Latanoprost rescues retinal neuro-glial cells from apoptosis by inhibiting caspase-3, which is mediated by p44/p42 mitogen-activated protein kinase

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Title page

Full title: Latanoprost rescues retinal neuro-glial cells from apoptosis by inhibiting caspase-3, which is mediated by p44/p42 mitogen-activated protein kinase.

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Abstract

The purpose of this study was to investigate whether latanoprost, a prostaglandin F2α analogue, has a direct anti-apoptotic effect both in retinal neuro-glial cells in culture and in diabetic retina. R28 cells, immortalized retinal neuroglial progenitor cells, were induced apoptosis by 24 h serum deprivation. Serum withdrawal made up to 15 % of R28 cells pyknotic and activated caspase-3 immunoreactive, and latanoprost acid suppressed apoptosis with dose dependency at an optimum concentration of 1.0 μM ($P<0.001$). UO126, a mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase kinase (MEK) 1 and 2 inhibitor reversed this effect. Streptozotocin induced one- or three-month diabetic rats received balanced-salt-solution (BSS) in the left eye and latanoprost eye drops in the right for 5 days. Retinal wholemount was subjected to terminal dUTP nick end labeling (TUNEL) staining, whereas eyeballs were enucleated for cleaved caspase-3 immunofluorescence. Retinal homogenates were probed for phospho- or total p44/p42 MAPK and Akt. One- and three-month diabetic retina had 30.2 ± 15.3 and 23.6 ± 9.0 TUNEL positive cells per 0.5 cm^2, respectively, whereas control retina had few TUNEL positive cells. Latanoprost instillation significantly reduced these

cells (10.0 ± 3.1 and 11.3 ± 3.1 cells per 0.5 cm^2 for 1 M and 3 M, respectively, P<0.01), whereas BSS did not. Latanoprost also significantly reduced cleaved caspase-3 immunoreactive cells in ganglion cell and inner nuclear layers (P<0.05). Latanoprost increased phosphorylated to total protein ratio of p44/p42 MAPK (P<0.05), but not of Akt. Taken together, the present findings suggest that latanoprost rescues retinal neurons and/or glial cells from apoptosis, which is probably mediated by p44/p42 MAPK through caspase-3 inhibition.

Keywords: retinal neurodegeneration, apoptosis, latanoprost, mitogen-activated protein kinase, diabetic retinopathy

Text

Introduction

Latanoprost is one of the prostaglandin-related anti-glaucoma medications with powerful ocular hypotensive effects (van der Valk et al., 2005) through facilitated uveoscleral outflow (Toris et al., 1993; Stjernschantz et al., 1998). Latanoprost is a prostaglandin F2α (PGF2α) analogue, has the high affinity to the specific receptor FP (Sharif et al., 2003), and is an isopropyl esterified prodrug, which is converted to latanoprost acid (LA) by endogenous esterase (Bito and Baroody, 1987). In addition to the intraocular pressure (IOP) lowering effect, a prior paper demonstrated the potential neuroprotective ability in retina of latanoprost (Drago et al., 2001). Latanoprost decreased lactate accumulation in the in vivo retina given ischemia / reperfusion injury, whereas LA reduced lactate dehydrogenase (LDH) release from primary cultures of human retinal cells in vitro exposed to glutamate or hypoxia / re-oxygenation (Drago et al., 2001). PGF2α is known to inhibit apoptosis of some types of cells and tissues including cortical neurons (Cazeville et al., 1994). In addition, the retina expresses FP receptor (Ocklind et al., 1996; Davis and Sharif, 1999; Ocklind et al., 1997). However, precise mechanism by which latanoprost and

its active metabolite LA exert the neuroprotective ability in retina is still open to debate.

Evidence is mounting that diabetic retinopathy affects not only vascular permeability and growth but also the neuro-glial metabolism and function in retina (Barber, 2003; Nakamura et al, 2005), including increased apoptosis of retinal inner neurons (Hammes et al., 1995; Barber et al., 1998; Agardh et al., 2001; Kanamori et al., 2004a; Martin et al., 2004), altered expression of glial fibrillary acidic protein (GFAP) in astrocytes and Müller cells (Lieth et al., 1998; Mizutani et al., 1998; Barber et al., 2000; Rungger-Brändle et al., 2000), microglia activation (Zeng et al., 2000), and impaired glutamate metabolism (Ambati et al., 1997; Lieth et al., 2000; Kowluru et al., 2001). Streptozotocin (STZ)-induced diabetic rat retina, which is IOP irrelevant neurodegenerative disease unlike glaucoma, is a useful model for assessing the neuroprotective ability of anti-glaucoma agents such as latanoprost, because one should distinguish the direct neuroprotective effects in the retina from IOP-lowering effects of the anti-glaucoma agents.

The p44/p42 mitogen-activated protein kinase (MAPK) is known to mediate the neurotrophic ability of several types of growth factors in various kinds of cells or tissues (Cobb, 1999). Upon binding of growth factors to their specific receptors, GDP

bound form of small molecular weight G protein Ras is transformed to GTP bound one, which subsequently activates Raf, mitogen-activated protein / extracellular signal-regulated kinase kinase (MEK) 1/2, and eventually p44/p42 MAPK by dual phosphorylation of threonine and tyrosine residues in the TEY motif. The activated p44/p42 MAPK phsophorylates and thus regulates downstream targets to modulate cellular function and gene expression (Cobb, 1999).

The purposes of this study were: 1) to test whether latanoprost and LA prevent retinal neurons and/or glial cells in vivo and in vitro, respectively, from apoptosis, which is the fundamental process of the various neurodegenerative diseases in retina including glaucoma (Levin, 2003) and diabetic retinopathy (Barber, 2003) and 2) if so, to elucidate which intracellular pro-survival pathway mediates the cytoprotective ability of latanoprost. In the present study, we tested whether LA and latanoprost eye drops could reduce apoptosis of serum-deprived R28 cells, an immortalized retinal neuro-glial progenitor cell line (Seigel, 1996), and that of cells in retinal ganglion cell layer (GCL) in STZ-induced diabetic rats, respectively, through the p44/p42 MAPK pro-survival signaling and eventual caspase-3 inactivation.

Methods

Specific Reagents

LA was purchased from Cayman Chemical (Ann Arbor, MI). Latanoprost eye drop (Xalatan®) was from Pfizer (Tokyo, Japan). LY 294002 was from Upstate Biotechnology (Lake Placid, NY). UO126, KT 5823, KT5720, and KN-93 were from Calbiochem (San Diego, CA). ApopTag Peroxidase In Situ Apoptosis Detection Kit was purchased from Chemicon International (Temesula, CA).

Cell culture and Induction of apoptosis

R28 cells, retinal progenitor cells that were immortalized by transfection with adenovirus 12S E1A into the neonatal rat retinal tissue (Seigel, 1996), were used in this study. These cells express both glial and neuronal, but not vascular, phenotypes such as GFAP, vimentin, S-100, GluR1 through 3, Thy-1, and NMDA and GABA receptors (Seigel et al., 1996, 2004; Sun et al., 2002). Because of their easiness to handle and established characterization as retinal non-vascular cells, R28 cells have been widely used to elucidate the molecular mechanisms of apoptosis and neurotrophic factor effects over the limitation due to the gene alteration via adenovirus-transfection (Seigel and Liu, 1997; Seigel et al., 2000; Tezel and Wax,

2000; Barber et al., 2001; Nakamura et al., 2001, Mukuno et al., 2004; Narayanan et al. 2006). R28 cells were seeded on glass coverslips at $2x10^5$ cells/cm^2 density and fed with Dulbecco's modified Eagle's medium supplemented with 10 % newborn bovine serum (GIBCO Inc., Rockville, MD) as described previously (Barber et al., 2001; Nakamura et al., 2001, Mukuno et al., 2004). When grown to 60 % confluency, they were deprived of serum for 24 h with or without varying concentrations of LA or DMSO (10 μl/ml medium) as a vehicle. Although glutamate toxicity is thought to be involved in pathogenesis of various neurodegenerative conditions, R28 cells are quite resistant to glutamate toxicity. The glial cell nature that R28 cells potentially hold may facilitate extracellular gutamate uptake. At least in part, for this reason, we chose the serum-deprivation as apoptosis induction, which is another established method (Seigel and Liu, 1997; Barber et al., 2001). Each one of the following inhibitors with indicated concentrations was added 15 min prior to addition of LA or DMSO in some groups of experiments. The inhibitors were LY294002, a phosphatidylinositol 3-OH kinase (PI3K) inhibitor; UO126, an MEK1/2 inhibitor; KT5823, a protein kinase G (PKG) inhibitor; KT5720, a protein kinase A (PKA) inhibitor; or KN-93, a Ca^{2+}/calmodulin kinase II (CaMK II) inhibitor.

CM-1 immunocytochemistry

CM-1 immunocytochemistry against activated caspase-3 in a combination with Hoechst nuclear staining was performed as previously described (Barber et al., 2001; Nakamura et al., 2001, Mukuno et al., 2004). In brief, the coverslips were fixed in 1 % paraformaldehyde for 10 min, and blocked in 10 % goat serum at room temperature for 1 h. They were then incubated with a rabbit polyclonal antibody against activated caspase-3 (1:1000, Idun Pharmaceuticals, La Jolla, CA) at room temperature for 1 h. The cells were washed and incubated with tetramethyl rhodamine (TRITC)-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) and bisbenzamide (Hoechst dye 33258, 0.5 μg/ml, Sigma, St Louis, MO) at room temperature for 1 h. Immunostaining with an exclusion of the primary antibody addition was also conducted as negative controls. Cells were viewed using a high power (40×) objective of an Olympus Provis fluorescence microscope (AX80; Olympus Optical, Tokyo Japan) mounted with a digital camera (DP50; Olympus Optical, Tokyo, Japan) attached to a SONY personal computer running image analysis software (Viewfinder Lite, Tokyo, Japan). Five visual fields were randomly sampled from each coverslip. The number of pyknotic cells with condensed or fragmented nuclei was summated in the five sampled

regions and expressed as percentage of apoptosis per coverslip (the number of pyknotic cells / total number of cells × 100) (Barber et al., 2001; Nakamura et al., 2001, Mukuno et al., 2004). Likewise, the percentage of CM-1 immunoreactive cells per coverslip was also counted.

The above experiments were done in a triplicate fashion and repeated three times.

Animal handling and induction of diabetes mellitus

Male Sprague-Dawley rats weighing 225-275 g (CREA, Osaka, Japan) were housed in the Kobe university animal facility under standard conditions of room-temperature and a 12:12 h light-dark cycle with an ad libitum access to food and tap water. The following experiments were in accordance with the Animal Care Committee of Kobe University Graduate School of Medicine and the Association of Research in Vision and Ophthalmology Resolution on Care and Use of Laboratory Animals.

Diabetes was induced by tail vein injection of 65 mg/kg streptozotocin (STZ; Sigma, St Louis, MO) in 1 mM citrate buffer (pH 4.5) under general anesthesia by intraperitoneal injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (10 mg/kg) (Kanamori et al., 2004a; Nakamura et al., 2005). At one or three months after STZ injection, rats received instillation of 10 μl latanoprost eye

drops in the right eyes and balanced-salt-solution (BSS)(BSS Plus®; Alcon Japan, Osaka, Japan) in the left as control once a day between 10 and 11 o'clock pm for five days.

At one week after injection and immediately before sacrifice, they were confirmed diabetes mellitus if their blood glucose level was above 250 mg/dl measured by Lifescan meter® (LifeScan Inc., Drive Milpitas CA). Concurrently, body weight was also measured.

Retinal preparation and terminal dUTP nick end labeling (TUNEL) staining

Rats were sacrificed under deep ether anesthesia following measurements of body weight and blood glucose as described above. Retinas were dissected and subjected to terminal dUTP nick end labeling (TUNEL) staining in flat-mounted retina preparations using ApopTag Peroxidase In Situ Apoptosis Detection kit as previously reported (Barber et al., 1998; Kanamori et al., 2004a, 2004b; Nakamura et al., 2005). For detection of TUNEL-positive cells, each retina was visually scanned with a high power (40×) objective under microscope in a masked fashion. The entire retina area was measured by tracing the outline of each retinal image using an image analysis system (Micro Analyzer®, Japan Poladigital, Tokyo, Japan). The total number of

TUNEL-positive cells was counted for each flat-mounted retina and was expressed as the number per unit area of 0.5 cm^2. The number of rats used was 4 at each sacrifice point.

Cleaved caspase-3 immunohistochemistry for retinal cryosection

For cleaved caspase-3 immunohistochemical study, 3-month diabetic rat eyes, which received 5-day instillation either with latanoprost eye drops or BSS, were enucleated, fixed in 20 % sucrose optimal cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA), and stored at –80 °C until use. Retinal cryosections with 5 μm thickness were collected onto silane-coated microscope slides. After blocking with 10 % goat serum in PBS, the sections were incubated overnight at 4 °C with rabbit anti-cleaved caspase-3 (1:800, Cell Signaling Technology, Beverly, MA). Following washing, the sections were then incubated with TRITC-conjugated goat anti-rabbit IgG (1:2000) together with nuclear counterstaining with bisbenzamide at room temperature for 1 h as described earlier. Immunostaining with an exclusion of the primary antibody addition was also conducted as negative controls.

Retinal images were captured as mentioned earlier. Ten serial sections with 100 μm distance apart, the center of which was assigned to the plane through the optic nerve, were selected. The ratio of cleaved caspase-3 positive cells to total cells stained with Hoechst dye was counted for each section and the average was defined as the ratio of cleaved caspase-3 positive cells for the specific retina. The mean from 3 retinas was compared between the latanoprost and BSS treated eyes.

Electrophoresis and western blotting

Protein preparation and immunoblotting for phosphorylated or total p44/p42 MAPK and Akt were performed as previously reported (Barber et al., 2001; Nakamura et al. 2001; Kanamori et al., 2004b). Retinas were dissected from diabetic rat eyes treated with 5-day instillation of latanoprost eye drops or BSS, which were immediately snap frozen and stored at –80 °C until use. The retina samples were homogenized in 250 μl lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 mM benzamidine, 1 % Triton, and 1 protease inhibitor tablet/10 ml).

Tissue homogenates were rocked in 4 °C for 15 min. After centrifugation at 15,000

rpm for 10 min, the supernatant was collected and protein concentration was determined using a BioRad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). A 50-μg portion of proteins from each sample was subjected to 10 or 15 % SDS-polyacrylamide gel electrophoresis (PAGE) and was transferred onto nitrocellulose membranes. Following 1 h blocking with 5 % non-fat milk, the membranes were incubated with primary antibodies diluted in 1:1000 at room temperature for 1 h. The primary antibodies used were rabbit anti-total or phospho (Ser473) Akt and p44/p42 MAPK (Cell Signaling Technology, Beverly, MA). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG diluted in 1:4000 at room temperature for 1 h. Proteins bands were visualized with enhanced chemiluminescence reagents (ECL plus; Amersham, Arlington Heights, IL) and exposed to instant films using ECL-mini-camera. Relative intensities of bands were quantified by densitometric analysis using NIH Image software. Phosphorylated ratio relative to total proteins was calculated and graphically demonstrated as arbitrary units. The number of experiments was three.

Statistical Analyses

Body weight and blood glucose at each sacrifice point were compared with Mann-Whitney nonparametric test (Table 1).

Time courses of changes in the number of TUNEL-positive cells, percentages of activated caspase-3 immunoreactive cells, and immunoblotting densities were analyzed using two-way non-repeated measures ANOVA with Bonferroni post hoc test. All statistical values were judged significant if a p value was less than 0.05.

Results

Cytoprotective ability of LA in R28 cells in a dose-dependent fashion through the MAPK pathway

As previously reported (Barber et al., 2001; Nakamura et al., 2001; Mukuno et al., 2004), up to 15 % of R28 cells became pyknotic and up to 40 % of these cells were immunoreactive for activated caspase-3 at 24 h after serum withdrawal (Fig 1). Addition of LA, but not vehicle, reduced the number of pyknotic (～6%) as well as activated caspase-3 immunoreactive (～11%) cells in a dose-dependent fashion up to 1.0 μM ($p<0.001$; Figs 1 & 2 Top). In higher concentration range, the cytoprotective ability of LA was reduced to null level (Fig 2 Top).

Several types of inhibitors were added 15 min prior to addition of 1 μM LA in order to elucidate which intracellular signaling pathway mediates the cytoprotection of LA. Inhibitors alone did not increase pyknotic or activated caspase-3 immunoreactive cells as previously reported (Nakamura et al., 2001; Mukuno et al., 2004)(data not shown). Neither the PKA inhibitor KT5720 (168 nM), the CaMKII inhibitor KN93 (1.1 μM), the PKG inhibitor KT5823 (10 μM), or the PI3K inhibitor LY294002 (4.2 μM) reversed the LA's rescue effect (Fig 2 Bottom). In a marked contrast, the MEK1/2 inhibitor UO126 completely blocked the anti-apoptotic effect of LA, since addition of this inhibitor increased the number of pyknotic and activated caspase-3 immunoreactive cells to the serum free condition level ($p<0.0001$; Figs 1 & 2 Bottom).

Thus, LA showed the anti-apoptotic effect on R28 cells with dose-dependency, which was probably mediated by the MAPK pathway.

Rat metabolic conditions

STZ-induced diabetic rats gained significantly less weight than the age-matched controls, whereas the former had the significantly higher blood glucose than the latter at 1 and 3 months after STZ injection (Kanamori et al., 2004a; Nakamura et al.,

2005) ($p<0.001$; Table 1).

Anti-apoptotic effect of latanoprost eye drops on diabetic rat retina

As previously reported (Kanamori et al., 2004a; Nakamura et al., 2005), retinas from rats without STZ injection had few TUNEL-positive cells (less than 2 cells / 0.5cm^2 retina), whereas those from rats with STZ injection and BSS instillation had 30.2 ± 15.3 and 23.6 ± 9.0 cells / 0.5cm^2 retina at 1 and 3 months, respectively, both of which were significantly higher than controls ($p<0.01$; Fig 3 Bottom). The majority of TUNEL positive cells were distinct from observable vasculature as previously reported (Kanamori et al., 2004a; Nakamura et al., 2005) (Fig 3 Top). On the other hand, retinas from STZ-injected rats which received latanoprost eye drops for 5 days before sacrifice had 10.0 ± 3.1 and 11.3 ± 3.1 cells / 0.5cm^2 retina at 1 and 3 months, respectively. Although these numbers were still higher than non-diabetic controls, those were statistically significantly lower than diabetic rat retinas with BSS instillation ($p<0.05$; Fig 3 Bottom).

Reduced immunoreactivity for cleaved caspase-3 in diabetic retina by latanoprost eye drop instillation

Since activation of caspase-3 is known to be one of the critical apoptosis execution steps, we looked at the effect of latanoprost eye drops on the caspase-3 activation in retinal cryosections of STZ-induced diabetic rat by immunofluorescene analyses. The mean ± SD of ratio of the cleaved caspase-3 positive cells relative to total cells determined by Hoechst staining in GCL and INL of 10 sections, the center of which was assigned to the plane through the optic nerve, was 17.4 ± 2.6 % in the retinas of eyes with BSS instillation and 12.8 ± 0.87 % in those with latanoprost ($p<0.05$, $n=3$; Fig 4). Thus, 5-day instillation of latanoprost eye drops caused approximately 26 % reduction of the caspase-3 activation as compared with BSS instillation in 3-month diabetic rat retina.

Taken together with the TUNEL study, latanoprost eye drops reduced or slowed down the in vivo retinal neurodegeneration due to diabetes.

Activation of p44/p42 MAPK, but not of Akt, by latanoprost eye drops in the in vivo retina

In order to test whether cytoprotective effect of latanoprost was mediated by the MAPK pathway in the in vivo retina, immunoblottings using phospho- and total p44/p42 MAPK antibodies and phospho- and total Akt antibodies were performed.

Three-month diabetic rats received latanoprost eye drops in the right eyes and BSS in the left for 5 days before sacrifice. As demonstrated in Fig 5, total contents of p44/p42 MAPK was not different between in BSS and latanoprost treated retinas, whereas the phosphorylated form of p44/p42 MAPK was increased in the latanoprost treated retina. Thus, the ratio of phosphorylated to total p44/p42 MAPK was significantly higher in the latanoprost treated retina than in the BSS treated (1.3 fold, $p<0.05$; Fig5 Bottom). In contrast, phospho- as well as total Akt contents were not different between the two groups ($p=0.27$; Fig 6). Thus, latanoprost, applied in the form of eye drops, specifically activated the p44/p42 MAPK pathway in the in vivo rat retina.

Discussion

The present study clearly demonstrated that LA, the de-esterified and thus activated form of latanoprost, rescued R28 cells, retinal neuro-glial progenitor cells, from apoptosis induced by serum deprivation in a dose-dependent manner through the MAPK pathway. Latanoprost eye drops reduced apoptosis in GCL and INL in STZ-induced diabetic rat retinas and activated retinal p44/42 MAPK.

As mentioned earlier, latanoprost is a PGF2α derivative and has the high affinity of binding to FP receptor (Sharif et al., 2002, 2003). Evidence is mounting that PGF2α and FP receptor system has an anti-apoptotic ability in the cortical neurons as well as kidney cells, cardiomyocytes, and hen granulose cells (Cazeville et al., 1994; Manchanda et al., 2001; Morissette et al., 2003; Nishimura et al., 2004) probably through the phosphoinositide-mediated intracellular calcium mobilization (Kitanaka et al., 1991; Chen et al., 1997; Griffin et al., 1998; Ansari et al., 2004). PGF2α is known to activate the p44/p42 MAPK pathway in bovine (Ansari et al., 2004) and cat iris sphincter muscle cells (Husain and Jafri, 2002). Autoradiogram (Davis and Sharif, 1999), in-situ hybridization and immunohistochemical studies (Ocklind et al., 1996, 1997) demonstrated that the FP receptor is expressed in the retina in rat, monkey, and human. In the current study, the MEK 1/2 specific inhibitor UO126 antagonized the rescue effect of LA in R28 cells. Further, latanoprost phosphorylated the p44/p42 MAPK, but not another well-known pro-survival mediator Akt, in the in vivo retina. These lines of evidence strongly indicate that latanoprost promotes neuro-glial survival in retina via the p44/p42 MAPK pathway coupled to FP receptor. In this regard, we previously demonstrated that one of the biologically active forms of unoprostone isopropyl (M1), which has been introduced as another PGF2α-related

ocular hypotensive, also showed the anti-apoptotic effect in R28 cells but through Akt or/and protein kinase G pathway unlike latanoprost (Mukuno et al., 2004). Thus, PGF2α-related compounds may have diverse intracellular signaling pathways depending on the structural modification specific for each derivative. Identifying the signaling molecules of other PGF2α analogues such as bimatoprost and travoprost may shed light on this issue.

We chose diabetes mellitus as a retinal neurodegeneration model in this study for the following reasons: First, the major purpose of this study was to test whether latanoprost and its metabolite have direct neuroprotective ability in retina irrespective of the IOP lowering property. If we had used the experimental glaucoma model, it might have been very difficult to distinguish the IOP reduction effects from direct neuroprotection. Second, we wanted to evaluate the neuroprotective ability of latanoprost in the chronic disease model. Although ischemia / reperfusion (Aviles-Triguneos et al., 2003) as well as glutamate agonist injection into the vitreous cavity (Manabe and Lipton, 2003) are excellent and widely used neurodegeneration models, these acute injuries are far from the chronic disease conditions we target to treat in the clinical situations.

Evidence is accumulating that glial and neuronal elements, which are major components of the retinal parenchyma, are functionally and metabolically altered by diabetes from a very early stage of the disease in addition to the prominent vascular changes (Bresnick, 1986; Gardner et al., 2002; Barber, 2003; Nakamura et al., 2005). Electrophysiological and psychophysical studies have clinically acknowledged the compromised neuro-glial functions (Simonsen, 1980; Della et al., 1985; Juen and Kiselbach, 1990; Dosso et al., 1998; Fong et al., 1999). Histologically, as early as in 1960's, Wolter (1961) and Bloodworth (1962) found pyknotic configurations of neuronal cells in retinas of postmortem eyes from people with diabetes. More recently, several independent groups revealed that STZ diabetes increased TUNEL positive cells in GCL and INL both in rats and mice (Hammes et al., 1995; Barber et al., 1998; Agardh et al., 2001; Kanamori et al., 2004a; Martin et al., 2004). Immunohistochemical studies demonstrated that expression of pro-apoptotic proteins such as BAX and caspase were also increased in retinas of diabetic animals (Podesta et al., 2000; Mohr et al., 2002; Martin et al., 2004). Regarding glial cells, diabetes increased GFAP expression in Müller cells, which accompanied the loss of GFAP immunoreactivity in astrocytes in experimental diabetic retina (Barber et al., 2000; Rungger-Brändle et al., 2000). A recent report using human donor eyes

showed that RGCs in diabetic retina expressed caspase-3, Fas, and BAX, whereas Müller cells amplified pro-survival Bcl-2 and p44/p42 MAPK expression in addition to pro-apoptotic FasL, indicating that glial cells may regulate the RGC survival in diabetic retina (Abu El-Asrar et al., 2004). Thus, experimental diabetes may be one of the appropriate chronic retinal neurodegeration models in assessing the direct neuroprotective ability of latanoprost and other anti-glaucoma eye drops. However, we cannot tell whether latanoprost eye drops have a beneficial effect on preventing or halting the development of diabetic retinopathy until the effect of latanoprost on the vascular changes in retina is evaluated, because retinal vascular endothelial cells express FP receptors (Ocklind et al., 1996) and latanoprost is known to induce cystoid macular edema (Warwar et al., 1998), a hallmark of the blood-retinal barrier breakdown.

On the other hand, diabetic retinopathy has been recently recognized as a chronic inflammation. Diabetes recruits leukocytes through adhesion molecules in retina at very early time course (Chen et al., 2003; Joussen et al., 2004). Hyperglycemia and diabetes were reported to increase expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in cultured Müller cells and retina, respectively (Joussen et al., 2002; Du et al., 2004). Previous papers demonstrated that

non-steroidal anti-inflammatory drugs and an iNOS inhibitor, aminoguanidine, reduced leukocyte adhesion, expression of iNOS and COX-2, vascular permeability, and glial cell death in experimental diabetic retinas (Joussen et al., 2002; Du et al., 2004). Since Drago et al. (2001) suggested that latanoprost also inhibited iNOS and COX-2 acvitity in the injured retina as described earlier, it is of particular interest to further research the relationship between the latanoprost's anti-inflammatory effect and the suppression of diabetic retinopathy for both aspects of vascular complications and neurodegeneration.

Whether and how latanoprost and its metabolites can reach the choroids and retina are important issues to be addressed. Although we did not examine the route of latanoprost delivery in this study, two prior papers (Aihara et al., 2001; Kim et al., 2002) demonstrated that the posterior part of sclera is highly permeable to PGF2α than the cornea. Latanoprost is reported to enhance human scleral permeability to macromolecules probably by altering the metalloproteinase expression (Aihara et al., 2001; Kim et al., 2002). In addition, evidence is emerging that eye drops are able to better reach the posterior part of eyeballs through the sub-Tenon route and more easily penetrate transsclerally into the choroids and retina than previously acknowledged (Ishii et al., 2001; Mizuno et al., 2002; Trier and Ribel-Madsen, 2004).

Trier and Ribel-Madsen (2004) recently demonstrated that 12-week treatment with latanoprost eye drops significantly increased uronic acid concentration in the rabbit posterior sclera, which indirectly indicated the accessibility of latanoprost to the posterior part of eyes. Further pulse-chase studies of radiolabeled latanoprost are needed to elucidate the route and the mode of scleral penetration of latanoprost and its metabolites.

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Figure legends:

Table 1. Metabolic condition of rats at sacrifice point.

* $p<0.001$, ANOVA with Bonferroni post hoc test (n=11). S.D., standard deviation.

Figure 1. Representative pictures of cytoprotective ability of latanoprost acid. R28 cells were deprived of serum for 24 h with or without 1 μM LA in the presence or absence of 216 nM UO126, a mitogen-activated protein kinase / extracellular signal-regulated kinase kinase (MEK) 1/2 inhibitor. Upper panel indicates Hoechst 33258 nuclear staining, whereas lower panel does immunocytochmsitry for activated caspase-3 (CM-1). Note that the number of pyknotic and CM-1 positive cells is increased by serum deprivation, which is reduced by LA and is reversed by UO126. LA, latanoprost acid; ICC, immunocytochemistry.

Figure 2. Dose- and p44/p42 MAPK pathway-dependent cytoprotective ability of latanoprost acid.

R28 cells were deprived of serum for 24 h in the presence of the indicated concentrations of LA with or without several types of signal transduction protein

inhibitors indicated. Percent of pyknoctic cells (Black bar) and activated caspase-3 immunoreactive cells (Gray bar) relative to total cells determined by Hoechst 33258 nuclear staining and CM-1 immunocytochemisrty, respectively, are plotted.Upper panel indicates dose response curve for the rescue effect of LA on serum deprivation-induced apoptosis of R28 cells. * $p<0.001$, ** $p<0.01$ versus serum deprivation without LA (ANOVA with Bonferroni post hoc test) (average ± S.D., n=3). Lower panel shows abrogation of the LA's rescue effect on R28 cells specifically by MEK 1/2 inhibitor. * $p<0.0001$ versus serum deprivation without LA (ANOVA with Bonferroni post hoc test) (average ± S.D., n=3). LA, latanoprost acid; UO126, MEK 1/2 inhibitor; KT5720, protein kinase A inhibitor; KN93, Ca^{2+}/calmodulin kinase II inhibitor; KT5823, protein kinase G inhibitor; LY294002, phosphatidylinositol 3-OH kinase inhibitor.

Figure 3. Inhibitiory effect of latanoprost eye drops on diabetes-induced apoptosis of retinal neurons and glial cells.

Upper panel shows a representative photograph of TUNEL staining of flat-mounted retina. An inner box demonstrates TUNEL-positive cells (arrow heads). One or three months after induction of diabetes by streptozotocin injection or after vehicle injected

as control, rats received balanced-salt-solution (BSS) in the left eye and latanoprost in the right for 5 days and then retinas were dissected for TUNEL staining. Lower panel shows quantification of TUNEL positive cell number in unit area of whole mounted retina. * $p<0.01$, ** $p<0.05$ (ANOVA with Bonferroni post hoc test) (average ± S.D., n=4). DM, diabetes mellitus.

Figure 4. Inhibitiory effect of latanoprost eye drops on diabetes-induced caspase-3 activation in ganglion cell layers of rat retinas.

At 3 months after induction of diabetes by streptozotocin injection, rats received Balanced-Salt-Solution (BSS) in the left eye and latanoprost in the right for 5 days and then eyeballs were enucleated to probe for cleaved caspase-3. Upper panel shows representative pictures of cleaved caspase-3 immunofluorescence (A, B) counterstained with Hoechst 33258 dye (C, D). Closed arrowheads indicate cleaved caspase-3 immunoreactive cells in GCL, whereas open ones in INL. Lower graphs quantify the of ratio of cleaved caspase-3 immunoreactive cells to total Hoechst-stained nuclei in GCL and INL in 10 retinal sections per eye. * $p<0.05$; Unpaired t-test (average ± S.D., n=3). RGC: retinal ganglion cell, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer

nuclear layer.

Figure 5. Activation of p44/p42 MAPK by latanoprost eye drops in rat retinas. Streptozotocin-induced diabetic rats received latanoprost eye drops for 5 days and retinal homogenates were probed for total and phosphorylated p44/p42 MAPK . Upper panels show representative immunoblots, whereas lower graphs show quantification of phosphorylation ratio relative to total contents of the proteins. * $p<0.05$ (Unpaired t-test) (average ± S.D., n=3).

Figure 6. Unchanged Akt phosphorylation by latanoprost eye drops in rat retinas. Streptozotocin-induced diabetic rats received latanoprost eye drops for 5 days and retinal homogenates were probed for total and phosphorylated (Ser 473) Akt. Upper panels show representative immunoblots, whereas lower graphs show quantification of phosphorylation ratio relative to total contents of the protein. N.S., not significant.

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Figure.1

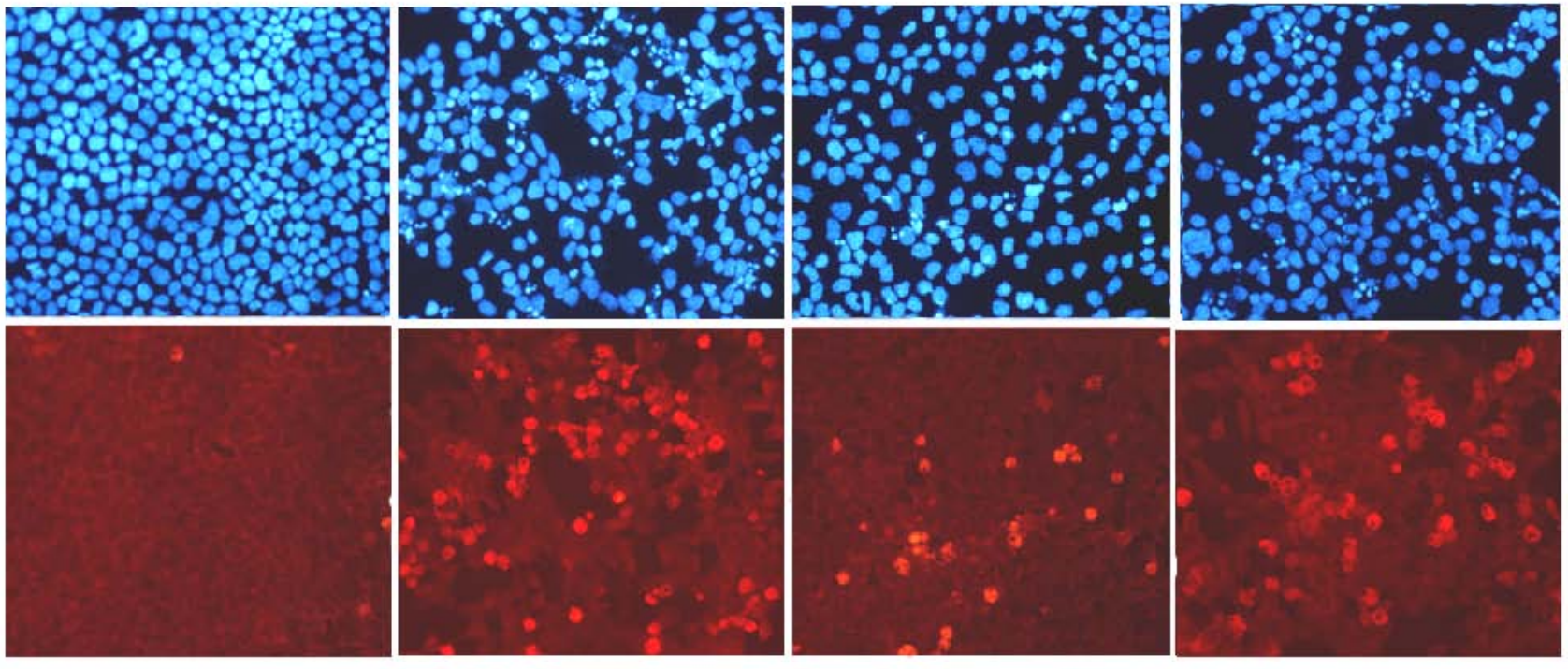
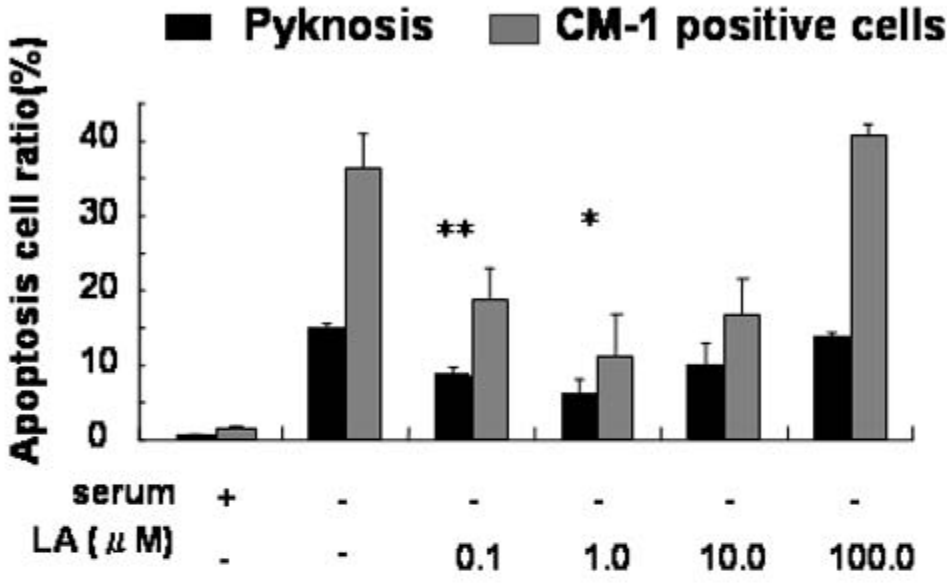


Figure.2


Pyknosis
CM-1 positive cells
Apoptosis cell ratio(%)
0
10
20
30
40
**
*
serum + - - - - -
LA (μM) - - 0.1 1.0 10.0 100.0

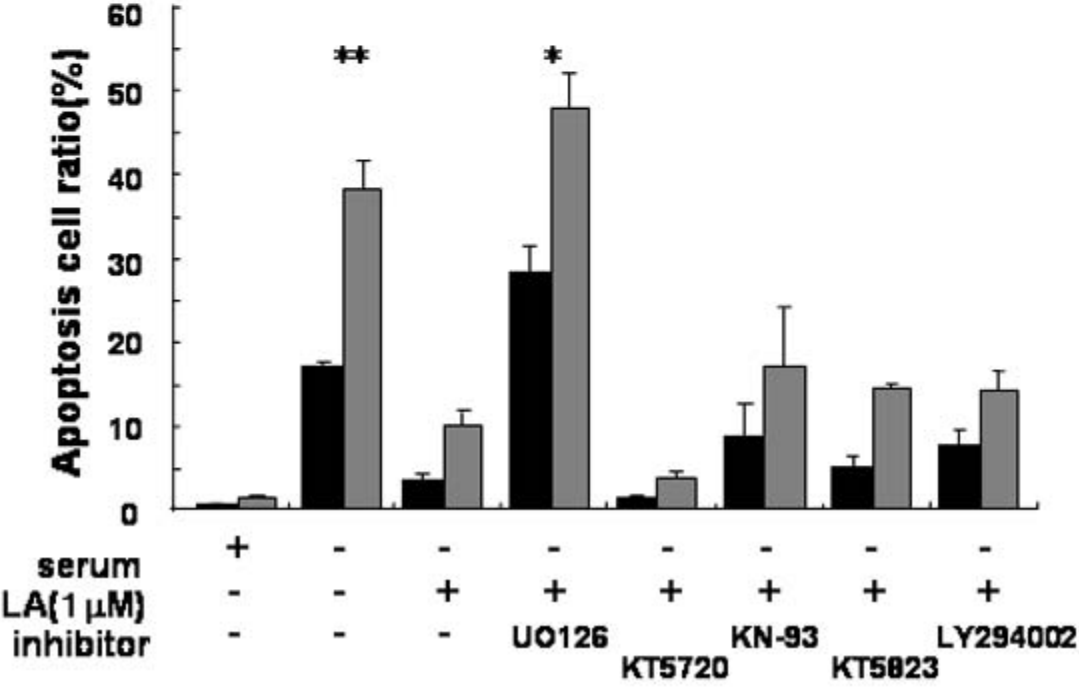
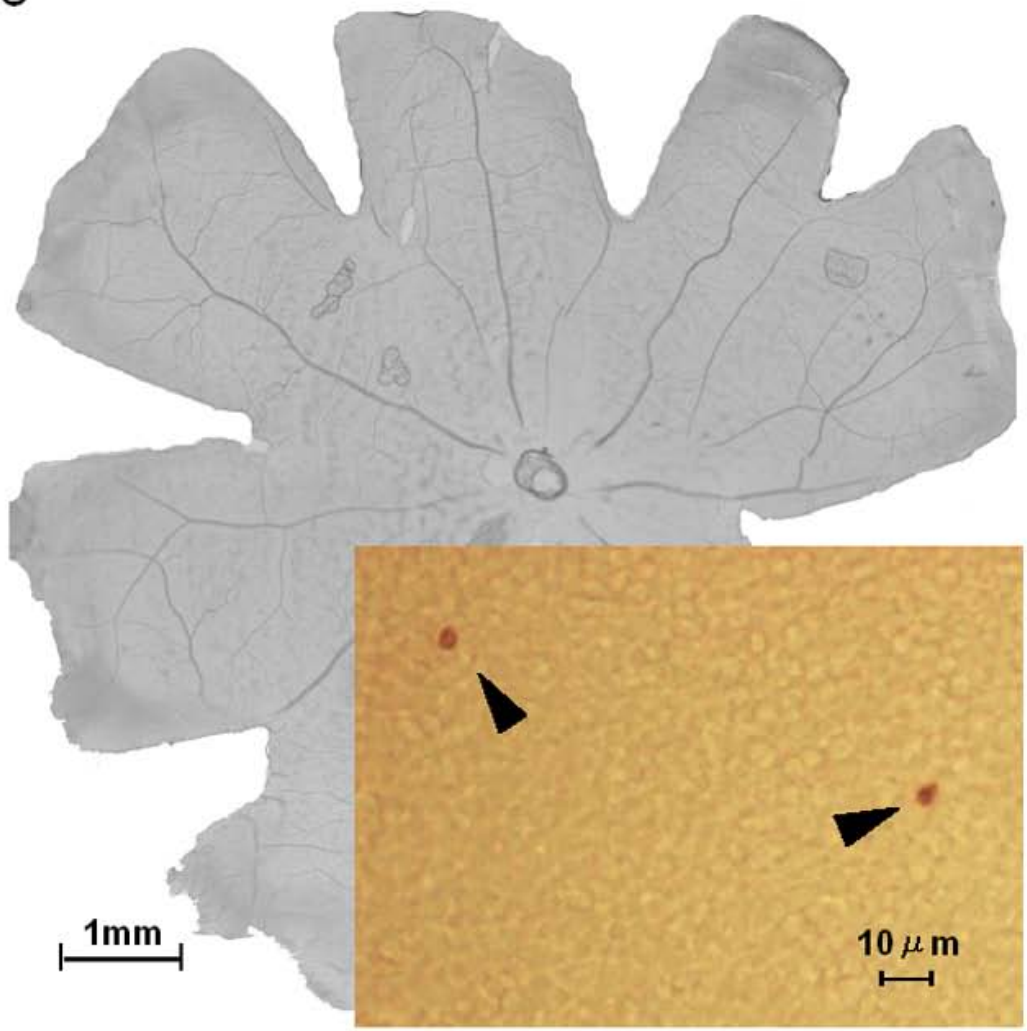

Apoptosis cell ratio(%)
0
10
20
30
40
50
60
**
*
serum + - - - - - - -
LA(1 μM) - - + + + + + +
inhibitor - - - UO126 KT5720 KN-93 KT5823 LY294002

Figure.3


1mm
10 μm

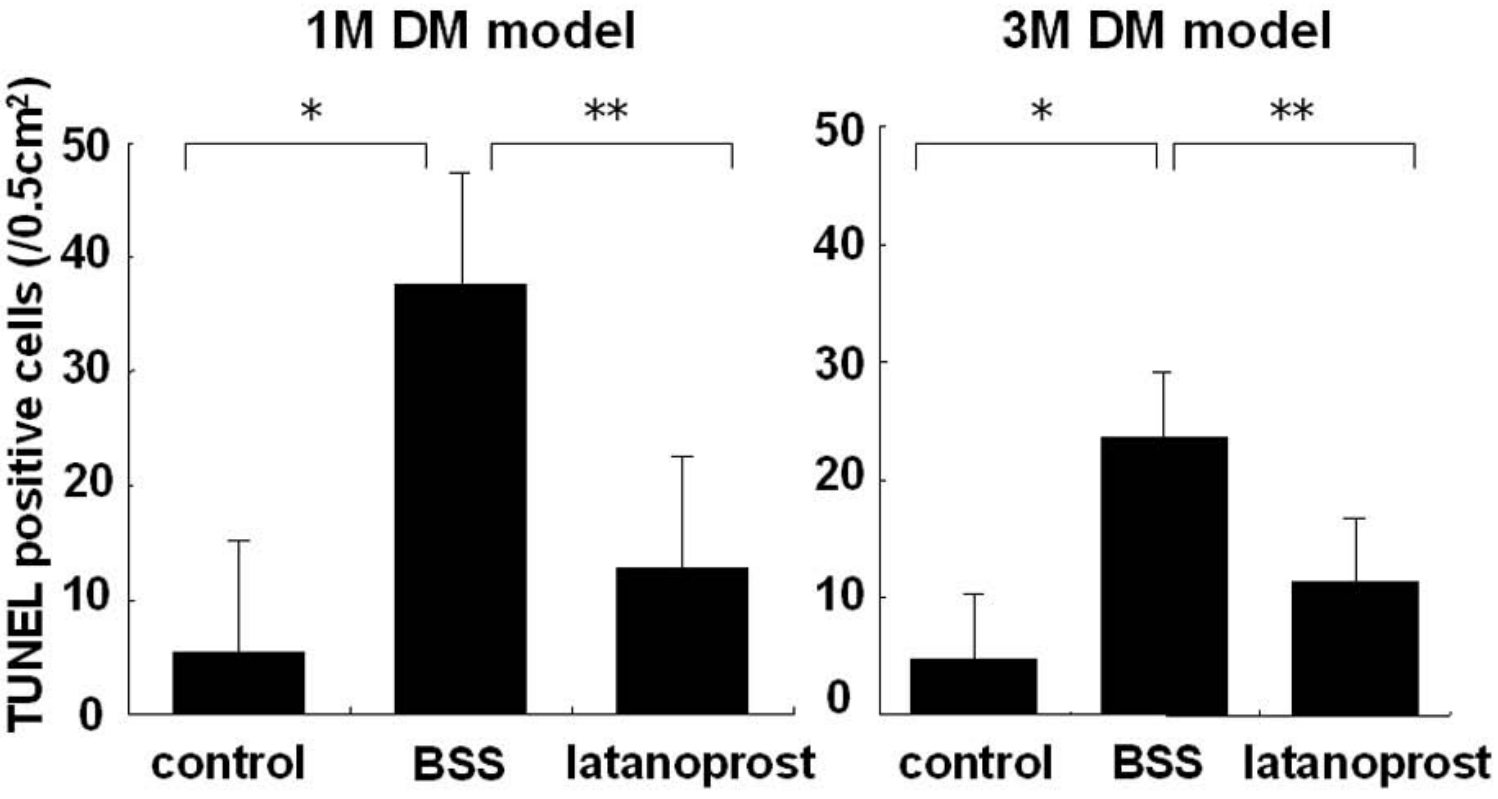
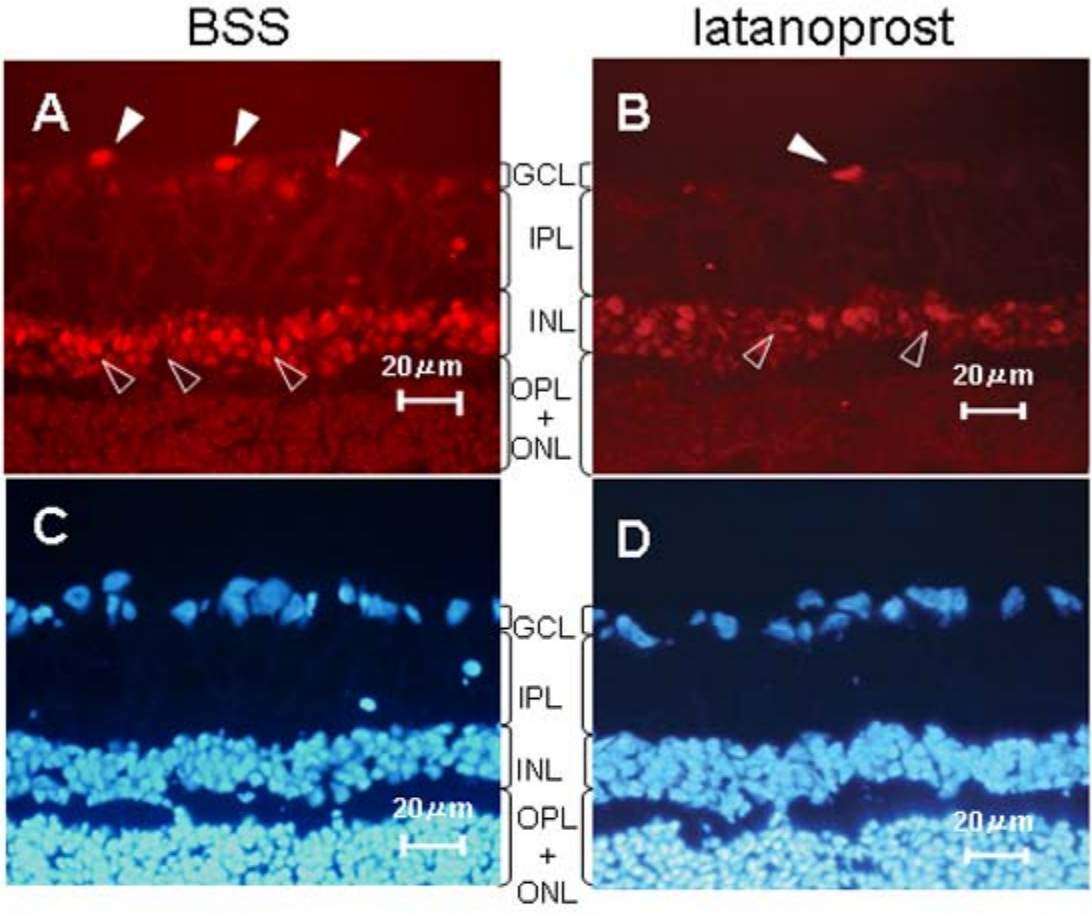

1M DM model
3M DM model
TUNEL positive cells (/0.5cm²)
0
10
20
30
40
50
*
**
control
BSS
latanoprost

Figure.4


BSS
latanoprost
A
B
C
D
GCL
IPL
INL
OPL
+
ONL
20μm

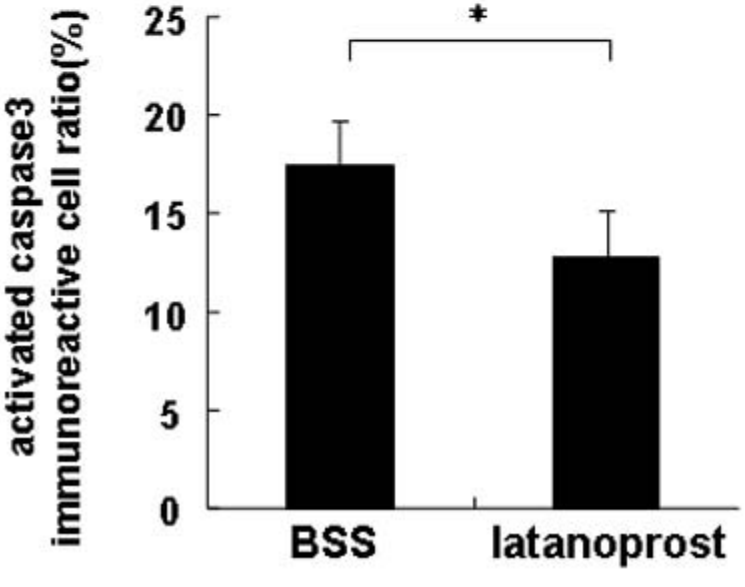
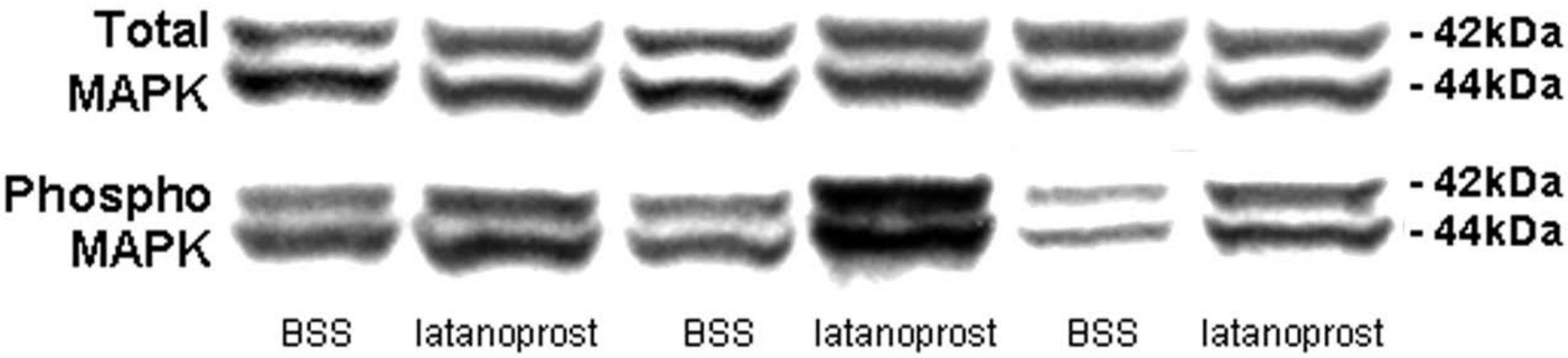

activated caspase3
immunoreactive cell ratio(%)
25
20
15
10
5
0
*
BSS
latanoprost

Figure.5


Total
MAPK
- 42kDa
- 44kDa
Phospho
MAPK
- 42kDa
- 44kDa
BSS
latanoprost
BSS
latanoprost
BSS
latanoprost

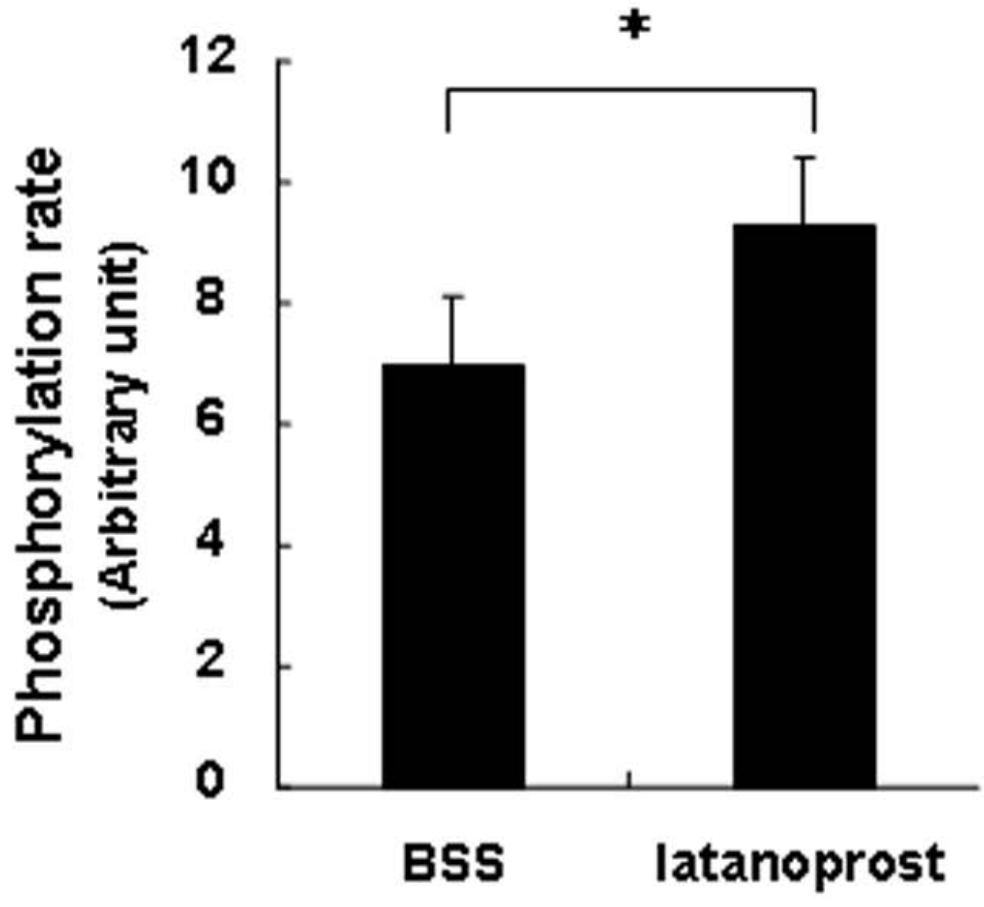
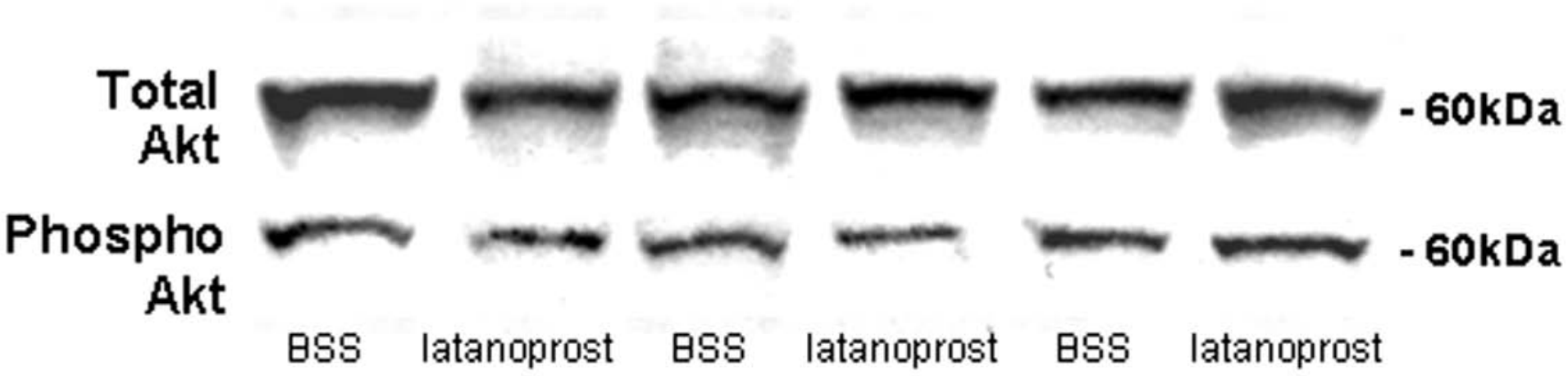

*
12
10
8
6
4
2
0
Phosphorylation rate
(Arbitrary unit)
BSS
latanoprost

Figure.6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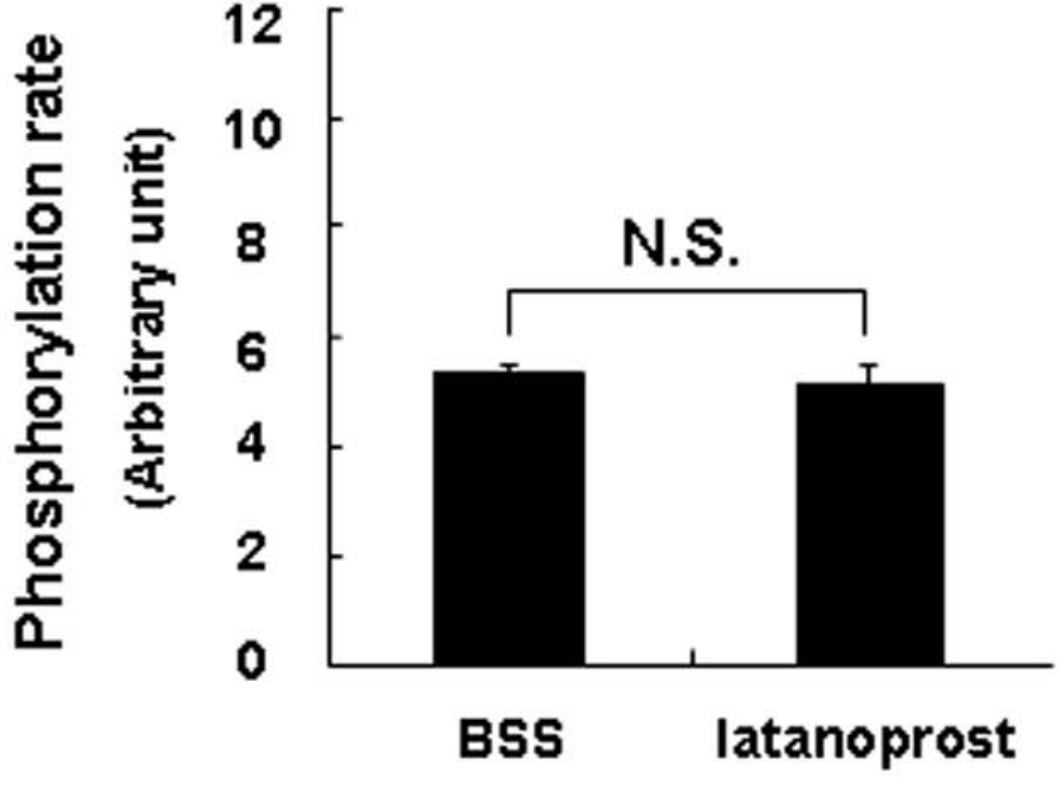
Phospho Akt
- 60kDa
- 60kDa
BSS
latanoprost
BSS
latanoprost
BSS
latanoprost


Phosphorylation rate
(Arbitrary unit)
12
10
8
6
4
2
0
N.S.
BSS
latanoprost