Development- and age-associated expression pattern of peroxiredoxin 6, and its regulation in murine ocular lens

Regulation and Development-and age-associated expression pattern of peroxiredoxin 6 in murine ocular lens

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Short title: Regulation and distribution pattern of PRDX6 in eye lens

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Abstract

Peroxiredoxin (PRDX) 6 is a unique member of the PRDX family of nonselenium glutathione peroxidases. Its antioxidant and signaling properties are related to its expression level in cells/tissues and its ability to limit H_2O_2. We studied development- and age-associated changes in PRDX6 expression in the murine lens. We also investigated the effects of dexamethasone (Dex), TGF-β1 and TNF-α on PRDX6 expression. Expression levels of PRDX6 mRNA in whole lenses isolated from postnatal day (PD)1 to 18-month-old mice, and the effects of Dex, TGF-β1 and TNF-α on the expression of PRDX6 in lens epithelial cells (LECs), were monitored using real-time reverse transcriptase-PCR or western blot. Localization of PRDX6 was studied using *in-situ* hybridization and immunohistochemistry. PRDX6 expression gradually increased in the lenses of 4-week- to 6-month-old mice and declined thereafter. PRDX6 was localized in the cytoplasm of LECs and in lens fibers. Intense PRDX6 staining was present in the whole lens on gestational days 14 and 18. The lenses of PD1 mice showed diminished nuclear fiber staining, while those of 4-week-old mice revealed lack of nuclear fiber staining but intense staining of the germinative zone. LECs treated with TNF-α or Dex showed higher PRDX6 expression, while TGF-β1 downregulated expression. The expression of PRDX6 in the lens during development and aging is regionally dynamic. Our results provide a topographic basis for understanding the role of PRDX6 in the lens.

Keywords: Lens; PRDX6; Anti-oxidant protein; Oxidative stress

Abbreviation: Ab, antibody; Dex, dexamethasone; DIG, digoxigenin; GD, gestational day; HA, His-Tag; hLEC, human lens epithelial cells; LEC, lens epithelial cell; LEDGF,

lens epithelium-derived growth factor; MAPK, mitogen-activated protein kinase; mLECs, mouse lens epithelial cells; PD, postnatal day; PRDX, peroxiredoxin; RT-PCR, real-time reverse transcriptase-polymerase chain reaction; ROS, reactive oxygen species; TGF-β1, transforming growth factor-beta1; TNF-α, tumor necrosis factor-alfa;

1. Introduction

Although age-related cataract is a multifactorial condition, the incidence and progression of which are modified by age, sex, and exposure to radiation (UV and X-ray), oxidation and biomolecules (such as transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α)(HardingCrabbe 1984; Lovicu et al. 2002; McAvoy et al. 2000), the most significant single factor is the oxidative load on the lenticular cells. Various antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione transferase, have been reported to be present in the lens and to be involved in maintaining lenticular homeostasis(Spector 1995b; 2000; Spector et al. 1995a).

Peroxiredoxins (PRDXs) are a newly identified family of nonselenium glutathione peroxidases that have been reported to be present in many major organs, including the lens(Chen et al. 2000; Fatma et al. 2001; Kang et al. 1998; Kang et al. 1998; Kim et al. 2002) (Fatma et al. 2005; Kubo et al. 2003; Kubo et al. 2004; Peshenko et al. 2001; Rhee 1999; Wood et al. 2003). Substantial evidence suggests that they are involved in balancing the oxidant-antioxidant system by removing or limiting reactive oxygen species (ROS), thereby acting as protector proteins. The mammalian PRDX family is composed of six members (PRDX 1-6) (Fatma et al. 2001; Lyu et al. 1999; Wood et al. 2003). 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. 2003). After peroxide reduction, oxidized PRDX1-5 are reduced via electron transfer from thiol-containing donor molecules such as thioredoxin and cyclophilin A (Lee et al. 2001; Wood et al. 2003). The electron donor for PRDX6 is not absolutely clear as yet. PRDX6 has been documented to exhibit peroxynitrite (PeshenkoShichi 2001; Peshenko et al. 2001) and phospholipid hydroperoxide reductase activities (Chen et al. 2000; Manevich et al. 2002). In addition, it is the only PRDX6 to have acidic calcium-independent phospholipase A2 activity (Kim et al. 2002). PRDX6 has been reported to be expressed in many rat tissues, such as the lung, brain, kidney, heart, liver and testis(Fujii et al. 2001; Kim et al. 2002; SparlingPhelan 2003; Wang et al. 2003). We have also cloned PRDX6 from a human lens epithelial cells (hLECs) cDNA library, and have demonstrated both its expression in hLECs(Fatma et al. 2001) and its ability to protect hLECs from H_2O_2-induced or hyperglycemia-induced apoptosis(Kubo et al. 2004).

Many growth factors and hormones, such as glucocorticoids, TNF-α and TGF-β, generate ROS (Dudek et al. 2001; Iuchi et al. 2003; Jang et al. 2002; Ohba et al. 1994; Oshima et al. 2004; Yasuda et al. 2003) and thus induce oxidative stress. PRDX 6 can reduce H_2O_2 generated in response to growth factors (Frank et al. 1997; Kang et al.

1998; Kim et al. 2003; Munz et al. 1997; SparlingPhelan 2003) and thus protects cells from such stress. Notably, as well as inducing ROS production, TGF-β and glucocorticoids have been implicated in causing cataract formation(Kosano et al. 2001; Lovicu et al. 2002; Murakami et al. 1996). We predicted that these cataract-inducing agents may affect PRDX6 regulation in the lens. Moreover, the protective and signaling functions of PRDX6(Fatma et al. 2005; Kubo et al. 2005; Kubo et al. 2004) may be associated with the cellular microenvironment and be dependent on the localization or expression pattern of PRDX6 in the cells/tissues at various ages. It is, therefore, important to characterize its spatial distribution in tissues/cells during development and aging in order to understand its functions.

In the present study, we investigated development- and age-associated changes in the mRNA and protein expression of PRDX6 in the murine lens. We also examined the effects of TNF-α, TGF-β1 and dexamethasone (Dex) on PRDX6 expression using mouse LECs (mLECs). Our aim was to provide new information regarding the development of antioxidant defenses in the lens and their regulation, thereby widening existing knowledge of the role of PRDX6 in lens physiology and pathophysiology.

MATERIALS AND METHODS

Animals

Adult female Balb/C mice and timed-pregnant female mice (gestational day [GD] 1 = day of mating; term = GD22) were obtained from Clea Japan Inc. (Osaka, Japan). For the developmental study, embryos were extracted from the pregnant mice at GD14 and 18. The day of birth was designated postnatal day (PD) 1. Neonatal mice were studied at PD1or 2, and adult mice at 4, 8 and 12 weeks, and 6, 8, 12 and 18 months. All animals were handled in accordance with the ARVO Policies on the Use of Animals in Research.

Cell culture

Cell culture studies were performed as described previously(Singh et al. 1999). Briefly, a mLEC cell line was cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) at 37°C in an air/CO^2 (19:1) atmosphere for 24 h. The cells were then were maintained for an additional 48 h in serum-free medium with or without added Dex (Sigma), TNF-α or TGF-β1 (R&D Systems, Inc., Minneapolis, MN). The medium (with or without supplements) was changed every 24 h.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Regulation and distribution pattern of PRDX6 in lens

The mice were humanely sacrificed with CO_2 at the appropriate stage of development. Their lenses were extracted and the total RNA from each lens was immediately extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The total RNA from mLECs was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The reverse transcriptase reaction was carried out using the Ready-To-Go You-Primed first strand cDNA Kit and $pd(T)_{12-18}$ primer (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's protocol.

To validate the expression patterns of PRDX6, relative quantification of mRNA was performed using Prism7000 (Applied Biosystems, Foster City, CA). PCR amplification was performed using TaqMan Universal Master Mix (Applied Biosystems). In brief, reactions were performed in triplicate in mixtures containing 2 X Universal PCR master mix, 5 μl of template cDNA, 20 X probe mix containing the PRDX6 primers (5'-CCCGGAGGGTTGCTTCTC-3' and 5'- GAATCTCCCAGGAAATCGTGGAA -3') and the PRDX6 probe (5'- FAM CTTTGAGGCCAATACCACC-3') to a final volume of 50 μl. The relative quantity of PRDX6 mRNA was obtained using the comparative CT method and was normalized using pre-developed TaqMan assay reagent rodent GAPDH as an endogenous control (Applied Biosystems).

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***In-situ* hybridization**

The embryos and eyes were fixed for 48 h in 10% neutral buffered formalin, embedded in paraffin and sectioned at approximately 4 μm. A 694-bp full-length mouse PRDX6 (mPRDX6) cDNA (Gene Bank accession number: NM_007453; 50-743 bp position) was isolated from the mLEC total RNA by RT-PCR using mPRDX6-specific primers (sense primer: 5’-atgcccggagggttgcttct-3’; antisense primer: 5’-aaagacttaaggctggggt-3’), and cloned into a pGEM-T vector (Promega, Madison, WI). The cDNA inserts were confirmed by DNA sequencing. Negative control hybridizations were conducted using a corresponding 1438-bp sense probe. Digoxigenin (DIG)-labeled antisense and sense mPRDX6-cRNA probes were generated according to the manufacturer’s instructions (DIG RNA labeling kit, Roche Diagnostics, Mannheim, Germany). The positive control hybridization was conducted using the DIG-labeled beta-actin probe supplied with the DIG RNA labeling kit. To ensure optimal penetration of the tissue sections, the RNA probes were subjected to alkaline hydrolysis. The tissues were digested with proteinase K (Sigma) for 10 min at 37°C. Pre-hybridization of the tissues with pre-hybridization buffer (Sigma) was performed following acetylation of the tissue with acetic anhydride (0.25%) in triethanolamine. Twenty μl of DIG-labeled mPRDX6 probe was diluted to

1.0 μg/ml in hybridization buffer (Sigma). The probe was placed onto the slides, which were then incubated at 42°C overnight. After incubation, the sections were washed at 42°C in 2 X SSC-50% formamide solution. They were then incubated with 20 μg/ml RNase in Buffer NTE (NaCl 0.5 M, Tris-HCl (pH 8.0) 10 mM and EDTA 1 mM), washed three times in 0.1 X SSC at 42°C for 20 min and equilibrated for 1 min in Buffer1 (Tris-HCl 100 mM and NaCl 150 mM, pH 7.5). The sections were further incubated for 30 min in Buffer 1 containing 1% blocking reagent (Roche), then for 2 h with alkaline phosphatase-coupled anti-DIG antibody (Ab) (Roche) diluted to 1:5000 in Buffer 1 containing 1% blocking reagent. Color development was performed in Buffer 2 (Tris-HCl 100 mM, NaCl 100 mM and $MgCl_2$ 50 mM, pH 9.5) containing NBT and BCIP (Roche).

Immunohistochemistry

Immunostaining was performed using the Tyramide Signal Amplification (TSA™) Kit (Molecular Probes Inc., Eugene, OR), following the manufacturer's protocol. The tissue sections were permealized with 0.2% Triton® X-100 (Sigma) for 10 min and endogenous peroxidase activity was quenched by incubating with Peroxidase Quenching Buffer (Molecular Probes). The specimens were incubated with 1% Blocking Reagent

(Molecular Probes). They were then exposed to the anti-rabbit PRDX6 polyclonal Ab (diluttion 1:2000) 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 for 10 min. Negative controls were incubated with the His-Tag (HA)-PRDX6 protein-neutralized preparation. Preparation of the anti-rabbit PRDX6 polyclonal Ab and the HA recombinant PRDX6 protein has been reported elsewhere(Fatma et al. 2001; Kubo et al. 2004). Cell nucleus of tissues were stained with Hoechst33342 (Molecular Probes)

Western Blotting

Cell lysates from mouse whole lenses or mLECs were prepared in ice-cold radioimmune precipitation buffer, as described previously(Kubo et al. 2002; Kubo et al. 2003). A 20-μg aliquot of protein was resolved on a 10–20% gradient sodium dodecylsulfate-polyacrylamide electrophoresis gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) and immunostained with anti-rabbit polyclonal PRDX6 Ab (dilution, 1:200) to monitor PRDX6 expression. The densities in each band were analyzed using Science Lab 2003 software (Fuji Photo Film, Tokyo, Japan).

Data analysis

All positive results were verified by performing an un-paired Student's *t*- test.

RESULTS

Expression profile of PRDX6 mRNA in the murine lens during development and aging.

We were interested to know whether its expression levels change during development and aging. We compared expression at 8 convenient time-points: PD1; 4, 8 and 12 weeks; and 6, 8, 12 and 18 months). To monitor the expression level of PRDX6, quantitative real-time RT-PCR was conducted (Fig. 1). The results disclosed that PRDX6 is expressed in the developing murine lens, and that levels increase gradually after birth. We surmise that this change in PRDX6 levels might be associated with its antioxidant activity, since the animals face increased environmental stresses after birth. Notably, the strongest expression of PRDX6 was observed in the lenses of 6-month-old mice ($p<0.0001$). A decline in expression was observed from 8 months of age onwards ($p<0.01$). Since PRDX6 is the most strongly expressed PRDX in the lens, we consider that diminution of this protein during aging may be a cause of catractogenesis.

Distribution patterns of PRDX6 mRNA in embryonic and aging murine lenses

Because the localization pattern of biomolecules is a good predictor of their biological importance, we utilized *in-situ* hybridization with a PRDX6-specific probe to determine the expression and possible functional sites of PRDX6. In GD14 mice, PRDX6 mRNA was already localized in the lens, especially in the primary lens fibers (Fig. 2A). A negative control using a DIG-labeled sense mouse PRDX6 RNA probe showed only background staining (Fig. 2B). By the adult stage (8 weeks of age), PRDX6 mRNA was localized in the cytoplasm of the LECs and in the surface cortical fibers (so-called secondary lens fibers) (Fig. 2C). Again, a negative control showed only background staining (Fig. 2D). However, the PRDX6 mRNA localization pattern indicated that PRDX6 transcripts are prevalent in the LECs as well as the fiber cells of GD 14 lenses. In contrast, in aged lenses (8 weeks), the distribution pattern indicated that PRDX6 transcripts were predominantly localized in the cortical region, and were strongly expressed in the bow region, a germinative zone (Fig. 2C). These results reveal that the expression of PRDX6 is dynamic, and might be associated with changing cellular requirements. It may also play a role in signaling by limiting ROS.

Immunohistochemical analysis of PRDX6 protein localization in embryonic and aging murine lenses

The localization of PRDX6 protein was investigated using a PRDX6-specific Ab. In the embryonic stages, PRDX6 was localized in the primary lens fibers (Figs 3A and B), whereas by the neonatal stage (PD1), expression had shifted to the surface fibers (Fig. 3C). In adult lenses (4 and 12 weeks, and 6 months onwards), PRDX6 was expressed in the cytoplasm of the LECs and in the secondary surface fibers; no staining was detected in the adult or aged lens nucleus (Figs 3D, F and G). These results are similar to those for the localization of PRDX6 mRNA obtained with *in-situ* hybridization (Fig. 2). Negative controls treated with neutralized PRDX6 protein Ab showed no staining (Fig. 3E). The intense staining of the lens bow region (a zone with high metabolic activity, reflecting high ROS production) suggests that PRDX6 may play a role in protecting lenticular cells against environmental and oxidative stresses, as well as in cellular signaling, by limiting ROS levels.

Regulation of PRDX6 expression in mLECs by Dex, TNF-α and TGF-β1

Biomolecules such as TGF-β and TNF-α are thought to modulate lens physiology by regulating various signaling pathways, and also induce apoptosis depending on ROS

levels. In the present study, we wished to determine the effects of these molecules on expression levels of PRDX6, an antioxidant. For this purpose, we used cultured mLECs (Kubo et al. 2003) as a model (Fig. 4).

Incubation of mLECs with 10^{-6}, 10^{-7} and 10^{-8} M Dex for 12 h significantly increased PRDX6 mRNA expression compared with untreated controls (incubated in the absence of Dex; $p<0.01$-0.001; Fig. 4A). With 10^{-7} M Dex, the PRDX6 mRNA level was increased to over 5 times that in the untreated control (Fig. 4A). Addition of 10^{-6} and 10^{-7} M Dex to the medium also increased PRDX6 protein expression after 12 h of incubation compared with untreated controls (Fig. 4B). With 10^{-7} M Dex, the PRDX6 protein level was approximately doubled compared with the control (Fig. 4B).

Next, we evaluated the influence of TNF-α and TGF-β1 on PRDX6 expression in mLECs. Since these molecules are present in aqueous and vitreous humor (Tripathi et al. 1991), we predicted that they would modulate the expression of PRDX6. Cells were treated with various concentrations of TNF-α (0, 5, 10 or 20 ng/ml) for variable time periods, or left untreated (Fig. 5). Addition of 10 or 20 ng/ml TNF-α to the medium significantly increased PRDX6 mRNA expression after 24 and 48 h of incubation ($p<0.005$) compared with untreated controls (Fig. 5). To determine the effects of TGF-β1, mLECs were treated with various concentrations of TGF-β1 (0, 0.1, 1.0 and 10.0 ng/ml)

or left untreated. Addition of 10.0 ng/ml TGF-β1 to the medium significantly decreased the PRDX6 mRNA level after 12 h of incubation compared with the untreated control (Fig. 6).

DISCUSSION

In the present study, we investigated development- and age-dependent changes in the expression pattern of PRDX6 in the murine lens. We also investigated the regulatory effects of TNF-α and TGF-β1 on PRDX6. Our results demonstrated that PRDX6 is more strongly expressed in the murine lens than any other known member of the PRDX family(Fatma et al. 2005). However, although PRDX6 is abundantly expressed in the lens, it is not specific to this organ(Fatma et al. 2005; Kim et al. 2002; Kim et al. 1998). During the study, we found that PRDX6 levels gradually increase after birth and reach a peak in the lenses of 6-month-old mice, suggesting its role as antioxidant. This up-regulation of PRDX6 might occur to counteract oxidative stress generated by various physiological and environmental factors. At birth, cells experience marked environmental changes, exposure to new stressors (including biomolecules such as TNF-α, TGF-β and corticosteroids as well as mechanical stress), and to an increased O_2 concentration. An increase in PRDX6, as demonstrated here (Fig. 1), might be an

important physiological step in resisting against and adapting to these stresses. Furthermore, the continuing changes in expression levels of PRDX6 mRNA (Figs 1 and 2) in the lenses throughout embryonic and postnatal life suggest that PRDX6 is regulated by trancriptional control rather than by translational efficiency. Figure 1 shows that the expression of PRDX6 is decreased in the lenses of aged mice. This may be due to inhibitory signaling by TGF-β, which is present in the lenticular microenvironment and has been implicated in the induction of cataractogenesis (McAvoy et al. 2000; Tripathi et al. 1991). Recently, we have shown that ROS is an activator of latent TGF-β1, and that TGF-β1 represses PRDX6 transcription(Fatma et al. 2005). Thus, enviromental stresses such as UV radiation, cytokines and drugs may induce ROS generation in the lenticular microenvironment and may suppress PRDX6 expression in aged animals. Interestingly, in the lenses of adult mice, PRDX6 was localized predominantly in LECs and surface cortical fibers. The LECs and superficial cortical lens contain many organelles. It appears that PRDX6 is necessary for their protection and normal function. Furthermore, superficial cortical fiber cells containing mitochondria may play an active role in metabolism in the lens(Bassnett et al. 1994). Mitochondria are associated with a high-energy-demand organelle system, it’s the electron transport chain of which produces ROS(Nishikawa et al. 2000). Our immunohistochemical studies indicated that

the localization of PRDX6 is similar to the mitochondrial distribution pattern in the lens(Bantseev et al. 1999), suggesting that PRDX6 is involved in the removal of ROS generated by mitochondria. The ocular lens is a potential target for ROS generated as by-products of cellular metabolism, as a result of photochemical reactions or due to environmental stresses. Thus, PRDX6 may play a significant role in an antioxidant defense system that protects adult LECs and surface cortical fibers against ROS.

In the developing lens, PRDX6 transcripts and protein were present in the proliferating anterior LECs and the terminally differentiated primary lens fiber cells at the gestational stage, and also in non-proliferating lens fiber cells in the equatorial zone (where LECs withdraw from the cell cycle and terminally differentiate into secondary lens fiber cells) at the early postnatal stage (Figs 2 and 3). This spatially restricted pattern of PRDX6 expression in the developing lens suggests that PRDX6 may play an important role in regulating lens maturation.

Figures 4A and B illustrate that Dex induces the expression of PRDX6 mRNA and protein in mLECs. Glucocorticoids are widely used in the treatment of various disorders, including autoimmune diseases, allergic diseases and lymphoproliferative disorders. However, therapy with agents such as prednisolone, methylprednisolone and Dex is often limited by adverse reactions associated with excessive glucocorticoid

levels, including truncal obesity, moon-face, striae, cataract, glaucoma, osteoporosis, diabetes mellitus, immunosuppression, and cardiovascular disorders such as hypertension and atherosclerosis (Iuchi et al. 2003; RenfroSnow 1992; Whitworth 1994). Dex has been shown to regulate to some antioxidant enzymes (Asayama et al. 1992; Frank et al. 1985; Tanswell et al. 1986) and, in excess, to induce ROS. Since PRDX6 is induced by oxidative stress (Kim et al. 2003), we speculate that it may be possible to use Dex to induce PRDX6 in LECs, in order to protect the lens against oxidative stress (inductive therapy). However, glucocorticoid excess can lead to steroid-induced cataracts, and it is also possible that PRDX6 expression would be attenuated. Thus, administration of PRDX6 would be required.

Our study also demonstrated that TNF-α (20 ng/ml) induced PRDX6 mRNA expression (Fig. 5). However, we cannot rule out the possibility that higher concentrations or longer exposure periods may down-regulate PRDX6 expression. TNF-α is a multifunctional cytokine which induces a variety of biological responses, such as the production of inflammatory cytokines, upregulation of adhesion molecules, cell proliferation and cell death(TraceyCerami 1994). It has been demonstrated that stimulation by TNF-α leads to the accumulation of ROS, which is essential for prolonged activation of mitogen-activated protein kinase (MAPK) and cell death(Sakon

et al. 2003). MAPK activation is associated with gene expression, proliferation or differentiation, whereas prolonged MAPK activation promotes cell death in a cell type- and stimulus-dependent manner(Chen et al. 1996; Guo et al. 1998; Xia et al. 1995). Thus, accumulation of ROS and/or activation of MAPK as a response to TNF-α may reduce PRDX6 expression.

In contrast to both TNF-α and Dex, TGF-β1 downregulated PRDX6 expression (Fig. 6). Lens epithelium-derived growth factor (LEDGF) is a transcriptional activator of PRDX6 (Fatma et al. 2001). We have previously shown that TGF-β1 inactivates LEDGF by repressing its transcription and attenuating its DNA binding(Sharma et al. 2003), suggesting that repression of LEDGF is a critical event in TGF-β1-induced changes that result in the downregulation of PRDX6.

The effects of oxidative stress upon cell behavior are variable. Low-level oxidative stress has been shown to stimulate the proliferation of many cell types in culture. In contrast, higher levels of oxidative stress usually paralyze cell growth, and have been implicated in the development of several diseases. Thus, a critical balance between the oxidant and antioxidant systems is required to maintain cellular homeostasis. Cells can usually tolerate mild oxidative stress, which often results in upregulation of the antioxidant defense systems in an attempt to restore the oxidant/antioxidant balance.

However, LECS are readily damaged by ROS, showing strand breakage and abnormalities in ion transport, e.g. damage to Na+, K+- ATPase(Spector 1995b; Spector et al. 1995a). Cells potentially have more than one major source of production of ROS. ROS can also be generated by external stimuli. Although receptor-mediated systems are likely to control certain sources of ROS production, only a few such systems have been characterized. Many of the known sources of ROS production are under the control of metabolic processes. Based on previous studies by ourselves and others, the ROS-producing systems most likely to be linked to cellular control mechanisms include the NAD(P)H oxidases, xanthine oxidases and peroxisomal oxidases. Thus, the control of ROS production is potentially linked to signaling systems in which intracellular ROS levels are of the utmost importance. High levels of ROS are deleterious to cells, while low levels are beneficial. In this regard, we consider that PRDX6 plays a pivotal role by controlling ROS levels.

In summary, we have characterized the expression and distribution of PRDX6 in the murine lens. PRDX6 is more strongly expressed in the murine lens than other members of the PRDX family. Expression of PRDX6 increases after birth and peaks at 6 months, then decreases in aging murine lenses. During the prenatal period, PRDX6 is localized in the primary lens fibers and LECs; however, it shifts to the surface cortical fibers

(secondary differentiated fibers) and LECs after birth and during aging, indicating that PRDX6 may have important biological functions associated with its antioxidant properties as well as in cellular signaling. In conclusion, the localization pattern of PRDX6 suggests that this molecule has important functions in the lens, which may include protection of the lenses against age-related as well as secondary cataracts.

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Figure Legends

FIGURE 1. Changes in expression levels of peroxiredoxin 6 (PRDX6) mRNA in the murine lens during development and after birth. The strongest expression of PRDX6 was observed in the lenses of 6-month-old mice (*$p<0.0001$). A decline in expression was observed from 8 months of age onwards (**$p<0.01$).

FIGURE 2. Localization of PRDX6 mRNA as determined by *in-situ* hybridization with a digoxigenin (DIG)-labeled-PRDX6 RNA probe. Intense hybridization signals (Dark grey color) were detected in the primary lens fibers of gestational day (GD)14 mice **(A)**. In 8-week-old mice, PRDX6 mRNA was localized in the cytoplasm of the lenticular epithelial cells (LECs) and the surface cortical fibers (so-called secondary lens fibers) **(C)**. No significant signals were detected using sense probes **(B, D)**. **A, B** Bar120μm. **C, D** Bar 80μm.

FIGURE 3. Immunohistochemical investigation of PRDX6 protein localization at various developmental and life stages. In GD14（**A**）and GD18（**B**）mice, green color of positive immunostaining was observed in the primary lens fibers. Expression of PRDX6 had shifted to the surface fibers by postnatal day (PD)1 **(C)**. In adult lenses (4 and 12 weeks, and 6 months) PRDX6 was localized in the cytoplasm of the LECs and in the secondary surface fibers. No staining was seen in the adult lens nucleus in aged mice **(D,F,G)**. No staining was visible in the lens after treatment with neutralized PRDX6 antibody **(E)**. Figures 3A-E were merged pictures with immunostaining and Hoechst nuclear staining. **A-E;** Bar120μm. **F, G;** Bar 80μm.

FIGURE 4. Time course of the effects of dexamethasone (Dex) on the expression of PRDX6 in cultured mouse LECs (mLECs). Treatment with Dex at concentrations of 10^{-6}, 10^{-7} or 10^{-8} M significantly increased PRDX6 mRNA expression (*p<0.005; **p<0.0001; ***p<0.01) after 12 h of incubation **(A)**. At concentrations of 10^{-7} and 10^{-6} M, Dex also increased PRDX6 protein expression after 12 h of incubation **(B)**.

FIGURE 5. Time course of the effects of TNF-α on the expression of PRDX6 mRNA in mLECs. MLECs were treated with 5, 10 or 20ng/ml TNF-α or left untreated. At 10 or 20 ng/ml, TNF-α significantly increased PRDX6 mRNA expression after 24 h of incubation. *p<0.05; **p<0.001; ***p<0.0001.

FIGURE 6. Time course of the effects of TGF-β1 on the expression of PRDX6 mRNA in mLECs. MLECs were treated with 0.1, 1.0 or 10.0ng/ml TGF-β1 or left untreated. At 10 ng/ml, TGF-β1 significantly decreased PRDX6 mRNA expression after 12 h of incubation (*p<0.001). At 1.0 ng/ml, TGF-β1 significantly decreased PRDX6 mRNA expression after 24 h of incubation (**p<0.04).

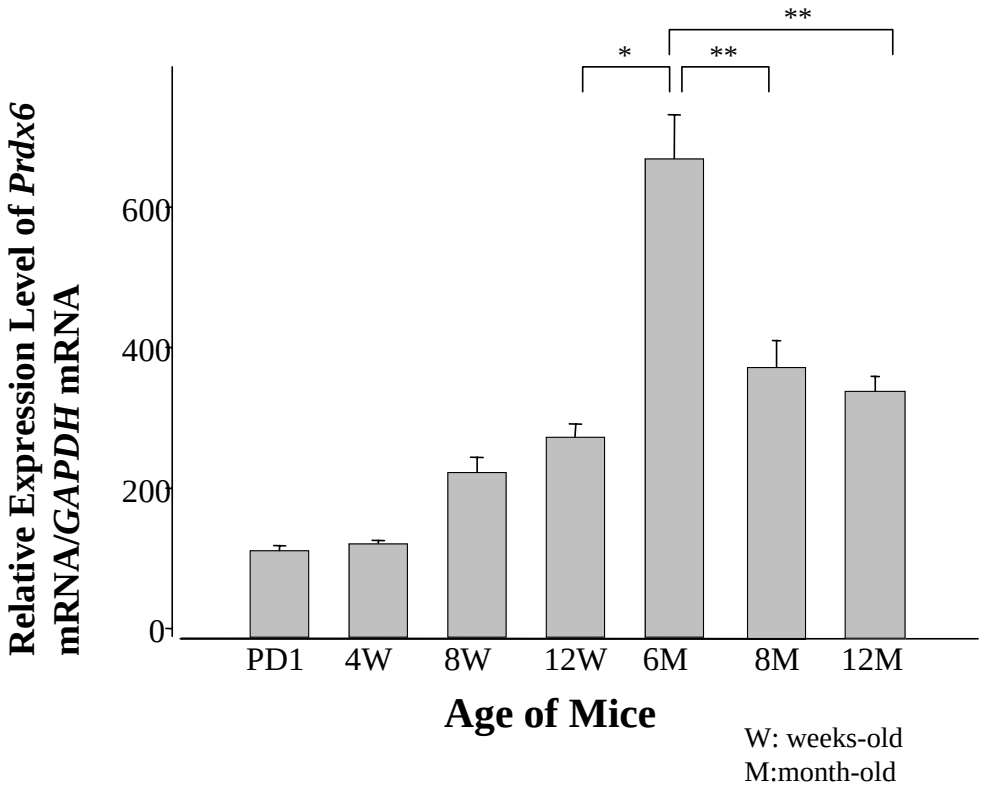
**
*
**
Relative Expression Level of Prdx6 mRNA/GAPDH mRNA
600
400
200
0
PD1
4W
8W
12W
6M
8M
12M
Age of Mice
W: weeks-old
M:month-old

Fig. 1

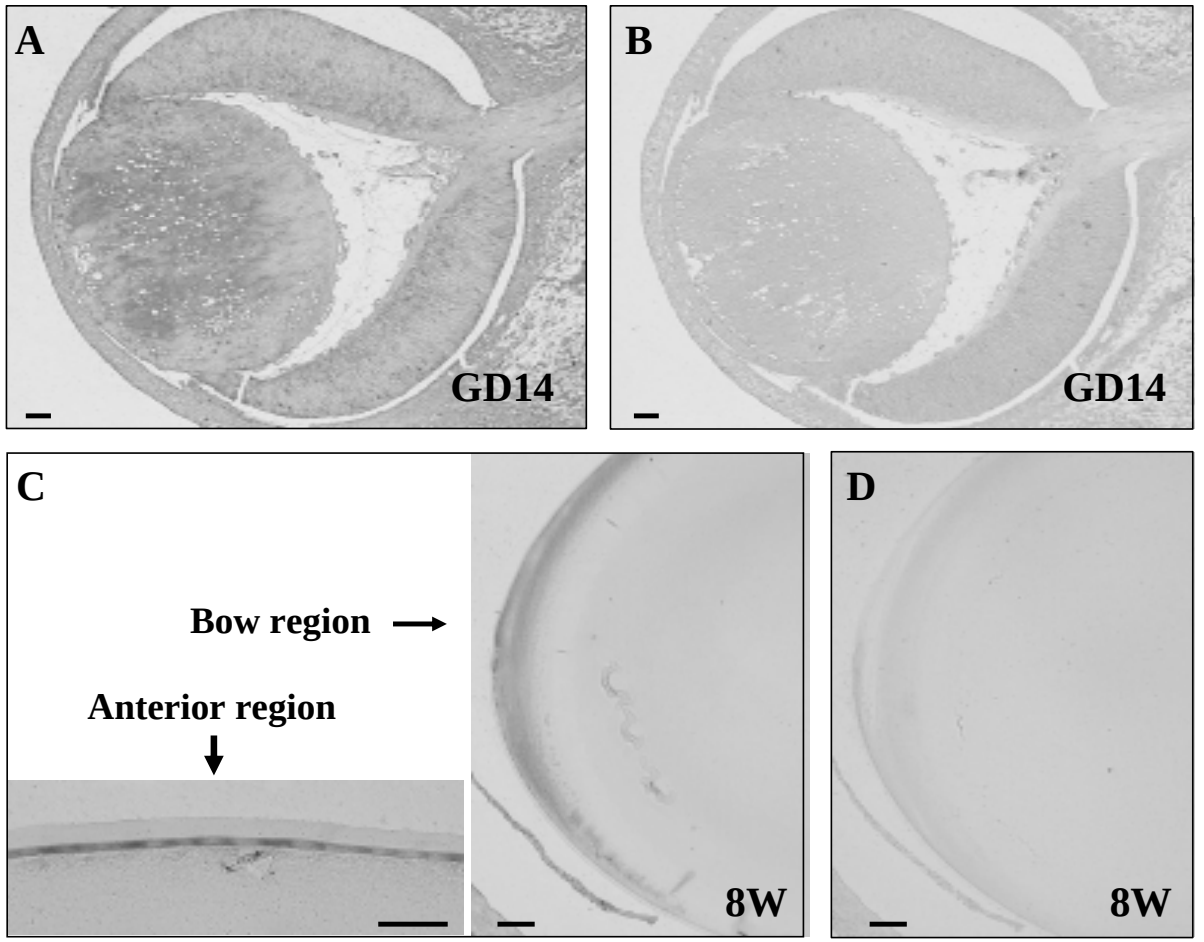

Fig. 2

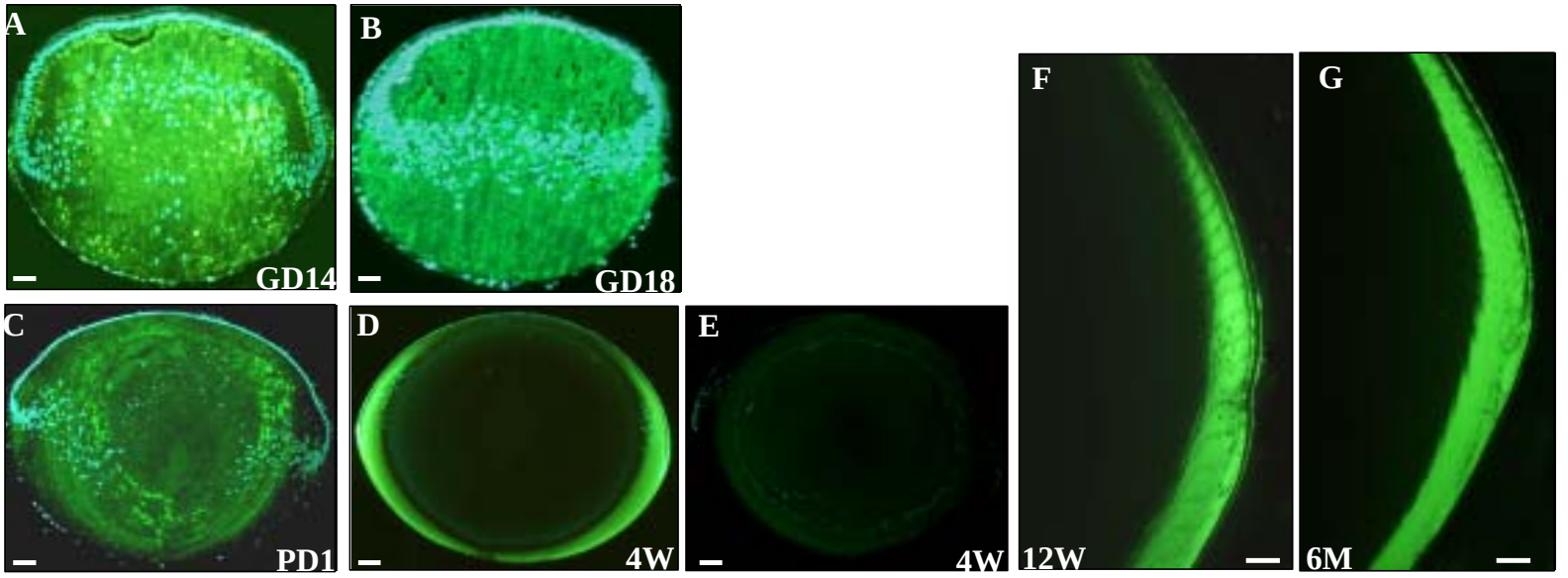

Fig. 3

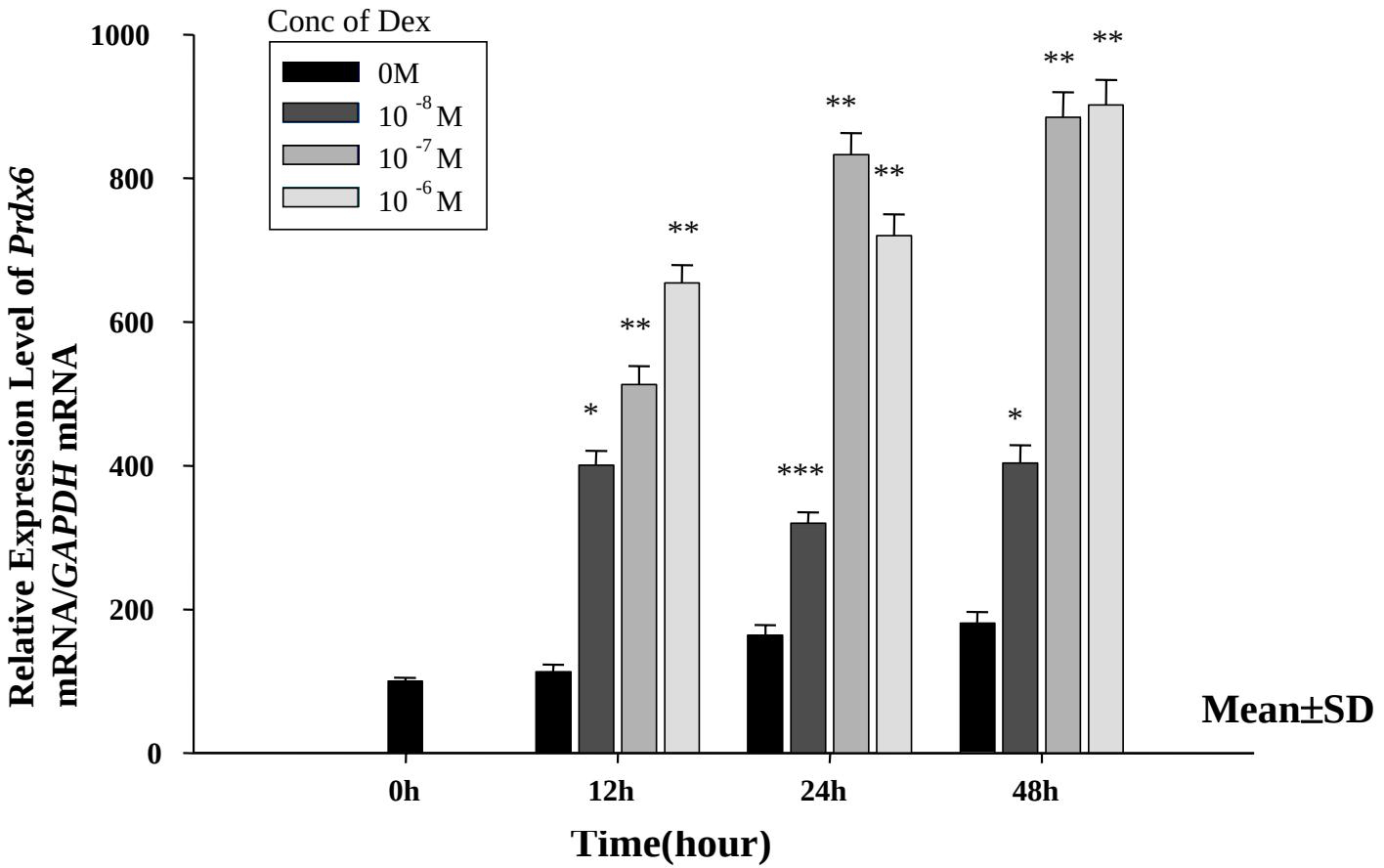
Conc of Dex
0M
10 -8 M
10 -7 M
10 -6 M
Relative Expression Level of Prdx6 mRNA/GAPDH mRNA
1000
800
600
400
200
0
*
**
**
**
***
**
**
*
**
**
0h
12h
24h
48h
Time(hour)
Mean±SD

Fig. 4A

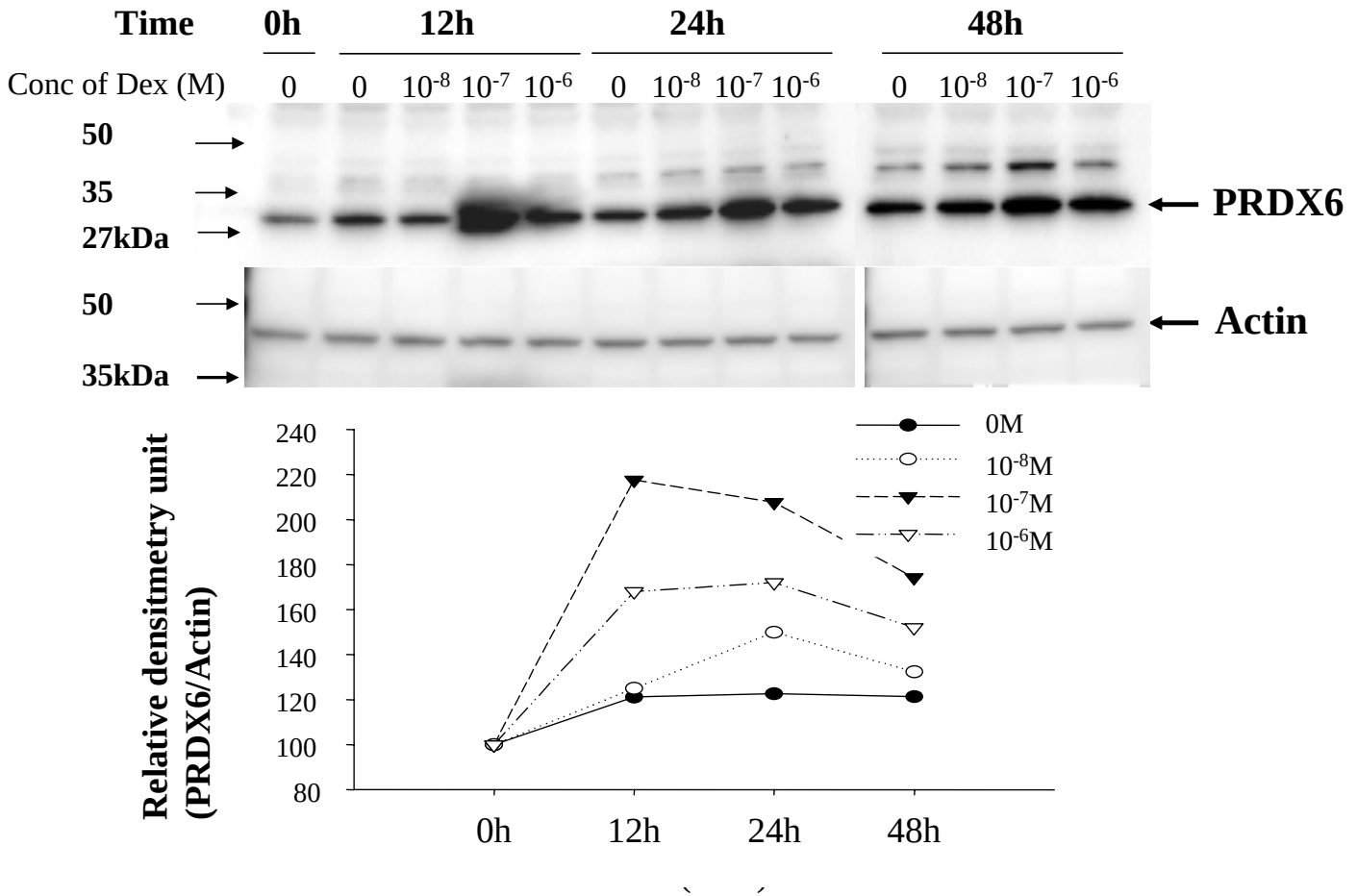
Time
0h
12h
24h
48h
Conc of Dex (M)
0
0
10-8
10-7
10-6
0
10-8
10-7
10-6
0
10-8
10-7
10-6
50
35
27kDa
PRDX6
50
35kDa
Actin
Relative densitmetry unit
(PRDX6/Actin)
240
220
200
180
160
140
120
100
80
0M
10-8M
10-7M
10-6M
0h
12h
24h
48h

Fig. 4B

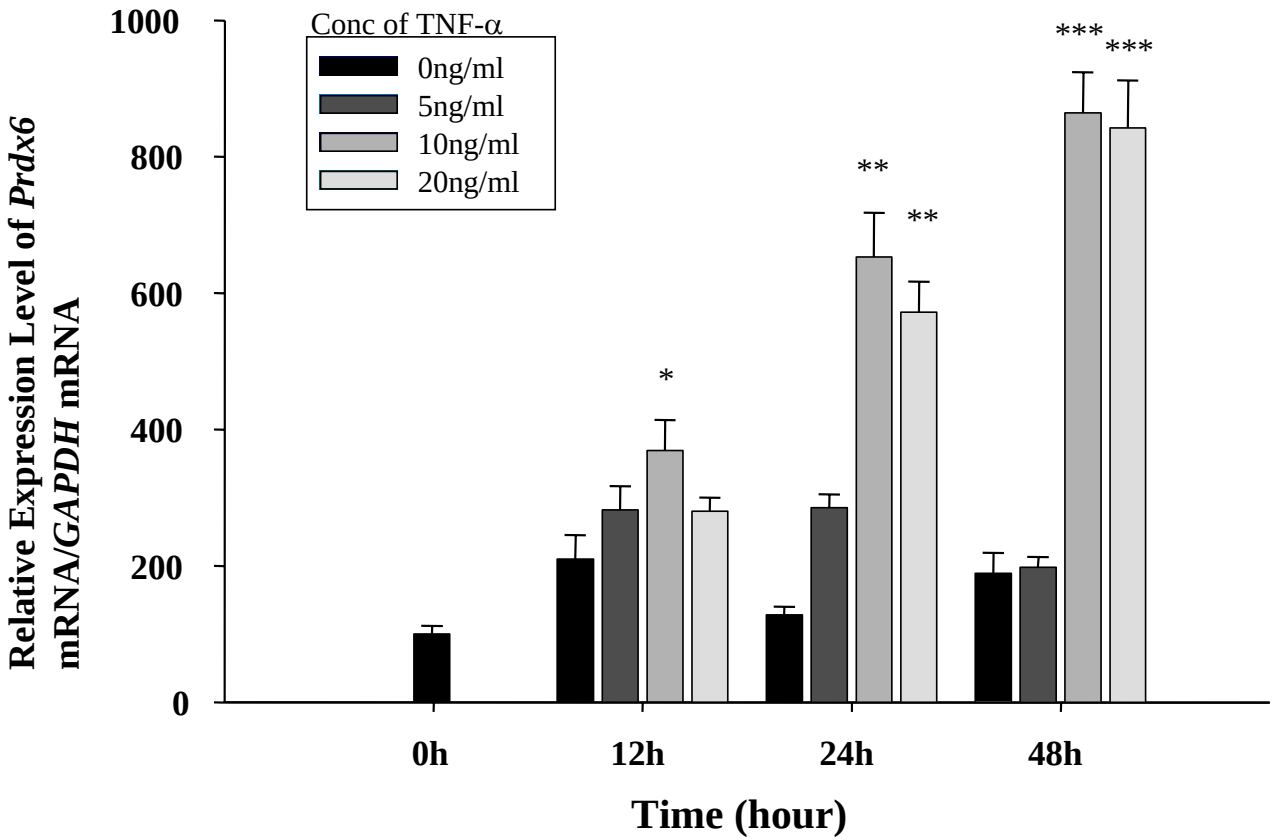
Conc of TNF-α
0ng/ml
5ng/ml
10ng/ml
20ng/ml
Relative Expression Level of Prdx6 mRNA/GAPDH mRNA
1000
800
600
400
200
0
*
**
**
***
***
0h
12h
24h
48h
Time (hour)

Fig. 5

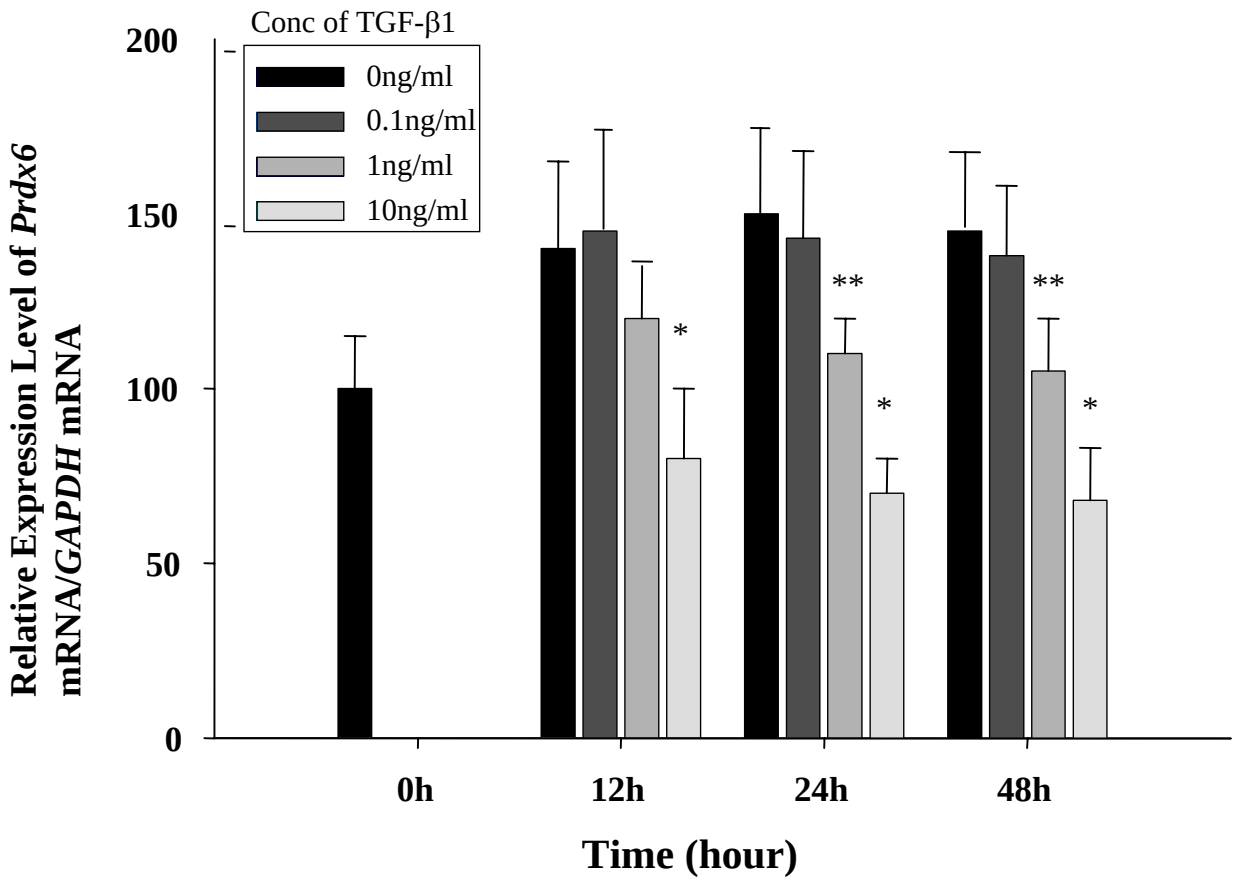
Conc of TGF-β1
0ng/ml
0.1ng/ml
1ng/ml
10ng/ml
200
150
100
50
0
Relative Expression Level of Prdx6 mRNA/GAPDH mRNA
*
**
*
**
*
0h
12h
24h
48h
Time (hour)

Fig. 6