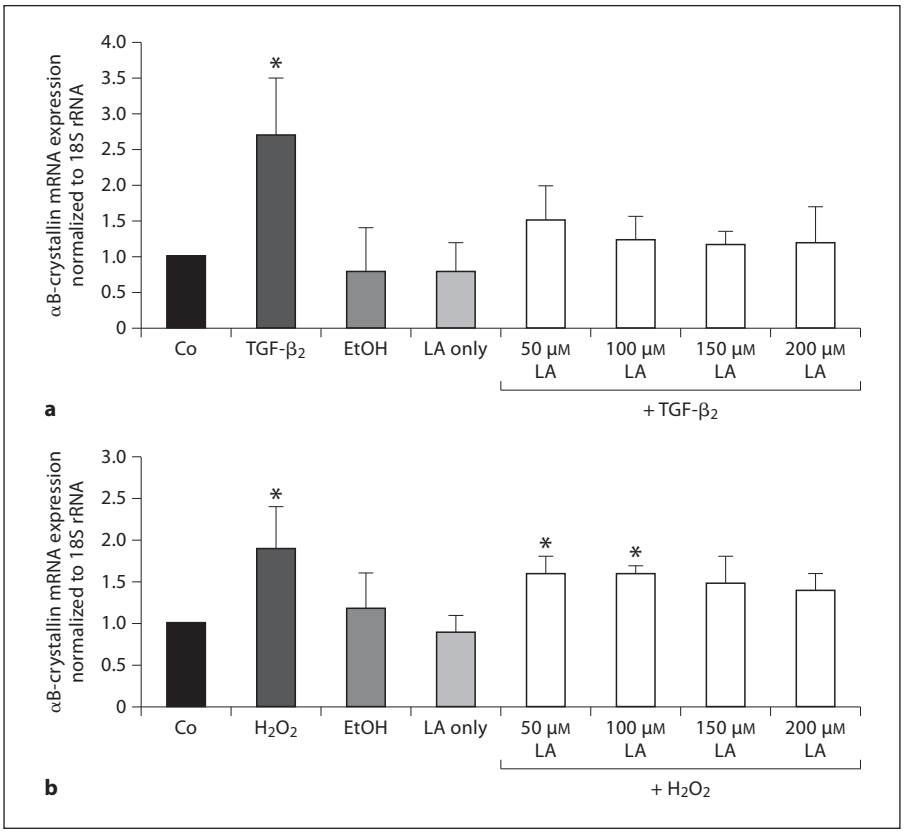

Fig. 3. Real-time PCR analysis of αB-crystallin mRNA expression. Cells were pretreated with 50, 100, 150 and 200 μM LA and then exposed either to 1.0 ng/ml TGF-β_2 (**a**) for 24 h or 200 μM H_2O_2 (**b**) for 1 h with subsequent incubation under serum-free conditions for 24 h. Results were normalized to 18S rRNA as reference. Data are expressed as x-fold changes compared to untreated control cells and represent the mean ± SD of results of 12 experiments with 4 different cell cultures from different donors (* $p < 0.05$). Co = Control.

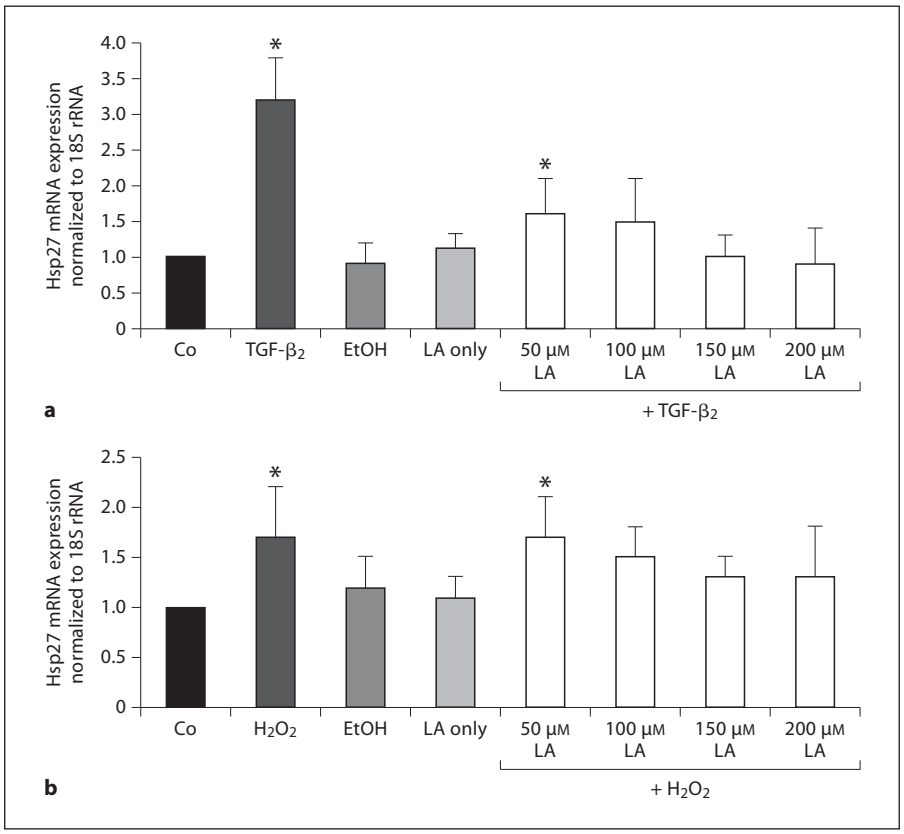

Fig. 4. Real-time PCR analysis of Hsp27 mRNA expression. Cells were pretreated with 50, 100, 150 and 200 μM LA and then exposed either to 1.0 ng/ml TGF-β_2 (**a**) for 24 h or 200 μM H_2O_2 (**b**) for 1 h with subsequent incubation under serum-free conditions for 24 h. Results were normalized to 18S rRNA as reference. Data are expressed as x-fold changes compared to untreated control cells and represent the mean ± SD of results of 12 experiments with 4 different cell cultures from different donors (* $p < 0.05$). Co = Control.

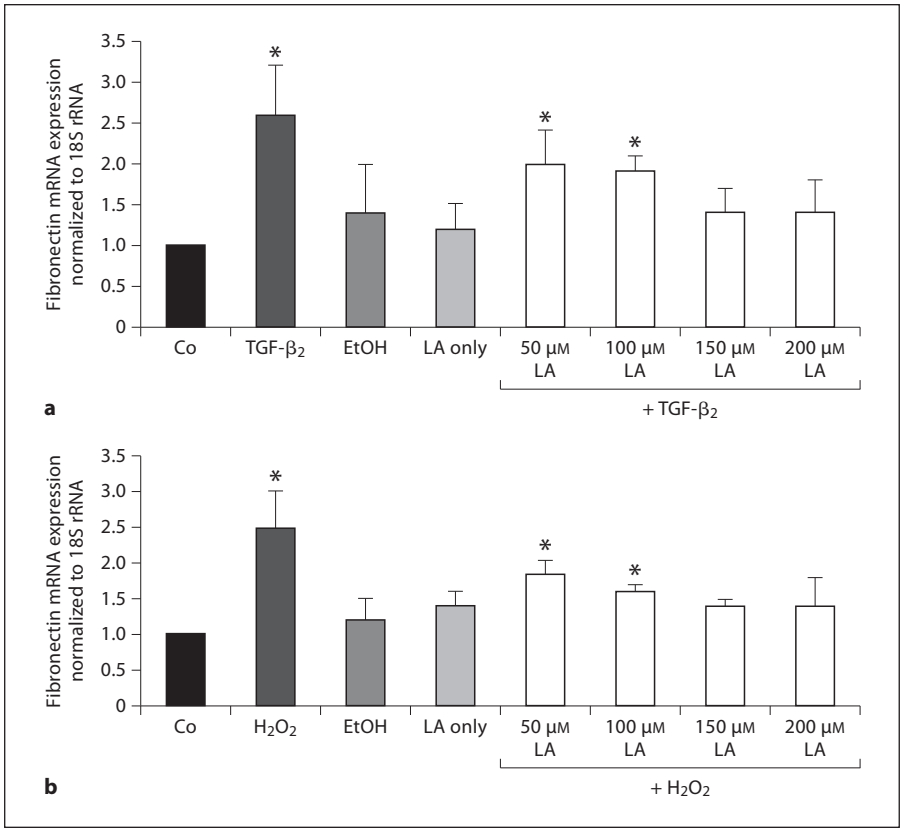

Fig. 5. Real-time PCR analysis of fibronectin mRNA expression. Cells were pretreated with 50, 100, 150 and 200 µM LA and then exposed either to 1.0 ng/ml TGF-β_2 (**a**) for 24 h or 200 µM H_2O_2 (**b**) for 1 h with subsequent incubation under serum-free conditions for 24 h. Results were normalized to 18S rRNA as reference. Data are expressed as x-fold changes compared to untreated control cells and represent the mean ± SD of results of 12 experiments with 4 different cell cultures from different donors (* $p < 0.05$). Co = Control.

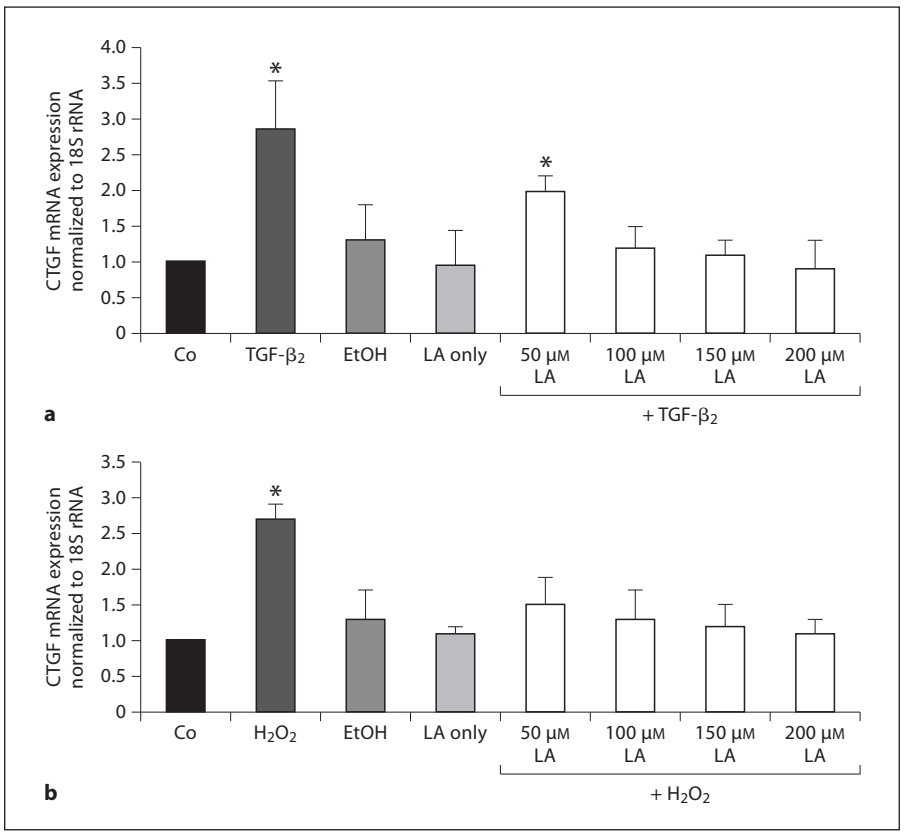

Fig. 6. Real-time PCR analysis of CTGF mRNA expression. Cells were pretreated with 50, 100, 150 and 200 µM LA and then exposed either to 1.0 ng/ml TGF-β_2 (**a**) for 24 h or 200 µM H_2O_2 (**b**) for 1 h with subsequent incubation under serum-free conditions for 24 h. Results were normalized to 18S rRNA as reference. Data are expressed as x-fold changes compared to untreated control cells and represent the mean ± SD of results of 12 experiments with 4 different cell cultures from different donors (* $p < 0.05$). Co = Control.

and after exposure of cells to 200 μM H_2O_2 for 1 h (1.7 ± 0.5-fold) (fig. 4b) compared to untreated control cells. The maximal LA concentration (200 μM) and its corresponding ethanol volume had no influence on Hsp27 expression (fig. 4a, b). However, pretreatment of astrocytes with different concentrations of LA could prevent the TGF-β_2- and H_2O_2-induced Hsp27 expression (fig. 4a, b). TGF-β_2-mediated Hsp27 upregulation could be most effectively blocked by pretreatment of astrocytes with 200 μM LA (0.9 ± 0.5-fold) (fig. 4a), while H_2O_2-mediated Hsp27 expression could be reduced by 150 and 200 μM LA to 1.3 ± 0.2 and 1.3 ± 0.5-fold (fig. 4b). Immunohistochemical stainings supported these observations (fig. 2h, i, j).

TGF-β_2- and H_2O_2-Induced Fibronectin Expression Could Be Blocked by Pretreatment with LA

To evaluate whether or not ECM components such as fibronectin can be induced by TGF-β_2 and H_2O_2 in cultured human ONH astrocytes, cells were treated either with 1.0 ng/ml TGF-β_2 for 24 h or 200 μM H_2O_2 for 1 h (fig. 5). The signals generated by real-time PCR analyses in untreated control cells were set to 100% (fig. 5). Fibronectin mRNA expression could be increased both by TGF-β_2 treatment (2.6 ± 0.6-fold) (fig. 5a) and after H_2O_2 exposure (2.5 ± 0.5-fold) (fig. 5b) compared to untreated control cells. To further examine whether or not ROS are involved in TGF-β_2- and H_2O_2-induced fibronectin expression, cultured human ONH astrocytes were pretreated with different concentrations of LA (fig. 5). TGF-β_2-mediated fibronectin upregulation could be most effectively prevented by pretreatment with 150 and 200 μM LA (1.4 ± 0.3- and 1.4 ± 0.4-fold) (fig. 5a). H_2O_2-stimulated fibronectin expression was markedly blocked by pre-exposure of cells to 150 and 200 μM LA (1.4 ± 0.1- and 1.4 ± 0.4-fold) (fig. 5b).

TGF-β_2- and H_2O_2-Induced CTGF Expression Could Be Prevented by Pretreatment with LA

To investigate CTGF mRNA expression, the signals generated by real-time PCR analyses in untreated control cells were set to 100% (fig. 6). Treatment with 1.0 ng/ml TGF-β_2 for 24 h could upregulate CTGF mRNA expression by 2.8 ± 0.7-fold (fig. 6a), while exposure of astrocytes to 200 μM H_2O_2 for 1 h induced a CTGF increase by 2.7 ± 0.2-fold compared to untreated control cells (fig. 6b). Pretreatment of astrocytes with 200 μM LA could markedly reduce TGF-β_2-induced CTGF expression to 0.9 ± 0.4-fold (fig. 6a), while H_2O_2-mediated CTGF upregulation was most effectively blocked by pretreatment with 200 μM LA (1.1 ± 0.2-fold) (fig. 6b).

Discussion

There is a growing body of evidence suggesting that glaucomatous optic neuropathy is not only attributed to elevated intraocular pressure but also to several concomitant factors such as TGF-β_2 [16–19] and oxidative stress [20–22]. Reactive astrocytes of the ONH are involved in cellular responses to both TGF-β_2 and oxidative stress [21, 26, 45]. In cultured human ONH astrocytes, TGF-β_2 expression could also be induced by oxidative stress [46]. Immunohistological investigations revealed increased levels of TGF-β_2 in the ONH of glaucomatous patients [45]. Furthermore, oxidative stress exposure increased antioxidant defense mechanisms of ONH astrocytes of glaucomatous donor eyes as compared to normal non-glaucomatous ONH astrocytes [21, 27]. Oxidative stress is a resultant of ROS overproduction, which, among other factors, contributes to optic neurodegeneration [22, 47]. Similarly, TGF-β_2 is also a potent inducer of ROS generation as demonstrated in a number of different in vitro studies [23, 48, 49]. In our first experiments, we detected elevated lipid peroxidation estimated by the loss of PNA fluorescence, a known marker of late oxidative stress [44], after treatment of cultured human ONH astrocytes with TGF-β_2 or hydrogen peroxide (H_2O_2). These observations suggest that both TGF-β_2 and H_2O_2 are capable of producing ROS in our in vitro model.

As mentioned before, the exposure of the optic nerve to increased ROS levels plays an important role in glaucomatous optic neuropathy [22, 24, 26, 27]. Therefore, we hypothesized that both TGF-β_2 and H_2O_2 may convey their involvement in the pathogenesis of primary open-angle glaucoma, at least in part, via ROS formation. It is known that increased ROS production can affect gene regulations including those of Hsps, ECM components and ECM-modulating proteins [50]. Thus, in our next experiments, we examined whether TGF-β_2- and H_2O_2-induced gene expressions in reactive human ONH astrocytes can be minimized by the use of antioxidants.

In the ONH, reactive astrocytes play a critical role in the defense system against ROS [50]. One defense mechanism of astrocytes to protect neurons is the generation of Hsps in response to oxidative stress [50]. Two factors, which have the ability to produce Hsps in reactive astrocytes, are TGF-β_2 and H_2O_2 [51, 52]. We could demonstrate that exposure to TGF-β_2 and H_2O_2 could increase the expression of the small stress-sensitive Hsps αB-crystallin and Hsp27. Furthermore, in our experiments, both TGF-β_2- and H_2O_2-induced upregulations of αB-crystallin and Hsp27 expression in cultured human ONH as-

trocytes could be prevented by pretreatment with LA. Interestingly, pretreatment of astrocytes with LA could also block the TGF-β_2- and H_2O_2-mediated increase in lipid peroxidation. It is known that LA is a natural, potent thiol antioxidant and capable of regenerating major physiological antioxidants of the lipid and aqueous phases [53]. LA has been widely used as a therapeutic agent in diseases associated with increased oxidative stress [53]. A similar effect of LA could be observed in the perfused amphibian heart, in which oxidative stress-induced Hsp27 expression could be reduced by LA [54]. In contrast, LA-mediated cytoprotection against oxidative stress was accompanied by Hsp upregulation in primary neuronal cell cultures in vitro [55] and in the diabetic rat liver in vivo [56]. One explanation for these contrary effects of LA might be that high-dose LA can enhance Hsp expression by activating the transcriptional factor heat shock factor-1, while lower doses of LA reduce the expression of ROS-sensitive Hsp based on their antioxidant properties [51, 53, 54]. Thus, increased αB-crystallin and Hsp27 concentrations, as found in the ONH of glaucomatous eyes [3, 4], may result from increased ROS production generated by TGF-β_2 and H_2O_2.

Another characteristic finding in the glaucomatous ONH are fibrotic changes [5–7]. ECM remodelling of the ONH appears to be primarily attributed to exogeneous or endogeneous oxidative stress [50]. Various in vitro studies have shown that ECM components such as fibronectin and ECM-modulating proteins such as CTGF are increasingly produced by human ONH astrocytes after TGF-β_2 treatment [10–13]. In this study, we could show that increased oxidative stress levels, generated by TGF-β_2 or H_2O_2, induced fibronectin and CTGF expression in cultured human ONH astrocytes, which could be prevented by pretreatment with the antioxidant LA. In a similar way, high glucose stress-induced fibronectin upregulation in ARPE-19 retinal cell cultures [57] have been reduced by LA. In accordance with its antioxidant effect, LA could also prevent hypertension-induced CTGF increase in the rat kidney and heart [58]. Based on these findings, both TGF-β_2 and H_2O_2 may convey their ECM-stimulated effects in human ONH astrocytes via ROS production.

In conclusion, our results support the hypothesis of ROS involvement in glaucomatous optic nerve changes. The protective effects of antioxidants such as LA on cellular stress responses of reactive human ONH astrocytes may provide further insights into new therapeutic approaches for glaucomatous neuroprotection.

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