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Effect of Antioxidant Enzymes on Hypoxia-Induced HIF-1α Accumulation and Erythropoietin Activity

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The mechanisms underlying the actions of the antioxidants upon reactive oxygen species (ROS) generation by NADPH oxidase complex have remained uncertain. In this study, we investigated NADPH oxidase activity and the role of antioxidant enzymes upon the generation of ROS during hypoxic stress. ROS generation was found to increase in the mouse kidney under hypoxic stress in a time-dependent manner. Moreover, we found in MCT cells that hypoxia-induced hydrogen peroxide production was decreased by NAC pretreatment. We further analyzed HIF-1α, PHD2 and VHL expression in the NAC-pretreated MCT cells and assessed the response of antioxidant enzymes at the transcriptional and translational levels. SOD3 and Prdx2 were significantly increased during hypoxia in the mouse kidney. We also confirmed in hypoxic Prdx2$^{-/-}$ and SOD3 transgenic mice that erythropoietin (EPO) is transcriptionally regulated by HIF-1α. In addition, although EPO protein was found to be expressed in a HIF-1α independent manner in three mouse lines, its activity differed markedly between normal and Prdx2$^{-/-}$/SOD3 transgenic mice during hypoxic stress. In conclusion, our current results indicate that NADPH oxidase-mediated ROS generation is associated with hypoxic stress in the mouse kidney and that SOD3 and Prdx2 cooperate to regulate cellular redox reactions during hypoxia.

Key words: hypoxia, reactive oxygen species, NADPH oxidase, peroxiredoxin 2, superoxide dismutase 3, erythropoietin.

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Introduction

Tissue hypoxia is the result of an inadequate supply of oxygen (O_2) that compromises biologic functions (Wang *et al*., 2006). Large-scale prospective studies, including the tubulo-interstitial injury and acute renal failure, have established the relationship between hypoxia and progressive renal disease (Nangaku, 2006; Higging *et al*., 2007; David *et al*., 2003). The kidney is rather sensitive to changes in oxygen delivery due to its structure that causes it to be at considerable risk during times of inadequate oxygenation (Eckardt *et al*., 2003). This sensitivity is also reflected by the ability of the kidney to adjust erythropoietin (EPO) production to compensate for changes in the oxygen supply. Our experiments focus on the redox regulation in mice kidney and mouse kidney proximal tubular cell lines (MCT) during hypoxia. Oxygen is converted to reactive oxygen species (ROS) including superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), in aerobic organs during respiration (Turrens, 2003).

NADPH oxidase is a membrane-bound complex, which consists of 2 membrane-bound subunits, $gp91^{phox}$ and $p22^{phox}$ that form the cytochrome b_{558} complex, together with the cytosolic subunits $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and Rac G-protein in phagocyte cells (Block, 2008). This complex is induced by assembly of the cytosolic and membrane-bound subunits and produce superoxide anion ($^{\cdot}O_2^-$) (Mittal, 2007). However, NOX4, a main NADPH oxidase in kidney, is not required among cytosolic subunits except $p22^{phox}$ (Shiose *et al*., 2001; Martyn *et al*., 2006; Kawahara *et al*., 2005). Compared to $^{\cdot}O_2^-$, H_2O_2 is much more stable and can diffuse through biological membranes, providing it with the potential to act as a long-range signaling molecule (Nangaku, 2006).

In a point of redox potential, antioxidant activity is important to cellular redox regulation. Three superoxide dismutase (SOD) isoenzymes convert $\cdot O_2^-$ to H_2O_2. The two intracellular isoenzymes, Cu, Zn-SOD and Mn-SOD, are both synthesized by virtually all cell types in the body. The extra-cellular SOD (EC-SOD, SOD3) as one of Cu, Zn-SOD is the major isoenzyme in extracellular fluids, such as plasma, lymph and synovial fluid, but has also been demonstrated in all investigated in mammalian tissues (Marklund, 1990). SODs play an important role to protect organs against superoxide. SOD3-disrupted mice are more sensitive to lung damage by hyperoxia (Carlsson *et al*., 1995). Peroxiredoxins (Prdxs) have received considerable attention as a new and expanding family of thiol-specific antioxidant proteins, also termed the thioredoxin peroxidases and alkyl-hydroperoxide-reductase-C22 proteins. Prdxs exert their protective antioxidant role in cells through their peroxidase activity (Wood *et al*., 2003). Six Prdx isoforms have been identified and characterized in mammals to act as regulators of hydrogen peroxide-mediated signal transduction (Kang *et al*., 1998; Kang *et al*., 1998) and protect cells from ionized radiation (IR). IR increased expression of Prdx2 in head-and-neck cancer cell lines, and antisense oligonucleotides against Prdx2 sensitized these cells to IR (Park *et al*., 2000). ROS is an oxygen sensor and thereby linked to the regulation of hypoxia response proteins under the limited oxygen supply.

The cellular response to hypoxia is stringently mediated by the transcription factor, hypoxia-inducible factor 1 (HIF-1), which is a heterodimer protein composed of HIF-1α and HIF-1β subunits and activates the transcription of many genes containing a hypoxia response element (HRE) in the promoter region. HIF-1α is a pO_2-sensitive partner, as it is accumulated under hypoxia and unstable under normal oxygen levels due to a pO_2-dependent degradation involving an ubiqutin-proteasomal pathway (Huang *et al*., 1998; Li *et al*., 2007). Target genes of HIF-1 regulate several metabolic processes including oxygen homeostasis, energy metabolism, growth, and differentiation (Marxsen *et al*., 2004). Several reports show that antioxidants might regulate HIF-1α and HIF-1α mediated genes. Prdx3 over-expressed cells are resistant to increases in H_2O_2 and apoptosis caused by hypoxia (Nonn *et al*., 2003). Attenuating thioredoxin-reductase activity blocked HIF-1 transactivation (Moos *et al*., 2003). But, the relation between the responsibility of Prdx2 and HIF-1 expression during hypoxia-exposed stress is yet unknown.

In this study, we investigated whether specific antioxidants are induced by hypoxic stress and affect the expression of HIF-1α and EPO. In addition, we also investigated whether EPO expression was HIF-1α dependent or not during hypoxia in SOD3 over-expressed transgenic mice or Prdx2 knock-out mice.

Materials and Methods

Animals

All animals in these studies were 7~8 weeks of age and included SOD3 over-expressed transgenic (O/E) mice (Kim *et al*., 2005), and Prdx2 knock-out (K/O) mice (Lee *et al*., 2003). The activity of the mice was not restricted, and they were maintained on a 12-hr light/dark schedule and bred in the specific-pathogen free (SPF) conditions. Experimental protocols were approved by the Animal Care Regulations (ACR) of Chonnam National University (accession number: CNU IACUC-YB-2008-4).

Cell culture

Mouse kidney proximal tubular cell lines (MCT) were provided by Dr. Woo JS (Soonchunhyang University Hospital, Korea) and maintained in REGM bullet kit medium (Cambrex Bio Science, NJ, USA). The medium was composed of REBM medium and REGM SingleQuots containing the following: rhEGF (0.1%), insulin (0.1%), hydrocortisone (0.1%), GA-1000 (0.1%), epinephrine (0.1%), T3 (0.1%), transferrin (0.1%), and FBS (0.5%). The cells were routinely cultured at 37°C in an atmosphere of humidified incubator with 95% air and 5% CO_2.

Hypoxia exposure

Mice were exposed to hypoxia in an altitude chamber with a flow of premixed gas containing 7 ± 0.5% O_2 and 93 ± 0.5% N_2 for periods of 2, 4, and 8 hrs. Cells were incubated with 1% O_2, 5% CO_2 and N_2 94% certified gas and in a standard humidified incubator. Parallel cultures were placed in normoxia (95% air and 5% CO_2) for all time points. Cells were tested after 0.5, 1, 2, and 4 hrs of hypoxia.

NADPH oxidase activity

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as previously described (Turrens, 2003). Cell and tissue were homogenized in a RIPA buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM $C_3H_7Na_2O_6P$, 1 mM Na_3VO_4, 2.34 μM leupeptin, and 1 mM PMSF. The homogenate was subjected to centrifugation at 13,600×g for 30 min to remove cellular debris. 100 μg aliquots of homogenates were added to RIPA buffer containing 0.5 uM Lucigenin (Fluka, USA) and 0.5 μM NADPH (Sigma, USA). Proton emission, in terms of relative light units, was measured in a luminometer (Berthold Technologies GmbH & co. KG, Germany) every 30 sec for 20 min. BCA protein assay reagent was used to measure protein content.

Flow cytometry assay

Flow cytometry measurement of carboxy-H_2DCFDA (H_2DCFDA) (Invitrogen Carlsbad, CA, USA), cell-permeable

probe, were used to measure cellular ROS levels. Monolayer cultures were rinsed with Phosphate-buffered saline (PBS), pre-incubated in hypoxic chamber, and harvested by trypsinizing the cells. Cells were incubated in PBS buffer containing 10 μM H_2DCFDA for 30 min. H_2DCFDA fluorescence was analyzed by flow cytometry (Beckman Coulter, Miami, FL, USA).

Nuclear extracts purification

Nuclear extracts were prepared by lysing cells in a hypotonic solution containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors for 10 min on ice. The disrupted cells were then centrifuged at 1,500×g for 5 min, and the pellet was washed five times with hypotonic buffer containing 30% sucrose. The pellet was resuspended in a high salt buffer containing 20 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitors in order to lyse the nuclear membrane. The suspension was then centrifuged at 13,600×g for 30 min, and the proteins in the supernatant were quantified by Bradford reagent (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis

To determine the expression levels of proteins, cells were rinsed 3 times with ice-cold PBS and immediately lysed in RIPA buffer. Protein was quantified using the BCA protocol (BCA protein assay kit, Pierce, USA). Then, cell lysates were denatured at 100°C for 5 min and separated on 8-15% acrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Saboratories, USA). Membranes were blocked in 5% skim milk in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20). Membranes then were analyzed with primary antibodies against the following proteins: Prdxs (1:2000, LabFrontier, Korea), GPxI (glutathione peroxidase I, 1:2000, LabFrontier, Korea), SOD1, 2 (1:2000, LabFrontier, Korea), SOD3 (1:1000, R&D Systems, Inc., USA), VHL (von hippel-lindau tumor suppressor, 1:1000, Cell Signaling, USA), PHD2 (prolyl hydroxylase 2, 1:500), HIF-1α (1:500, R&D Systems, Inc., USA), β-actin (1:5000, Sigma-Aldrich, USA). Membranes were probed with rabbit polyclonal antibodies and mouse monoclonal antibodies (1:2000, cell signaling technology, USA). After incubation with the corresponding HRP-conjugated secondary antibodies, the specific bound antibody was visualized using a Super Signal chemiluminescence detection kit (Intron Biotechnology, Korea).

RNA extraction and RT-PCR

Cells exposed to hypoxia were used for total RNA extraction (Invitrogen, USA) according to the manufacturer's instructions and the integrity of all tested total RNA was verified by agarose gel electrophoresis and quantified by spectrophotometer. A manufactured protocol was used to converts 1 μg of RNA for cDNA synthesis using reverse transcriptase (Roche Applied Science, France). The cDNA was submitted to PCR using the primer pairs as given below. The following primer sets for the target site of each gene were: Prdx1, 5'-cct gct ccc aac ttc-3' and 5'-agg ccc ctg aaa gag-3'; and Prdx2, 5'- tac ccc acg gaa gga-3' and 5'-ggg ctt gat ggt gtc-3'; and Prdx4, 5'-gct ctg ttg act ctc ag-3' and 5'-cta cca ggt ttc cag cc-3'; and Prdx6, 5'-tta ggg gtc acc gca ac-3' and 5'-cag tga ggt gcc tcc ta-3'; and SOD3, 5'-ccc gag ctc gga tcc ag-3' and 5'-ggg tct aga tga att gct g-3'; and EPO, 5'-cag gcc ata gaa gtt tgg ca-3' and 5'-gct ccc agt acc cga agc ag-3' ; and β-actin, 5'-ctg tcc acc ttc cag cag atg t-3' and 5-aca gtc cgc cta gaa gca ctt g-3'. Ex *Taq* Polymerase (TaKaRa, Japan) was used to perform PCRs at 95°C for 5 min, and 28 cycles at 94°C for 30 sec, 53-55°C for 30 sec, 72°C for 30 sec, 72°C for 7 min. The amplified DNA was separated on 1.5% agarose gel and visualized by staining with ethidium bromide. For quantification, the target gene was normalized to the internal standard gene β-actin.

Immunohistochemical staining

Immunohistochemical staining of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and antioxidant enzymes were performed to assess the topography and extent of oxidative damage (Kim *et al*., 2008). Kidney was fixed in 4% neutral buffered formalin (Sigma, USA) overnight at room temperature, paraffin-embedded, and sectioned at 3 μm. Immunoperoxidase staining was performed at room temperature using a Histostain-Plus kit (Zymed Laboratories Inc., USA), in accordance with the manufacturer's instructions. In brief, the deparaffinized sections were treated with 3% H_2O_2 in methanol for 5 min to reduce the endogenous peroxidase activity. The sections were incubated with serum block solution for 30 min, followed by incubation with one of the diluted primary antibodies, Prdx2 (1:100) and SOD3 (1:100) overnight at 4°C, and washing in PBS. The specimens were subsequently incubated with the corresponding biotinylated anti-rat secondary antibodies (1:200) for 10 min, and then with HRP-streptavidin complex (1:300, Santa Cruz Biotechnology, USA) for an additional 10 min. Color was developed using an HRP substrate for 3 min. The sections were counterstained with haematoxylin. All magnifications were at 400×. Scale diagrams made of immunopositive tissue for 8-OHdG (1:50, Santa Cruz Biotechnology, USA) and immunohistochemistry were transcribed to determine hypoxic damage.

EPO activity assay

The mice were exposed to hypoxia for times indicated and isolated kidney and serum. The kidney (40 mg) was homogenized within PBS and then centrifuged for 5 min at 5000×g after two cycles of freeze-thaw. Serum was prepared by centrifugation of blood for 20min at 1000×g, removing of

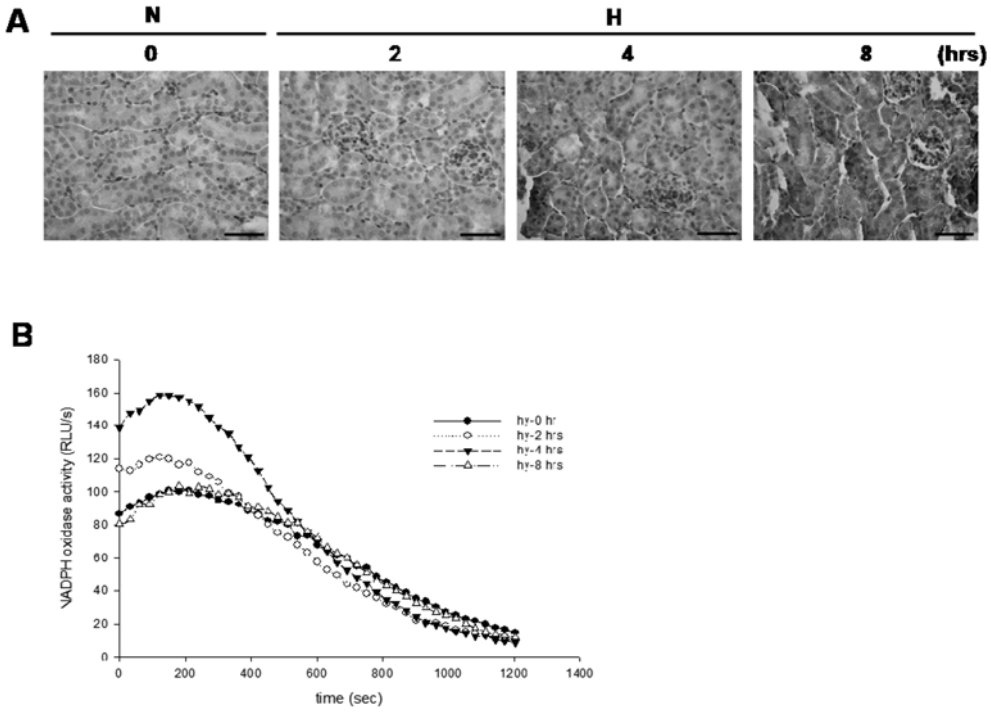

Fig. 1. ROS was generated during hypoxia in mice kidney and MCT cell lines. (A) Immunohistochemistry for 8-HdG in normal mice kidney during normoxia (N) or hypoxia (H). Bars = 50 μm. (B) Superoxide production was measured by lucigenin-enhanced chemiluminescence. Mice kidney was isolated during normoxia and hypoxia after the indicated times. RLU/s, relative light units per second.

serum, and then EPO activity was determined by enzyme-linked immune absorbance assay kits (R&D Systems, Inc., Minneapolis, USA). Assays were performed 3 times.

Results

Generation of ROS during hypoxia in mice kidney and MCT cell lines

Our result showed that the 8-OHdG, an oxidative DNA damage marker, was elevated continuously during hypoxia (Fig. 1A), confirming that the ROS was generated during hypoxia. We also investigated NADPH oxidase activity in mice kidney. As shown in Fig. 1B, the NADPH oxidase activity was increased by hypoxic stress and was maximum after hypoxia exposure for 4 hrs. However that activity was recovered at 8hrs after hypoxic stress similar to normoxia levels. In addition, we investigated the generation of intracellular ROS in MCT cells by flow cytometry analysis (Fig. 2A). The generation of H_2O_2 was increased about 60% during hypoxia in 0.5 hr compared to normoxic cells. Our results indicated that the ROS, $\cdot O_2^-$ and H_2O_2, might be generated by NADPH oxidase in hypoxia exposed mice kidney.

Inhibition of HIF-1α expression by NAC during hypoxia in MCT cell lines

MCT cells pre-incubated with 20 μM NAC for 1hr, followed by exposure to hypoxia for 0.5 hr. The flow cytometry analysis indicated that H_2O_2 was reduced after NAC treatment by approximately 40% than under hypoxic conditions (Fig. 2A). To determine the ROS influenced hypoxia-related proteins, we confirmed HIF-1α accumulation in the nucleus by western blot in MCT cells. The HIF-1α level was elevated 4-fold during hypoxia (Fig. 2B). In contrast, the HIF-1α reduced the almost normoxia exposed cell levels after pre-treatment of NAC under hypoxic conditions. In addition, to determine the proteins participating in HIF-1α degradation, we confirmed PHD2 and VHL levels. PHD2 level in hypoxic condition was decreased to one third of that in normoxia condition. In NAC pretreated cells, however, PHD2 level in hypoxia was similar that in normoxia. VHL was reduced slightly during hypoxic conditions rather than under conditions of normal oxygenation, decreasing approximately 2.5-fold in NAC pretreated cells. Consequently our results suggested that ROS, especially H_2O_2, influence HIF-1α regulation by influencing PHD2 production in MCT cells.

Increase of specific antioxidants, Prdx2 and SOD3, in mice kidney during hypoxia

Our data showed $\cdot O_2^-$ and H_2O_2 were generated during hypoxia. We anticipate some antioxidants would be involved for cellular redox homeostasis (Fig. 2.). Of the antioxidants, we confirmed the membrane and cytosolic antioxidants. RT-PCR data indicated that *Prdx2* and *Prdx4* transcripts were increased 1.2-fold during hypoxic stress after 2 hrs, and then recovered to levels similar to those found under normal

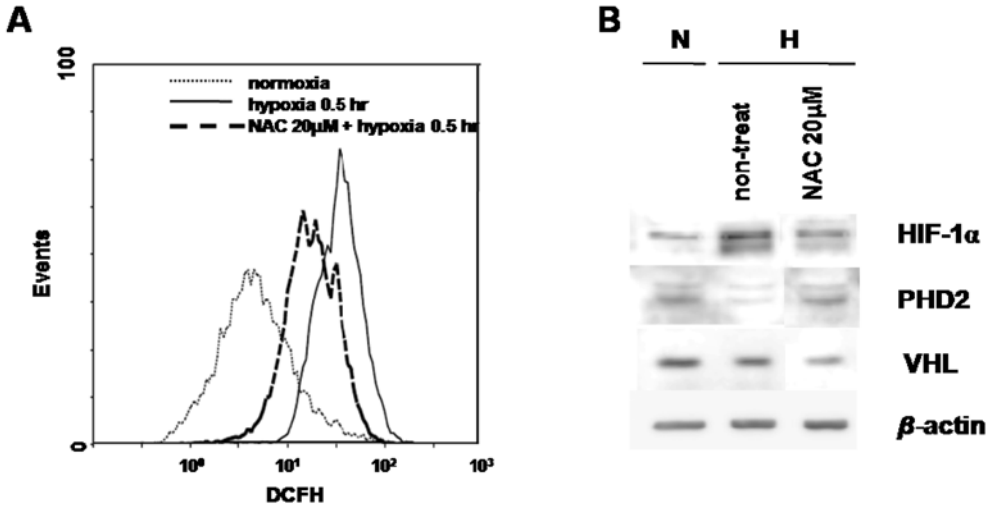

Fig. 2. Hypoxia-induced HIF-1α expression was inhibited by NAC in MCT cell line. (A) Detection of intracellular generation of ROS was performed by using the DCFH probe after hypoxia 0.5 hr in MCT cells. In order to evaluate the role of ROS, cells were pre-treated for 1 hr with the inhibitor, NAC. Cells were observed using flow cytometry analysis. (B) Protein expression level of HIF1α, PHD2, and VHL was determined by Western blot analysis. HIF1α level was examined using nuclear extracts whereas the level of PHD2 and VHL using whole cell lysates. β-actin was used as a loading control. N-normoxia; H- hypoxia.

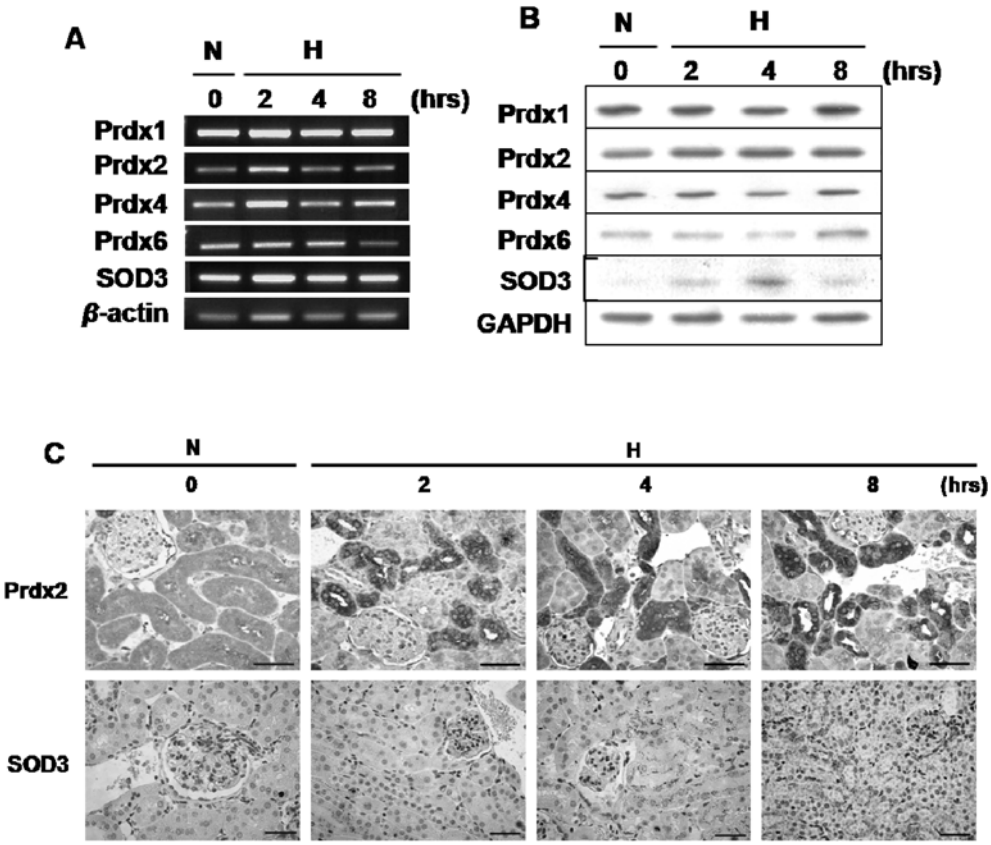

Fig. 3. Specific antioxidants, Prdx2 and SOD3, were increased in mice kidney during hypoxia. (A & B) RT-PCR and Western blotting analysis were performed using the indicated primers and antibodies. (C) Comparison of Prdx2 and SOD3 protein distribution in normal mice kidney during normoxia (N) and hypoxia (H). Bars = 50 μm.

conditions of oxygenation (Fig. 3A). The other antioxidant enzyme transcripts studied were decreased based on β-actin expression during hypoxia. However, protein expression data showed that SOD3, the scavenger of $\cdot O_2^-$ on cellular membrane, was increased from 2 to 4 hrs during hypoxia (Fig. 3B). Prdx2 was also up regulated from 2 to 4 hrs

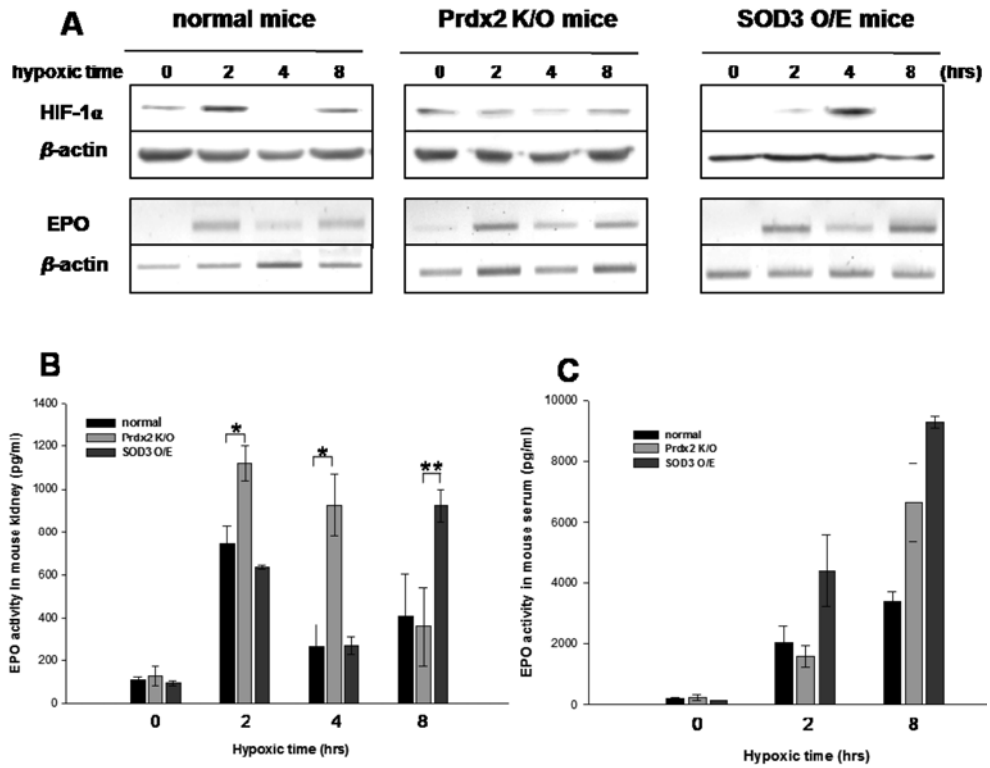

Fig. 4. HIF-1α accumulation, EPO activity and expression were disregulated in Prdx2 K/O and SOD3 O/E transgenic mice. (A) To confirm hypoxia *in vivo*, we analyzed HIF-1α protein level by western blot analysis of nuclear extracts, and HIF-1α downstream *EPO* mRNA expression by RT-PCR. We compared HIF-1α and *EPO* mRNA expression in Prdx2 and SOD3 modified mice kidney during hypoxia. (B & C) EPO activity assay was confirmed in normal and transgenic, SOD3 O/E, and Prdx2 K/O mice kidney and serum during hypoxia. It was determined with the Quantikine mouse EPO ELISA kits. The vales are the means ± SEM. n = 3 mice per group, *p < 0.001; **p < 0.05 between groups.

maximally 2-fold beyond hypoxic stress. The other antioxidants include Prdx4 were decreased during hypoxia. We also determined Prdx2 and SOD3 expression were increased in mice kidney by Immunohistochemical analysis (Fig. 3C). In particular, the expression of SOD3 was moved from cortex to the medulla of the kidney during hypoxic conditions. These results suggest that Prdx2 and SOD3 were specifically used as scavengers of ROS, which were generated during hypoxic condition in the mice kidney.

Abnormal regulation HIF-1α accumulation and EPO activity in Prdx2 K/O and SOD3 O/E transgenic mice

We investigated HIF-1α levels in the mice kidney during hypoxia. In the non-transgenic mice, HIF-1α accumulation was 2-fold increased at 2 hrs after hypoxia, but reduced at 4 hrs (Fig. 4A). SOD3 O/E mice showed similar results to non-transgenic mice at normal oxygen levels. In contrast, Prdx2 K/O mice showed that the HIF-1α was highly accumulated in nucleus at normoxia and gradually degraded under hypoxic conditions for 4 hrs. In addition, SOD3 O/E mice showed that HIF-1α accumulation was increased at 4 hrs. Whether the differential expression of HIF-1α will regulate downstream molecules, we confirmed the *EPO* transcript level in the mice kidney. *EPO* was increased at 2 hrs and at 8 hrs after hypoxia in the non-transgenic mice. It means that *EPO* regulation depends on HIF-1α. On the other hand, the pattern of *EPO* expression in transgenic mice was similar to non-transgenic mice, independent of HIF-1α. We also checked EPO activity during hypoxia in mice kidney and serum (Fig. 4B and 4C). The EPO activity increased about 7-fold at 2 hrs, then decreased at 4 hrs during hypoxia in normal mice kidney. The Prdx2 K/O mice showed an approximate 9-fold increase in EPO at 2 hrs hypoxia and then decreased gradually. In the SOD3 O/E mice, EPO activity was increased again after hypoxia exposure for 8 hrs (**P < 0.05). The EPO activity was increased in all normal and transgenic mice by hypoxic stress for 2 hrs. But the rate of increase was specifically higher in the Prdx2 K/O mice (*P < 0.001). The EPO activity in mice serum was increased under hypoxic conditions in a time dependent manner. Especially, the EPO activity was 70-fold increased in SOD3 O/E mice at 8 hrs during hypoxia. These results showed that Prdx2 deficiency induced HIF-1α accumulation during normoxia, but reduced the accumulation during hypoxia. EPO activity was regulated by HIF-1α in normal mice but, when the antioxidants level was changed, the transcriptional expression and activity of EPO was regulated independently of HIF-1α accumulation during hypoxia.

Discussion

The major findings of present study were that ROS production and the NADPH activity were increased by hypoxia, and the specific antioxidants, Prdx2 and SOD3, were highly expressed effecting the expression of hypoxia-response proteins.

Recent evidence supports that hypoxia causes production of ROS in skeletal muscle and intrapulmonary artery via NADPH oxidase and/or mitochondria (Zuo *et al*., 2005; Guzy and Schumacker, 2006; Liu *et al*., 2006). The production of $^{\cdot}O_2^-$ in kidney was reported to be different depending on the regions examined. The production of $^{\cdot}O_2^-$ via NADH oxidase was greater in the renal cortex and in the outer medulla than in the papilla (Zou *et al*., 2001a). Our study also showed that ROS was generated by hypoxic stress and the NADPH oxidase activity was increased in a time-dependent manner under hypoxic stress. Since NADPH oxidase is known to be one of the major sources of ROS during hypoxia in kidney, our data suggest that hypoxia-induced ROS may be mainly produced by the NADPH oxidase. In addition, NAC pretreatment suppressed hypoxia-induced increase in DCFH fluorescence (Jun *et al*., 2008), suggesting that $^{\cdot}O_2^-$ generated by NADPH oxidase is subsequently converted to H_2O_2 via cellular redox regulation system.

HIF-1 regulates the transcription of hypoxia-responsive gene as EPO (Stockmann and Fandrey, 2006). Under normoxia, HIF-1 binds to the VHL protein by PHD2, which is rapidly degraded by the ubiqutin-proteosome pathway (Salceda *et al*., 1997). Our results showed that accumulation of HIF-1α was decreased after pretreatment of NAC in MCT cell line during hypoxia (Fig. 2). First, our results showed that PHD2 expression was increased, but VHL was not increased after pretreatment with NAC during hypoxic conditions. These data suggested that ROS affected HIF-1 expression via PHD2 suppression. Callapina *et al*. showed the DMNQ-induced ROS induced the HIF-1α accumulation via decreased PHD activity as seen in the in vitro pVHL-HIF-1c binding assay (Callapina *et al*., 2005). Tajima *et al*. (2009) suggested that *HIF-1α* mRNA was reduced after treatment with NAC or with membrane-permeable GSH analog. NAC scavenges free radicals, and can suppress the elevation of HIF-1α induced by H_2O_2 (Salceda *et al*., 1997). Previous report has shown that under hypoxia, the elevated ROS leads to HIF-1α stabilization in the kidney region exposed to low Po_2 (Zou *et al*., 2001b).

Antioxidants control the ROS levels in cellular homeostasis; we hypothesize that the expression level of antioxidants will be changed during hypoxia. Tajima *et al*. suggested that HIF-1 activation in HSC-2 cells was not inhibited by many antioxidants (Tajima *et al*., 2009). Because the connections of HIF-1 and hypoxia-related proteins process in cytosol and NADPH oxidase complex is located in the plasma membrane, we focused on $^{\cdot}O_2^-$ and H_2O_2 scavenger in plasma membrane and cytosol, but not mitochondrial antioxidants. Among the investigated antioxidants, SOD3 and Prdx2 were specifically increased during hypoxia (Fig.3).

In addition, the specific antioxidant regulates HIF-1α expression as a major transcription factor for EPO (Fig. 4). HIF-1α induction in nucleus and EPO expression were decreased in SOD3 knock-out mouse during hypoxia (Suliman *et al*., 2004; Zelko and Folz, 2005). Our data showed that EPO expression and activity were not related with HIF-1α in SOD3 O/E mice, because HIF-1α level was increased only at 4 hrs of hypoxia but EPO activity was highest at 8 hrs of hypoxia.

Previously, our data showed EPO protein expression in serum increased in Prdx2 knock-out mice under conditions of normal oxygenation (Lee *et al*., 2003). Our result showed that EPO activity was almost not changed between normal and the Prdx2 K/O mice during normoxia. But after hypoxia, the activity was significantly increased approximately 7-fold at 2 hrs, and then decreased in the mice kidney. The activity increased at 8 hrs in mice serum, later than the time response in the kidney. HIF-1α expression level was also high during normoxia and down during hypoxia. We suggest that disruption of Prdx2 function might increase H_2O_2 (Lee et al., 2003), so HIF-1α level is high during normoxia. Increased HIF-1α regulates a significant increase in EPO activity during short-term expose of hypoxic stress in Prdx2 K/O mice.

In accordance with our data, we predict that EPO level was regulated by H_2O_2. In the SOD3 O/E mice, H_2O_2 was generated more than in non-transgenic mice. But it will be converted to H_2O by Prdx2, so an increase in EPO activity is revealed under long-term hypoxic stress (more than 8 hrs). In contrast, in the case of the Prdx2 deficient mice, since H_2O_2 can't be converted, high levels of H_2O_2 could increase EPO activity during short-term exposure to hypoxia. These results were found in the mice kidney, the main source of EPO production (Weidemann *et al*., 2009). In the serum, EPO activity was more sensitively demonstrated in SOD3 O/E mice. It means that mature and activated EPO should respond directly when H_2O_2 is increased.

In conclusion, our results show that specific antioxidants are involved in the ROS mediated hypoxic response to regulate HIF-1α or EPO for homeostasis. Importantly, Prdx2 and SOD3 are major antioxidants used during hypoxia in mice kidney. Additionally, we further consider the relationship between antioxidants and proteosomal destruction of HIF-1α related proteins.

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