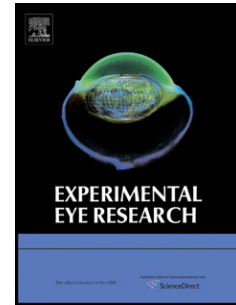


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Title: Simvastatin Upregulates Bcl-2 Expression and Protects Retinal Neurons from Early Ischemia/reperfusion Injury in the Rat Retina

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I. Highlights

Simvastatin has been shown to enhance retinal ganglion cell (RGC) survival against ischemia-reperfusion (IR) injury. > Simvastatin treatment increased RGC survival by proximately 40%. > Simvastatin upregulated the expression of Bcl-2 in the retina as shown by western blot analysis. > The expression of Bax was unaffected. > The mechanism of simvastatin-mediated neuroprotection likely involves the apoptotic pathway.

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**Simvastatin Upregulates Bcl-2 Expression and Protects Retinal Neurons from
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IV. Abstract

Simvastatin has been shown to enhance the survival of retinal ganglion cells (RGCs) following ischemia-reperfusion (IR) injury by mediating the expression of stress proteins. The purpose of this study was to investigate the effect of simvastatin on retinal neurons and the expression of apoptotic proteins in a rat IR model. Wistar rats received intravitreal injection of simvastatin immediately after retinal reperfusion. Retinal ischemia was induced by increasing intraocular pressure to 150 mmHg for 60 min. The number of viable RGCs was measured after retrograde labeling with Fluoro-Gold. Ischemia-induced apoptotic cell death was studied using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). We found that simvastatin treatment enhanced RGC survival after retinal ischemia by

approximately 40% and decreased retinal neuronal apoptosis. Using western blot analysis, we found that simvastatin upregulated the expression of Bcl-2 in the retina. In contrast, the level of the protein Bax was unaffected by simvastatin treatment. Our results suggest that RGC loss induced by retinal IR may be prevented by simvastatin and that the mechanism underlying this process possibly involves an alteration in the apoptotic pathway.

V. Highlights

Simvastatin has been shown to enhance retinal ganglion cell (RGC) survival against ischemia-reperfusion (IR) injury. > Simvastatin treatment increased RGC survival by approximately 40%. > Simvastatin upregulated the expression of Bcl-2 in the retina as shown by western blot analysis. > The expression of Bax was unaffected. > The mechanism of simvastatin-mediated neuroprotection likely involves the apoptotic pathway.

VI. Keywords:

Retinal ganglion cell, Simvastatin, Bcl-2, Bax, ischemia/reperfusion

VII. Abbreviations

retinal ganglion cell (RGC), ischemia-reperfusion (IR), Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), 3-hydroxy- 3-methylglutaryl coenzyme A (HMG-CoA), ganglion cell layer (GCL), inner nuclear layer (INL), Tris-buffered saline (TBS), normal goat serum (NGS),

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1. Introduction

Compromised retinal circulation can lead to retinal thinning and the loss of neurons, in particular the death of retinal ganglion cells (RGCs) (Hughes, 1991). The complex biochemical processes that follow ischemia-reperfusion (IR) have been widely investigated and include energy depletion, glutamate-mediated excitotoxicity and subsequent calcium overload, oxidative stress, inflammation and eventual cellular necrosis, apoptosis (Buchi, 1992; Osborne et al., 2004) and necroptosis (Rosenbaum et al., 2010). Animal models of transient IR have been established for mimicking clinical diseases such as central retinal artery occlusion and ischemic optic neuropathy to elucidate the pathological mechanisms underlying IR and to help develop feasible treatments.

Apoptosis, a programmed form of cell death following a destructive cascade, is triggered by a number of death receptor signaling pathways (Qu et al., 2010). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-stained cells in the retinal ganglion cell layer (GCL) and the inner nuclear layer (INL) of the retina are present approximately 2 h following IR and reach their peak at 24 h (Lam et al., 1999). In addition, the expression of certain genes that are related to the apoptotic pathway is initiated secondary to IR (Nickells et al., 2008). Studies regarding neuroprotectants that may help rescue retinal neurons from

apoptosis by mediating the expression of genes that are associated with the apoptotic pathway are ongoing (Asomugha et al., 2010; Shen et al., 2010).

Statins, which inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have been described extensively for the treatment of hyperlipidemia since 1987. However, their pleiotropic properties beyond cholesterol reduction, including anti-inflammation (Miyahara et al., 2004), immunosuppression (Kwak et al., 2001), cytoskeletal modulation (Song et al., 2005) and protection against certain neurological diseases (Menge et al., 2005), have gained increasing attention over the past decade. Honjo et al. first used pravastatin and cerivastatin to investigate their neuroprotective ability following retinal IR injury. They demonstrated that, following IR, statin treatment preserved the retinal histo-architecture and ameliorated RGC apoptosis by inhibiting leukocyte–endothelium interactions, in particular through nitric oxide produced by the endothelium (Honjo et al., 2002). Another statin, pitavastatin, has been shown to protect RGCs after retinal IR by modulating intercellular adhesion molecules (Kawaji et al., 2007). Simvastatin is one of the most potent members of the statins and was first marketed in the early 1990s. The protective action of simvastatin for RGCs has been demonstrated in various experimental models. Intravitreal injection of simvastatin has been shown to enhance RGC survival by 90 and 19% at 7 and 14 days, respectively, after optic nerve axotomy

by promoting heat shock protein expression and Akt activation (Kretz et al., 2006).

The same researchers also applied four different statins by subcutaneous injection after retinal ischemia and found that the rescue rates for RGCs were 18.8, 58.3, 29.6 and 57.7% for mevastatin, pravastatin, lovastatin and simvastatin, respectively.

Modulating stress protein expression played an important role in the neuroprotective effect of simvastatin in their study (Schmeer et al., 2008). In contrast, in a rat model of autoimmune optic neuritis, simvastatin oral therapy failed to increase RGC survival or visual function as assessed by visual evoked potential tests.

In addition to the above pathways through which simvastatin exerts its neuroprotective action, regulation of genes that are related to cell death and survival has been observed in cortical neurons following simvastatin treatment (Franke et al., 2007; Johnson-Anuna et al., 2007). Among these genes, *Bcl-2* is critical in the apoptotic process (Reed, 1994). The ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax plays an important role in cell fate (i.e., survival or death) following an apoptotic stimulus (Oltvai et al., 1993). However, whether simvastatin mediates the expression of these factors in the retina has not yet been investigated. Accordingly, the aim of this study was to examine the effects of simvastatin on RGCs in a rat model of retinal IR, with particular emphasis on the expression of proteins that are related to the apoptotic pathway.

2. Material and Methods

2.1 Animals

Eight-to-ten-week-old female Wistar rats weighing 200-250 g were used in this study. All animal experiments and animal care were conducted in accordance with the International Guiding Principles for Animal Research adopted by the Laboratory Animal Center, National Taiwan University. The rats were housed under a 12-h light/12-h dark cycle. The experiments were conducted under general anesthesia induced by an intraperitoneal injection of a mixture of ketamine (50 mg/kg; Pfizer, New York, NY, USA) and xylazine (8 mg/kg; Sigma, St. Louis, MO, USA).

2.2 Transient Retinal Ischemia

Under anesthesia, the animals were placed on a stereotaxic frame, and their core body temperature was maintained at 37°C with a heating pad throughout the experiment. A 30-gauge needle that was connected to a saline bottle was inserted into the anterior chamber of the left eye. Acute retinal ischemia was induced by elevating the bottle to a height that produced an intraocular pressure (IOP) of 150 mmHg for 60 min. The corneal puncture site was sealed with glue to ensure that the increased IOP was maintained. In eyes that were subjected to a sham IR treatment, the procedure performed was the same as above but without the elevation of the saline bottle.

2.3 Drug Administration

The animals were randomly assigned to four groups: sham □ solvent, sham □ simvastatin, retinal IR □ solvent and retinal IR □ simvastatin. The rats were treated with simvastatin (1.6 µg/µl) or vehicle (solvent, a mixture of ethanol and NaOH) via intravitreal injection immediately after reperfusion.

2.4 Retrograde Labeling of RGCs and Counting

The head of the anesthetized rat was immobilized in a stereotaxic frame. The scalp was incised, and two small holes were drilled into the skull above each superior colliculus (bilaterally 6 mm posterior to Bregma and 1.5 mm lateral to the midline). Three microliters of 5% Fluoro-Gold (Sigma-Aldrich) in sterilized distilled water was injected with a micropipette at various depths in both superior colliculi.

RGC counting was performed three days after IR or sham injury. Rats were euthanized with an overdose of chlorohydrate (Kanto Chemical, Tokyo, Japan). After enucleation, the retina was gently peeled off and placed in a well containing 0.1 M phosphate-buffered saline (PBS; pH 7.4) and then fixed in 4% paraformaldehyde for 1 h. After rinsing three times with PBS, each retina was divided equally into four quadrants, flat-mounted on slides, and examined under fluorescence microscopy.

Each retinal quadrant was divided into three zones: central, middle, and peripheral, approximately 1, 2, and 3 mm from the optic disc, respectively. In each

zone, six microscopic fields of $430\ \mu\text{m} \times 285\ \mu\text{m}$ each along the medial line were counted. In total, 72 fields were counted for each entire retina. The average RGC density was derived from the total number of RGCs, which was then divided by the total area of each retina that was counted.

2.5 TUNEL staining method

We used the TUNEL assay to evaluate apoptosis of retinal neurons after IR injury. The eyes were enucleated 24 h after IR injury, and the retina was dissected as described above. The tissue was then fixed with 10% formaldehyde for 24 h. The whole retina was divided into two parts through the optic disc and then dehydrated and embedded in paraffin. Sagittal sections ($5\ \mu\text{m}$ in thickness) were cut through the optic disc and mounted. The deparaffinized sections were treated with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). This kit is based on the binding of digoxigenin-dUTP to the 3'-OH end of DNA by terminal deoxynucleotidyl transferase (TdT) followed by incubation with an anti-digoxigenin antibody that is conjugated to peroxidase. The sections were examined under $40\times$ magnification. Six microscopic fields of each eye—three adjacent areas on both sides of the optic nerve head (1 mm from the optic nerve head)—were used to count the TUNEL-positive cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL). The average number of TUNEL-positive cells in these two layers per field was used

for analysis.

2.6 Western Blotting Analysis

The detached retina was homogenized for 30 min on ice in buffer containing proteinase inhibitors (Sigma-Aldrich). The samples were boiled for 5 min in a water bath and then centrifuged at 14,000×g for 30 min at 4°C. A Bradford assay (BioRad, Hercules, CA, USA) was used to measure the protein concentration. Retinal proteins (30 µg per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) acrylamide gels and electroblotted onto a nitrocellulose membrane. The membrane was blocked with non-fat dry milk (5% w/v) in Tris-buffered saline (TBS) for 1 h and then incubated with a primary antibody against BAX (1:250 in 2% normal goat serum [NGS] in TBS; Stressgen Bioreagents, Victoria, BC, Canada) or Bcl-2 (1:200 in 2% NGS in TBS; Stressgen Bioreagents, Victoria, BC, Canada). After three washes with Tween-TBS (BioRad, Hercules, CA, USA), the secondary antibody (biotinylated anti-mouse IgG, 1:1000 in 2% NGS; Vector, Burlingame, CA, USA) was applied for 1 h at room temperature. β -actin was used as a loading control. Labeled proteins were detected using an enhanced chemiluminescence western blotting system (ELC; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and the signals were quantified using computer-assisted densitometry (Gel Pro 3.1; Media Cybernetics, Bethesda, FL, USA). The band

densities of each sample were normalized to the β -actin band.

2.7 Statistical Analysis

All of the data in the text and figures are presented as mean \pm SEM. Statistical analysis was performed using the Student's *t*-test for paired comparisons between groups. Differences with a p-value of less than 0.05 were considered statically significant.

3. Results

3.1 Evaluation of Retrograde Labeling of Retinal Ganglion Cells

RGCs that were labeled with Fluro-Gold in a retrograde manner were counted 3 days following retinal IR or sham surgery with or without simvastatin treatment. The mean densities of RGCs in the sham + solvent and sham + simvastatin groups were 1373.1 ± 18.7 and 1338.4 ± 45.3 cells/mm², respectively (n = 7 and 4, respectively; Fig. 1). Three days after the ischemic episode, the density of RGCs was 1096.9 ± 37.3 cells/mm² in the IR + solvent group, a decrease of approximately 20% compared with the sham + solvent group ($p < 0.001$; n = 8 per group). In the IR-induced eyes that were treated with simvastatin, the mean density of RGCs was 1208.6 ± 40.3 cells/mm² (approximately 88% of control), which was significantly higher than the eyes from the IR + solvent group ($p = 0.025$; n = 8 per group).

3.2 The Effect of Simvastatin Treatment on Apoptosis in the Retina Following Ischemic Injury

To further test whether simvastatin protects RGCs from apoptosis, TUNEL staining was used to detect DNA fragmentation of cells undergoing apoptosis (Fig. 2; n = 6 per group). Fig. 2A-D shows representative pictures of TUNEL staining from the different groups. TUNEL-positive cells in the GCL and INL of the retinas 24 h after IR were counted. Significantly more TUNEL stained cells were found in the GCL and INL

following IR (16.0 ± 6.7 vs. 0.2 ± 0.4 cells/field; $p < 0.0001$ compared with the sham + solvent group). In rats subjected to IR with simvastatin treatment, the number of TUNEL-positive cells was 9.2 ± 1.2 cells/field ($p = 0.006$ compared with the IR + solvent group).

3.3 Bcl-2 and Bax protein levels Following Retinal Ischemia-reperfusion

The temporal expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax following retinal IR was examined by western blot analysis. No significant change in Bcl-2 protein levels in the retina was observed 8, 16 or 24 h following reperfusion ($n = 3$ at each time point; Fig. 3A). On the other hand, an approximately 3-fold increase in retinal Bax protein level was found at 8 and 24 h after reperfusion ($n = 4$ at each time point; $p = 0.042$ and 0.044 , respectively, compared with the normal control retinas; Fig. 3B). There was no significant difference in Bax expression 48 h after reperfusion in comparison with the normal control.

3.4 Bcl-2 protein levels are Upregulated after Simvastatin Treatment

The time course of changes in Bcl-2 protein levels in the retina after simvastatin administration is shown in Fig. 4 ($n = 4$ at each time point). The level of Bcl-2 protein in the retina was increased at 4 h after simvastatin treatment and reached a peak at 24 h ($p = 0.034$). The level of expression then declined and no significance difference

was found 48 h after administration of simvastatin.

Fig. 5 shows the effect of simvastatin on Bcl-2 protein levels 24 h after retinal IR was induced (n = 4 in each group). In the sham groups, the eyes that received simvastatin treatment had a significantly higher level of Bcl-2 protein than those that received solvent ($p = 0.018$). After retinal IR, the simvastatin-treated eyes also showed a significant increase in Bcl-2 protein levels compared with solvent-treated eyes ($p = 0.044$).

3.5 Bax Protein Levels are not Affected by Simvastatin Treatment

The level of the pro-apoptotic protein Bax in rat retinas with or without simvastatin treatment 24 h after IR is shown in Fig. 6 (n = 4 in each group). Retinal ischemia drastically upregulated Bax protein expression at 24 h ($p = 0.021$ between the sham + solvent and IR + solvent groups and $p = 0.025$ between the sham + simvastatin and IR + simvastatin groups). No significant difference in Bax protein levels was found after simvastatin treatment.

4. Discussion

In this study, we investigated the effects of simvastatin on RGCs following a transient ischemic challenge. Our results suggest that simvastatin treatment prevented the death of a proportion of RGCs following IR insult. Furthermore, the increase of Bcl-2 protein level and amelioration of apoptotic neuronal death appear to be associated—at least partially—with the neuroprotective effects of simvastatin.

The cholesterol-independent neuroprotective actions conferred by statins involve a variety of putative mechanisms, including anti-inflammatory, antioxidant, anti-apoptotic and endothelial protection (Schmeer et al., 2007). Simvastatin is hydrophobic, which means it can cross the blood-brain barrier and possibly the blood-retinal barrier more easily than hydrophilic statins. The above properties and its safety suggest the potential neuroprotective benefit of simvastatin for long-term clinical use. Long-term oral statin use reduces the risk of open-angle glaucoma, in particular among patients with cardiovascular and lipid disorders. Improving ocular blood flow and/or enhancing aqueous outflow via inhibition of rho kinase activity were proposed to play a role in this protection (McGwin et al., 2004). In a 3-year cohort study, stabilization of normal tension glaucoma after taking simvastatin was demonstrated (Leung et al., 2010). An additional ocular beneficiary of statins is their ability to prevent myofibroblast transdifferentiation in Tenon's tissue. This effect

might provide a promising strategy for inhibiting wound scarring after glaucoma filtration surgery (Meyer-Ter-Vehn et al., 2008). On the other hand, contradictory results regarding the protective effects of statins have been reported. For example, statins failed to protect RGCs in a rat model of autoimmune optic neuritis (Sattler et al., 2005), and an inhibition of remyelination by blocking cholesterol synthesis was proposed as a possible reason for this lack of protection. Moreover, differences in the immunomodulatory effect of statins in various strains of animals and in humans is another concern (Sattler et al., 2005).

Programmed cell death, or apoptosis, has been identified in the retina following various injuries, including ischemia (Buchi, 1992; Berkelaar et al., 1994; Quigley et al., 1995; Barber et al., 1998). Following an apoptotic stimulus, the 21 kDa pro-apoptotic protein Bax is translocated to the mitochondria. The subsequent opening of the mitochondrial permeability transition pore and release of cytochrome c ultimately leads to a unidirectional apoptotic cascade. Bcl-2 can counter the actions of Bax and thereby prevent the cell from succumbing to apoptosis. Thus, the balance between Bcl-2 and Bax leads a cell toward survival or death. Two theories have been proposed to explain the switch of apoptosis. In the direct activation model, Bcl-2 Homology 3 (BH3)-only proteins are classified as activators (which bind directly to BAX and BAK and drive their activation) or sensitizers (which bind only to the Bcl-2 homologs)

(Letai et al., 2002). Because recent data challenge the first model, the indirect activation model suggests that the BH3-only proteins induce apoptosis by engaging the various pro-survival proteins that guard BAX and BAK (Willis et al., 2007).

Regarding the response of Bcl-2 family members to retinal IR, ischemia-induced BAX expression is increased, as demonstrated at both the mRNA and protein level (Kaneda et al., 1999; Zhang et al., 2002; Ju et al., 2008). However, the biological activity of Bcl-2 following retinal IR remains inconclusive (Kaneda et al., 1999; Ju et al., 2008). A recent study unraveled the sequential regulation of Bcl-2 family members in the ischemic retina (Produit-Zengaffinen et al., 2009). There was no obvious change in *Bcl-2* mRNA levels from 3 to 24 h after reperfusion, whereas the protein level was significantly increased 3 h after reperfusion. Overall, the Bax/Bcl-2 balance was not affected in the initial phase following an ischemic challenge. Our findings support a dysregulation of Bax protein levels and an unchanged Bcl-2 level 24 h after reperfusion.

Upregulation of Bcl-2 expression that gives RGCs protection from ischemia-induced damage has been reported for an alpha-2 adrenoceptor agonist (Lai et al., 2002). Moreover, our finding that simvastatin increases Bcl-2 levels in the ischemic retina is consistent with similar findings in brain neurons. Johnson-Anuna et al. found that simvastatin treatment resulted in increased Bcl-2 mRNA and protein

levels and reduced cytotoxicity. Additionally, an antisense oligonucleotide directed against *Bcl-2* abolished simvastatin-induced *Bcl-2* overexpression and neuroprotection (Johnson-Anuna et al., 2007). In addition to an upregulation of *Bcl-2* expression, a downregulation of *Bax* was observed in a rat model of Huntington's disease and in brain cells isolated from guinea pigs after challenge with the *Bcl-2* inhibitor HA 14-1 or a nitric oxide donor (Franke et al., 2007; Patassini et al., 2008). Despite our observation that simvastatin increased *Bcl-2* upregulation in the retina, *Bax* protein levels after simvastatin treatment were not reduced, as was seen in brain neurons. This discrepancy might be a result of the different tissues studied and/or the method of simvastatin delivery (intraperitoneal vs. intravitreal injection).

5. Conclusions

Taken together, the level of the anti-apoptotic protein Bcl-2 was increased by simvastatin in ischemic retinas. In contrast, the level of the pro-apoptotic protein Bax was not affected by simvastatin treatment. Therefore, this increased Bcl-2/BAX ratio might have contributed to decreased retinal neuron degeneration following ischemia. Further studies exploring the long-term benefit of this treatment, as well as studies detailing the mechanisms of its actions, are clearly needed.

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Fig. 1. Density of retinal ganglion cells (RGCs) treated with simvastatin or solvent in rats subjected to retinal ischemia at 60 min. The number of viable RGCs is significantly decreased following reperfusion at 3 days. Treatment with simvastatin partially inhibits ischemia-induced RGC loss. Data are expressed as mean \pm SEM. IR, ischemia-reperfusion; Sim, simvastatin. N = 7, 4, 8, 8, in sham + solvent, sham + Sim, IR + solvent, and IR + Sim group, respectively. * $p < 0.05$, *** $p < 0.001$.

Fig. 2. Simvastatin treatment inhibits retinal neuron apoptosis after ischemic injury. (A-D) Representative photomicrographs showing TUNEL-labeled cells in retinal sections from various treatment groups. The labeled nuclei were visualized as brown granules by streptavidin-DAB staining and then counterstained with 1% Methyl Green. Barely visible TUNEL-reactive cells in the retinas of the sham + solvent (A) and sham + simvastatin (B) groups. (C) A robust increase of TUNEL positive cells is noticed in the ganglion cell layer (GCL) and the inner nuclear layer (INL). (D) Treatment with simvastatin significantly reduces TUNEL positive cells in both the GCL and INL. (E) Quantitation of TUNEL-labeled cells in each treatment group. Data are expressed as mean \pm SEM. IR, ischemia-reperfusion; Sim, simvastatin. N = 6 in each group. ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. Western blot analysis of Bcl-2 and Bax protein expression after retinal ischemia. (A)

No detectable change in Bcl-2 expression is observed following reperfusion at 8 to 24 hours.

N = 3 at each time point. (B) Retina ischemia results in significant upregulation of Bax protein expression at 8 and 24 hours following reperfusion. Data are expressed as mean \pm SEM. C: normal control retinas. N = 4 at each time point. * $p < 0.05$ when compared with the normal control retinas.

Fig. 4. Western blot analysis showing the temporal pattern of protein expression of Bcl-2 after simvastatin treatment. (A) Representative blots of two samples. (B) Twenty-four hours following simvastatin administration the protein expression of Bcl-2 in the retina is significantly upregulated. Data are expressed as mean \pm SEM. C: normal control retinas. N = 4 at each time point. * $p < 0.05$ when compared with the normal control retinas.

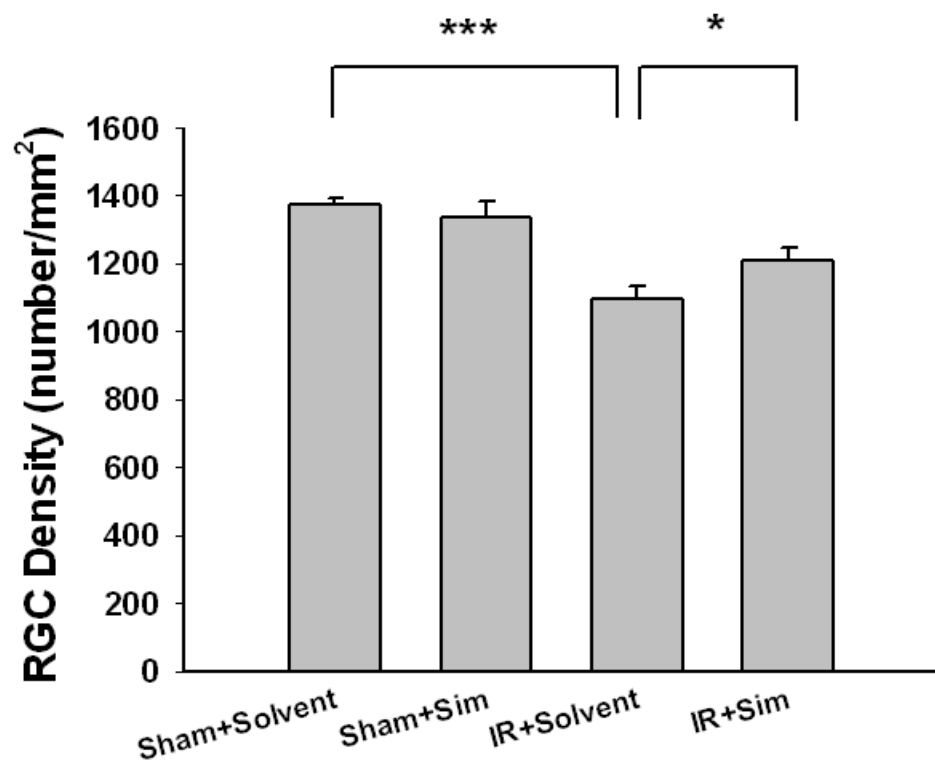
Fig. 5. Protein expression of Bcl-2 at 24 hours after retinal ischemia with or without simvastatin treatment. (A) Upper panel shows the representative protein bands. (B) Densitometry reveals a significant induction of Bcl-2 protein expression in the retinas of rats treated with simvastatin after sham operation or retinal ischemia. Data are expressed as mean \pm SEM. IR, ischemia-reperfusion; Sim, simvastatin. N = 4 in each group. * $p < 0.05$.

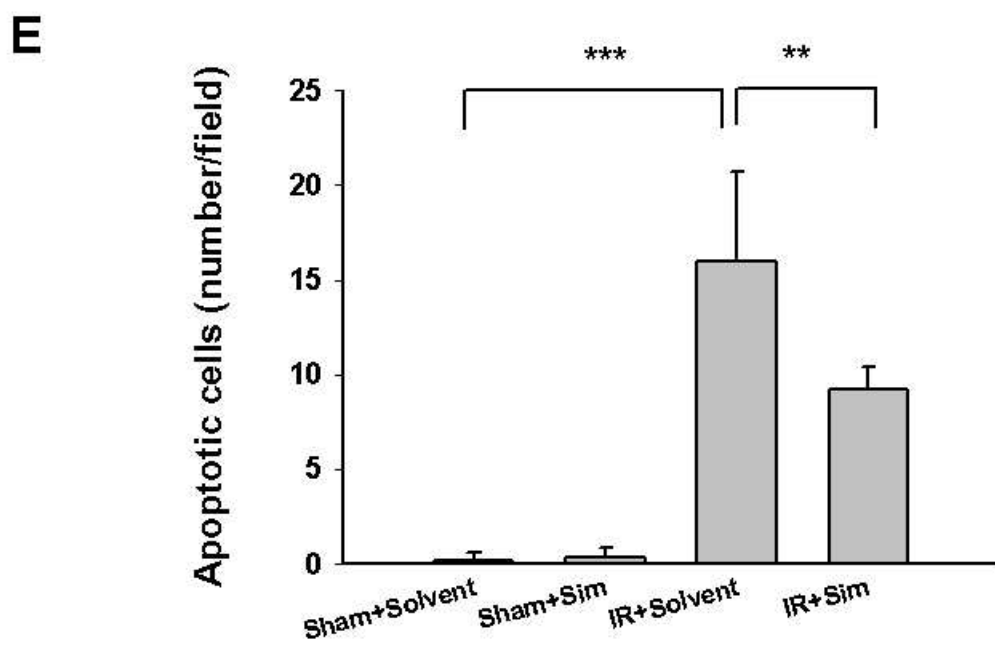
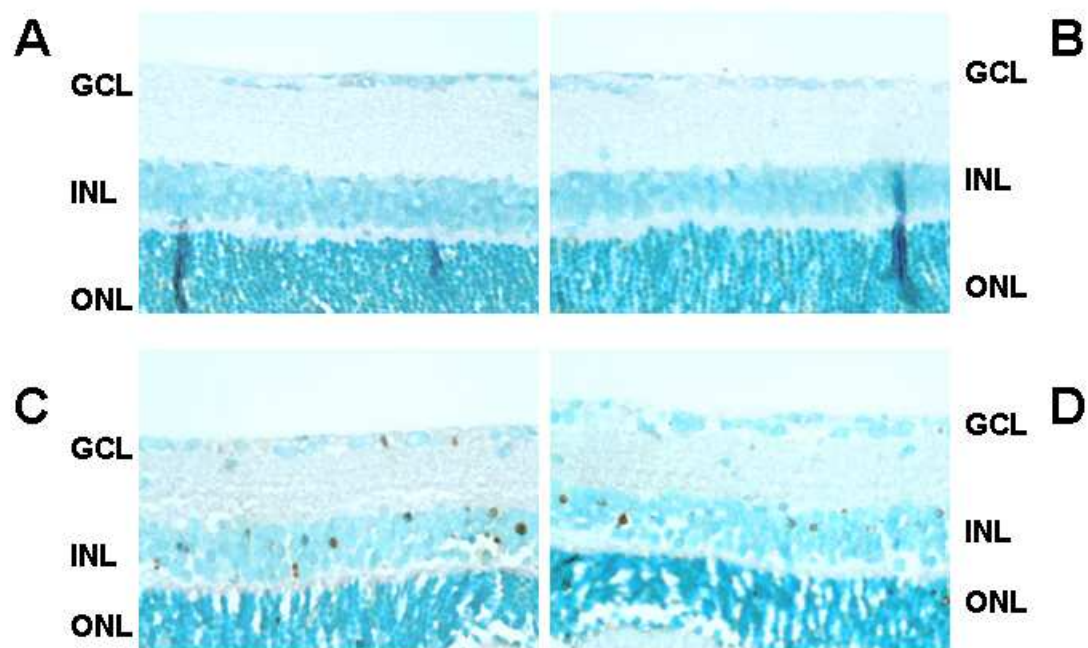
Fig. 6 There is no effect of simvastatin on Bax protein expression in the retina after ischemia

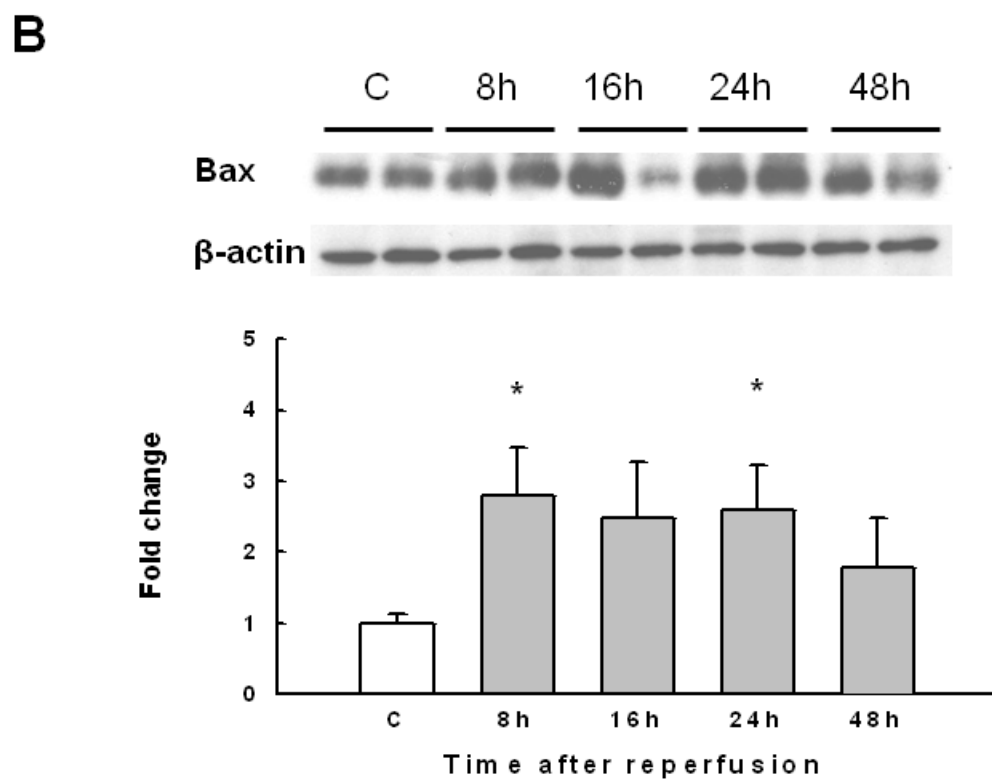
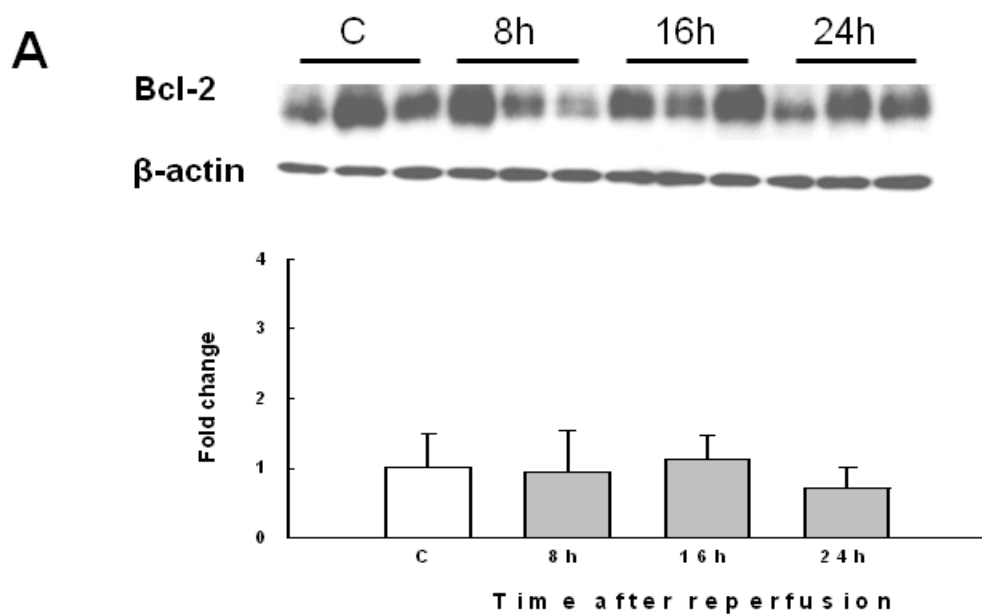
at 24 hours. Data are expressed as mean \pm SEM. IR, ischemia-reperfusion; Sim, simvastatin. N

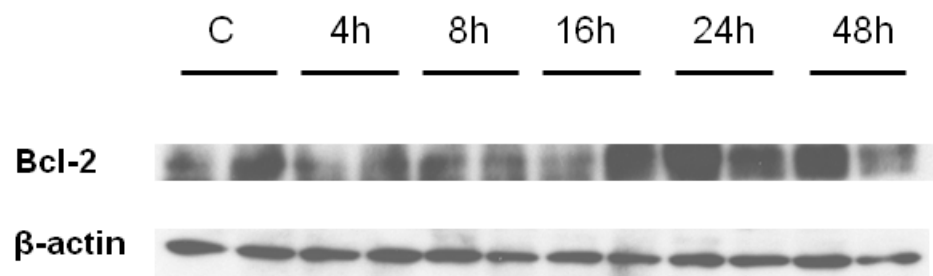
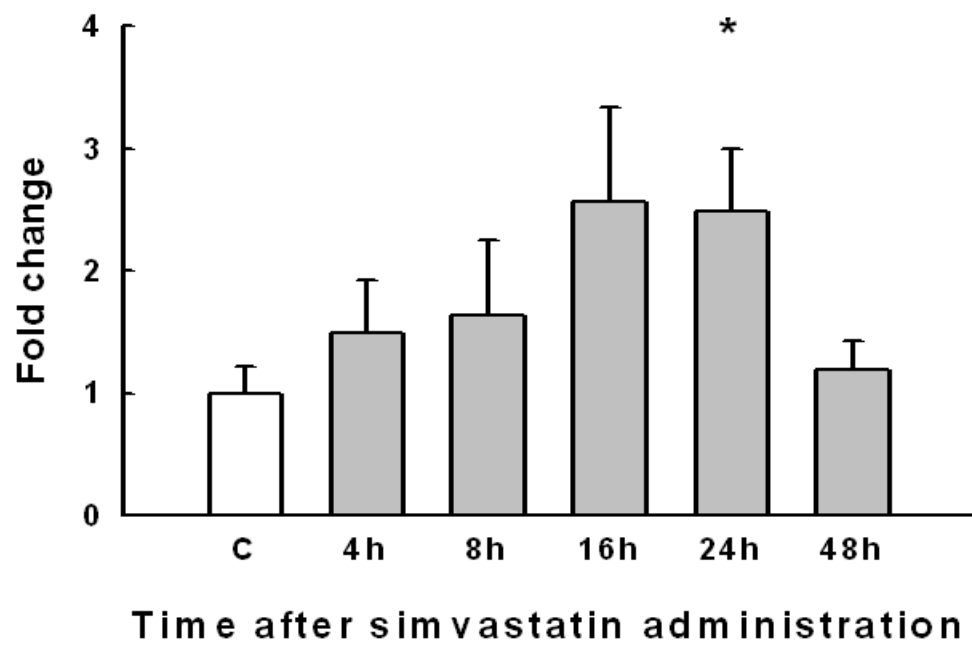
= 4 in each group. ** $p < 0.01$.

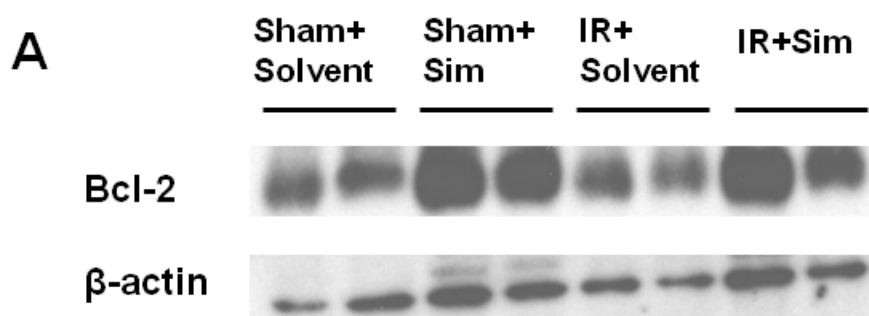
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