

Comparison between axonal and retinal ganglion cell gene expression in various optic nerve injuries including glaucoma

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Purpose: The pathogenesis of retinal ganglion cell loss in glaucoma remains incompletely understood. Current evidence suggests that the optic nerve (ON) head and axons are the main site of injury in glaucoma. This study compares changes in pro-survival and proapoptotic gene expression in ONs with those in retinas in three models of ocular injury, specifically ON transection (ONTX), N-methyl-D-aspartate (NMDA) retinal toxicity, and experimental glaucoma.

Methods: Rats (n=240) were divided into three models (ONTX, NMDA retinal toxicity, and experimental glaucoma). The experimental model was induced unilaterally and the contralateral eye served as control. Rats were sacrificed at 4–5 different time points specific for each model. ONs and retinas were isolated for real-time PCR investigation of expression of selected genes. Immunohistochemistry localized changes in inhibitor of apoptosis (IAP)-1 and X-linked IAP (XIAP) proteins in retinas and ONs. Colocalization was measured using Imaris colocalization software (three-dimensional analysis).

Results: The earliest changes in gene expression occurred in ONs in the ONTX model and in retinas in the NMDA model, as expected. However, some gene changes occurred first in ONs, while others occurred first in retinas in the glaucoma model. The expression patterns of the pro-survival genes IAP-1 and XIAP differed between retinas and ONs of glaucomatous eyes: Both were upregulated in the retinas, but XIAP was downregulated in the ONs, while IAP-1 stayed unchanged. Colocalization of IAP-1, XIAP, glial fibrillary acidic protein, and Thymus cell antigen-1 (Thy-1) suggested that IAP-1 was colocalized mostly with Thy-1 and XIAP with glial fibrillary acidic protein in the ONs. Members of the B-cell lymphoma 2 (BCL-2) family were similarly involved in the ONs and retinas of glaucomatous, transected, and NMDA-injected eyes. The expression of the pro-survival genes, Bcl-2 and Bcl-x1, decreased significantly in both the ONs and retinas of injured eyes. The proapoptotic genes, BCL2-associated X protein (BAX) and Bcl-2-associated death promoter (BAD), were significantly upregulated in both injured retinas and ONs.

Conclusions: The overexpression of XIAP and IAP-1 genes in the retinas was not associated with similar changes in the ONs of glaucomatous eyes. The lack of activation of these pro-survival genes in the ONs may explain the increased vulnerability of ONs to elevated intraocular pressure.

The pathogenesis of retinal ganglion cell (RGC) loss in glaucoma remains incompletely understood. A diverse range of mechanisms has been suggested, but it is still uncertain whether the primary site of damage is the RGC bodies or the RGC axons. Current evidence suggests that the optic nerve (ON) head and axons are the main site of injury in glaucoma [1-3]. It is believed that axonal degeneration occurs separately from and before somal degeneration, as has been observed in many other neurodegenerative diseases [4-15].

It is now known that neurons have distinct classes of self-destruct programs that are spatially compartmentalized [16]. In addition to the well-described intracellular suicide machinery in the neuronal soma, which is responsible for apoptosis, there is another, molecularly distinct, self-destruct

program localized in the axon [9]. Recent data from in vitro studies and from an inherited mouse model of glaucoma suggest that molecularly distinct degenerative pathways underlie the destruction of RGC somata and RGC axons [14,17]. Axons, dendrites, and synapses often degenerate well before the cells die in various neurodegenerative diseases, and there is increasing evidence that this is important for the production of clinical symptoms and signs. In search of more information on axonal damage in optic neuropathies, including glaucoma, we designed this study to compare three models of ocular injury—ON transection (ONTX), in which the initial site of injury is in the axons; N-methyl-D-aspartate (NMDA)-induced retinal toxicity, in which the initial site of injury is in the retinas; and experimental glaucoma, in which the initial site of injury is still unclear.

RGC damage due to glaucoma involves multiple molecular pathways, including pro-survival and proapoptotic genes [15,18-20]. Our group recently demonstrated that glaucoma and ONTX induce simultaneous upregulation of proapoptotic

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and prosurvival genes in the retina, suggesting that elevated intraocular pressure (IOP) induces an endogenous neuro-protective mechanism by upregulation of prosurvival genes, such as inhibitor of apoptosis (IAP)-1 [19,21]. However, the extent of information on changes in gene expression and the involvement of an endogenous neuroprotective mechanism in ON axons is minimal.

This study investigates a unique group of genes of particular relevance to glaucoma, including those from the B-cell lymphoma 2 (Bcl-2) and IAP family. Investigating changes in selected pro- and antiapoptosis genes that occur in different models and at different time points will provide more much-needed information on the pathogenesis of glaucoma and enable the exploration of novel therapeutic targets.

METHODS

Animals: Wistar rats (375–425 g) were used in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research in protocols approved and monitored by the Animal Care Committee of the Tel-Aviv University School of Medicine. The animals were housed with a 14 h:10 h light-dark cycle with standard chow and water ad libitum.

General design: Three models (ONTX, NMDA retinal toxicity, and experimental glaucoma) were used and retinas and ONs were removed separately for each of these (n=80 for each model, total of 240 rats). The experimental model was induced unilaterally and the contralateral eyes served as controls. Rats were sacrificed at 4–5 different time points specific for each model and chosen according to the rate of RGC and axonal loss in each model. The first time point was chosen when early gene changes were expected to occur. The last time point was chosen when about 50% of the RGCs were expected to be lost, which was 30 days for the glaucoma model [22], 8 days for the ONTX model [19], and 6 days for the NMDA model [23].

Optic nerve transection model: ON transection was performed unilaterally under anesthesia. Using an operating microscope, the superior conjunctiva was incised and the intraorbital ON was transected 1–2 mm behind the globe, taking care not to damage its blood supply. The retinas were examined ophthalmoscopically to assure blood vessel patency. Rats were sacrificed at four time points (4 h, 12 h, 3 days, and 8 days).

NMDA retinal toxicity model: The rats in this model were sacrificed at 3 h and 12 h and at 1, 3, and 6 days following a unilateral single intravitreal injection of 2 μ l of 4 mM NMDA. A single injection of NMDA was administered

intravitreally with a heat-pulled glass capillary connected to a microsyringe.

Experimentally induced glaucoma model: Elevated IOP was induced unilaterally by laser treatment of the outflow channels of the eye through the peripheral cornea using the translimbal photocoagulation model [22]. The treatment was repeated at 1 week. IOP was measured under anesthesia, and the average of 10 readings was recorded with a TonoLab tonometer (TioLat, Helsinki, Finland). IOP measurements were taken immediately before and after the laser treatment, 1 day after each treatment, and then weekly. The animals were sacrificed at five time points, specifically 1, 4, 8, 14, and 30 days.

Real-time reverse transcription polymerase chain reaction: ONs and retinas were removed separately and instantly frozen in dry ice. The ONs were removed from the optic chiasm to the eye. Total RNA was isolated from the retinas and ONs of control and experimental eyes at various time points using the Trizol total RNA isolation kit (Invitrogen, Paisley, UK). RNA was then reverse transcribed into cDNA by MMLV reverse transcriptase (Invitrogen). cDNA samples were analyzed by real-time PCR using the Quanti Tect SYBR Green reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen, Valencia, CA). Amplification reactions were performed by 40 cycles of 20 s at 94 °C and of 1 min at 60 °C in the ABI/Prism 7700 Sequence Detector System (Applied Biosystems, Perkin Elmer, UK). All samples were tested in triplicate. A standard curve for each gene was created using three 10-fold dilutions of a cDNA sample prepared from the retina of a naïve animal. The results were analyzed using the Sequence Detector Software V1.6 (Applied Biosystems), and β -actin messenger RNA (mRNA) was used as an endogenous control. Primers were purchased from Sigma-Aldrich (Rehovot, Israel; Table 1).

Immunohistochemistry: The eyes of each animal with glaucoma were enucleated and cryopreserved 8 days after lasering (Sakura Finetek, USA Inc.; Torrance, CA) in sucrose/optimum cutting temperature (OCT). At least three cryosections of 10 μ m thick were examined from each eye. For IAP, X-linked IAP (XIAP), Thymus cell antigen-1 (Thy-1), a marker of RGC and glial fibrillary acidic protein (GFAP), sections were incubated with goat antirat IAP (1:100, Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-XIAP (1:100, R&D Systems, Minneapolis, MN), mouse antirat Thy-1 (1:100, Biologend, San Diego, CA), and mouse anti-GFAP (1:500, mouse monoclonal [Sigma Aldrich] and rabbit polyclonal [Millipore, Billerica, MA]). The secondary antibody was Alexa Fluor 633- or 488-conjugated antigoat immunoglobulin G (1:500), Alexa Fluor F68 antirabbit (1:500), or

TABLE 1. PRIMERS USED FOR qPCR ANALYSIS OF GENE EXPRESSION

Gene	Primer (5'-3')
IAP	Left: TGTGCATCTGGGCCCTG
Gene ID: 78971	Right: CTGACCGTCCTGTAGTTCTCA-3'
XIAP	Left: GACAAATGTCCCAT
Gene ID: 63,897	Right: CTAATGGACTGCGA
BCL-2	Left: ATAACCGGGAGATCGTGAG
Gene ID: 24,224	Right: CAGGCTGGAAGGAGAAAGATG
Bcl-xl	Left: GGTGAGTCGGATTGCAAG
Gene ID: 24,888	Right: GGCAGTTAGGGATCTCCA
BAD	Left: CAGGCAGCCAATAACAGT
Gene ID: 64,639	Right: CCATCCCTTCATCATCTTCCTC
BAX	Left: GCTGACATGTTTGCTGATGG
Gene ID: 24,887	Right: GATCAGCTCGGGCACTTTAG
GADD45	Left: TAACTGTGGCGTGACGAG
Gene ID: 25,112	Right: GCAACAGAAAGCACGAATGA
β -ACTIN	Left: GCTACAGCTTCTCCACCACA
Gene ID: 81,822	Right: TCTCCAGGGAGGAAGAGGAT

Alexa Fluor 488 or 633 antimouse (1:500; Invitrogen). Negative controls included nonimmune serum of the same species as the primary antibody at the same protein concentration as the secondary antibody only.

Confocal images were acquired with a Zeiss LSM 750 (Carl Zeiss, Thornwood, NY) confocal microscope using objectives of X63 oil with X3 optical zoom (numerical aperture 1.4). Images were acquired as confocal stacks of 60–80 optical sections of 1024×1024 pixels. Each Z-stack consisted of a depth of optical sections collected at 0.2 μ m increments along the Z-plane. Excitation light was provided by the 488 nm line of argon lasers for the Alexa-488 fluorophore, the 561 nm line of diode lasers for the Alexa-568 fluorophore, and the 633 nm line of HeNe lasers for the Alexa-633 fluorophore. The images were further improved using Huygens deconvolution software (Scientific Volume Imaging b.v., The Netherlands) [24,25].

In this study, colocalization of two labels means that they are present in the tissue with enough proximity that they cannot be resolved optically. This was interpreted as indicating that the targets of the antibodies are located in the same small neuronal compartment, that is, the ON. Colocalization was measured using Imaris colocalization software (X63, 6.1.5, Bitplane, Zurich, Switzerland), which analyzes two-channel confocal stacks in their entirety by measuring the intensity of each label, voxel by voxel. Performing the analysis in three dimensions obviates the generation of

false-positive results in projection images by projecting voxels in different Z positions into the same X-Y position.

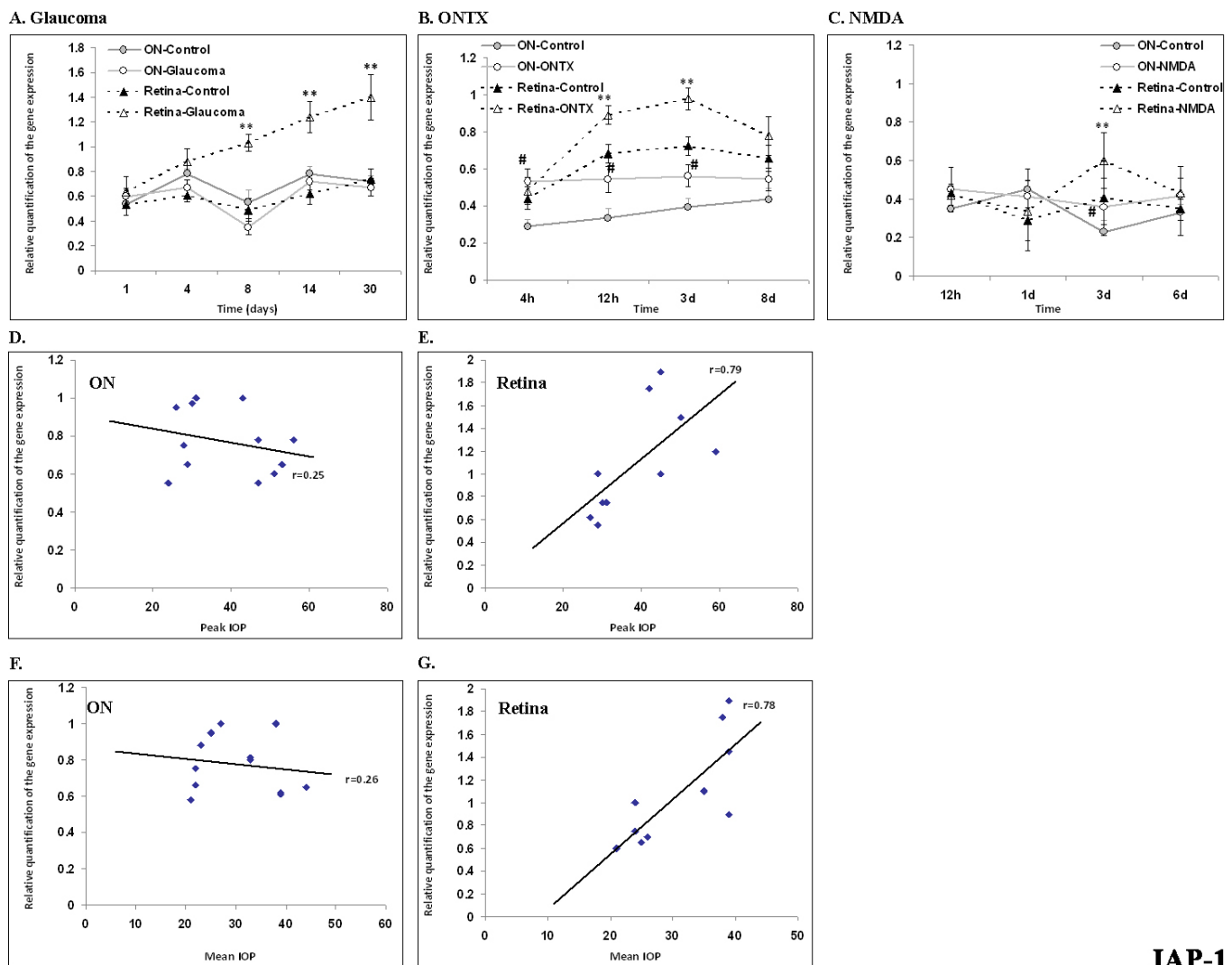
RESULTS

The expression of seven different representative genes known to be involved in glaucoma and other ocular injuries was investigated in the retinas and ONs of eyes by means of three ocular injury models at multiple time points. These genes were carefully selected based on our earlier studies [19,26,27], and their involvement had been investigated in various settings of ON injuries. A total of 240 rats were involved in this study.

The involvement of IAP and XIAP, members of the inhibitor of apoptosis gene family: Real-time PCR and immunohistochemistry were performed to determine whether members of the IAP family are involved in axonal degeneration of various ocular injuries and to compare their involvement with RGC body death. The glaucoma model was the only one of the three models in which the prosurvival gene IAP-1 was not overexpressed in the ON. IAP-1 was significantly upregulated in the retinas of glaucomatous eyes, supporting our previously published data [19,27]; however, IAP-1 mRNA levels stayed unchanged in glaucomatous ONs (Figure 1A). Peak and mean IOPs were significantly correlated with IAP-1 expression in the retinas (Figure 1E,G, $r=0.79$, 0.78 , $p<0.05$, respectively), but not in the ONs (Figure 1D,F, $r=0.25$, 0.26 , respectively). At the protein level, staining for IAP-1 was similar in the ONs

of the glaucomatous and control eyes, but it was more intense in the retinas of the glaucomatous eyes (Figure 2A and Figure 3A). IAP-1 was upregulated in both the ONs and retinas of the ONTX and NMDA models. In the ONTX model, IAP-1 was first upregulated in the ONs 4 h after transection ($p=0.011$, $n=6$), and later at 12 h ($p=0.035$, $n=5$) and 3 days ($p=0.047$, $n=5$) in the retinas (Figure 1B). In the NMDA model, IAP-1 was significantly upregulated in both retinas and ONs on day 3 (Figure 1C, $p=0.026$ and $p=0.05$ respectively, $n=5$ for each group).

The pattern of XIAP expression in the retinas and ONs was reversed. The prosurvival gene XIAP was significantly upregulated in the retinas of glaucomatous eyes at 2 days ($p=0.003$, $n=5$) and stayed elevated for 2 weeks. However, it was significantly downregulated in the ONs of the glaucomatous eyes beginning as early as day 1 ($p=0.02$, $n=6$) and lasting for 4 days ($p=0.001$, $n=5$; Figure 4A). Peak and mean IOPs were significantly correlated with XIAP expression, positively in the retinas (Figure 4E,G, $r=0.71$, 0.64 , $p<0.05$, respectively) and negatively in the ONs (Figure 4D,F, $r=0.74$, 0.80 , $p<0.05$, respectively). These results, taken together with



IAP-1

Figure 1. Real-time polymerase chain reaction analysis of the prosurvival gene inhibitor of apoptosis-1 (IAP-1) from the optic nerves and retinas of three models of ocular injury. **A:** Inhibitor of apoptosis (IAP)-1 gene expression increased significantly in the retina, while it stayed unchanged in the ON in the glaucoma model. **B** and **C:** IAP-1 increased significantly in both the ONs and retinas in the ONTX and NMDA models. **D-G:** Peak and mean intraocular pressures (IOPs) were significantly correlated with IAP-1 expression in the retina (**E, G:** $r=0.79$, 0.78 , $p<0.05$, respectively) but not in the ON (**D, F:** $r=0.25$, 0.26 , respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly higher at $p<0.05$ than ON controls, **significantly higher at $p<0.05$ than retina controls.

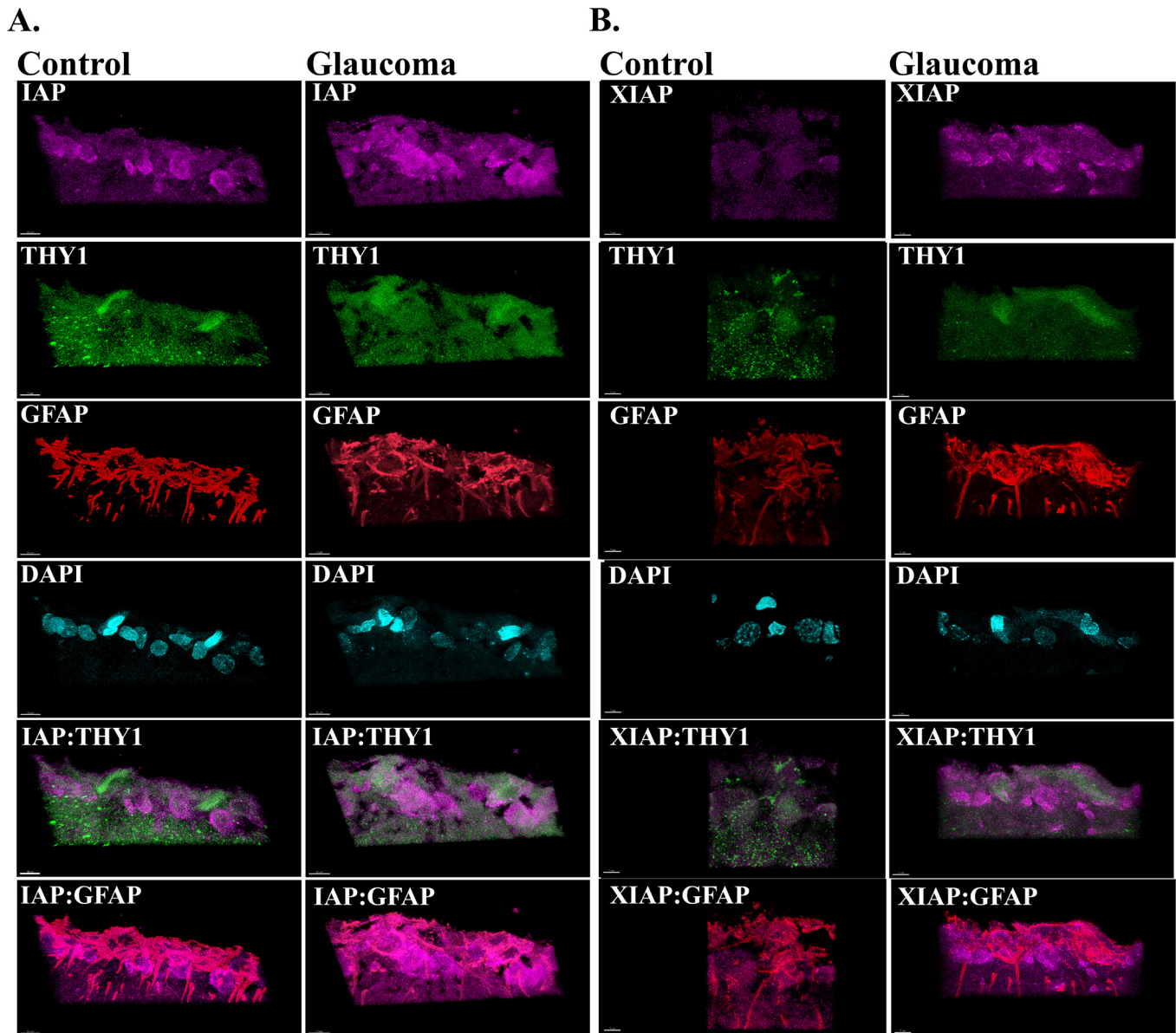


Figure 2. X-linked inhibitor of apoptosis (XIAP) and inhibitor of apoptosis-1 (IAP-1) protein expressions increased in retinas of glaucomatous eyes compared to controls. **A** and **B**: Both were expressed in the retinal ganglion cell (RGC) layer and in other retinal layers. Colocalization with glial fibrillary acidic protein (GFAP; a glial marker) and Thy-1 (an RGC marker) at 8 days after the induction of elevated intraocular pressure (IOP) suggests that both inhibitor of apoptosis (IAP)-1 and X-linked IAP (XIAP) secretion in the retina originate from both RGC bodies and glial cells.

the lack of IAP-1 upregulation in the ONs, indicate deficiency of prosurvival signaling in the ONs of glaucomatous eyes. At the protein level, staining for XIAP was less intense in the ONs of glaucomatous eyes than controls, but it was more intense in the retinas of the glaucomatous eyes (Figure 2B and Figure 3B).

To localize changes in IAP-1 and XIAP expression in the ONs, we evaluated confocal images with Imaris software.

Colocalizations for IAP-1 and XIAP proteins and for Thy-1 and GFAP were investigated (Figure 5), and IAP-1 was found to be colocalized with neurofilament-H (NFH, a neuronal marker) and GFAP (a glial marker) in the ONs. However, the degree of colocalization of IAP-1 with NFH was significantly higher in the control and glaucomatous ONs compared to its colocalization with GFAP, suggesting that most of the IAP-1 in the ONs comes from axons (Figure 5A,C). Interestingly, most of the XIAP protein was colocalized with GFAP,

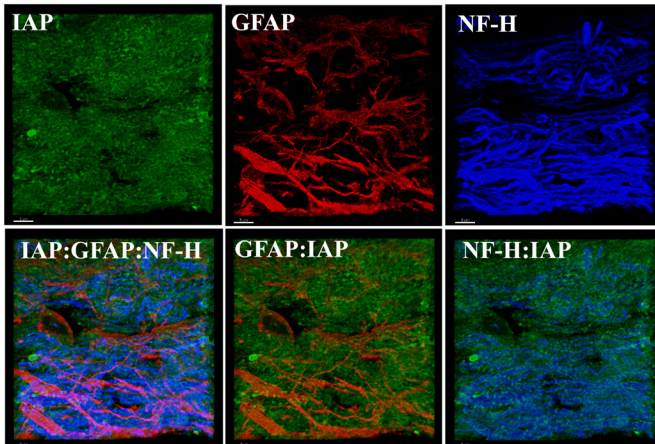
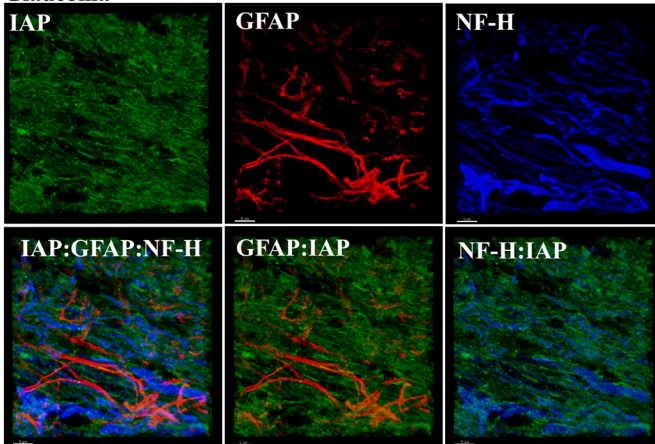
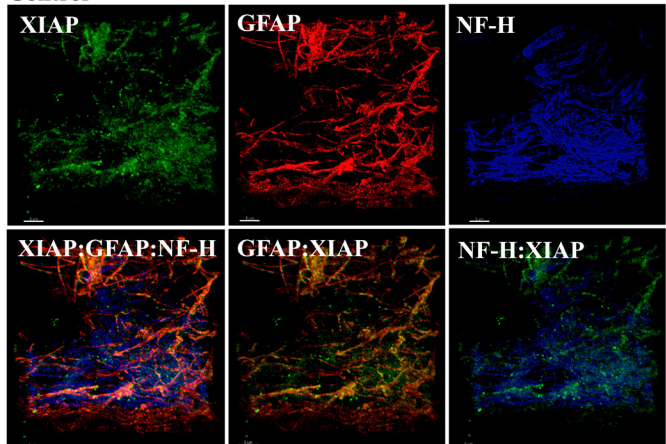
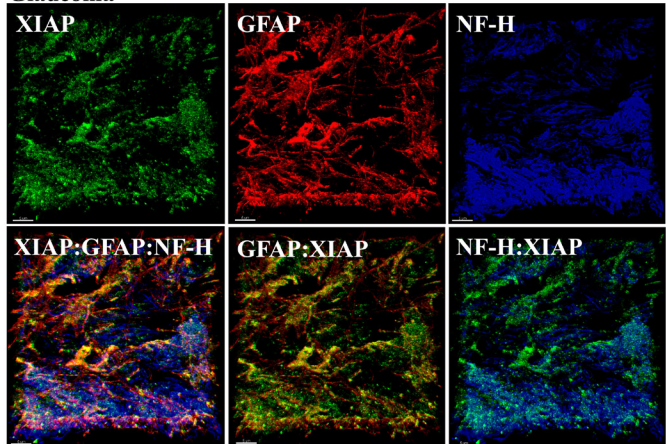
A. IAP expression in Optic Nerve**Control****Glaucoma****B. XIAP expression in Optic Nerve****Control****Glaucoma**

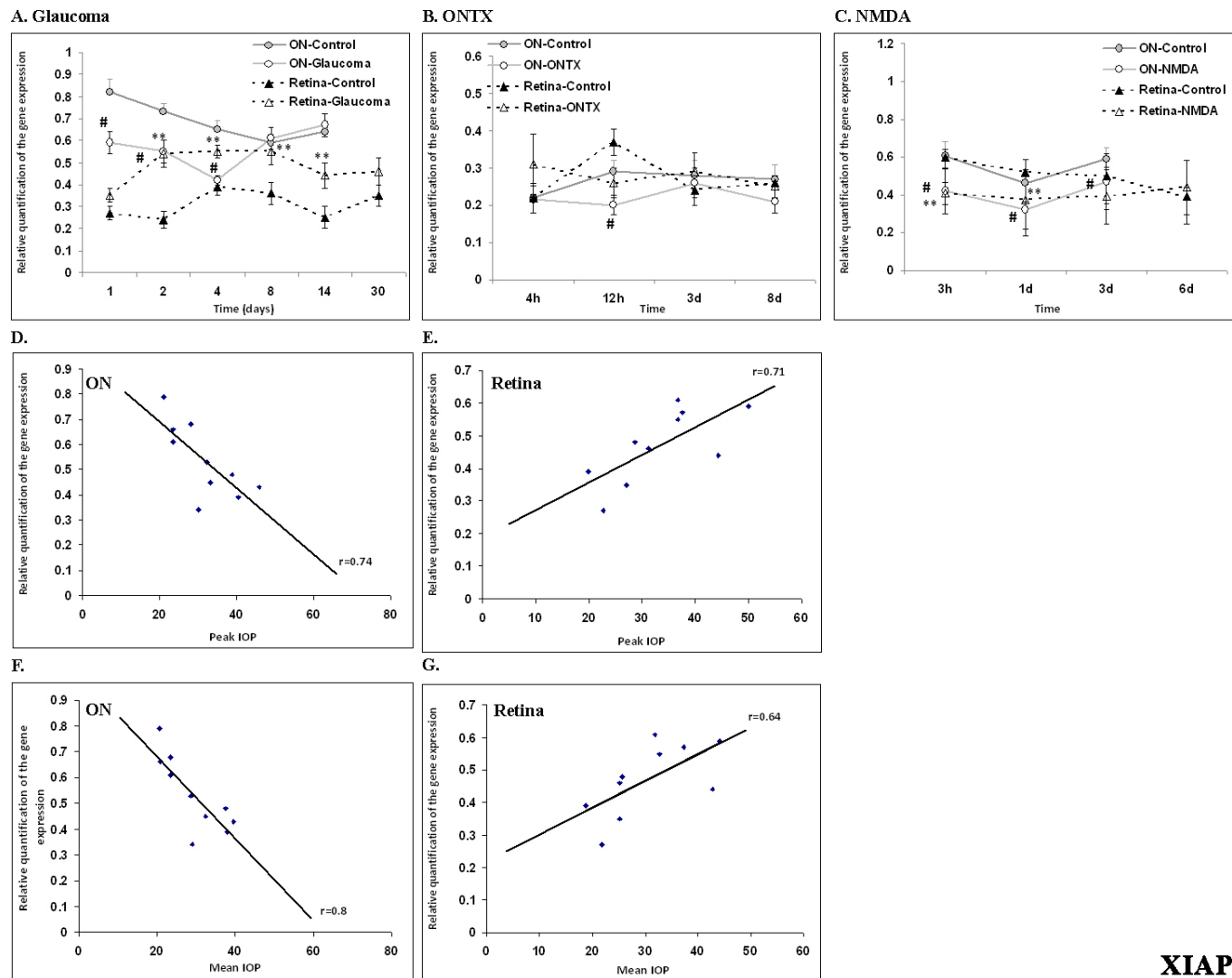
Figure 3. X-linked inhibitor of apoptosis and inhibitor of apoptosis-1 protein expressions in the optic nerves of glaucomatous eyes and their controls. **A** and **B**: Colocalization of X-linked inhibitor of apoptosis (XIAP) and IAP-1 with glial fibrillary acidic protein (GFAP; a glial marker) and neurofilament-H (NFH, a neuronal marker) at 8 days after the induction of elevated intraocular pressure (IOP) suggests that both XIAP and IAP-1 are present in optic nerve (ON) axons and glia. IAP-1 was more colocalized with NFH and XIAP was more colocalized with GFAP.

suggesting that most of its expression comes from glial cells (Figure 5D). XIAP expression was significantly downregulated in the ON at 12 h in the ONTX model (Figure 4B) and stayed unchanged in the retinas ($p=0.06$ at 12 h). There was a significant downregulation of XIAP mRNA in the ONs and retinas in the NMDA model (Figure 4C).

The involvement of Bcl-2 family members in this multimodel study: The expression of the prosurvival gene Bcl-2 decreased significantly in both ONs and retinas of the glaucoma and ONTX models. Interestingly, changes appeared earlier in the retinas of glaucomatous eyes and in the ONs of transected eyes (Figure 6A,B). There was a significant negative correlation between peak and mean IOPs and Bcl-2 gene expression in the retinas and ONs (Figure 6C-F). There was no significant difference in mRNA Bcl-2 levels in the retinas or

ONs of the NMDA eyes (Figure 6C). The proapoptotic gene BAX was significantly upregulated in both retinas and ONs of glaucomatous eyes on day 14 ($p=0.006$ and $p=0.01$, $n=5$ respectively), returning to normal levels on day 30 ($p=0.5$, $n=5$, and $p=0.5$, $n=5$, respectively; Figure 7A). There was a significant correlation between IOP and Bax expression in ONs and retinas (Figure 7D-G). Bax was significantly upregulated first in the ONs of eyes with ONTX followed by a concomitant increase in the retinas (Figure 7B). The increased Bax level returned to normal in both ON and retina samples after 8 days. Bax expression increased in both ONs and retinas at 12 h in the NMDA model ($p=0.0002$, $n=5$, and $p=0.006$, $n=5$; Figure 7C).

We investigated two more members of the Bcl-2 family—the prosurvival gene Bcl-xl and the proapoptotic



XIAP

Figure 4. Real-time polymerase chain reaction analysis of the prosurvival gene X-linked inhibitor of apoptosis (XIAP) from the optic nerves and retinas of three models of ocular injury. **A**: X-linked inhibitor of apoptosis (XIAP) gene expression increased significantly in the retinas and decreased significantly in the optic nerves (ONs) in the glaucoma model. **B** and **C**: XIAP decreased significantly in the ONs and retinas in the ONTX and NMDA models. **D-G**: The peak and mean intraocular pressures (IOPs) were significantly and positively correlated with XIAP expression in the retinas (**E**, **G**: $r = 0.71$, 0.64 , $p < 0.05$, respectively) and inversely correlated in the ONs (**D**, **F**: $r = 0.74$, 0.80 , $p < 0.05$, respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n = 5-7$ rats were used at each time point. Data represent means \pm standard error of the mean (SEM); #significantly different at $p < 0.05$ than ON controls, **significantly different at $p < 0.05$ than retina controls.

gene BAD—in models of glaucoma and NMDA. Similarly to the *bcl-2* gene, *Bcl-xl* was significantly decreased in glaucoma, first in the retinas on day 8 ($p = 0.0003$, $n = 5$) and later in a significant decrease in both retinas and ONs at day 14 ($p = 0.02$, $n = 5$ and $p = 0.04$, $n = 5$, respectively; Figure 8A). *Bcl-xl* mRNA levels stayed unchanged in the ONs and retinas of the NMDA model (Figure 8B). There was a significant negative correlation between peak and mean IOPs and *Bcl-xl* retinal expression, but not with *Bcl-xl* ON expression (Figure 8C-F). The *Bcl-xl* expression behavior was similar

to *Bcl-2*, with the first appearance of change in the retinas of the glaucoma model and no change in the retinas of the NMDA model. The expression of the proapoptotic gene BAD increased significantly, first in the retinas of the glaucomatous eyes ($p = 0.02$, $n = 5$; Figure 9A), and then in a concomitant increase in the ONs on day 14 ($p = 0.006$, $n = 6$). There was a significant correlation between peak and mean IOPs and BAD expression (Figure 9C-F). BAD expression increased significantly in the retinas and ONs, mimicking the behavior of BAX, in the NMDA model (Figure 9B).

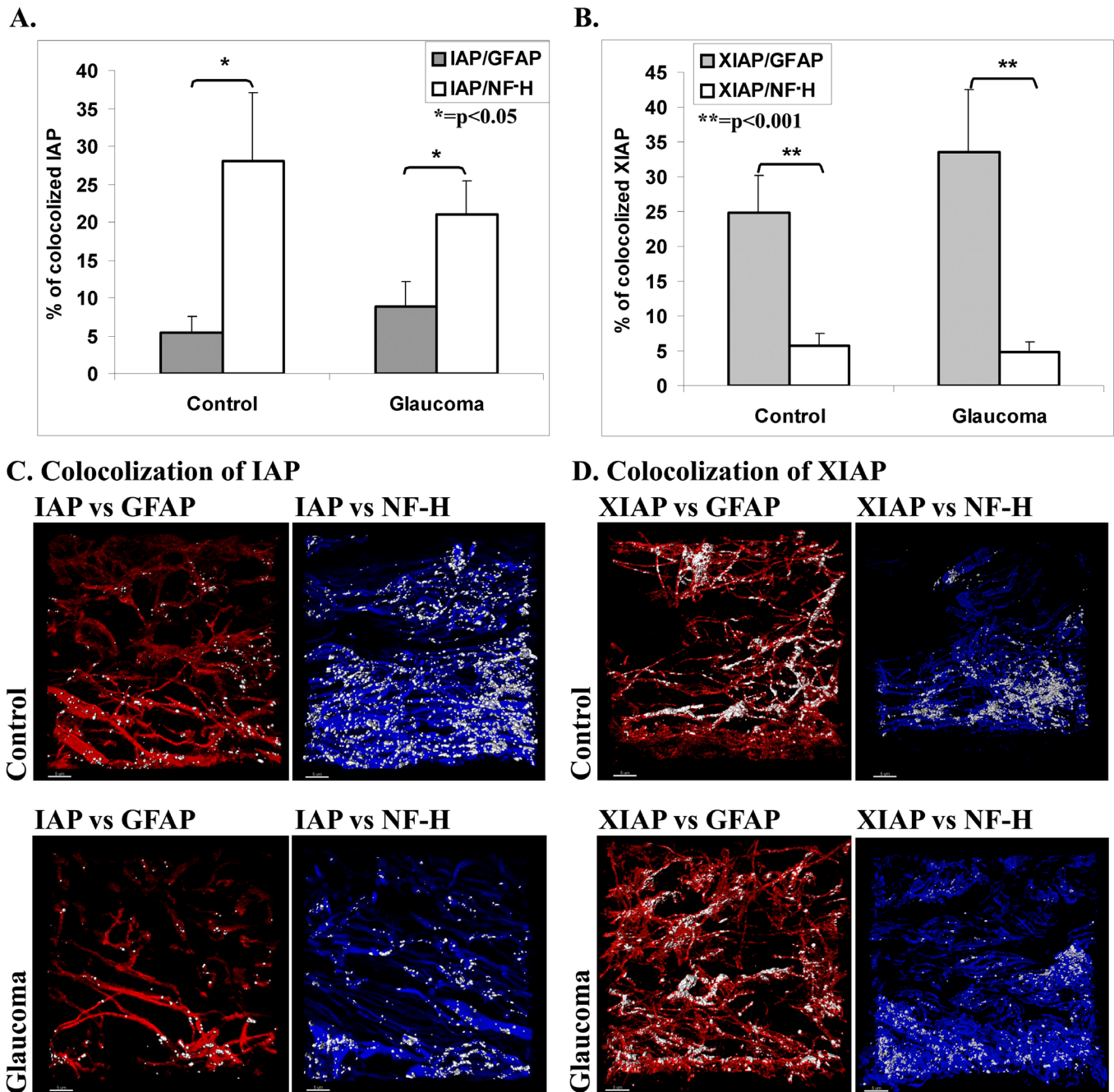
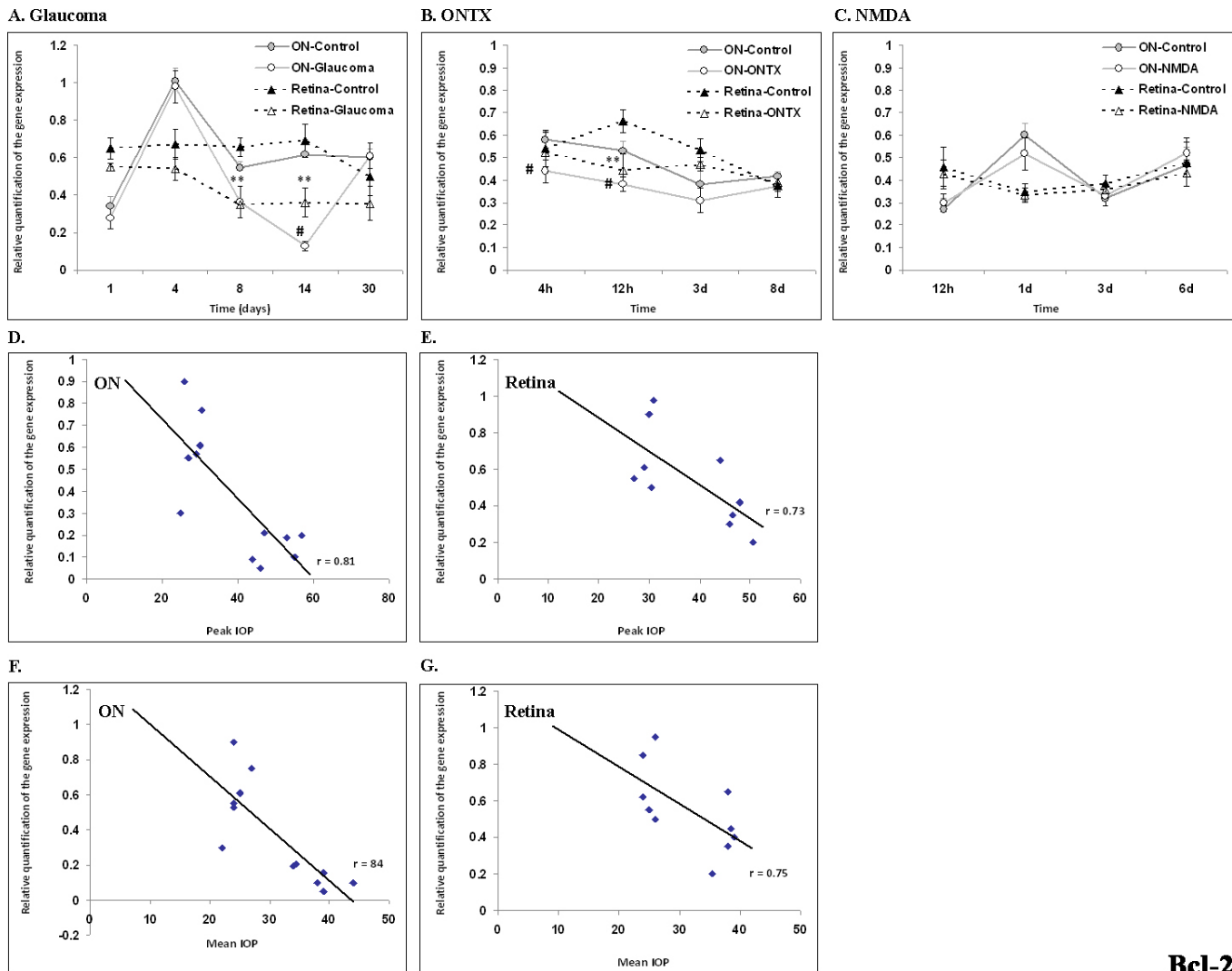


Figure 5. Colocalization of inhibitor of apoptosis and X-linked inhibitor of apoptosis proteins with neurofilament H (a neuronal marker) and glial fibrillary acidic protein (a glial marker). Triple immunostaining of X-linked inhibitor of apoptosis (XIAP) and IAP with glial fibrillary acidic protein (GFAP) and neurofilament H (NFH) was performed on optic nerves (ONs) at 8 days after the induction of elevated intraocular pressure (IOP). **A:** Colocalization of IAP-1 with NFH and GFAP suggests that IAP-1 in the ON is mostly neuronal. **B:** Colocalization of XIAP with NFH and GFAP suggests that XIAP in the ON is mostly glial. **C, D:** These images represent colocalization of IAP-1 and XIAP with NFH and GFAP (white areas). The analysis was done after the pictures were deconvoluted using Hyugens program with the Imaris Bitmate, as described in Material and Methods; n=5 for each staining, *p<0.05, **p<0.001.

The involvement of the proapoptotic gene, *GADD45a*, a member of the *p53* pathway: *GADD45a* mRNA levels increased significantly in ONs and retinas of glaucomatous

eyes beginning at day 4 (p=0.005, n=6 and p=0.02, n=5, respectively) and were back to baseline at day 30 in the ONs (p=0.9, n=7), while they stayed elevated in the retinas



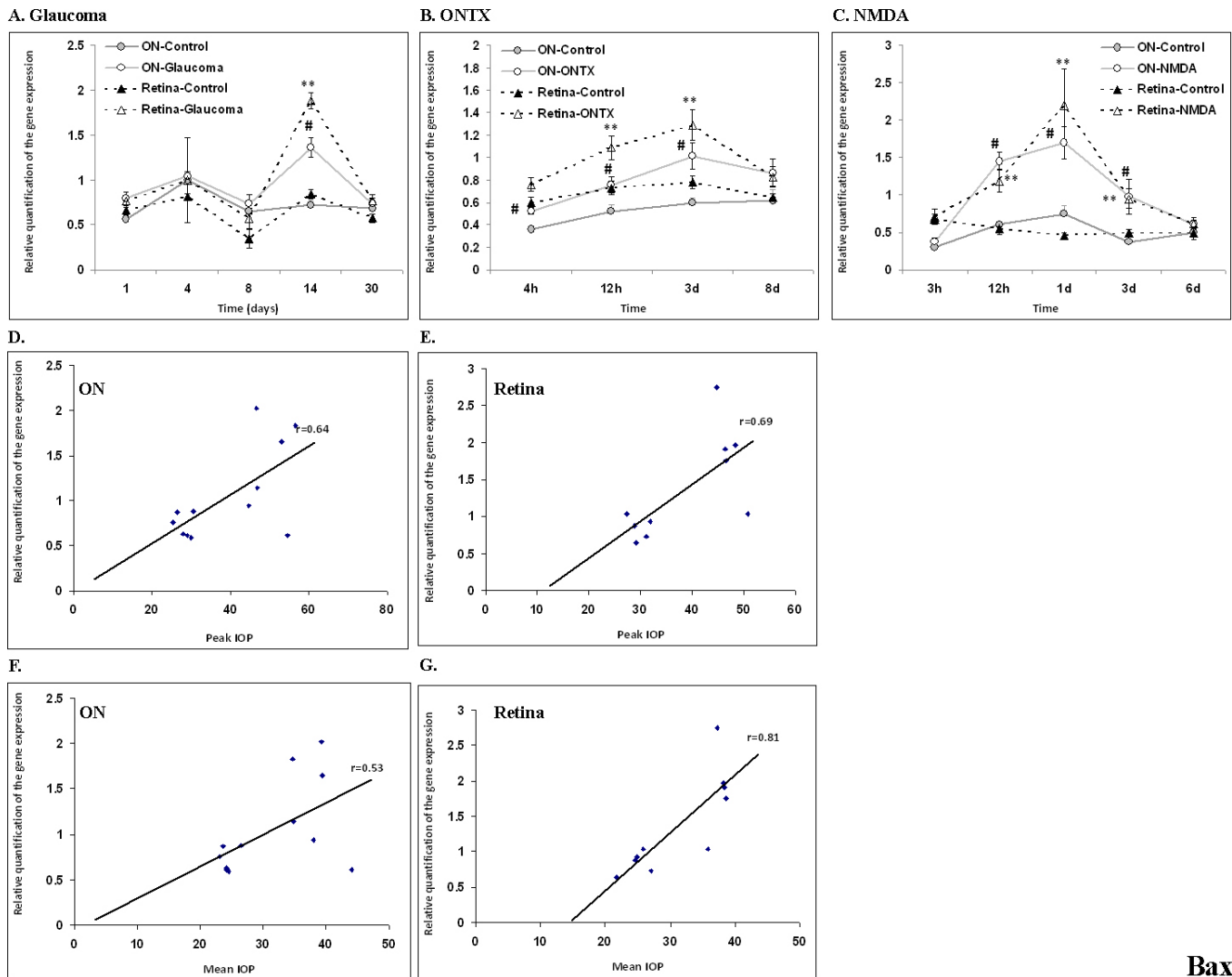
Bcl-2

Figure 6. Real-time polymerase chain reaction analysis of the prosurvival gene, BCL-2, in optic nerves and retinas of three ocular injury models. **A-C**: The Bcl-2 gene expression decreased significantly in both the optic nerves (ONs) and retinas in the glaucoma and ONTX models, while it stayed unchanged in the NMDA model. **D-G**: The peak intraocular pressure (IOP) was significantly correlated with Bcl-2 expression in ONs and retinas (**D, E**: $r=0.81, 0.73, p<0.05$, respectively), as was the mean IOP (**F, G**: $r=0.84, 0.75, p<0.05$, respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly lower at $p<0.05$ than ON controls, **significantly lower at $p<0.05$ than retina controls.

($p=0.02, n=5$; Figure 10A). There was a significant positive correlation between GADD45 α expression and peak and mean IOP (Figure 10D-G, $p<0.05$ for all correlations). GADD45 α levels increased first in the ONs (4 h, $p=0.01, n=6$) and later in both ONs and retinas in the ONTX model (day 3, $p=0.019, n=5$, and $p=0.03, n=6$, respectively; Figure 10B). Both ON and retinal GADD45 α mRNA levels increased significantly from 12 h ($p=0.006, n=5$, and $p=0.0005, n=5$, respectively) and remained high for 3 days in the NMDA model ($p=0.002, n=5$ and $p=0.009, n=5$; Figure 10C).

DISCUSSION

The focus of this paper is on evaluating and comparing changes in gene expression between ONs and retinas at multiple time points to determine where these changes begin and why the ON is more vulnerable to elevated IOP than the retina. We used three models of optic neuropathy and retinal damage (glaucoma, NMDA retinal toxicity, and ONTX), and the expression of selected genes was evaluated in the ONs and retinas separately and at multiple time points in each. The glaucoma model used in this study is similar to acute-onset



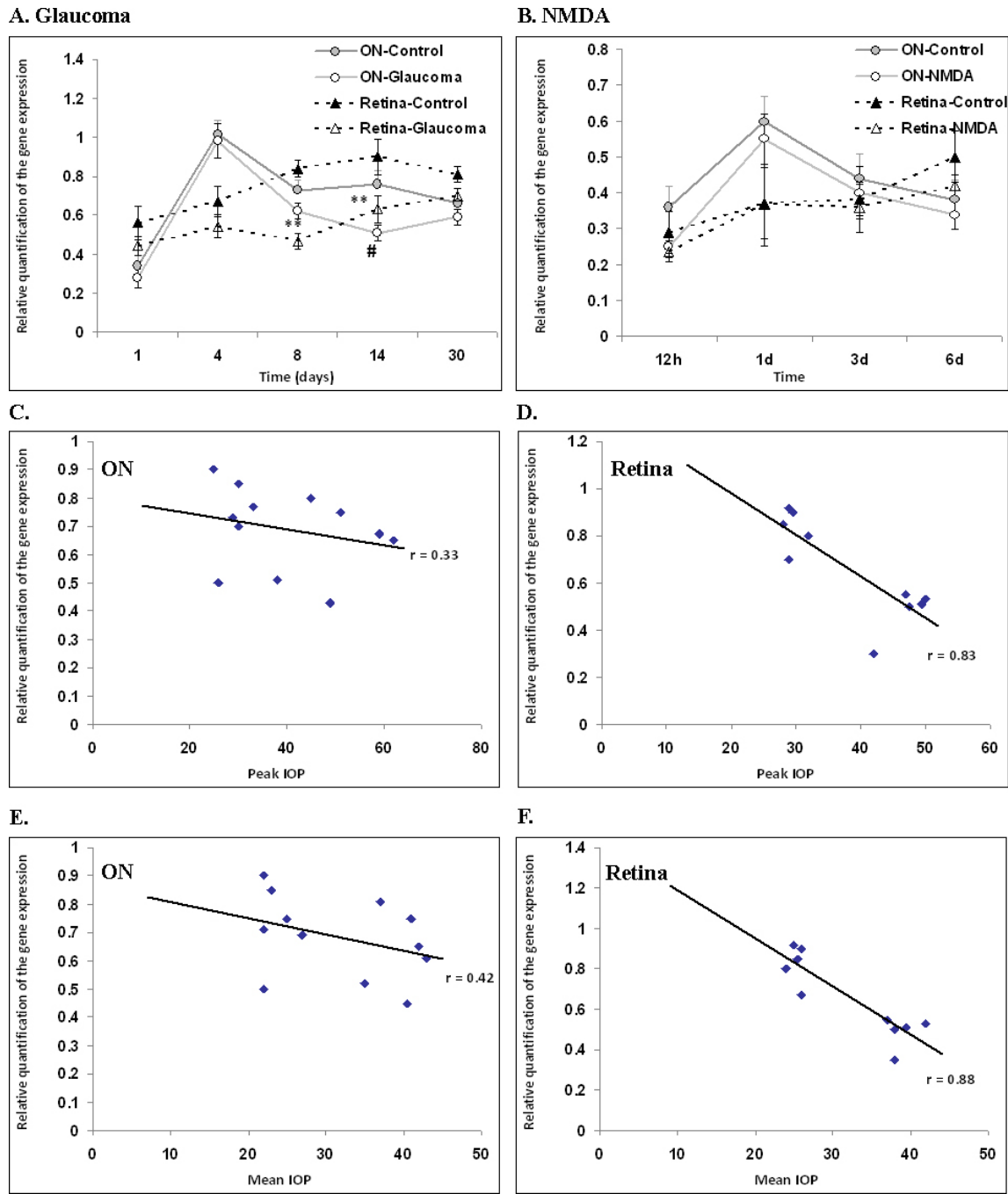
Bax

Figure 7. Real-time polymerase chain reaction analysis of the proapoptotic gene, Bax, from the optic nerves and retinas of three ocular models: (A) Glaucoma (B) ONTX and (C) NMDA retinal toxicity. Bax expression increased significantly in the optic nerves (ONs) and retinas of all three models. The peak intraocular pressure (IOP) was significantly correlated with Bax expression in ONs and retinas (D, E: $r=0.64$, 0.69 , $p<0.05$ respectively), as was the mean IOP (F, G: $r=0.53$, 0.81 , $p<0.05$, respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly higher at $p<0.05$ than ON controls, **significantly higher at $p<0.05$ than controls.

glaucoma with IOP elevation for about 3 weeks. Selection of genes was based on our previously published results [19,27]. We chose to investigate this particular group of genes because of their unique involvement in glaucoma and other ocular injuries, including secondary degeneration [26]. The results of the current study suggest that the earliest changes in gene expression occur in the ON in the ONTX model and in the retina in the NMDA model, as expected. However, some of these changes occurred first in the ONs and others in the retinas in the glaucoma model, suggesting that the site of early insult can originate from both the retina and the ON,

a heretofore unreported finding in the pathophysiology of glaucoma.

The involvement of three major signaling pathways was investigated in this multimodel study, specifically the bcl-2 family, the IAP family, and the p53 pathway. Changes in gene expression were detected in the retinas and ONs for all pathways, suggesting that there is active involvement of this group of genes in both retinas and ONs for all models. However, changes in the expression of the prosurvival genes IAP and XIAP were different between ONs and retinas in the glaucoma model. We found upregulation of members of

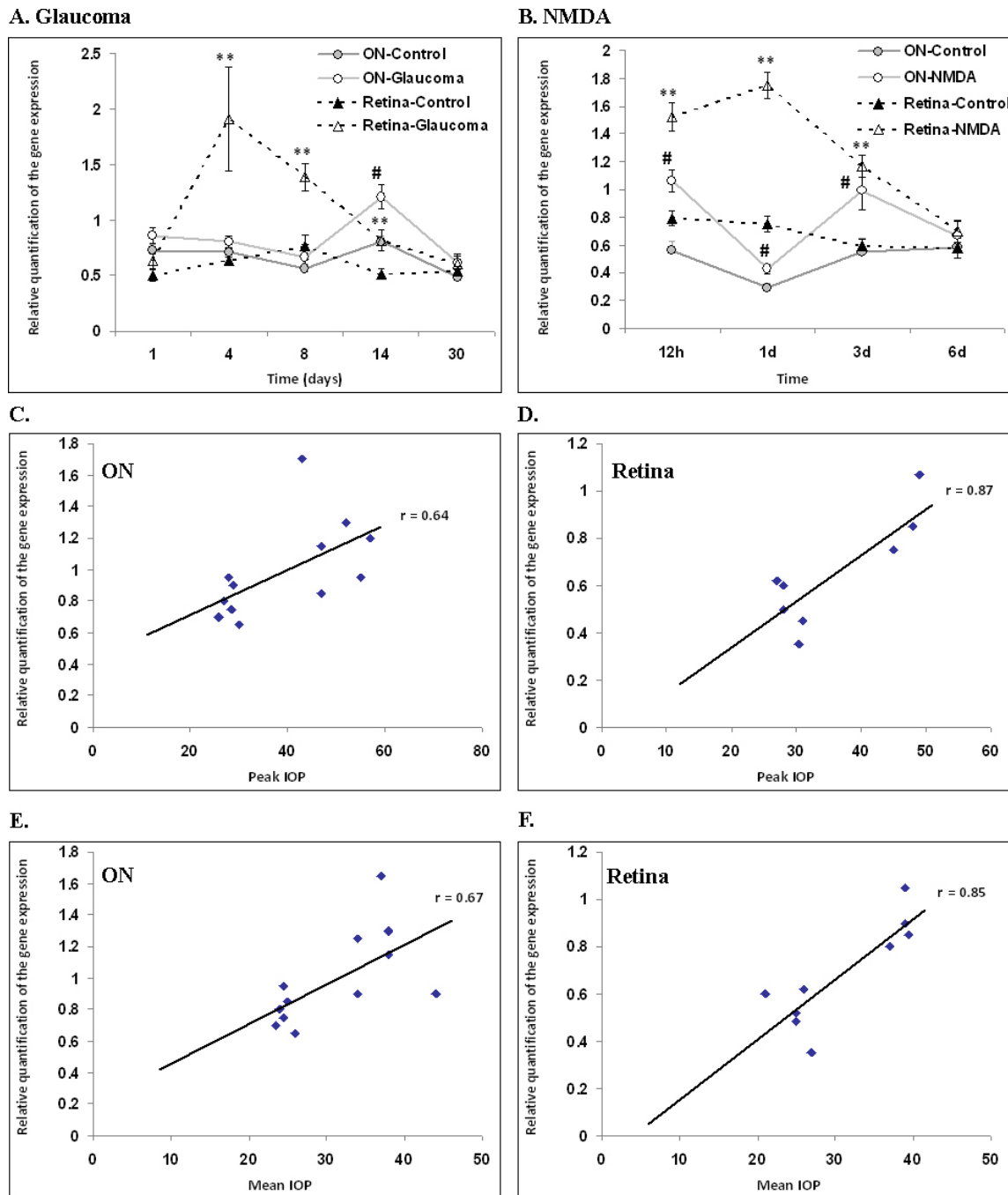


Bcl-xl

Figure 8. Real-time polymerase chain reaction analysis of the prosurvival gene, Bcl-xl, from optic nerves and retinas of glaucoma (A) and NMDA retinal toxicity (B) models. Bcl-xl gene expression decreased significantly in both the optic nerves (ONs) and retinas of glaucomatous eyes, while it stayed unchanged in the NMDA model. The peak and mean intraocular pressures (IOPs) were significantly correlated with Bcl-xl expression in the retinas (D, F: $r=0.83$, 0.88 , $p<0.05$, respectively), but not in the ONs (C, E: $r=0.33$, 0.42 , respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly lower at $p<0.05$ than ON controls, **significantly lower at $p<0.05$ than retina controls.

the IAP family (the IAP-1 and XIAP genes) in the retinas of glaucomatous eyes, suggesting an active endogenous

neuroprotective mechanism in the retina as a response to elevated IOP. This mechanism was most probably inactive

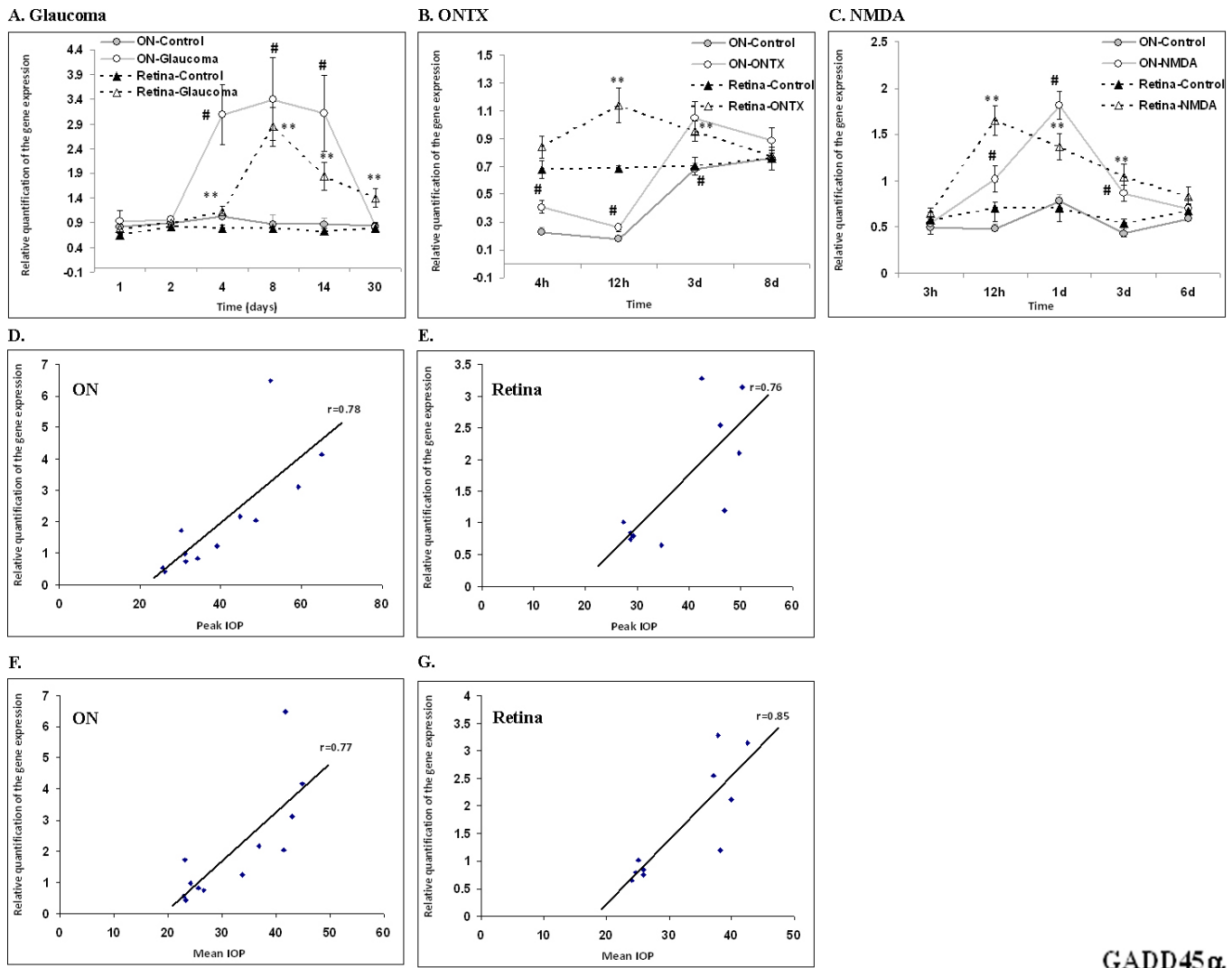


Bad

Figure 9. Real-time polymerase chain reaction analysis of the proapoptotic gene BAD from the optic nerves and retinas of the glaucoma model (A) and the NMDA retinal toxicity model (B). BAD gene expression increased significantly in both the optic nerves (ONs) and retinas of both models. The peak intraocular pressure (IOP) was significantly correlated with BAD expression in the ONs and retinas (C, E: $r=0.64, 0.87, p<0.05$ respectively), as was the mean IOP (D, F: $r=0.67, 0.85, p<0.05$, respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly lower at $p<0.05$ than ON controls, **significantly lower at $p<0.05$ than retina controls.

in the ON because there was downregulation of XIAP and no change in the IAP-1 expression level. Our hypothesis is that the absence of an active endogenous neuroprotective

mechanism in the ON makes it vulnerable to elevated IOP, thus providing molecular-based evidence in support of many



GADD45 α

Figure 10. Real-time polymerase chain reaction analysis of the proapoptotic gene, GADD45 α , from the optic nerves and retinas in three ocular models: (A) Glaucoma (B) ONTX, and (C) NMDA retinal toxicity. The GADD45 α expression increased significantly in the optic nerves (ONs) and retinas of all 3 models. The peak intraocular pressure (IOP) was significantly correlated with GADD45 α expression in both the ONs and retinas (D, E: $r=0.78, 0.76, p<0.05$, respectively), as was the mean IOP (F, G: $r=0.77, 0.85, p<0.05$, respectively). Values are expressed as fold change of gene expression compared to a calibrator (endogenous control, β -actin); $n=5-7$ rats at each time point. Data represent means \pm standard error of the mean (SEM); #significantly higher at $p<0.05$ than ON controls, **significantly higher at $p<0.05$ than retina controls.

studies that suggest that the ON is the main site of injury in glaucoma.

IAP proteins, as their name implies, confer protection from death-inducing signals by inhibition of diverse apoptosis mediators such as caspase, 3, 6, 7, and 9 [28,29]. XIAP is the best-characterized IAP and the most powerful suppressor of apoptosis [30,31]. In the current study, XIAP and IAP-1 expressions were increased in glaucomatous retinas, whereas XIAP and IAP-1 expressions in the ON were decreased or stayed unchanged, suggesting that inhibition of apoptosis is

compromised in the ON. The response of members of the IAP family was unique and required further attention. As opposed to other prosurvival genes like bcl-2 and bcl-x1, which are downregulated in both the retinas and the ONs, the prosurvival genes IAP-1 and XIAP were upregulated in the retina, as we previously showed [19]. Interestingly, they had the opposite reaction in the ON, suggesting that they had no active participation in protecting the axons from degeneration. The difference in the IAP family response between retinas and ONs was unique to the glaucoma model, leading

to the hypothesis that RGC bodies and axons react differently in their response to elevated IOP.

Until recently, axonal degeneration was considered a passive mechanism induced by disconnection of the axon from the cell body and essential proteins, which resulted in starvation [2]. Current evidence suggests that local signaling pathways may determine whether axons, dendrites, and their synapses remain connected or degenerate with consequent effects on neuronal function and survival [32]. The data presented herein provide support for the hypothesis that the axonal degeneration in glaucoma is a molecularly distinct degenerative process, and that compartmentalized and autonomous programs are of critical importance in the pathophysiology of glaucoma. Libby et al. and others [13-15] found that degeneration in Bax knockout mice proceeds in the axon while RGC somata survive, further illustrating the compartmentalization of degenerative changes that can occur. Soto et al. [11] suggested that there are two distinct degenerative processes that contribute to the ultimate loss of the majority of RGCs in glaucoma. The first process involves linked declines in RGC gene expression and retrograde transport that are probably chronic and widespread across the retina. The second process apparently involves damage to the axon that results in the rapid degeneration of the distal portion while there is still preservation of the proximal portion for as long as one month. Our immunohistochemistry studies suggested that IAP-1 and XIAP proteins are expressed in RGCs and glial cells in both the retina and the ON. However, when colocalization measures were used, the origin of most IAP-1 in the ON emerged as being neuronal, while XIAP protein was mostly glial. Further studies are required to address this issue.

Members of the IAP gene family stand out in this study because of their unique involvement in glaucoma. These genes were also conspicuous in an earlier study of our group that compared gene expression response between young and old glaucomatous eyes: They demonstrated upregulation in young glaucomatous eyes and downregulation in old ones. The lack of their overexpression with aging, as well as in ON axons, may have implications for our understanding of the molecular basis for glaucoma.

This paper once again confirms that various types of retinal and ON injuries share similarities while also being unique and having distinct properties. ONTX is often used as a model for other ON diseases, including glaucoma, but the current study demonstrates the differences between the two, suggesting that it should be kept in mind that ONTX is a model for ON trauma and that glaucoma is a different entity altogether. Elevated IOPs affect both the retina and the ON.

The belief that axonal degeneration occurs separate from and before RGC somal degeneration in glaucoma suggests that some RGCs maintain their cell bodies within the retina while their axons suffer from dysfunction at various time points of this chronic disease. The lack of an endogenous neuroprotective mechanism in the ON of glaucomatous eyes may provide a molecular explanation as to why ON axonal damage precedes RGC body death in glaucoma. Finally, in this study, members of the IAP family were shown to play a major and interesting role in protecting RGC cell bodies from elevated IOP. Their lack of involvement in the ON response to elevated IOP and with aging suggests that they have critical importance in the pathophysiology of glaucoma. Thus, novel treatment modalities that enhance IAP family expression should be developed as neuroprotective therapies for advanced optic nerve damage in glaucoma.

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