Title: TAT-mediated peroxiredoxin 5 and 6 protein transduction protect against high glucose-induced cytotoxicity in retinal pericytes

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Abstract

Aims: Hyperglycemia-induced oxidative stress is implicated to be involved in pericyte apoptosis seen in diabetic retinopathy. The six mammalian Peroxiredoxins (PRDXs) comprise a novel family of antioxidative proteins that negatively regulate oxidative stress-induced apoptosis by controlling reactive oxygen species (ROS) levels.

Main methods: Sprague Dawley rats were used to detect the retinal expressions of PRDXs1-6. Pig pericytes cultured in high-glucose medium were used to monitor the protective effect of PRDX5 and 6 against high-glucose- associated change. Recombinant PRDX5 and 6 proteins were linked to the Trans-Activating Transduction (TAT) domain from HIV-1 TAT protein for their efficient delivery into cells/tissues.

Key findings: We found higher expression of *PRDX5* and 6 mRNAs and PRDX5 and 6 proteins in retina than the other *Prdx*s (*Prdx* 1–4). Western blotting affirmed the intracellular presence of TAT-linked proteins and revealed the efficient transduction of TAT-HA-PRDX5 and 6 in these cells. Extrinsic supply of TAT-HA-PRDX5 and 6 proteins inhibited the oxidative stress induced DNA damage after high-glucose exposure in pig pericytes. The cell survival and apoptosis assay revealed that extrinsic supply of TAT-HA-PRDX5 and 6 proteins were responsible for inhibiting hyperglycemia-induced pericyte

apoptosis.

Significance: Results suggest that delivery of PRDX5 and 6 might protect hyperglycemia-induced pericyte loss to inhibit oxidative stress.

Key Word: Retinopathy; Pericyte; Oxidative stress; Diabetes; Peroxiredoxin

Introduction

Hyperglycemia-induced oxidative stress is known to play a critical role in the development and progression of diabetic retinopathy (Baynes 1991; Kowluru et al. 1998; Kowluru et al. 2001 Rosen et al. 2001; Setter et al. 2003). Studies have shown that as retinal pericytes undergo oxidative stress, several molecular and biochemical changes under high-glucose conditions eventually cause apoptosis and accelerated cell death (Amano et al. 2005; Kowluru 2003; Lorenzi and Gerhardinger 2001). ROS production mediated by hyperglycemia is thought to be generated via a number of mechanisms including auto-oxidation of glucose, non-enzymatic glycation of proteins, glucose-induced activation of protein kinase C, increased polyol pathway activity, and the impaired anti-oxidant enzymes and alterations in mitochondria (Chung et al. 2003; Inoguchi et al. 2003; Kanwar et al. 2007; Nishikawa et al. 2000; Sakurai and Tsuchiya 1988). High glucose levels attenuate the function of pericytes through the generation of free radicals, suggesting a possible pathophysiological linkage to diabetic retinopathy with the oxidant-antioxidant balance.

Peroxiredoxins (PRDXs), a new family of antioxidants, function in connect to detoxify ROS and thus provide cytoprotection from internal/external environmental stress (Peshenko

et al. 2001; Wood et al. 2003b). The mammalian PRDX family is composed of six members (PRDX1–6) (Fatma et al. 2001; Lyu et al. 1999; Wood et al. 2003a; Wood et al. 2003b). All PRDXs have two catalytically active cysteines, except PRDX6, a cytosolic antioxidant protein, which contains only one (Fatma et al. 2001; Lyu et al. 1999; Wood et al. 2003a; Wood et al. 2003b). PRDX5 is a novel and unusual PRDX with mitochondrial and peroxisomal targeting signals (Verdoucq et al. 1999; Zhou et al. 2000) and characterized as a thioredoxin peroxidase. PRDX6 has been documented to inhibit peroxynitrite (Peshenko and Shichi 2001; Peshenko et al. 2001) and phospholipid hydroperoxide reductase activities (Chen et al. 2000; Manevich et al. 2002). We hypothesized that PRDXs may be able to remove H_2O_2 or capture ROS in pericytes under hyperglycemic conditions thereby protecting the cells from hyperglycemia-induced pericyte loss.

Advances in gene/protein delivery, and identification of several protein transduction domains has made possible delivery of proteins to cells or organs (Frankel and Pabo 1988; Green and Loewenstein 1988). HIV-Trans-Activating Transduction (TAT) domain has 11 amino acids (aa; YGRKKRRQRRR) and has 100% potential for intracellular delivery of proteins across the plasma membrane and the blood brain barrier (Becker-Hapak et al. 2001; Kubo et al. 2008; Mann and Frankel 1991; Nagahara et al. 1998; Rusnati et al. 1997).

Our previous study demonstrated that this recombinant PRDX6 protein linked to TAT is internalized into lens epithelial cells (LECs) and is biologically active (Kubo et al. 2008).

Taking advantage of the ability of TAT domain to reach into cells, in the present study, we examined the effects of adding TAT-linked PRDX5 and 6 on high-glucose- induced cell death and oxidative stress using pig pericytes. Our aim was to provide new information on the antioxidant defenses against pericyte loss in diabetic retinopathy and efficacy of PRDX5 and 6 proteins in diabetic pericyte loss.

Materials and methods

Animals and culture

All animal experiments were accepted the committee of animal research in University of Fukui, Japan and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the animal protocol in University of Fukui.

We used 4 and 7-week-old, Sprague-Dawley albino rats, which were obtained from a local animal dealer (Clea Japan Inc., Osaka, Japan). To measure the expressions of PRDXs1-6, 7-week-old, female rats were used for the real-time PCR (n=8), protein blot (n=8) and the immunohistochemistry (n=6). Diabetes were induced in female-, 4-week-old

rats (n=8) by a single intraperitoneal injection of 80 mg/kg body weight streptozotocin (STZ) (Sigma, St. Louis, MO, USA) in 0.05mM citrate buffer (pH 4.5) after they had been fasted overnight (Rakieten N et al. 1963). Female, 4-week-old rats (n=8) were used as normal control animal. These control and STZ-injected rats were given *ad libitum* access to regular chow consisting of 25% (w/w) protein, 53% carbohydrate, 6% fat, and 8% water (Oriental Yeast Co. Ltd., Osaka, Japan) for 31 weeks. In the 4^{th} week after STZ injection, all rats had blood glucose levels >600 mg/ml (33.4 mmol/l). At 31 weeks after STZ injection, these 35-week-old rats were used for experiments as STZ rats. Other 35-week-old rats were used as control.

Primary cultures of retinal capillary pericytes were isolated from pig retinal microvessels as described previously (Gitlin and D'Amore 1983). Briefly, porcine eyes were obtained from the local abattoir and retinas isolated and homogenized in Dalbeco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA). The homogenate was filtered through a 100μm Nylon cell strainer (Beckton Dickinson, Bedford, MA). The trapped microvessels were digested in the collagenase and deoxyribonuclease (Sigma) in phosphate buffered saline (PBS; pH7.4) for 20 minutes at 37°, filtered through a 70μm Nylon cell strainer (Beckton Dickinson), and plated in 25cm^2 tissue culture flasks (Nalge

Nunc, Rochester, NY). Cells were cultured in DMEM supplemented with 15% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 1% antibiotic-antimycotic (penicillin G sodium, streptomycin sulfate, and amphotericin B) (Sigma). The retinal pericytes in culture were identified by positive staining for α-smooth muscle actin (α-SMA) antigen and negative staining for factor VIII-related antigen (Sigma). Cells generated after 3 to 6 such passages were used in the experiments.

Pericyte were cultured in DMEM supplemented with 15% FBS and antibiotics (100μg/ml streptomycin, 100U/ml penicillin) at 37°C in 6% CO_2. To monitor the expression of PRDX5 and 6 after high glucose culture, pig pericytes were cultured with DMEM containing 5.5 mM (5G) or 30or 50 mM (30G or 50G) D-glucose (Sigma) supplemented with 5% FBS medium. For osmotic control, pig pericytes were grown in 5.5 mM D-glucose medium containing 24.5 or 44.5 mM (30M or 50M) mannitol (Sigma). For transduction of TAT-HA-PRDX5 and 6, cells were grown overnight on 6-well plate, and then 10 μg/mL of TAT-HA-PRDX5 and 6 proteins were added in culture media. After incubation periods of 24 hr, cells were washed and incubated further 0 or 24 hr and harvested for the preparation of cell extracts. Three experiments have done for each assay using 4 different isolates.

Prokaryotic Expression of PRDX5 and 6

The cDNA encoding the open reading frame of PRDX5 or 6 were isolated from human LEC cDNA library (Fatma et al. 2001) using PRDX6 specific sense (5'GTCGCCATGGCCGGAGGTCTGCTTC-3' contained *Nco1* site) and antisense primer (5'AATTGGCAGCTGACATCCTCTGGCT C-3') , and PRDX5 specific sense (5'GCTGGTACCATGGCCCCAATCAAGGTGGG A-3' contained *kpnI* site) and antisense primer (5'TAGAATTCAGAGCTGTGAGATGA TATTGG-3'). The cDNA encoding the open reading frame of PRDX5 or 6 are subcloned in-frame downstream of the N-terminal 6xHis–TAT protein transduction domain sequences in the pTAT-HA (hemaglutinin-tagged) expression vector as described in our previous study (Kubo et al. 2008). For the preparation of TAT-HA-PRDX6 constructs, we followed the method of Dowdy et al. (Schwarze and Dowdy 2000; Vocero-Akbani et al. 2000). Expression and purification of TAT-HA-PRDX5 and 6 fusion proteins were followed as described in our previous study (Kubo et al. 2008).

Real-time RT-PCR.

To monitor the levels of PRDX1-6 in rat retina, total RNA was isolated from 6 rats

using the single-step guanidine thiocyanate/phenol/chloroform extraction method (Trizol Reagent, Invitrogen) and converted to cDNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ). To monitor the levels of PRDX5 and 6 in pig pericytes, total RNA was isolated using RNeasy® Mini Kit (Qiagen Inc., Turnberry Lane, Valencia) and converted to cDNA using Ready-To-Go You-Prime First-Strand Beads (Amersham). To validate the expression patterns of pig PRDX5 and 6, relative quantification of mRNA was performed using Prism 7000 (Applied Biosystems, Foster City, CA). PCR amplification was performed with TaqMan Universal Master Mix (Applied Biosystems). The relative quantities of rat or pig PRDX1-6 mRNA were obtained using the comparative Ct method and was normalized using pre-developed TaqMan assay reagent human ribosomal RNA as an endogenous control (Applied Biosystems).

In each group, the Cts of target genes were normalized to the levels of ribosomal RNA, used as an endogenous control. The ΔCt for each gene was calculated as described previously (Kubo et al. 2005). Three experiments have done for the each assay using 4 different isolates in each group.

Western blot analysis

Protein lysates of rat retinal tissue or pig retinal pericytes were prepared in ice-cold radioimmune precipitation buffer as described previously (Kubo et al. 2003). Twenty micrograms of protein were loaded and run on a 10–20% SDS-PAGE gradient gel and transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA). The membranes were blocked with 5% milk and were incubated overnight at 4°C with anti-PRDX1-6 monoclonal Ab (LabFrontier, Seoul, Korea) (dilution 1:3000). After being washed, the membranes were incubated with anti-mouse IgG labeled with horseradish peroxidase (diluted 1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and visualized by the enhanced chemiluminescence method according to the manufacturer's protocol (Santa Cruz Biotechnology). The same dilutions of absorbed Abs to the recombinant PRDX5 and 6 proteins were used as negative controls. Anti-rabbit β-actin Ab (Sigma) was used to show that equal amount of protein was loaded in each lane. We used LAS-3000mini (Fujifilm, Tokyo, Japan), which is an image analysis system dedicated to chemiluminescence applications in all experiments for western blot. Densities (pixels) of captured images were analyzed using Science Lab software (Fujifilm) and Multigauge software (Fujifilm) and relative densities to control samples were calculated using Multigauge software (Fujifilm).

Three independent experiments were obtained in the each protein blot assay using four different isolates in each group.

Immunohistochemical localization of PRDX5 and 6 in rat retina

The 4 rat eye was fixed in 4% palaformaldehyde in phosphate buffered saline and embedded in paraffin and sectioned at 4 μm.

Immunostaining was performed using the Tyramide Signal Amplification (TSA™) Kit (Molecular Probes Inc., Eugene, OR), following the manufacture's protocol. The tissue sections were exposed to the anti- PRDX5 or 6 monoclonal Ab (LabFrontier) (dilution 1:2000) or α-smooth muscle actin (α-SMA) Ab (dilution 1:500) (Sigma) overnight, followed by incubation in horseradish peroxidase-conjugated goat anti-rabbit IgG (Molecular Probes) diluted to 1:100. Tyramide working solution was applied to the specimens. The same dilutions of absorbed Abs to the recombinant PRDX5 and 6 proteins were used as negative controls. Preparation of HA recombinant PRDX6 protein has been reported elsewhere (Fatma et al. 2001; Kubo et al. 2008). Staining of nuclei was performed using Hoechst solution (Molecular Probes) following immunostainings.

Cell viability assays and TdT-mediated dUTP-biotin Nick End Labeling (TUNEL)

For cell survival assays, pig pericytes were cultured with 5.5, 30 or 50mM D-glucose (5G, 30G or 50G) for 2 days and then cultured with 5.5, 30 or 50mM D-glucose (5G, 30G or 50G) in the presence of 10 μg/mL of PRDX5 or bovine serum albumin for 4 (Day6) and 8 (Day10) days Then, cell proliferation assay using 3-(4,5-dimethylthiazol -2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)- 2H- tetrazolium, inner salt (MTS) (Promega, Madison, WI) was performed to monitor the number of surviving cells in each group. Absorbance of pig pericytes cultured with 5.5 mM D-glucose was measured as 100% cell survival, and the percentage cell survival was then calculated for each group. Three experiments have done for each assay using 4 different isolates in each group.

The TUNEL assay was performed to assess apoptotic cell death. Pig pericytes were cultured for 6 and 10 days with DMEM/5% FBS medium containing 5.5 mM (5G), 30 mM (30G) or 50 mM D-glucose (50G) treated with/without 10 μg/mL TAT-HA-PRDX5 or 6 recombinant protein in 4-well chamber slides (Nalge Nunc International Corp., Naperville, IL) and then subjected to TUNEL staining (ApoAlert DNA fragmentation assay, BD Bioscience) and staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo Laboratories, Kumamoto, Japan). Four experiments have done for each assay

using different isolates. The total number of cells and the number of apoptotic cells in each group were estimated. The percentage of apoptotic cells (TUNEL-positive cells) per total number of cells was counted from the number of DAPI-stained nuclei in six different fields of each slide for each group using the Scion Image software. Three experiments have done for each assay using 4 different isolates in each group.

In our preliminary unpublished data and our previous study (Kubo et al. 2008), 2.5-20μg/mL TAT-HA-PRDX5 or 6 recombinant proteins were effective to reduce ROS level in human cultured LECs. We used 10 μg/mL TAT-HA-PRDX5 or 6 recombinant proteins in this study as this concentration was found optimum to protect pericytes from glucose-induced apoptosis.

Detection of oxidative stress-induced DNA damage using 8-hydroxy-2'- deoxyguanosine (8-OHdG) immunohistochemistry

Pig pericytes were cultured for 10 days with DMEM/5% FBS medium containing 5.5 mM (5G) or 30 mM D-glucose (30G) treated with/without 10 μg/mL TAT-HA-PRDX5 and 6 recombinant proteins in 4-well chamber slides (Nalge Nunc) and were then subjected to anti-8-OHdG immunostaining. Four experiments have done for each assay using different

isolates. Immunostaining was carried out using a DAKO LSAB Kit for rabbit and mouse primary Abs (DAKO, Carpinteria, CA) according to the company's protocol. They were then exposed overnight to the anti-8OHdG monoclonal Ab (JaIKA, Nikken SEIL Co., Ltd, Shizuoka, Japan) (dilution 1:500). The Ab complex was visualized by adding a 0.02% (v/v) solution of 3,3 diaminobenzidine (Bio-Rad). Three experiments have done for each assay using 4 different isolates in each group.

Statistical analysis

The results are reported as means ± standard deviation and were analyzed statistically using ANOVA with Fisher's test.

Results

PRDX5 and 6 are highly expressed in rat retina

According to a recent classification, PRDX family consists of six members (PRDX 1–6). An initial examination of all the members using real-time RT-PCR and western blot analysis revealed higher expressions of PRDX5 and 6 in the rat retina (Fig. 1A and B) than other PRDX members (PRDX1-4). On the basis of these results, we selected PRDX5 and 6

for the following study using pig pericyte.

Immunohistochemistry was performed to analyze the expressions and localizations of PRDX5 and 6 in the sections of 7 week-old rat eyes. PRDX5 and 6 were expressed in the cytoplasm of whole layers of retina (Fig. 1C-a and -b, respectively). α-SMA expresses in pericyte of rat retinal vessels (Fig. 1C-c). PRDX5 and 6 were co-localized with α-SMA in pericyte of rat retina (Fig. 1C-d and-e, respectively). These results implicate that PRDX5 and 6 were expressed in rat pericyte.

To examine the effect of hyperglycemia to the expressions of PRDX5 and 6, we measured the levels of PRDX5 and 6 proteins in 35-week-old STZ and age-matched control rats using real time PCR and protein blot method. Levels of PRDX5 and 6 mRNA and protein expressions did not alter in whole retina of 35 weeks–old STZ rats comparing with control 35 weeks–old rats with significance.

Expression of PRDX5 and 6 in high-glucose-exposed pig pericytes

We have previously shown that PRDX6 expression is reduced in high-glucose-exposed human LECs in comparison to normal (5mM)-glucose exposed LECs, leaving them vulnerable to oxidative stress (Kubo et al. 2004). Therefore, we investigated the expression

levels of PRDX5 and 6 proteins in high-glucose-exposed pig pericytes. Expressions of PRDX5 mRNA and protein was down-regulated in pig pericytes cultured with high-glucose medium for 10 days compared to those cultured with normal glucose medium (5G) with significance (Fig. 2A and B). In contrast, PRDX6 mRNA level was not decreased in pig pericytes cultured in high-(30G) and normal (5G) glucose media for 10 days (Fig. 2A and B).

TAT-HA-PRDX5 and 6 fusion proteins were able to enter cultured pig pericyte

Western blot analysis revealed that TAT-HA-PRDX5 and 6 were transduced into the cells after 24 and 48 hr (Fig. 3; TAT). We could not detect any band when HA-PRDX6 with flag tag (HA) only was added to the culture medium as a control (Fig. 3; NC).

TAT-HA- PRDX5 protected pig pericyte against high-glucose induced cell death.

A cell viability assay (MTS assay) revealed that cell survival gradually decreased in pig pericytes cultured with 30mM D-glucose (30G) for 10 days and 50mM D-glucose (50G) for 6 and 10 days (Fig. 4). Addition of PRDX5 significantly blocked the increased inhibitory effect of high glucose (30G or 50G) on pericytes at 6 and 10 days after culture. A

hyperosmolar condition (30M; 5.5 mM glucose + 24.5 mM mannitol, 50M; 5.5 mM glucose + 44.5 mM mannitol) had no effect on cell viability (Fig. 4). These results suggest that PRDX5 prevented high–glucose-induced cell death through its antioxidant property.

Next, we investigated whether PRDX5 and 6 could protect against high-glucose (30G and 50G)-induced apoptotic cell death in pericytes using the TUNEL assay. As shown in Fig. 5, PRDX5 and 6 significantly inhibited apoptotic cell death in the group cultured with 50 mM D-glucose (50G) after 6 days and high-glucose (30G and 50G) -exposed pericytes after 10 days. A hyperosmolar condition (30M; 5.5 mM glucose + 24.5 mM mannitol, 50M; 5.5 mM glucose + 44.5 mM mannitol) had no effect on cell apoptosis. These observations suggest that PRDX5 and 6 could protect retinal pericytes against high-glucose-induced apoptosis through its antioxidant property.

TAT-HA- PRDX5 and 6 protected pig pericytes against oxidative stress-induced DNA damage in high- and normal glucose exposure

We also investigated whether PRDX5 and 6 could protect against oxidative stress-induced DNA damage in pericytes using anti-8-OHdG Ab. 8-OHdG is known as a biomarker of oxidative stress-induced DNA damages (Morita et al. 2005; Nishigori et al.

2005; Sato et al. 2005; Tarng et al. 2000), because deoxyguanosine (dG) is one of the constituents of DNA, and when oxidized, it is converted into 8-OHdG. Ten days after high-glucose (30G) culture, 8-OHdG positive-pericytes were significantly increased in high-glucose (30G)-exposed pericytes, however, PRDX5 and 6 significantly inhibited oxidative stress-induced DNA damage in high glucose culture (Fig.6). A hyperosmolar condition (30M; 5.5 mM glucose + 24.5 mM mannitol) had no effect on oxidative stress-induced DNA damages.

Discussion

The importance of PRDX enzymes is underlined by the high abundance in the cytosol and involvement in multiple cellular processes ranging from antioxidant defenses (Kim et al. 2000; Neumann et al. 2003; Wood et al. 2003a) parasite drug resistance (Sherman et al. 1996), cancer (Chung et al. 2001; Neumann et al. 2003; Park et al. 2000) to H_2O_2-mediated cellular signaling (Choi et al. 2005; Vivancos et al. 2005), and regulation of cell proliferation. PRDXs are known as stress-response proteins. Expressions of PRDXs are induced by oxidative stress mediated by H_2O_2, glucocorticoids, and ultraviolet irradiation {Fatma, 2005 #5;Fatma, 2001 #4;Kubo, 2006 #86}. In mouse lenses and retinal ganglion

cells, the expression of PRDX6 is highest compared to other PRDXs (1-5) (Fatma et al. 2008; Fatma et al. 2005; Kubo et al. 2006). In lens, expression of PRDX5 is higher than PRDXs1-4, however, in cultured retinal ganglion cells, expression of PRDX5 was lowest in PRDXs1-6 (Fatma et al. 2008; Fatma et al. 2005). In the rat retina, gene expressions of PRDX5 and 6 are higher than those of other PRDX members. However, PRDX2 protein is also present at significant level, we selected PRDX5 and 6 in this study because expression of PRDX2 was lower in comparison to other PRDX5 or 6 in lens and retinal ganglion cells in our previous studies (Fatma et al. 2008; Fatma et al. 2005). Our previous study revealed decreased expression of *Prdx6* mRNA and protein in rat lenses with diabetes or galactose-induced cataracts (Kubo et al. 2005; Kubo et al. 2004). Hyperglycemia causes a decrease of this protein in LECs and that these cells subsequently undergo apoptosis (Kubo et al. 2005; Kubo et al. 2004). To study the protective ability of PRDXs in the total diabetic eye complications including diabetic cataract, rubeotic glaucoma and diabetic retinopathy, we selected PRDX5 and 6 in this study. Figure 5 demonstrates that these proteins have potential to protect pericite from high glucose-induced cell death in vitro. However, we recognize that the concentration of glucose used in the study is unphysiological. In reviewing the literature on the success of in vitro model systems for defining functions of

molecule, we found sufficient examples of reproducibility with similar activity in vivo. For instance, in vitro model systems were the first to show the activity of several molecules, such as Gpx, SOD, Cat, C. elegans-Prdx1-3, HO-1 (Amano et al. 2005; Fatma et al. 2008; Fatma et al. 2005; Fatma et al. 2001; Isermann et al. 2004; Kubo et al. 2008; Kubo et al. 2004; Kwong et al. 2000; Nishikawa et al. 2000; Orhan et al. 1999; Plaisant et al. 2003; Ribeiro et al. 2003). However, initially we used lower concentration of glucose (below 15-20mM) to induce apoptosis in pericytes, but failed to induce significant cell death in vitro (data not shown). In past several cell-culture based experiments have been conducted to disclose the biological protective function of and mechanism of actions of chemical/biomolecules, and have been found to reveal the same function, however, it requires higher concentration of biomolecules/chemical. It is documented that sensitivity of cell is reduced when cultured in vitro and this is associated with overexpression of protective genes (Dickinson et al. 1995; Karsan et al. 1996). We think that pericyte cultured in vitro are less susceptible to stressors due to overexpression of survival proteins.

The present study demonstrates that PRDX5 and 6 plays an important role in protecting the retinal pericytes from the high glucose induced oxidative damage (30 and/or 50mM). The expression of PRDX5 was decreased in high-glucose cultured pig retinal

pericytes. The reduction of PRDX5 expression may induce the production of ROS in pig pericytes, however, the expression of PRDX5 was not modified in retinal tissues obtained from STZ-induced diabetic rats. Because the population of pericytes in retinal cells in retinal tissues, we cannot estimate the expression levels of PRDX5 in retinal pericytes using the total retina. Further studies may be required using vascular tissues obtained from the retinal trypsin digests to analyze it. However, it is clear that PRDX5 and 6 significantly prevented the high-glucose-induced apoptotic cell death, a predictor of retinopathy (Kern et al. 2000; Mizutani et al. 1996), and the early signs of retinal pathology in diabetic rats. Increased oxidative stress in diabetes is considered a contributing factor in the development of diabetic complications, including retinopathy (Baynes and Thorpe 1999; Haskins et al. 2003; Kowluru et al. 2001). Superoxide levels are elevated (Du et al. 2003), mRNA levels of SOD are downregulated (Li et al. 1999), and glutathione levels are decreased in diabetic retina or high-glucose induced rat or pig pericyte (Kowluru 2003; Manea et al. 2004; Sharpe et al. 1998) suggesting overwhelming of the endogenous defense system. Similarly, we found the oxidative stress-induced DNA damage was elevated in pig pericytes cultured with high-glucose medium. In this regard, we evaluated the antioxidant potency of PRDX5 and 6, and found that the addition of PRDX5 and/or PRDX6 to cell culture enhances

cellular survival. TUNEL and DAPI assays demonstrated that pericytes treated with high glucose concentration underwent apoptosis, and a supply of PRDX5 or 6 could attenuate the process, suggesting that PRDX5 or 6 protects cells by attenuating apoptotic pathways. Several studies have shown that PRDXs are down regulator of apoptotic pathway. Recently, our group has shown that PRDX6 has anti-necrotic property (Fatma et al. 2008). We believe that glucose-induced toxicity of pericytes was inhibited by PRDX6, and was associated with its anti-apoptotic and anti-necrotic property. However, we do not know whether glucose treatment of pericytes could induce necrosis. Moreover, it is difficult to assess the relative contribution of the PRDX system compared with other peroxidase; however, we can consider their distribution, turnover, and abundance. Catalase is an abundant, high-turnover enzyme, but it is localized to the peroxisomes and is relatively inefficient at low concentrations of hydrogen peroxide. PRDX is efficient for the removal of hydrogen peroxide at low concentrations because of its greater abundance and low Michaelis constant (<20μM) (Chae et al. 1999). In various rat tissue, including lung, PRDX comprise ~1-10μg/mg of soluble protein, and the cellular concentration of glutathione peroxidase is much lower than PRDX in most cell except hepatocytes (Chae et al. 1999). Thus high abundance of PRDX in the cytoplasm allows it to be an important player in the

detoxification of hydrogen peroxide. We have measured PRDX5 and 6 levels in human retina samples. The levels of retinal PRDX5 and 6 were about 0.2-2.0 μg/mg and 1.0-6.0 μg/mg of soluble protein, respectively (unpublished data).

8-OHdG is a product of oxidative DNA damage and is a sensitive marker of increased oxidative stress (Morita et al. 2005; Nishigori et al. 2005; Sato et al. 2005; Tarng et al. 2000). Increased number of 8-OHdG positive cells cultured with high-glucose medium implicates that high glucose induces oxidative stress in pig pericytes. 8-OHdG levels are increased in the diabetic retina, which is inhibited by the same antioxidant therapy that inhibits diabetic retinopathy in rats (Kowluru and Odenbach 2004). We have previously reported that PRDX 6 prevents high-glucose-induced cell death in human LECs overexpressing aldose reductase (AR) (Kubo et al. 2004). Our findings revealed that PRDX6 is a negative regulator of the death pathway induced in hyperglycemia (Kubo et al. 2004). Because of these properties, PRDXs could be an important molecule in the prevention of hyperglycemia-induced complications. Thus, the therapies that inhibit ROS production via PRDX5 and 6 also may inhibit the pericyte loss in diabetic retinopathy. Moreover, in cultured pig pericytes, the expressions of PRDX5 mRNA and protein were down-regulated in pig pericytes cultured with medium containing 30 mM (30G) D-glucose

for 10 days. The depletion of PRDX5 protein may induce the production of oxidative stress and cell death. Our study shows that biologically active recombinant PRDX5 and 6 proteins bearing the protein transduction domain TAT can be entered into cells and protects them from high glucose-induced cell apoptosis and ROS elevation. The applicability of this new approach has been demonstrated for inter/intramolecular targeting of TAT-fusion proteins capable of modulating mitochondrial function and cell survival (Kubo et al. 2008; Shokolenko et al. 2005). Also, the intravitreous injection of TAT-HA-PRDX5 and 6 proteins can be transduced into rabbit retina (unpublished data of the department of ophthalmology in University of Fukui, Japan).

Conclusion

Administration of intravitreous injection of TAT-linked PRDX5 or 6 proteins may be useful for the therapeutic approach in retinal eye disease. However, our present investigation reveals that delivery of PRDX5 and 6 can inhibit apoptosis and oxidative damage to DNA in cultured pig pericytes. Thus, supplementation with PRDX5 and 6 may represent an achievable adjunct therapy to help a delay of the early progression of diabetic retinopathy.

Figure Legends

Fig. 1. PRDX5 and 6 are highly expressed in rat retinas

(A) We used female, 7-week-old, Sprague-Dawley (SD) albino rats. Total RNA from rat retina (n=6) was isolated and transcribed into cDNA. Quantitative real-time PCR was carried out using specific primers. mRNA expression of each PRDX was adjusted to the mRNA copies of glyceraldehyde-3-phosphate dehydrogenase.(GAPDH). Results indicated that mRNA expression level of PRDX5 and 6 were significantly high in comparison to other PRDXs (*$p<0.0001$). (B) Protein from female, SD rat retinas (7-week-old, n=8) was extracted and protein blot was performed using anti-PRDX1-6 Abs. Protein blot for all six PRDXs revealed greater abundance of PRDX5 and 6 proteins in rat retina, and the expression levels of PRDX5 protein were higher than that of the other PRDXs. Results are derived from 4 different isolates (2 rats were used for each isolate) (*$p<0.0001$). (C) Immunohistochemical localization of PRDXs 5 and 6 and α-SMA in rat retina. Seven-week-old, rat eyes (n=4) were paraffin-embedded and sectioned after being fixed in 4% paraformaldehyde, and sections were immunostained using Abs specific to PRDXs 5 and 6 and αSMA. Green color of positive staining of PRDX5 (a) and PRDX6 (b) was observed. Red color of positive staining of αSMA was localized in vessels suggesting that

pericytes were visualized using anti-αSMA Ab (c). Hoechst stained nuclei were observed in blue color in each panels.

Fig. 2. Effect of D-glucose on expressions of PRDX5 and 6 mRNA and proteins in cultured pig pericytes.

Pig pericytes were cultured in a medium containing 5.5 mM (5G) or 30mM (30G) levels of D-glucose for 6 and 10 days. Four independent samples (n=4) were used in each treatment group. (A) Total RNA from pig pericyte was isolated and transcribed into cDNA. Real-time PCR was carried out using specific primers. mRNA expression of each PRDX was adjusted to the mRNA copies of ribosomal RNA. (B) Protein was extracted from pig pericyte and used for the protein blot. Cells cultured in high-glucose (30G) medium showed apparent diminution of PRDX5 mRNA (A; $p<0.005$) and protein (B; $p<0.02$) on the 10^{th} day, while no change was detected in the expression of β-actin level, suggesting high glucose specifically modified the expression of PRDX5 (A and B). However, the expression of PRDX6 mRNA did not change on the 6^{th} and 10^{th} day in high (30G) glucose medium groups in comparison to normal (5G) glucose medium groups. Results are derived from 4 different isolates.

Fig. 3. Transduction of TAT-HA-PRDX5 and 6 into the pig pericytes

Cells ($2X10^5$ cells) were cultured in 60mm plates. The next day, 5μg/ml recombinant TAT-HA-PRDX5 or 6 protein was added to the culture medium, and transduction of TAT-HA-PRDX5 or 6 was assessed after 24 and 48 hours. Cells were washed, protein was extracted, and protein blot was performed using anti-HisG antibody (Invitrogen). Results revealed the intracellular transduction of TAT-HA-PRDX5 and 6 (lane; TAT) where as (HA)-PRDX5 and 6 with flag tag (HA) only could not internalize into cells (lane; NC). Three experiments have done for each assay using 4 different isolates.

Fig. 4. Effect of PRDX5 treatment on cellular survival of pig pericytes

Cells were cultured in a medium containing 5.5 mM (5G), 30mM (30G) or 50 mM (50G) D-glucose supplemented with 10μg/ml TAT-HA-PRDX5 or bovine serum albumine for 6 and 10 days in 96 well-plates. Six or 10 days later, cell viability was estimated using colorimetric MTS assay. Addition of PRDX5 significantly protected the inhibition of cell growth in the high-glucose (30G and 50G) medium at 6 and 10 days after culture. In

hyperosmolar condition (30M; 5.5 mM glucose + 24.5 mM mannitol, 50M; 5.5 mM glucose + 44.5 mM mannitol,), there was no significant changes of cell viability in comparison to normal (5G) group. *$p<0.003$; ** $p<0.01$; ***$p<0.001$; ****$p<0.0005$. Results were derived from 4 different samples.

Fig. 5. Effect of PRDX5 and 6 treatments against high-glucose-induced apoptosis in pig pericytes

Cells were cultured in a medium containing 5.5 mM (5G) 30mM (30G) or 50mM (50G) D-glucose supplemented with 10μg/ml TAT-HA-PRDX5 or -PRDX6 or bovine serum albumin as control for 6 and 10 days. Six or 10 days later, apoptotic cell death was estimated using TUNEL assay. On the 6^{th} day after high-glucose culture, percentages of TUNEL positive cells were significantly increased in high glucose (50G) medium group. On the 10^{th} day after high-glucose culture, percentages of TUNEL positive cells were significantly increased in high glucose (30G and 50G) medium group. Addition of PRDX5 and 6 significantly inhibited apoptotic cell death in high-glucose-exposed pericytes. *$p<0.0004$; **$p<0.00001$; ***$p<0.0007$. Results were derived from 4 different samples.

Fig. 6. Effect of PRDX5 and 6 treatment against oxidative stress-induced DNA damage in high-glucose-exposed pig pericytes

Cells were cultured in a medium containing 5.5 mM (5G) or 30mM (30G) D-glucose supplemented with 10μg/ml TAT-HA-PRDX5 or bovine serum albumine for 10 days. Ten days later, oxidative stress-induced DNA damage was estimated using immunolocalization of 8-OHdG. On the 10^{th} day after high-glucose culture, 8-OHdG positive-pericytes (arrows) were significantly increased in comparison to control (5G). Addition of PRDX5 and 6 significantly inhibited oxidative stress-induced DNA damage in high-glucose-exposed pericytes. *$p<0.00001$. Results were derived from 4 different samples.

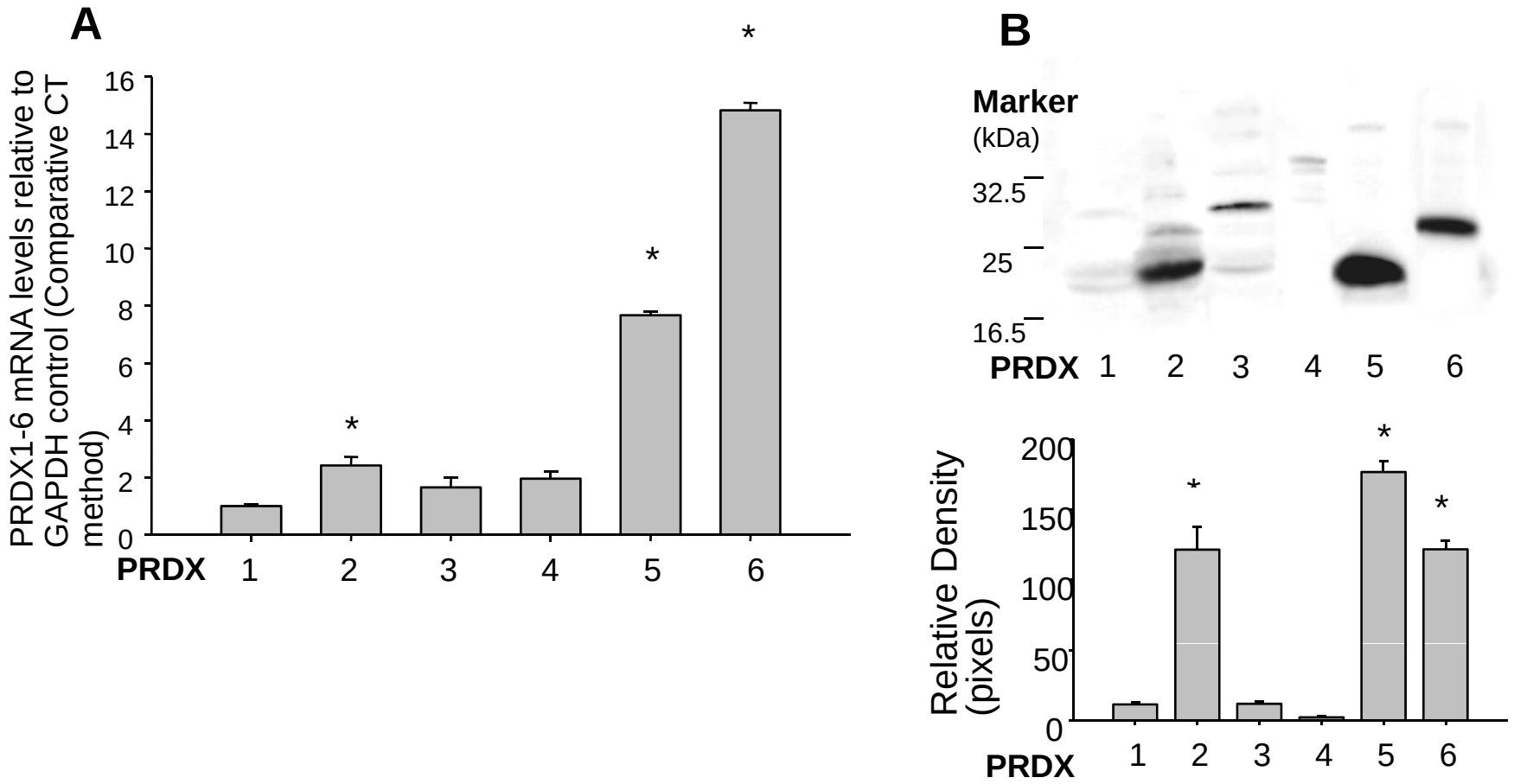
A
PRDX1-6 mRNA levels relative to GAPDH control (Comparative CT method)
16
14
12
10
8
6
4
2
0
*
*
*
PRDX 1 2 3 4 5 6
B
Marker
(kDa)
32.5
25
16.5
PRDX 1 2 3 4 5 6
Relative Density (pixels)
200
150
100
50
0
*
*
*
PRDX 1 2 3 4 5 6

Fig. 1.

C

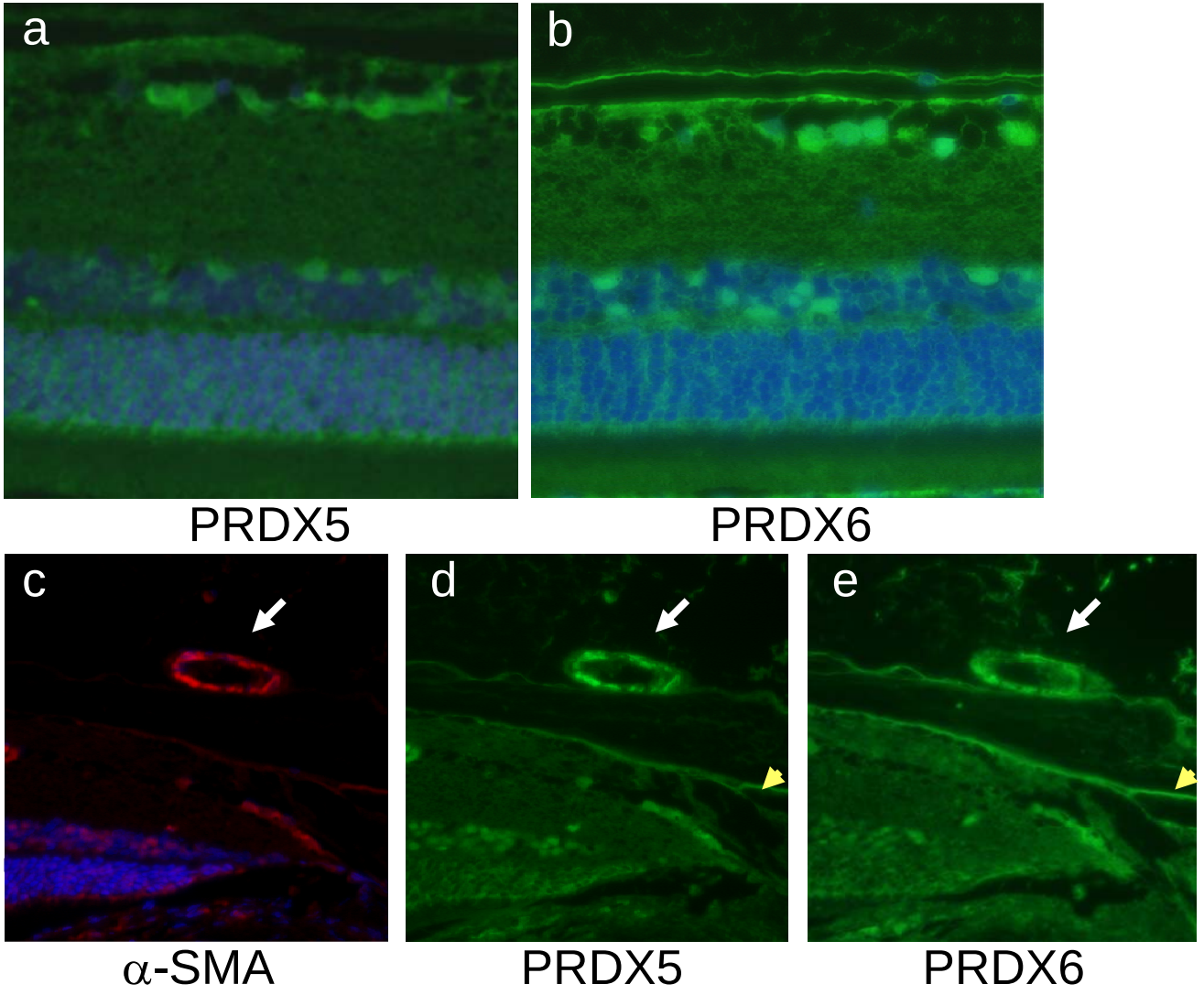

Fig. 1.

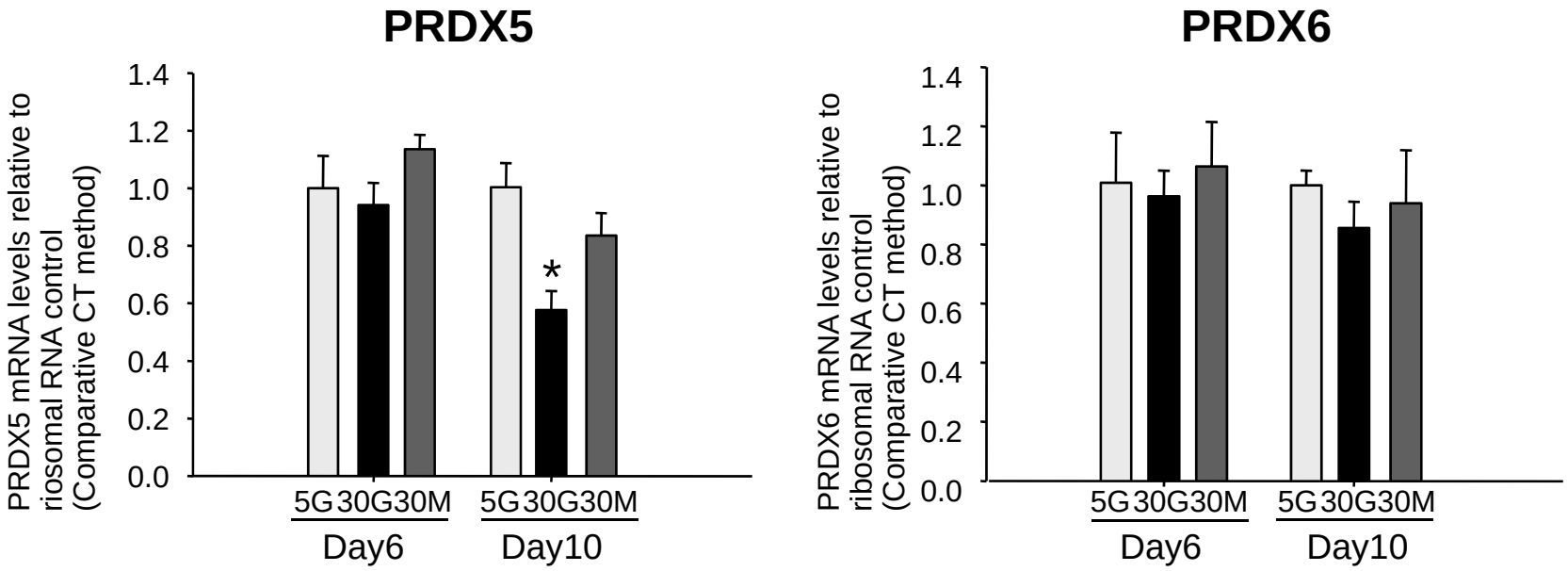
PRDX5
PRDX5 mRNA levels relative to riosomal RNA control (Comparative CT method)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
5G 30G 30M
5G 30G 30M
Day6
Day10
PRDX6
PRDX6 mRNA levels relative to ribosomal RNA control (Comparative CT method)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5G 30G 30M
5G 30G 30M
Day6
Day10

Fig. 2A.

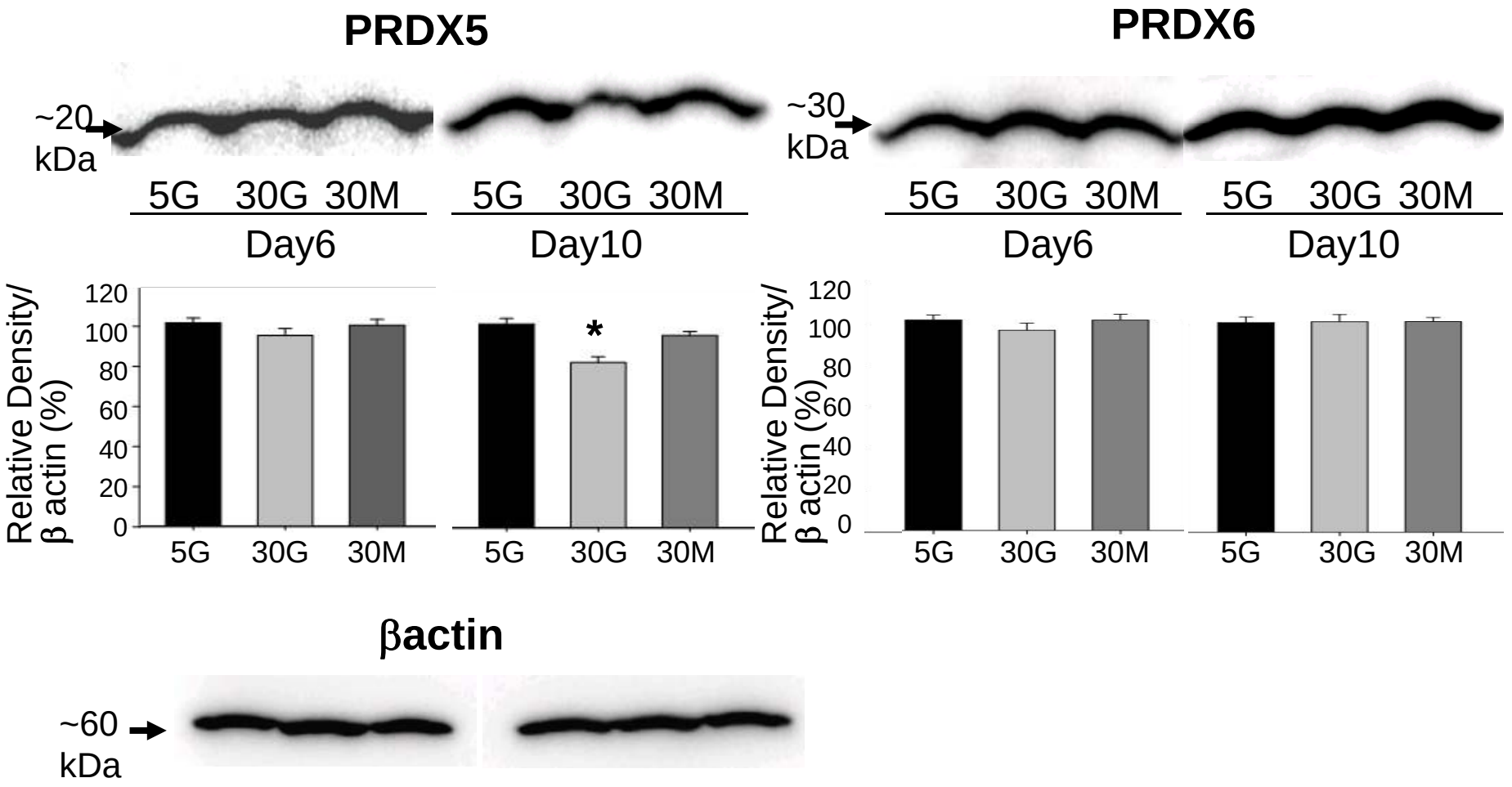
PRDX5
PRDX6
~20 kDa
~30 kDa
5G 30G 30M
5G 30G 30M
Day6
Day10
Relative Density/ β actin (%)
120
100
80
60
40
20
0
*
5G 30G 30M
βactin
~60 kDa

Fig. 2B.

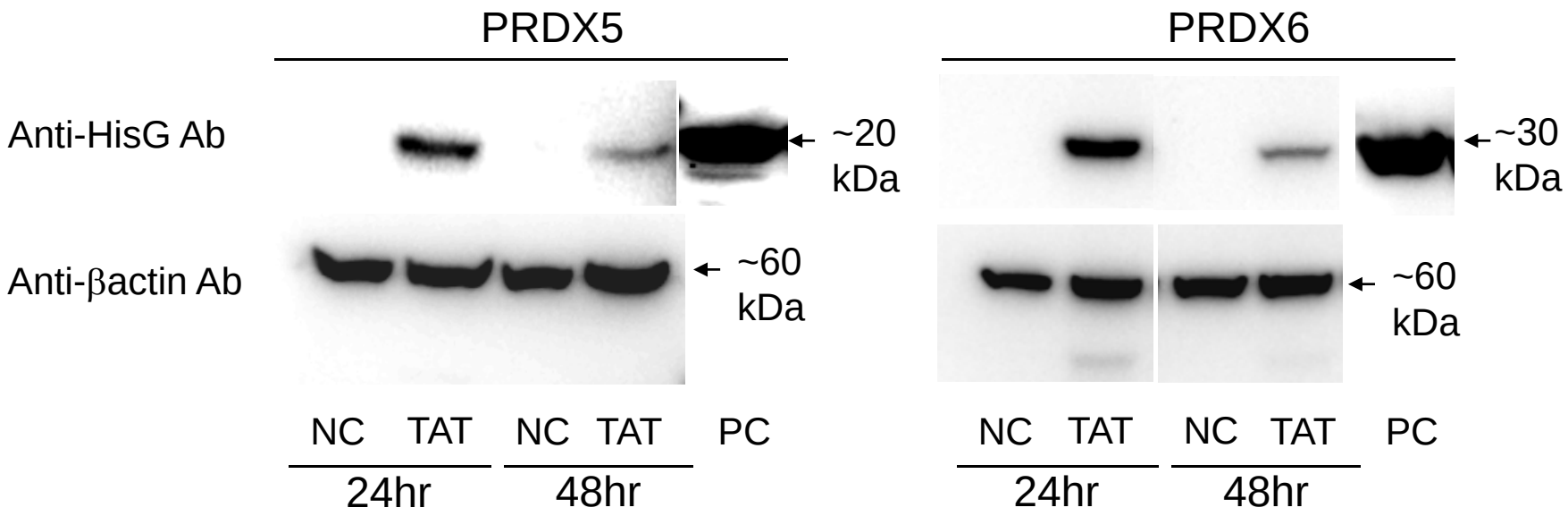
PRDX5
PRDX6
Anti-HisG Ab
Anti-βactin Ab
~20 kDa
~30 kDa
~60 kDa
~60 kDa
NC TAT NC TAT PC
NC TAT NC TAT PC
24hr
48hr
24hr
48hr

Fig. 3.

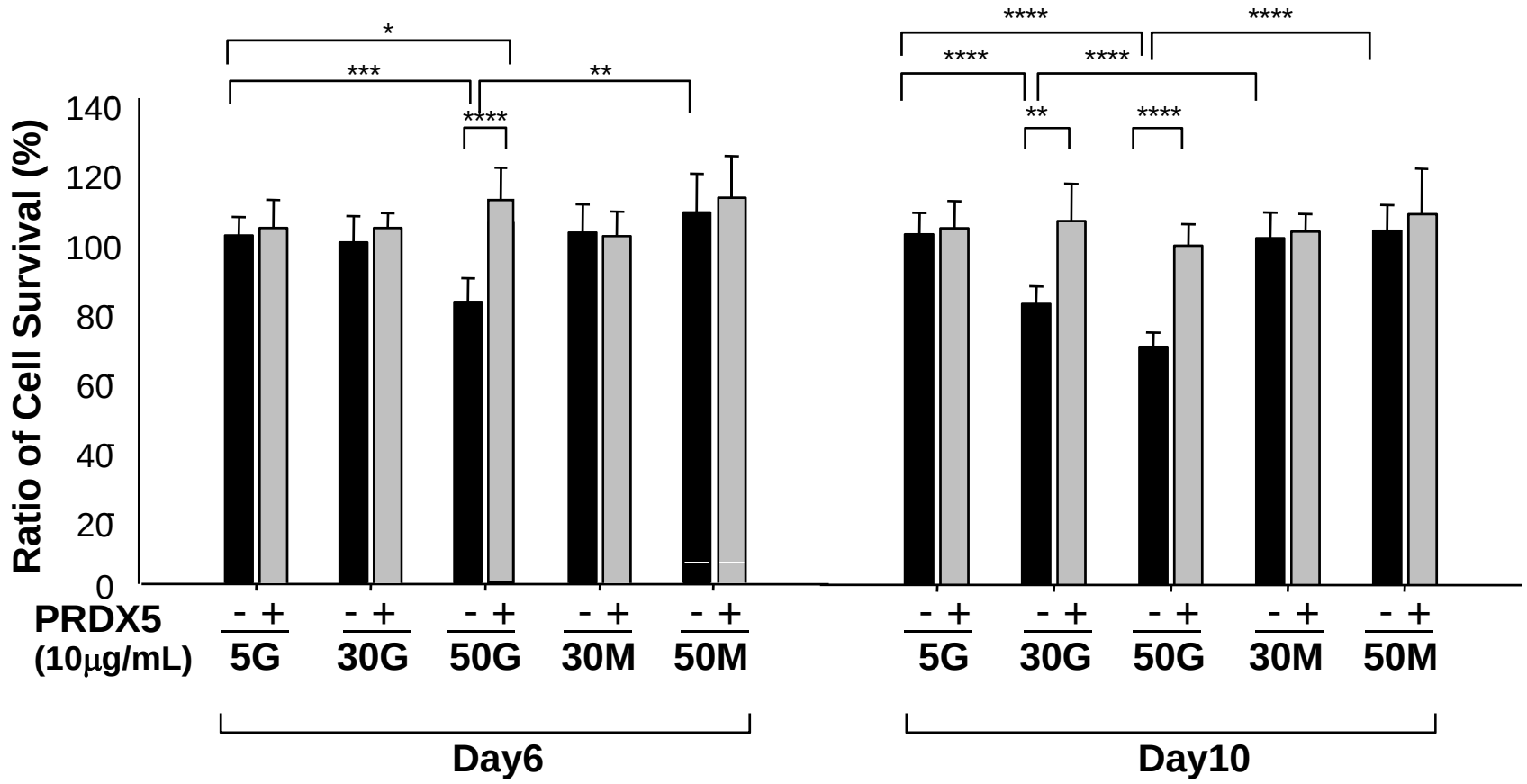
Ratio of Cell Survival (%)
140
120
100
80
60
40
20
0
*
***
**
****
****
****
****
**
****
PRDX5
(10μg/mL)
- + - + - + - + - +
5G 30G 50G 30M 50M
- + - + - + - + - +
5G 30G 50G 30M 50M
Day6
Day10

Fig. 4.

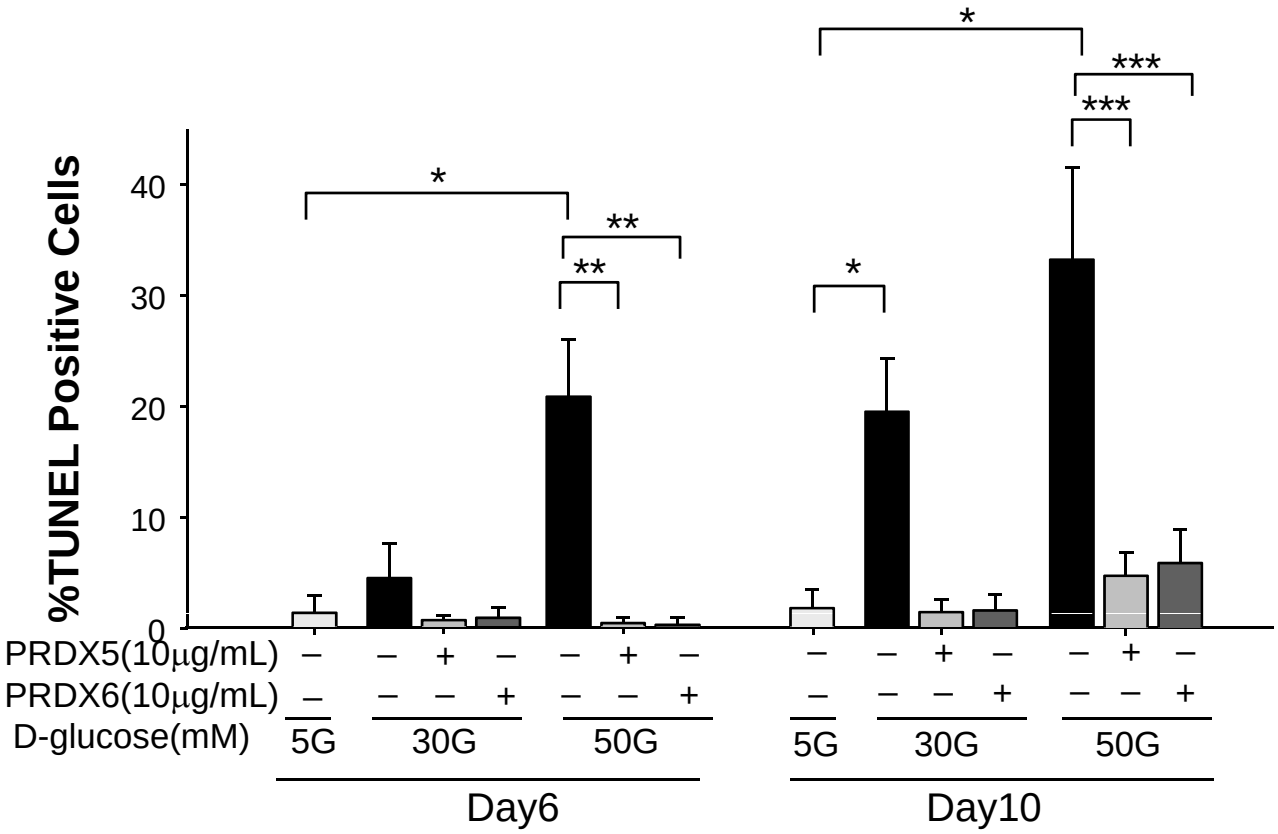
%TUNEL Positive Cells
0
10
20
30
40
*
**
**
*
*
***
***
PRDX5(10μg/mL) – – + – – + – – – + – – + –
PRDX6(10μg/mL) – – – + – – + – – – + – – +
D-glucose(mM) 5G 30G 50G 5G 30G 50G
Day6
Day10

Fig. 5.

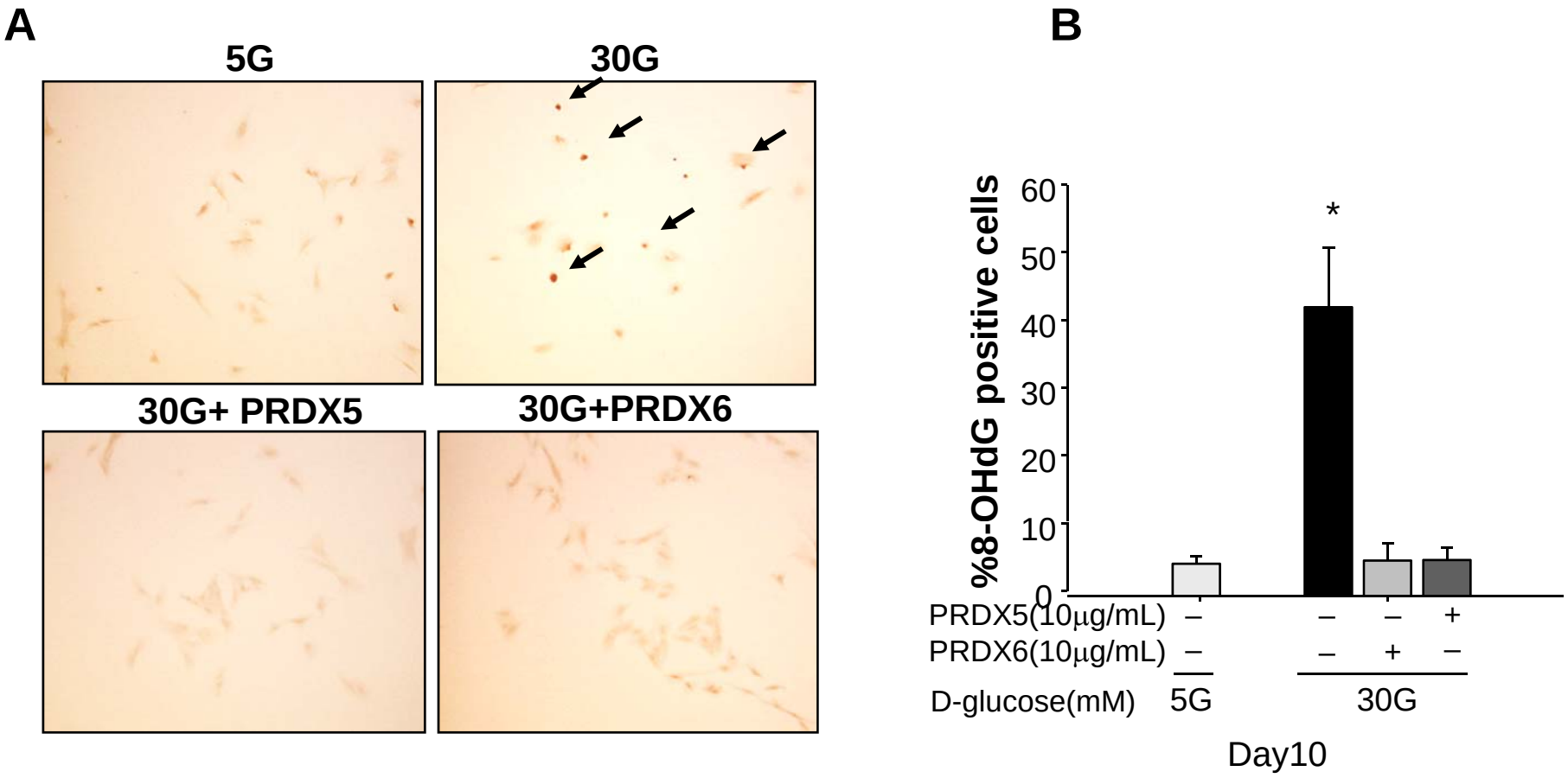
A
5G
30G
30G+ PRDX5
30G+PRDX6
B
%8-OHdG positive cells
60
50
40
30
20
10
0
*
PRDX5(10μg/mL) – – – +
PRDX6(10μg/mL) – – + –
D-glucose(mM) 5G 30G
Day10

Fig. 6.