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Interference of the Apoptotic Signaling Pathway in RGC Stress Response by SP600125 in Moderate Ocular Hypertensive Rats

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Abstract

The aim of the present study was to investigate the effect of SP600125 (1,9-pyrazoloanthrone), an inhibitor of JNK, on apoptosis of retinal ganglion cells (RGCs) induced by moderate elevation of intraocular pressure (IOP) in male rats. IOP was elevated by suture-pulley compression on eyeballs. Cell apoptosis, expression of phosphorylated JNK (p-JNK) and cleaved caspase-3 in retina were studied by TUNEL staining and immunohistochemistry. The expression of c-Jun in retina was assayed by Western blot. Following IOP elevation (about 45 mmHg) for 6 h, the number of TUNEL, p-JNK and cleaved caspase-3 positive cells and the amount of c-Jun expression in retina were significantly increased. All these changes were reversed by SP600125 treatment. The immune positive cells for TUNEL, p-JNK and cleaved caspase-3 following IOP elevation were localized at the RGC layer. We conclude that moderate elevation of IOP for 6 h induced apoptosis of RGCs, and SP600125 treatment attenuated this process by suppressing c-Jun expression.

Key Words: apoptosis, IOP, glaucoma, retinal ganglion cell

Introduction

Glaucoma is a serious and common clinical problem. Vision loss in glaucoma is attributed to retinal ganglion cell (RGC) death, and elevation of intraocular pressure (IOP) is the strongest risk factor (21). RGC stress responses induced by ocular hypertension are critical to the health and survival of RGC in glaucoma. In experimental glaucoma, c-Jun is activated (14) and phosphorylated c-Jun N-terminal protein kinase (JNK) is expressed (12) in rat RGCs. c-JNK has been implicated in the response of apoptosis and well established that JNK plays a critical role in death receptor-initiated extrinsic as well as mitochondrial intrinsic apoptotic pathway. Most commonly, JNK is thought to induce mitochondria-dependent apoptosis mainly through direct or indirect activation Bax and a pro-apoptotic Bcl-2 family member. Bcl-2 protein modulates the release of mitochondrial apoptogenic factors like cytochrome c or apoptosis-inducing factor that activates caspases and other proteases during the regulation of apoptosis. SP600125 (1,9-pyrazoloanthrone) is a small-molecule inhibitor of JNK (2), it reversed neuronal death in ischemic brain (7) and partly inhibited the RGC death induced by NMDA (17). SP600125 is commonly used to diminish JNK activity after traumatic optic neuropathy and retinal ischemia, but the role of

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SP600125 during an apoptotic signaling pathway in RGC after moderate elevation of IOP remains unknown.

Li *et al*. (15) described the pulley method, an easy, non-invasive, and highly reproducible technique for increasing IOP and inducing retinal ischemia in rats. Our data showed that when 15 g of the counterpoise was chosen, pulley method can induce a moderate increase of IOP that results in apoptosis in RGC by pressure- and time-dependent manner (unpublished data).

To explore whether JNK mediates RGC apoptosis, this report assesses the action of the recently described JNK inhibitor SP600125 in RGC during moderate ocular hypertension for six h in rats. Our data is the first to demonstrate that SP600125 interfered with signaling pathway in RGC apoptosis induced by moderately elevated IOP.

Materials and Methods

Experimental Animals

All experimental procedures were performed according to the guidelines approved by the Animal Ethics Committee of Shandong University School of Medicine. Male Wistar rats weighing 200-250 g were provided by the Animal Center of Shandong University. Each rat was anesthetized by chloral hydrate (400 mg/kg, intraperitoneal injection). Supplemental doses were administered as needed during the experiments. Corneal anesthesia was achieved with repeating treatment of one drop of lidocaine hydrochloride (20 g/l) with 1-h interval. Body temperature was maintained between 36°C and 38°C by a heating pad (ZH-BXT-3, Zhenhua, Yuanyang, Province Henan, Xinxiang, PRC).

IOP Elevation

Moderate elevation of IOP was induced by the suture-pulley compression method as described previously (15). Briefly, a suture thread (4/0 silk) of approximately 70 cm long was connected with two counterpoises (15 g) at both ends. It was placed as a loop around the circumference of the eyeball approximately 2 mm behind the limbus of one eye of the anesthetized rat. The other untreated eye acted as the negative control. A circumferential compression of the globe symmetrical to the optical axis was produced by passing both ends of the suture thread through a series of pulleys. The IOP of the eyes was measured by a TonoLab rebound tonometer for rodents (Colonial Medical Supply, Franconia, NH, USA). The elevation of IOP was maintained for 6 h. During the circumferential compression, the cornea was intermittently irrigated with normal sodium and erythromycin to maintain adequate moistening and to prevent infection. The eyes were treated with erythromycin twice a day before the rats were sacrificed. The eyeballs were enucleated and prepared for immunohistochemistry and Western blot analysis.

Administration of SP600125

SP600125 (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS, pH 7.4) to the final working concentration (17). Intravitreal administration of SP600125 (0.04 mM, 5 µl) (17) or the same volume of vehicle was performed by a microsyringe immediately after the elevation of IOP.

In Situ *TUNEL Staining*

In situ terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) was performed on sections of the retina from 24 rats. Twelve h after moderate IOP elevation, the rats were deeply anesthetized and perfused pericardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The eyes were enucleated, and the anterior segments of the eyeballs were removed. The posterior portions of the eyeballs were fixed overnight in 4% paraformaldehyde and immersed in 25% sucrose with PBS for two days. Ten-micrometer-thick sections, along the vertical meridian and through the optic nerve, were cut with a cryostat. The TUNEL assay was performed with the *in situ* Cell Death Detection Kit, POD (Cat. No. 11684817910, Roche, Penzberg, Germany). After two PBS washes, sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme (or vehicle control) at 37°C for 60 min. After three rinses with PBS, the sections were treated with 3,3'-diaminobenzidine tetrahydrochloride substrate (ZSGB-BIO, Beijing, PRC) to reveal peroxidase activities. For nuclear staining, the sections were counterstained with hematoxylin (Sigma). The number of cells in the RGCL layer was counted using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan).

Immunohistochemistry of Phosphorylated JNK and Cleaved Caspase-3

The rats were sacrificed at 3, 6 and 12 h after moderate IOP elevation. The retinal sections were prepared for the *in situ* TUNEL assay. After three rinses with PBS, the sections were treated with 10% normal goat serum for 15 min, then incubated with primary antibody against phosphorylated JNK (p-JNK) (1:100, #9251, Cell Signaling Technology,

Denver, CO, USA) or cleaved caspase-3 (1:100, #9661, Cell Signaling Technology) at 4°C overnight in a moist chamber. After being washed with PBS, the sections were incubated with polymer peroxidase–anti-mouse serum (ZSGB-BIO) for 30 min at room temperature, followed by several rinses. The peroxidase on the sections was revealed with 3,3'-diaminobenzidine tetrahydrochloride substrate. For nuclear staining, the sections were counterstained with hematoxylin (Sigma). In the sections of negative controls, rabbit IgG was applied instead of primary antibodies. The p-JNK- or cleaved caspase-3-positive cells in the RGCL of each section were counted manually under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan).

Western Blot Analysis

Following 6 h of moderate IOP elevation, the eyes were enucleated at various time points (n = 8 at each time point). The retinas were removed and homogenized by a glass homogenizer (DY89-II, Ningbo Scientz Biotechnology Co. Ltd., Ningbo, Zhejiang, PRC) in 1: 9 (w/v) lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS and 1 mM PMSF, P0013B, Beyotime Institute of Biotechnology, Suzhou, Jiangsu, PRC) at 4°C. The homogenates were centrifuged at 12,000 rpm for 10 min at 4°C, and the protein content in the supernatants was evaluated according to the method of Bradford (3) using a Protein Quantitative Analysis Kit (k3001-BCA; Shenergy Biocolor, Shanghai, PRC). Supernatants containing 100 μg protein were diluted in reducing 2× Laemmli's sample buffer (20% Glycerol, 5% β-mercaptoethanol, 2% SDS, 0.02% Bromophenol Blue and 0.3 M Tris-Cl), separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (5% nonfat dried milk/TTBS) for 3 h at room temperature, washed in TTBS (0.1% Tween 20, 50 mM Tris, 150 mM NaCl), and incubated overnight with a primary antibody for c-Jun (H-79) (1:800, sc-1694; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or β-actin (1:800, sc-1616; Santa Cruz). After the sections were washed, they were incubated with a polymer peroxidase anti-rabbit serum (ZSGB-BIO) for 30 min at room temperature. Finally, immunoreactive proteins were revealed by a Twinplate Color Scanner (T1200; AGFA, Shenzhen, Guangdong, PRC) and quantitatively analyzed for the ratio (treatment/control) of the value of the grayscale division using the ScanImage software (Scion Corporation, Frederick, MD, USA).

Statistical Analysis

The data are presented as means ± SEM with n indicating the number of rats. Data were analyzed with SigmaStat 3.5 software (SPSS Inc., Chicago, IL, USA). Differences among groups were analyzed by Student's *t*-test or one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

IOP Elevation during Circumferential Compression on Eyeballs

In the study, the normal IOP was 10.49 ± 0.16 mmHg in the adult male rats. IOP increased quickly at the beginning of circumferential compression and reached the highest level at 15 min (54 ± 1.25 mmHg, n = 17). At 30 min, IOP dropped to 45.1 ± 0.57 mmHg (n = 17) and remained at this level until the end of the circumferential compression.

TUNEL-Positive Cells

TUNEL staining is a marker of apoptotic cells (6). To investigate the effect of circumferential compression on the apoptosis of retinal cells, we labeled the apoptotic cells by TUNEL assay. There were few TUNEL-positive cells in the RGCL of the control retina (Fig. 1A). Twelve hours after the 6 h compression, the number of TUNEL-positive cells was significantly increased. Most of the TUNEL-positive cells were located at the RGCL. In the control eyes, the percentage of TUNEL-positive cells in the RGCL was 1.49% ± 0.57%. Twelve hours after compression, the percentage of TUNEL-positive cells increased to 52.4% ± 1.84%, significantly higher than that of the control eyes ($P < 0.05$, n = 8) (Fig. 1B). Administration of SP600125 (0.04 mM, 5 μl) significantly diminished the increase of TUNEL-positive cells following moderate IOP elevation, but the vehicle of SP600125 did not exert any effect (Fig. 1).

p-JNK Positive Cells

Moderate IOP elevation on eyeballs upregulated the number of p-JNK positive cells on the retina. The immunoreactivity of p-JNK was faint in RGCs of the control groups but became intense 6 h after moderate IOP elevation. Most of the p-JNK–positive cells following IOP elevation were localized at the RGCL (Fig. 2A). The percentage of p-JNK positive cells increased from 2.94% ± 1.84% to 90.83% ± 1.65%, significantly higher than that of the control group ($P < 0.05$, n = 6) (Fig. 2B). After administration of SP600125 (0.04 mM, 5 μl), the percentage of p-JNK positive cells was 25.14% ± 6.61%, significantly lower than that of compression + vehicle group ($P < 0.05$, n = 6) (Fig. 2B).

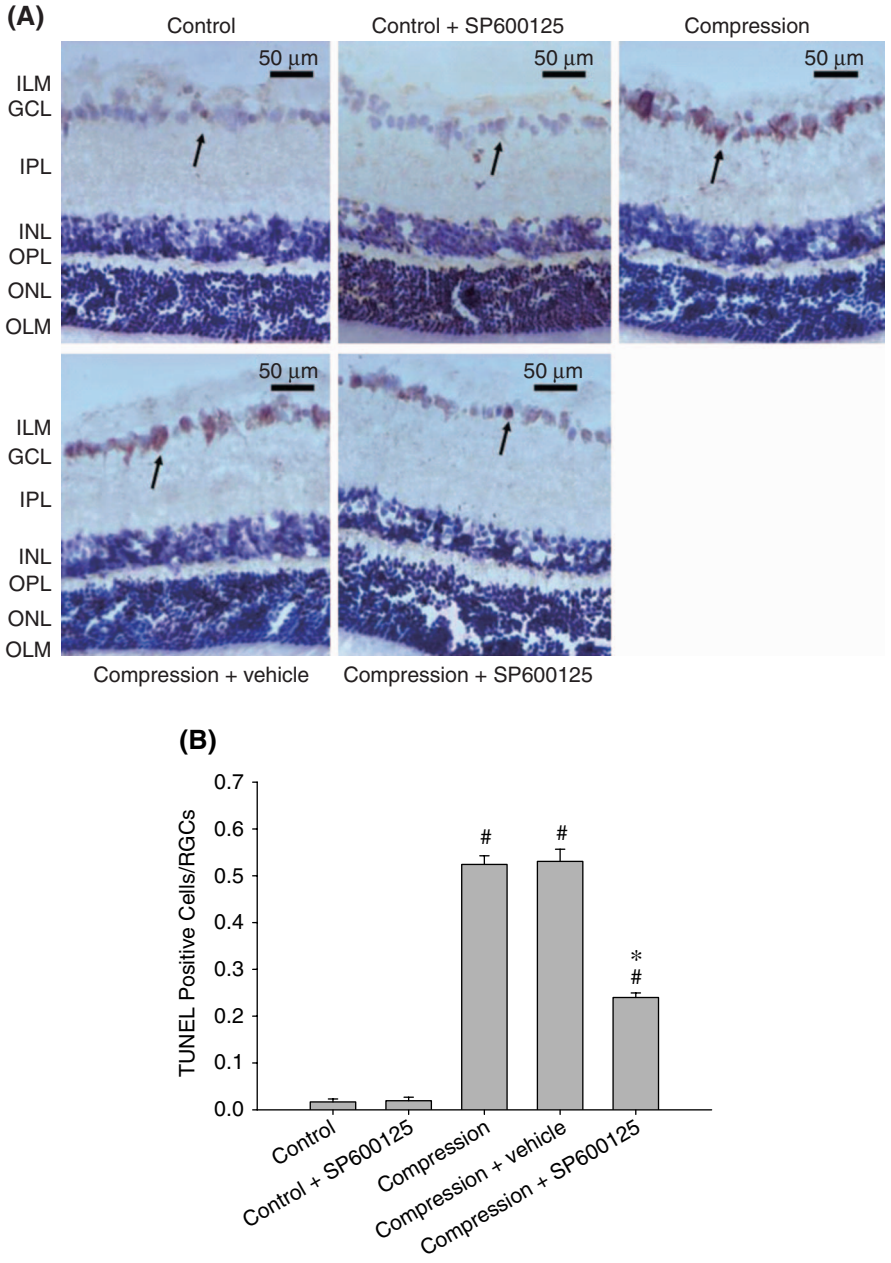

Fig. 1. *In situ* TUNEL assay on retina. (A) Representative photomicrographs of the retina in rats of different groups; (B) analysis of relative number of TUNEL-positive cells (TUNEL-positive cells/total number of the cells) in the retinal ganglion cells layer (RGCL). Circumferential compression significantly increased the number of TUNEL-positive cells in RGCL. Pretreatment of SP600125 partially reversed this effect. The arrows indicate the TUNEL-positive cells. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OLM, outer limiting membrane. Scale bar = 50 µm. $^{\#}P < 0.05$ *versus* control. $^{*}P < 0.05$ *versus* compression + vehicle.

c-Jun Expression

Moderate IOP elevation significantly increased the expression of c-Jun in retina. As shown in Fig. 3 (A and B), the amount of c-Jun expressed in the retina began to increase at 30 min following moderate IOP elevation, stayed at a high level for more than 12 h and returned to normal at 24 h. The normalized density of the c-Jun band increased from 1 (negative control) to 1.82 ± 0.13 ($P < 0.05$, n = 8) at 30 min (Fig. 3B). Intravitreal administration of SP600125 (0.04 mM, 5 µl) attenuated the elevated IOP induced increase of c-Jun expression in retina. As shown in Fig. 3 (C and D), at 3, 6 and 12 h following moderate IOP elevation, the amount of c-Jun expressed in the retina of compression + SP600125 group was significantly lower compared with that of the compression + vehicle group at the same time points ($P < 0.05$, n = 8)

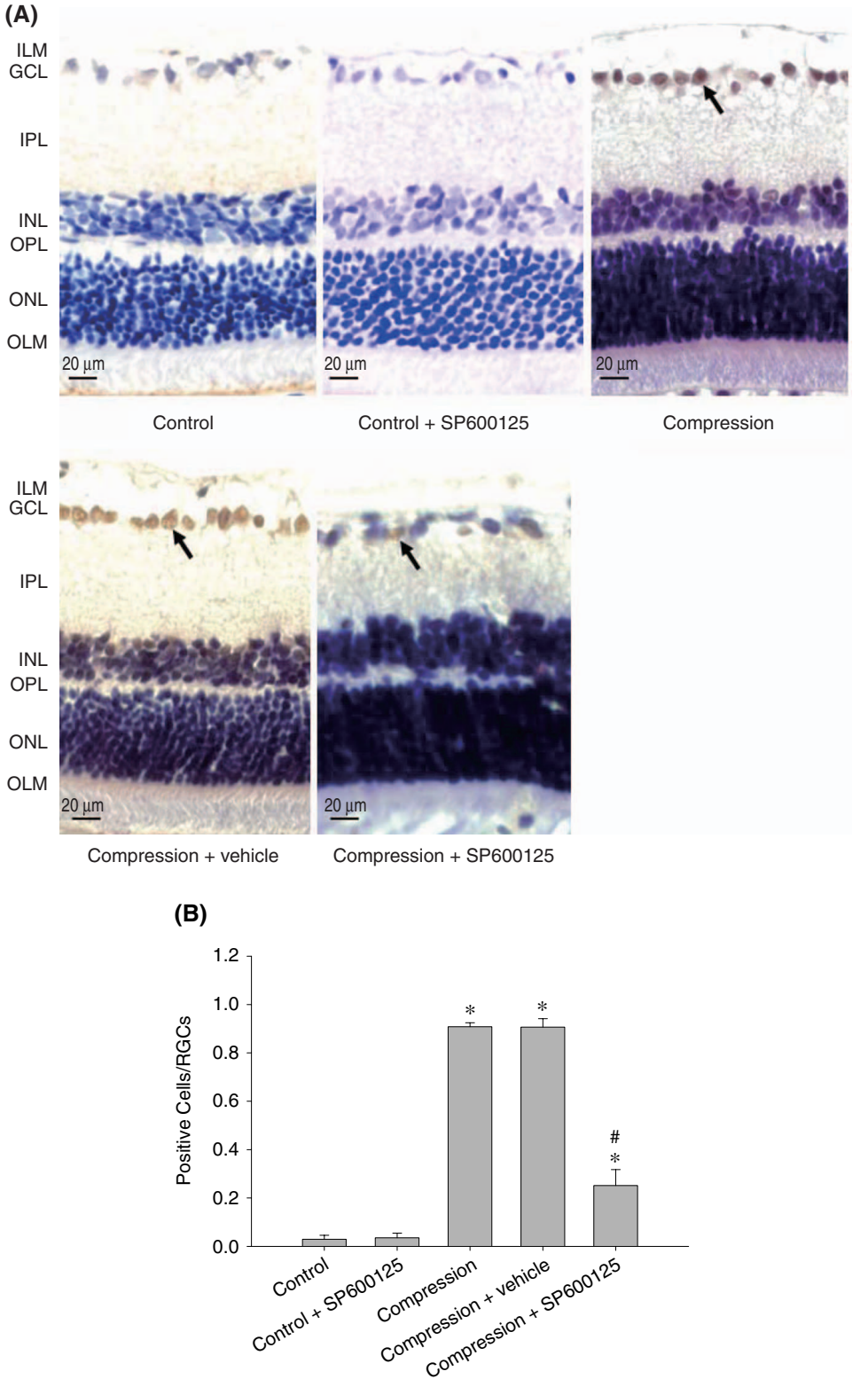

Fig. 2. Phosphorylated JNK (p-JNK) expression in retina. (A) Representative photomicrographs of the retinal section in different groups. Except for the control, the other specimen was prepared 6 h following circumferential compression. (B) Summarized data of different groups in panel (A) with the Y axis indicating the ratio of p-JNK cells (number of p-JNK positive cells/number of total cells) in retinal ganglion cells layer (RGCL). Circumferential compression significantly increased the number of p-JNK positive cells in RGCL and treatment of SP600125 reversed this effect. Arrows indicate p-JNK positive cells. Other abbreviations are as in the legend to Fig. 1. Scale bar = 20 µm, $^{*}P < 0.05$ *versus* control and $^{\#}P < 0.05$ *versus* compression + vehicle.

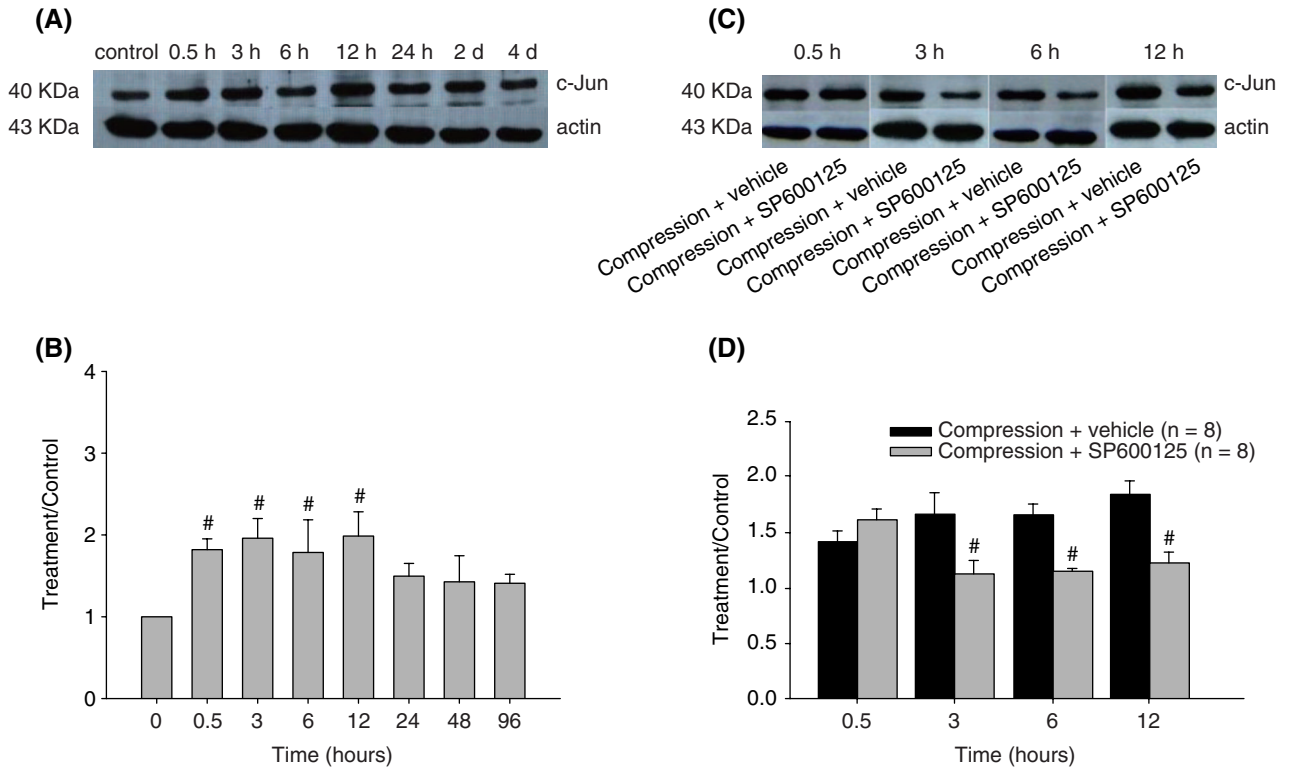

Fig. 3. Time-course of c-Jun expression in retina following 6-h circumferential compression on globes (A and B) and pharmacological manipulation by SP600125. (A) Representative immunoblot of c-Jun in protein extracted from retina. The upper panel shows a protein band with a molecular mass of approximately 40 kDa; β-actin was the internal control. (B) Quantitative analysis of c-Jun expression at different times after the 6-h circumferential compression. $^{\#}P < 0.05$ versus control. (C) Representative immunoblot of c-Jun in protein extracted from retina. The upper panel shows a protein band with a molecular mass of approximately 40 kDa; β-actin was the internal control. (D) Quantitative analysis of retinal c-Jun expression in the rats of different groups. From 0.5 to 12 h following circumferential compression, the expression of c-Jun in retinal was significantly increased. Pretreatment of SP600125 reversed these changes. $^{\#}P < 0.05$ *versus* compression + vehicle.

Cleaved Caspase-3-Positive Cells

Moderate IOP elevation for 6 h significantly increased the percentage of cleaved caspase-3-positive cells in the retina (Fig. 4A). The percentage of cleaved caspase-3-positive cells was 51.23% ± 2.29%, 76.82% ± 3.51% and 78.17% ± 5.29% at 3, 6 and 12 h, respectively, significantly higher than that of the control group (3.87% ± 1.94%) ($P < 0.05$, n = 6) (Fig. 4B). Treatment of SP600125 (0.04 mM, 5 μl) attenuated the increase of the cleaved caspase-3-positive cells in retina induced by circumferential compression (Fig. 4C). At 6 h after moderate IOP elevation, the percentage of cleaved caspase-3-positive cells treated by SP600125 was 30.20% ± 4.60%, significantly lower than that of compression + vehicle group (76.61% ± 5.52%) ($P < 0.05$, n = 6) (Fig. 4D).

Discussion

It is widely recognized that the vision loss of glaucoma is attributed to the apoptosis of RGCs, and IOP elevation is the major risk factor for RGC loss (21). As the marker of apoptosis, TUNEL-positive cells have been observed on the RGCL following IOP elevation (8, 20).

Severe IOP elevation rarely occurs in glaucoma. In animals, high IOP elevation induces not only death of ganglion cells but also damage of cells at all retinal layers. In patients with glaucoma, the cell apoptosis is mainly associated with retinal ganglion cells (1, 5, 19). We found that moderate IOP elevation significantly increased the number of TUNEL-positive cells in the RGCL. Therefore, we believe that the modified rat model of moderate IOP elevation used in this study partly mimicked the natural pathogenesis of glaucoma caused by IOP elevation.

The cause of apoptosis can be attributed to two signaling cascades, the mitochondrial pathway and the death receptor pathway. The mitochondrial pathway is activated by the release of cytochrome *c*, an important pro-apoptotic molecule, from the mitochondria. Cytochrome c activates Apaf-1, which in

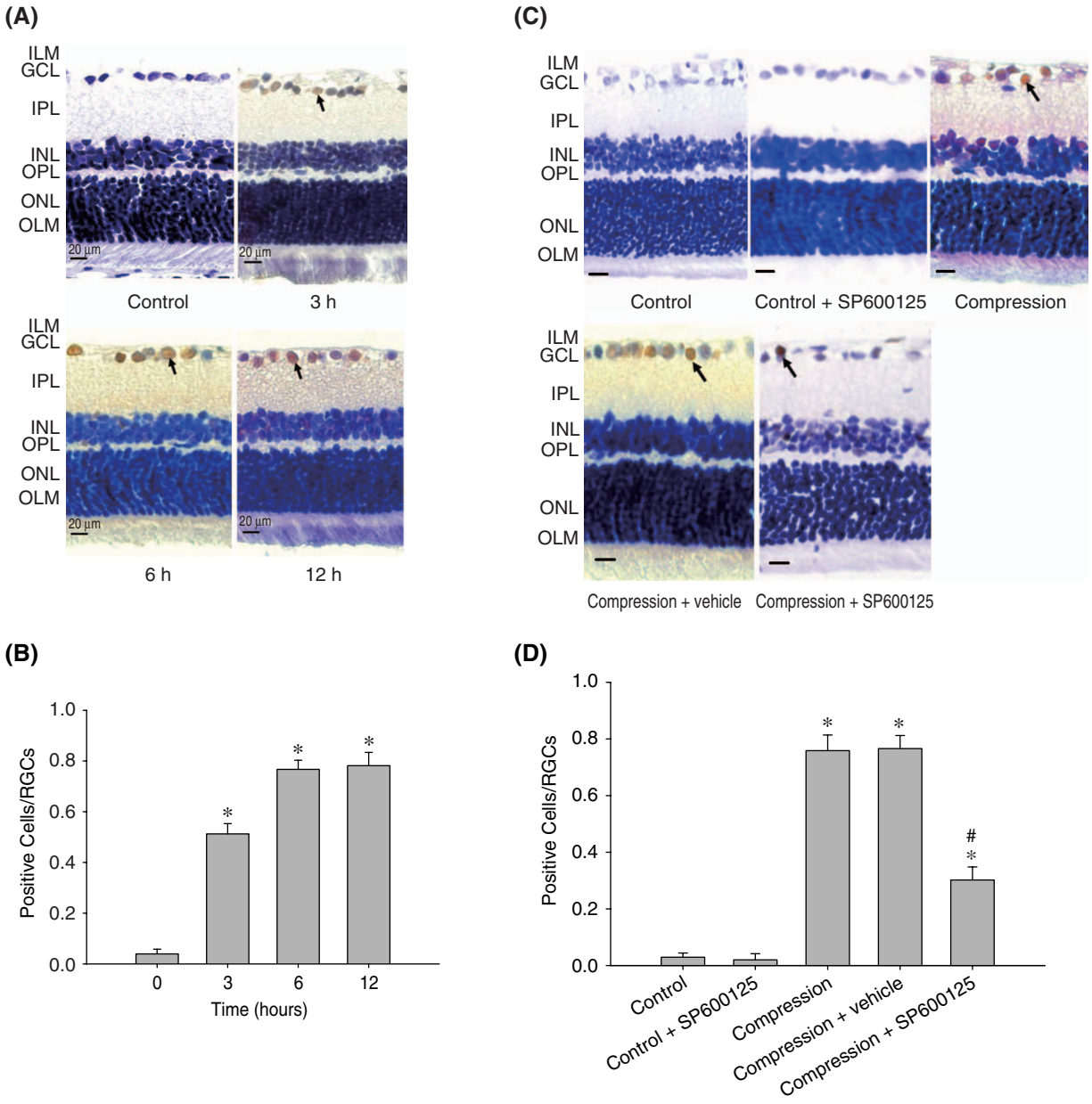

Fig. 4. Cleaved caspase-3 expression in retina following 6-h circumferential compression (A and B) and pharmacological manipulation by SP600125 (C and D). (A) Representative photomicrographs of the retina sections at different time points following circumferential compression. Arrows indicate positive cells. (B) Summarized data of different groups with the Y axis indicating of the ratio of cleaved caspase-3-positive cells (number of cleaved caspase-3 positive cells/total number of the cells) at the retina ganglion cells layer (RGCL). (C) Representative photomicrographs of the retina sections 6 h following circumferential compression; (D) Summarized data as in panel B. Circumferential compression significantly increased the ratio of cleaved caspase-3 positive cells in RGCL. Pretreatment of SP600125 reversed this effect. See legend to Fig. 1 for abbreviations used. Arrows indicate cleaved caspase-3-positive cells. Scale bar = 20 µm. $^{*}P < 0.05$ *versus* control, $^{\#}P < 0.05$ *versus* compression + vehicle.

turn activates caspase-9 in the presence of dATP. Activated caspase-9 activates downstream effector caspases such as caspase-3, -6 and -7. Caspase-3 is the key effector of the execution phase of apoptosis (4, 23). Activation of caspase-8 by death receptors on the cell surface also induces apoptosis (16). Death receptors belong to the tumor necrosis factor (TNF) receptor superfamily that includes CD95, TNF receptor-1 and the TNF-related apoptosis-inducing ligand receptors DC4 and DC5. Binding of death ligands to death receptors leads to activation of procaspase-8, which self-activates and activates caspase-3 to induce apoptosis (18).

JNK is one member of the MAPK family and

is a serine/threonine protein kinase. After double phosphorylation on tyrosine and threonine residues, JNKs phosphorylate c-Jun, ATF-2 and Elk-1 (13). Phosphorylation of JNK may induce apoptosis through mitochondrial mechanisms (11, 22).

Consistent with Kwong and Caprioli (12), in this study, we also observed increased number of p-JNK–positive cells in the RGCL after moderate IOP elevation. Besides this, we found that the expression of transport factor c-Jun, the downstream effector of the JNK signaling pathway, and cleaved caspase-3 were increased in retina following IOP elevation. These data are consistent with the reports of other groups which indicate that c-Jun is activated in RGCs of rat retina after IOP elevation or after optic nerve transaction (14), and that JNK upregulates the expression of caspase-3 in manganese-induced apoptosis in PC12 cells (10). These results indicate that the JNK signaling pathway participates in the mitochondrial-dependent apoptosis of RGCs induced by moderate IOP elevation.

SP600125 is a potent, cell-permeable, selective and reversible inhibitor of JNK with over 300-fold greater selectivity for JNK than ERK1 and p38 MAP kinases (2). SP600125 inhibits neuronal cell death in rat hippocampus (CA1) caused by transient brain ischemia/reperfusion (9) and the apoptosis of PGCs induced by NMDA (17). In the present study, we found that SP600125 inhibited RGC apoptosis induced by moderate IOP elevation in rats. Administration of SP600125 attenuated the increase of c-Jun and the cleaved caspase-3 expression in retina following IOP elevation. Therefore, we believe that SP600125 inhibited the JNK pathway and prevented RGC apoptosis induced by IOP elevation. Inhibiting JNK activity by SP600125 or similar chemicals may be a novel and effective strategy to treat glaucoma.

In summary, we found in this study that moderate elevation of IOP for 6 h induced apoptosis in rat retina. The apoptosis was localized to the RGCL. SP600125 partially reversed RGC apoptosis by suppressing the expression of c-Jun and the downstream activation of caspase-3. It is possible that SP600125 is a potential drug for the treatment of glaucoma.

Acknowledgments

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