Role of cytosolic NADP⁺-dependent isocitrate dehydrogenase in ischemia-reperfusion injury in mouse kidney

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Role of cytosolic NADP⁺-dependent isocitrate dehydrogenase in ischemia-reperfusion injury in mouse kidney. Am J Physiol Renal Physiol 296: F622–F633, 2009. First published December 23, 2008; doi:10.1152/ajprenal.90566.2008.—Cytoplasmic isocitrate dehydrogenase (IDPc) synthesizes reduced NADP (NADPH), which is an essential cofactor for the generation of reduced glutathione (GSH), the most abundant and important antioxidant in mammalian cells. We investigated the role of IDPc in kidney ischemia-reperfusion (I/R) in mice. The activity and expression of IDPc were highest in the cortex, modest in the outer medulla, and lowest in the inner medulla. NADPH levels were greatest in the cortex. IDPc expression in the S1 and S2 segments of proximal tubules was higher than in the S3 segment, which is much more susceptible to I/R. IDPc protein was also highly expressed in the mitochondrion-rich intercalated cells of the collecting duct. IDPc activity was 10- to 30-fold higher than the activity of glucose-6-phosphate dehydrogenase, another producer of cytosolic NADPH, in various kidney regions. This study identifies that IDPc may be the primary source of NADPH in the kidney. I/R significantly reduced IDPc expression and activity and NADPH production and increased the ratio of oxidized glutathione to total glutathione [GSSG/(GSH+GSSG)], resulting in kidney dysfunction, tubular cell damage, and lipid peroxidation. In LLC-PK₁ cells, upregulation of IDPc by IDPc gene transfer protected the cells against hydrogen peroxide, enhancing NADPH production, inhibiting the increase of GSSG/(GSH+GSSG), and reducing lipid peroxidation. IDPc downregulation by small interference RNA treatment presented results contrasting with the upregulation. In conclusion, these results demonstrate that IDPc is expressed differentially along tubules in patterns that may contribute to differences in susceptibility to injury, is a major enzyme in cytosolic NADPH generation in kidney, and is downregulated with I/R.

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Cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDPc) is a member of the isocitrate dehydrogenases (ICDHs) that catalyze oxidative decarboxylation of isocitrate into α-ketoglutarate (42). The family of ICDHs is classified on the basis of their cofactors and intracellular localization: IDPc encoded by Idh1 gene, mitochondrial NADP⁺-dependent ICDH encoded by Idh2 gene, and mitochondrial NAD⁺-dependent ICDH encoded by Idh3 gene (16, 23). IDPc is responsible for production of reduced NADP (NADPH) in cytoplasm (37). NADPH is an essential cofactor for the maintenance of glutathione in its reduced state (GSH) (45). GSH is the most abundant low-molecular-mass thiol in mammalian cells and plays an important role as an antioxidant in the oxidative stress defense system (5, 43). In addition, NADPH is used to convert not only oxidized glutathione (GSSG) to GSH but also oxidized thioredoxin to reduced thioredoxin, which also plays a role in the antioxidant system (10, 44). Recently we found that induction of IDPc protects cells against oxidative stress and ultraviolet radiation stress in fibroblasts (17, 21) and that IDPc mRNA is highly expressed in the liver and kidney (26). These observations suggest that IDPc may be involved in oxidative stress-related states such as ischemia-reperfusion (I/R) injury.

I/R markedly increases the production of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H₂O₂), hydroxyl radicals, and peroxyxinitrite, to levels above the normal scavenging capacity of the organ (15, 19). The abnormal excessive generation of ROS causes lipid peroxidation, disruption of the cellular cytoskeleton and integrity, and DNA breakdown, leading to cell damage (9, 20). Thus antioxidant agents have been developed to treat I/R-related diseases, and data suggest that treatment with antioxidant agents and activation of antioxidant enzymes ameliorate I/R injury (13, 25, 28). However, the role of IDPc in I/R injury, the pathogenesis of which is associated with ROS stress, has not yet been reported in any organ.

I/R results in acute kidney injury (AKI), which has high mortality and morbidity, and effective therapeutics against this disorder have not yet been developed (7). We found that IDPc is a major enzyme for cytosolic NADPH production, and its expression and activity differ in various kidney regions. Expression is low in the S3 segment of the proximal tubule, which is particularly sensitive to I/R-induced injury. Furthermore, I/R reduces the activity and expression of IDPc, decreasing NADPH production. Upregulation of IDPc expression in cultured kidney epithelial cells decreases cell susceptibility to oxidative stress, and downregulation of IDPc expression increases cell susceptibility. These results indicate that IDPc is a critical enzyme in the pathogenesis of I/R injury to kidney epithelial cells.

Materials and Methods

Animal preparation. Experiments were performed in 10-wk-old male C57BL/6 mice. Mice were allowed free access to water and food, and water was available for 10 wk before and 7 days after the experiment.
standard mouse chow. In all cases, studies were reviewed and approved by the Kyungpook National University Institutional Animal Care and Use Committee. Each animal group consisted of at least six mice. Kidney ischemia was carried out as described previously (33). Mice were anesthetized with pentobarbital sodium (60 mg/kg body wt) and then subjected to either 30 min of bilateral renal ischemia or sham operation. Kidneys were either perfusion fixed in 4% paraformaldehyde, 75 mM t-Lysine, 10 mM sodium periodate (PLP; Sigma, St. Louis, MO) for histological study or snap-frozen in liquid nitrogen for biochemical study. For histological studies, kidneys fixed in PLP were washed with phosphate-buffered saline (PBS) three times for 5 min each, embedded in oxtetraacyclene compound (Sakura FineTek, Torrance, CA) at −20°C or in paraffin at room temperature, and then cut into 4-μm cryosections and 2-μm paraffin sections with a cryotome (CM1850; Leica) and a microtome (RM2165; Leica, Bensheim, Germany), respectively.

Plasma creatinine and blood urea nitrogen concentration. Seventy microfilters of blood was taken from the retroorbular vein plexus at the times indicated in Fig. 4. Plasma creatinine concentration was measured with a Beckman Analyzer II (Beckman). Blood urea nitrogen (BUN) concentration was measured with a BUN assay kit (ASAN PHARM, Gyeonggi-do, Korea) according to the manufacturer's protocol.

Periodic acid Schiff stain. Two-μm paraffin sections were stained with periodic acid Schiff (PAS) according to a standard protocol.

Nitro blue tetrazolium stain. As described previously (15, 20), kidney sections were cut with a cryotome, incubated in 1 mg/ml nitro blue tetrazolium (NBT, Sigma) in PBS for 2 h at 37°C, and then washed with PBS. Signals were obtained with a light microscope.

Measurement of lipid peroxidation and H2O2. Thiobarbituric acid-reactive substances (TBARS) were determined as a measure of lipid peroxidation. Samples were evaluated for malondialdehyde (MDA) production with a spectrophotometric assay for TBARS (20, 40). H2O2 levels were measured with a ferric iron-sensitive dye, xylene orange. H2O2 oxidizes iron(II) to iron(III) in the presence of sorbitol, which acts as a catalyst. Iron(III) forms a purple complex with xylene orange as previously described (20, 40).

Cell culture. LLC-PK1 cells, a porcine tubular epithelial cell line, were grown in phenol red-free DMEM containing 10% fetal bovine serum (BFS, Invitrogen, Carlsbad, CA) at 37°C in an incubator with a 5% CO2 atmosphere. Cells were treated with either 1 mM H2O2 or vehicle for 2 h. Cell viability was evaluated by Trypan blue exclusion assay. Apoptotic and necrotic cells were analyzed by flow cytometry (FASCaria, BD Bioscience, San Jose, CA) with an annexin V-FITC/propidium iodide (PI) detection kit (ApoScan, LS-02-100; BioBud, Seoul, Korea).

Gene and small interference RNA transfer. LLC-PK1 cells were transfected with either the LNCX vector encoding the mouse IDPc gene (LNCX-IDPc) or with LNCX vector alone (LNCX-null) (26). Other cells were transfected with either scrambled small interference RNA (siRNA) or siRNA for IDPc (IDPc-siRNA), which were designed as follows: scrambled siRNA, 5'-CUGAUGAGCUAGAGUAAUGT-3'; IDPc-siRNA, 5'-GGACUUGGCUAGUGCUAGUUTT-3'. Cells were incubated with 100 or 200 pm siRNA and 4 or 8 μg of the LNCX vectors in 5 nl of culture medium with 30 μl of Lipofectamine 2000 (Invitrogen) and 90 μl of PolyFect Reagent (Qiagen, Valencia, CA) according to the manufacturers' protocols, respectively.

Antibodies. An antibody against IDPc was previously generated and characterized (21, 22). Tubes were identified with antibodies against tubule-specific proteins. Descending thin limbs (DTLs) were identified with an antibody against aquaporin-1 (AQPI) (AQP-001; Alomone Labs, Jerusalem, Israel) (31). An antibody against gp330 was used to label the apical plasma membranes of proximal tubule (PT) cells (36). Distal tubules (DTs) and thick ascending limbs (TALs) were identified with an antibody against Na+-K+-ATPase (C2A4) (C2A4-2; DakoCytomation) and an antibody against Na⁺-K⁺-Cl⁻ cotransporter-2 (NKCC2) (NKCC21-A; Alpha Diagnostic International, San Antonio, TX) (48). Intercalated cells were identified with an antibody against vacuolar-type H⁺-ATPase B1/2 (H⁺-ATPase) (sc-21209; Santa Cruz Biotechnology, Santa Cruz, CA) (46). Principal cells in the collecting duct (CD) were identified with an antibody against AQP2 (AQP-002; Alomone Labs) (31). An antibody against Na⁺-K⁺-ATPase was used to label the tubule cells (36). For Western blot analysis, polyclonal anti-histone H1 (sc-8616; Santa Cruz Biotechnology), anti-copper-zinc superoxide dismutase (CuZnSOD) (AB1237; Chemicon, Temecula, CA), anti-manganese superoxide dismutase (MnSOD) (574596; Calbiochem, San Diego, CA), and monoclonal anti-β-actin (sc-8432; Santa Cruz Biotechnology) antibodies were used.

Immunofluorescence. Immunofluorescence staining was performed as described previously (15, 36). Depending on the immunoreactivity of antibodies, immunofluorescence staining was carried out on microtome-cut tissue slices of paraffin-embedded tissues or cryo-cut slides of oxytetracycline compound-embedded tissues. The paraffin-embedded tissue sections were deparaffinized with xylene, rehydrated with 100%, 95%, and 80% ethanol, and then washed with PBS for 10 min each. Briefly, cryosections or deparaffinized paraffin sections were incubated in PBS containing 0.1% sodium dodecyl sulfate (SDS) for 5 min and washed in PBS for 10 min. To unmask antigen epitopes, sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 1 min in a microwave oven, cooled at room temperature (RT) for 20 min, and then washed with PBS three times for 5 min. Sections were blocked with PBS containing 1% bovine serum albumin (blocking buffer) for 30 min at RT, incubated with polyclonal anti-IDPc antibody (1:100) in blocking buffer overnight at 4°C, and washed with PBS three times for 5 min. After that, sections were incubated with FITC-conjugated goat anti-rabbit IgG (1:100; Fi-1000, Vector Laboratories, Burlingame, CA) for 60 min at RT and then washed with PBS three times for 5 min. For double staining, monoclonal anti-gp330 antibody (1:200) was repeatedly used in cryosections and monoclonal anti-Na⁺-K⁺-ATPase (1:50) and polyclonal anti-H⁺-ATPase (1:50) were used in paraffin sections. Polyclonal anti-NKCC2 (1:50), anti-AQP1 (1:50), or anti-AQP2 (1:200) antibody was used in serial paraffin sections. To detect cell nuclei, 4',6-diamidino-2-phenylindole (DAPI; Sigma) was placed on sections for 1 min. Finally, cryosections and paraffin sections were mounted with Prolong Gold antifade reagent (Invitrogen) and observed under an Axioskop epifluorescence microscope (Carl Zeiss, Munich, Germany). Images were collected with a digital camera (Carl Zeiss) and merged with Adobe Photoshop 7.0 software.

Western blot analysis. Western blot analyses were performed as described previously (33). Briefly, renal tissue or cell lysates (30 μg protein/lane) were separated on 10% SDS-PAGE gels and then transferred to Immobilon membranes (Millipore, Bedford, MA). The membranes were incubated with anti-IDPc, anti-histone H1, anti-MnSOD, anti-CuZnSOD, and anti-β-actin antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against the appropriate primary antibodies (1:4,000; PI-1000, Vector Laboratories), exposed to Western Lighting Chemiluminescence Reagent (NEL101; PerkinElmer, Boston, MA), and then developed with X-ray film. The area of each band was analyzed with LabWorks 4.5 software (UVP, Upland, CA).

Preparation of cytosolic and mitochondrial fractions. Cytosolic and mitochondrial fractions were prepared as described previously (17, 20). Briefly, frozen tissues or cells were homogenized in sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4; Sigma) on ice with Dounce homogenizers. The homogenate was centrifuged at 1,000 × g for 5 min, and then the supernatant was centrifuged at 15,000 × g for 30 min. The supernatant was the cytosolic fraction and was used to measure IDPc activity. The pellet was washed twice with sucrose buffer to collect mitochondrial pellets. The pellet was suspended in PBS containing 0.1% Triton X-100, disrupted twice with a sonicator (4710 series; Cole-Palmer, Chicago, IL) at 40% of maximum setting for 10 s, and centrifuged at 15,000 × g for 30 min. The supernatant was the cytosolic fraction.

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the mitochondrial fraction and was stored at −70°C. These fractions were confirmed by Western blot analysis using antibodies against CuZnSOD for the cytosolic fraction and against MnSOD for the mitochondrial fraction (Fig. 1A).

**IDPc and glucose-6-phosphate dehydrogenase activity assays.** The activities of both IDPc and glucose-6-phosphate dehydrogenase (G6PD) were measured as described previously (26, 41). Briefly, IDPc activities in the cytosolic fraction (50 μg protein) were measured in a reaction mixture containing (in mM) 40 Tris (pH 7.4), 2 NADP+, 2 MgCl2, and 50 threo-DS-isocitrate (Sigma). One unit of IDPc activity was defined as the amount of enzyme catalyzing the production of 1 μmol NADPH/min as measured by the absorbance at 340 nm at 37°C (n = 6). G6PD activities in the cytosolic fraction were measured in a reaction mixture containing (in mM) 55 Tris (pH 7.8), 3.3 MgCl2, and 4 glucose-6-phosphate, with 240 μM NADP+ (Sigma). One unit of G6PD activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol NADP+min as measured by the absorbance at 340 nm at 37°C.

**Measurement of cytosolic NADPH and total NADP levels.** The cytosolic NADPH level was measured as described previously (26, 49). Briefly, NADPH in the cytosolic fraction was induced by exclusion of NADPH through heating for 30 min at 60°C in a dry heating bath. Cytosolic fractions (200 μg protein each) for NADPH and total NADP (NADPt) levels were preincubated in a reaction mixture containing (in mM) 100 Tris (pH 8.0), 2 phenazine ethosulfate, 5 ethylenediaminetetraacetic acid (EDTA), and 0.5 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), with 1.5 U of G6PD (Sigma) for 5 min at 37°C. The reactions were started by the addition of 1 mM glucose-6-phosphate. NADPH and NADPt levels were defined as the change in optical density (OD) at 570 nm for 1 min at 37°C.

**Measurement of oxidized glutathione to total glutathione.** GSSG/(GSH+GSSG) was measured as described previously (1, 26). The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid. GSSG was measured by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay after removal of GSH from 2-vinylpyridine. Total glutathione and GSSG levels were defined as the change in OD at 412 nm for 1 min at 37°C.

**Statistics.** Results are expressed as means ± SE. Statistical differences among groups were calculated with analysis of variance (ANOVA) followed by least significant difference post hoc comparison with the SPSS 12.0 program. Differences between groups were considered statistically significant at a P value of <0.05.

**RESULTS**

**Expression and activity of IDPc in kidney.** The level of IDPc expression was the highest in the cortex, modest in the outer medulla (OM), and lowest in the inner medulla (IM) (Fig. 1, B).
and C). Consistent with the expression of IDPc, IDPc activity also was highest in the cortex, modest in the OM, and lowest in the IM (Fig. 1D). The level of cytosolic NADPH, a product of IDPc, was significantly higher in the cortex compared with the OM and the IM (Fig. 1E). Since NADPH is generated by not only IDPc but also G6PD (37), we determined the activity of G6PD. Compared with IDPc activity, G6PD activity for NADPH production was about 28-fold lower in the cortex, 13-fold lower in the OM, and 6-fold lower in the IM (Fig. 1D and F). In contrast to IDPc activity, G6PD activities were not significantly different in the various kidney regions (Fig. 1F). These results indicate that IDPc serves as a major enzyme for cytosolic NADPH production in the kidneys.

Distribution of IDPc in mouse kidney tubules. Kidney tubular cells of different nephron segments perform unique functions and are differentially susceptible to a variety of physiological and pathophysiological conditions. Consistent with the immunoblot results (Fig. 1B and C), nephron IDPc expression was highest in the cortex, modest in the OM, and lowest in the IM (Fig. 2, A–C). IDPc was expressed in the cytoplasm of cells (Fig. 2, D–V). Expression of IDPc in the S1 and S2 segments of the PTs (Fig. 2, D–I) was greater than that in the S3 segment in the outer stripe of the OM (OSOM) (Fig. 2, M–R). The expression of IDPc in the PTs was greater than that in the DTs (Fig. 2, D–I). IDPc expression was weak in the TAL (Fig. 2, J–L) and the DTL (Fig. 2, S–V) compared with that in the PTs. IDPc was highly expressed in the intercalated cells in the connecting tubule (CNT) and the CD (Fig. 3, A–T) but weakly expressed in the CNT cells and the principal cells (Fig. 3, A–T). In the intercalated cells in the CD, IDPc was expressed in the apical or basolateral domain (Fig. 3, G–T). Kidney tubular segments were identified by immunostaining using anti-Na+/K+-ATPase (47), anti-gp330 (6), anti-NKCC2 (48), anti-AQP1 (31), anti-H+/K+-ATPase (46), or anti-AQP2 (31) antibodies. The immunoreactivity for Na+/K+-ATPase is high in the basolateral domain of the CNT cells, moderate in the principal cells, and weak in the intercalated cells (Fig. 3, B and H) (38). H+/K+-ATPase is expressed in the diffuse and/or apical domain of the intercalated cells (Fig. 3, E, K, N, and Q) (46). AQP2 is expressed in the apical membrane of the principal cells (Fig. 3I) (31). As summarized in Fig. 3U, the distribution of IDPc in kidney tubules differed in various tubular segments, indicating that IDPc may have different effects on oxidative stress along the tubules.

Expression and activity of IDPc in kidneys subjected to ischemia-reperfusion injury. To investigate whether IDPc may play a role in I/R injury, IDPc activity and expression were determined in kidneys of mice that were subjected to 30 min of bilateral renal ischemia. Thirty minutes of ischemia resulted in significant increases of plasma creatinine and BUN concentrations 4 and 24 h after ischemia (Fig. 4, A and B). I/R resulted in significant increases of plasma creatinine and BUN concentrations 4 and 24 h after ischemia (Fig. 4, A and B). I/R resulted

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Fig. 2. Expressions of IDPc in mouse kidney tubular epithelial cells. Kidney sections were immunostained with antibodies described in MATERIALS AND METHODS. A–C: kidney sections were stained with anti-IDPc antibody. D–I: kidney sections were double-stained with anti-IDPc and then with anti-Na+/K+-ATPase or anti-gp330 antibodies. Pictures were taken of the cortex. IDPc is highly expressed in the cytoplasm of proximal tubule (PT, *) cells but weakly in distal tubule (DT, #) cells. J–L: serial kidney sections were mounted on the same slide for staining with anti-IDPc, anti-Na+/K+-ATPase, or anti-Na+/K+–Cl– cotransporter 2 (NKCC2) antibodies. Pictures were taken of the inner stripe of the OM. IDPc expression is weak in the cytoplasm of tubules. *, Thick ascending limb (TAL). M–R: kidney sections were double-stained with anti-IDPc antibody and then with either anti-Na+/K+-ATPase or anti-gp330 antibodies. S1–2 segment cells of the PT in the cortex express IDPc to a much greater extent than S3 segment cells in the outer stripe of the OM. *, S3 segment of the PT; S and T: serial kidney sections were stained with anti-IDPc and anti-AQP1 antibodies. Pictures were taken of the OM. Descending thin limb (DTL) cells faintly express IDPc in the cytoplasm. *, DTL in the IM (omDTL). U and V: serial kidney sections were stained with anti-IDPc and anti-AQP1 antibodies. Pictures were taken of the IM. Insets in A, D, S: kidney treated with secondary antibody alone. *, DTL in the IM (omDTL). D–I and M–R: pictures taken of the double staining merged with Photoshop software. Scale bars: 500 (A–C) and 50 (D–V) μm.
in deposition of tubular casts in the tubular lumens, disruption of tubular epithelial cells, and infiltration of leukocytes into the interstitium (Fig. 4C). The tubular epithelial cell damage was most severe in the S3 segments of PT in the OSOM 24 h after ischemia (Fig. 4C).

IDPc expression was significantly decreased in the kidneys 4 and 24 h after ischemia compared with that in kidneys subjected to sham operation (sham-operated controls) or those harvested 4 h after the surgery (Fig. 5, A and B). Consistent with the decrease of IDPc expression, IDPc activity in the kidney tissues was also decreased 4 and 24 h after ischemia compared with that in sham-operated control kidneys (Fig. 5C). The ratio of NADPH to total NADP (NADPH/NADPt) in the cytosolic fraction of the kidney was significantly decreased 4 and 24 h after ischemia compared with that in sham-operated control kidney (Fig. 5D). After 4 and 24 h after I/R, GSSG/GSH (GSH+GSSG) in the cytosolic fraction of the kidney was significantly increased (Fig. 5E). Postischemic total glutathione levels in the cytosolic fraction of the kidneys were gradually decreased below normal level over time (Fig. 5F). In contrast, postischemic cytosolic GSSG levels (0.016 OD/mg protein at 4 h and 0.018 OD/mg protein at 24 h after I/R) were gradually increased above the normal range (0.011 OD/mg protein) over time (Fig. 5E). These results indicate that the increases of GSSG/GSH may be caused by both decreases of total glutathione and reduction of GSSG to GSH due to the decrease of IDPc activation. IDPc expression and NADPH/NADPt level were consistent with the expression of IDPc (Fig. 6, C and D). To evaluate whether I/R injury was associated with oxidative stress in our experiments, lipid peroxidation, superoxide, and tissue H2O2 levels were measured. Superoxide and H2O2 levels were significantly increased 4 and 24 h after ischemia (Fig. 7, A and B). Lipid peroxidation was significantly higher at both time points in the ischemic kidney compared with the sham-operated control kidney (Fig. 7C).

Effect of up- and downregulation of IDPc expression against oxidative stress in cultured tubular epithelial cells. To evaluate the role of IDPc in oxidative stress, the levels of IDPc expression in LLC-PK1 cells, an established proximal tubular epithelial cell line, were increased or decreased by using the LNCX vector with IDPc (LNCX-IDPc) or the siRNA against IDPc (IDPc-siRNA), respectively; we then treated these cells with...
1 mM H$_2$O$_2$ for 2 h. The transfection of LNCX-IDPC significantly increased IDPC expression compared with LNCX vector alone (LNCX-null) (Fig. 8). IDPC expression was significantly lower in LLC-PK$_1$ cells transfected with IDPC-siRNA than in cells transfected with a scrambled siRNA (Fig. 8). To evaluate whether modulation of IDPC expression influenced other antioxidant enzymes, the levels of MnSOD, which is present in the mitochondria, and CuZnSOD, which is located in the cytoplasm, were determined in these transfected cells. The transfections of LNCX-IDPC or IDPC-siRNA did not influence the expression of either MnSOD or CuZnSOD compared with their respective controls (Fig. 8), indicating that expression of IDPC did not affect the expression of SOD enzymes.

Consistent with IDPC protein expression, IDPC activity was significantly increased by LNCX-IDPC transfection compared with LNCX-null transfection (Fig. 9A). When cells were treated with 1 mM H$_2$O$_2$ for 2 h, IDPC activity was significantly decreased (Fig. 9A). Two hours after 1 mM H$_2$O$_2$ treatment, IDPC activity was significantly greater in LNCX-IDPC-transfected cells than in LNCX-null cells (Fig. 9A). IDPC activity after H$_2$O$_2$ treatment was significantly lower in LNCX-null- and IDPC-siRNA-transfected cells than in LNCX-IDPC- and scrambled siRNA-transfected cells (Fig. 9C), indicating that IDPC regulated the ratio of antioxidant glutathione through NADPH production.

To evaluate whether up- or downregulation of IDPC expression influenced the activity of G6PD, which is another NADPH producer, we measured the activity of G6PD in LLC-PK$_1$ cells transfected with LNCX-IDPC or IDPC-siRNA. Consistent with the IDPC expression shown in Fig. 8, transfection of LNCX-IDPC or IDPC-siRNA did not influence the activity of G6PD compared with their respective controls (Fig. 9D). Two hours after 1 mM H$_2$O$_2$ treatment, G6PD activity decreased in all experimental groups (Fig. 9D). The decreases of G6PD activities were not significantly different among the experimental groups (Fig. 9D), indicating that G6PD was not associated with IDPC expression.

H$_2$O$_2$ treatment significantly increased lipid peroxidation (Fig. 10A). The increases in lipid peroxidation in the LNCX-IDPC-transfected cells were significantly less than those in LNCX-null-transfected cells (Fig. 10A). In contrast, IDPC-siRNA transfection resulted in significantly increased H$_2$O$_2$-induced lipid peroxidation compared with the cells transfected with scrambled siRNA (Fig. 10A). Cellular H$_2$O$_2$ levels significantly increased 2 h after H$_2$O$_2$ treatment (Fig. 10B). The increases of tissue H$_2$O$_2$ levels were significantly lower in LNCX-IDPC-transfected cells than those in LNCX-null-transfected cells (Fig. 10B). IDPC-siRNA transfection resulted in
greater H₂O₂-induced lipid peroxidation compared with scrambled siRNA transfection (Fig. 10B).

When cell viabilities were evaluated by Trypan blue exclusion, 1 mM H₂O₂ treatment significantly increased cell death (Fig. 10C). LNCX-IDPc transfection protected the cells against H₂O₂-induced cell death (Fig. 10C). In contrast, IDPc-siRNA transfection resulted in enhanced susceptibility to injury with H₂O₂ compared with scrambled siRNA-transfected cells (Fig. 10C). When apoptotic and necrotic cells were analyzed by flow cytometry with annexin V-FITC/PI staining, H₂O₂ treatment significantly induced both apoptosis and necrosis (Fig. 10, D–F). The increase of apoptosis in the LNCX-IDPc-transfected cells was significantly less than that in LNCX-null-transfected cells (Fig. 10E). In contrast, IDPc-siRNA transfection resulted in significantly increased H₂O₂-induced apoptosis compared with the cells transfected with scrambled siRNA (Fig. 10E). Consistent with the pattern of apoptosis, necrotic cells were significantly increased by LNCX-null and IDPc-siRNA transfection compared with LNCX-IDPc and scrambled siRNA transfection, respectively (Fig. 10F). These data indicate that IDPc expression and activation affect cell viability during oxidative stress through regulation of the cellular antioxidant capacity.

**DISCUSSION**

In this study we demonstrated that IDPc expression varies in different kidney regions and in different parts of the nephron.
Kidney I/R decreases the activity and expression of IDPc, NADPH levels, and GSH levels. The levels of IDPc expression positively correlate with kidney epithelial cell resistance to oxidative stress. NADPH is used to convert not only GSSG to GSH, which is the most abundant antioxidant in mammalian cells, but also oxidized thioredoxin to reduced thioredoxin, which also plays a role in the antioxidant system (10, 44). At least four enzymes produce NADPH: G6PD, the key regulatory enzyme of the pentose phosphate pathway; phosphogluconate dehydrogenase; malate dehydrogenase; and ICDH (12).

Fig. 6. Expression (A and B) and activity (C) of IDPc, and NADPH/NADP+ (D) in cortex, OM, and IM of kidneys of mice subjected to either 30 min of bilateral ischemia or sham operation. Kidneys were harvested 24 h after reperfusion. A: IDPc expression was determined by Western blot analysis using anti-IDPc antibody. Antibody against β-actin was used as an equal loading marker. B: densities of blots were quantified with the Lab Works program (n = 3). IDPc activity (C) and NADPH/NADP+ (D) were determined in cortex, OM, and IM of kidneys as described in MATERIALS AND METHODS. Results are means ± SE (n = 3). NS, no significant difference. *P < 0.05 vs. respective cortex; #P < 0.05 vs. respective OM.

Fig. 7. Levels of superoxide (A), H2O2 (B), and lipid peroxidation (C) in kidneys of mice subjected to either 30 min of bilateral ischemia or sham operation. Kidneys were harvested 4 and 24 h after surgery. A: superoxide formation was determined by conversion of nitroblue tetrazolium (NBT) to formazan (dark brown color). Levels of H2O2 (B) and lipid peroxidation (C) were determined in kidneys as described in MATERIALS AND METHODS. Results are means ± SE (n = 6).
G6PD has long been regarded as the primary source of NADPH (44). The activities of these four enzymes in NADPH production differ among organs, however. Some studies have demonstrated that IDPc is responsible for more NADPH production than G6PD in liver and ovary (11, 45). In contrast, Frederiks et al. (12) reported that G6PD activity was 14-fold greater than that of ICDH in the adrenal gland. In this study we found that IDPc activity for NADPH production was 10–30 times higher than G6PD activity in various kidney regions, suggesting that IDPc is a major enzyme for the generation of NADPH in the kidney and plays a critical role in I/R injury. Jain and colleagues demonstrated (14) that reduction of NADPH by G6PD gene deficiency increased myocardial dysfunction after I/R in mice.

In a previous study, we demonstrated (18) that IDPc mRNA is highly expressed in the liver and kidney, suggesting that IDPc protein expression may be high in those organs. In the present study, we confirmed the presence of IDPc protein in kidney. In addition, the levels of IDPc expression differed in various kidney regions and tubules as summarized in Fig. 3U. Nephron segments of the kidneys possess remarkably different susceptibilities to I/R injury. S3 segment cells of the PT are most susceptible to I/R injury and oxidative stress (27, 35). IDPc expression was lower in the cells of S3 segments than in the cells of S1 and S2 segments. The different distribution of IDPc expression may provide an explanation for the different susceptibilities of kidney tubular cells to I/R injury. Interestingly, the pattern of IDPc expression was different in different segments of the nephron: IDPc was expressed diffusely in the cytoplasm in the PTs, thin limbs, and DT cells, whereas IDPc was expressed intensively in the apical and/or basolateral parts of the CNT and CD cells. This suggests that IDPc may play a different role in each tubular cell type and that the pattern of IDPc expression may serve as a marker to identify each tubule type. Further studies are required to fully explore the potential differential roles of IDPc in tubular segments.

I/R results in excessive ROS production and decreases of antioxidants (3, 8, 9). Recently we also reported (20) that I/R decreased the expression of antioxidant enzymes, and that administration of a MnSOD mimetic reduced renal I/R injury in mice. In the present study, I/R resulted in reduced IDPc expression and activity as early as 4 h after ischemia, when cell death and renal functional impairment were mild, as well as in later phases (24 h), when cell death and renal functional impairment were substantial (34), suggesting that the reduction of IDPc activity may be not only a result of I/R injury but also...
a cause. Since effective IDPc mimetics and genetically modified animals have not been developed, the effect of IDPc in animal studies could not be fully explored in this study. Thus we included in vitro studies using LLC-PK₁ cells, an established epithelial cell line with characteristics of the PT, in the present investigation. Upregulation of IDPc protein increased NADPH production and decreased GSSG/GSH. A number of data have demonstrated that GSH reduces kidney cellular damage against oxidative stress (29, 32). In our present study, upregulation of IDPc reduced the increases of lipid peroxidation and cellular H₂O₂ levels and cell death including apoptosis and necrosis. Downregulation of IDPc protein presented results contrasting with the upregulation, indicating that IDPc overexpression protected cells from oxidative stress by increasing the GSH in the kidney tubular epithelial cells. In a previous study, we reported (21, 26) that IDPc upregulation in mouse embryonic fibroblasts reduced cell susceptibility to H₂O₂ and menadione. In a subsequent study, we found (17) that IDPc upregulation in fibroblasts reduced the cells’ suscep-

Fig. 10. Levels of lipid peroxidation (A), H₂O₂ production (B), cell death (C), flow cytometric analysis of annexin V-FITC/PI staining (D), and numbers of apoptotic cells (E) and necrotic cells (F) in LLC-PK₁ cells transfected with LNCX-IDPc, LNCX-null, IDPc-siRNA, or scrambled siRNA 2 h after 1 mM H₂O₂ treatment. LLC-PK₁ cells were treated with either 1 mM H₂O₂ or vehicle. Two hours after treatments, levels of lipid peroxidation (A) and H₂O₂ production (B) and cell death (C) were determined as described in MATERIALS AND METHODS. Apoptotic cell (E) and necrotic cell (F) numbers were determined by flow cytometric analysis using annexin V-FITC positive/PI-negative cells and annexin V-FITC positive/PI-positive cells, respectively (D). Results are means ± SE (n = 6). *P < 0.05 vs. respective vehicle.
tibility to irradiative stress. Thus IDPc expression controls the cell fate of nonepithelial cells as well as renal epithelial cells.

Numerous animal experiments have demonstrated that antioxidant treatments significantly reduce renal I/R damage (20, 25, 39). Antioxidant therapeutics have also been used in patients (2, 4). Since cellular antioxidants and antioxidant enzymes act in a concerted manner, multiple treatments using different kinds of antioxidants and antioxidant enzymes have been suggested for antioxidant therapy (24, 30). In the present study, changes in IDPc gene expression did not affect the levels of other antioxidant enzymes such as CuZnSOD and MnSOD, suggesting that treatment with an IDPc mimetic in combination with other antioxidants may exert a synergistic effect against oxidative stress-related diseases.

In conclusion, our results demonstrate that IDPc is a major enzyme for NADPH generation in kidney epithelial cells and is reduced in expression in renal I/R injury. Reduction of IDPc expression in vitro increases the susceptibility of epithelial cells to oxidant injury, whereas increased expression is protective against oxidant injury. Furthermore, reduced IDPc in S3 segment cells of the PT may contribute to that segment’s increased susceptibility to I/R-induced injury. By recognizing the importance of IDPc and considering approaches to enhance cellular levels we suggest a new strategy for the development of therapeutic agents for AKI.

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