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Molecules and Cells

Upregulation of Cytosolic NADP$^+$-Dependent Isocitrate Dehydrogenase by Hyperglycemia Protects Renal Cells Against Oxidative Stress

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Hyperglycemia-induced oxidative stress is widely recognized as a key mediator in the pathogenesis of diabetic nephropathy, a complication of diabetes. We found that both expression and enzymatic activity of cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc) were upregulated in the renal cortexes of diabetic rats and mice. Similarly, IDPc was induced in murine renal proximal tubular OK cells by high hyperglycemia, while it was abrogated by cotreatment with the antioxidant N-Acetyl-Cysteine (NAC). In OK cells, increased expression of IDPc by stable transfection prevented hyperglycemia-mediated reactive oxygen species (ROS) production, subsequent cellular oxidative stress and extracellular matrix accumulation, whereas these processes were all stimulated by decreased IDPc expression. In addition, production of NADPH and GSH in the cytosol was positively correlated with the expression level of IDPc in OK cells. These results together indicate that upregulation of IDPc in response to hyperglycemia might play an essential role in preventing the progression of diabetic nephropathy, which is accompanied by ROS-induced cellular damage and fibrosis, by providing NADPH, the reducing equivalent needed for recycling reduced glutathione and low molecular weight antioxidant thiol proteins.

INTRODUCTION

Diabetic nephropathy is one of the major microvascular complications of both type 1 and type 2 diabetes. As the most common cause of end-stage renal disease, it accounts for a signifycant increase in morbidity and mortality in patients with diabetes. Diabetic nephropathy is characterized by excessive deposition of extracellular matrix (ECM) with thickening of the glomerular and tubular basement membranes and an increased amount of the mesangial matrix, which ultimately progresses to glomerulosclerosis and tubulo-interstitial fibrosis (Kanwar et al., 2008).

It is generally accepted that overproduction of reactive oxygen species (ROS) is a direct consequence of hyperglycemia and that ROS is a leading cause of the progression and development of diabetic complications, including diabetic nephropathy (Forbes et al., 2008; Ha et al., 2008). ROS, including superoxide anion (O_2^-), hydrogen peroxides, hydroxyl radical and peroxynitrite, directly oxidize and damage DNA, proteins, lipids and carbohydrates (Djordjevic, 2004), thus inducing renal injury. In addition, ROS mediate hyperglycemia-induced activation of signaling cascades and transcription factors that activate the expression of profibrotic genes (Kanwar et al., 2008).

In biological defense systems, superoxide anions are rapidly reduced to H_2O_2 by superoxide dismutase and then H_2O_2 is further reduced to water and oxygen by catalase and/or glutathione peroxidase (Gpx). Reduced glutathione (GSH) is readily oxidized to glutathione disulfide (GSSG) by Gpx as well as by reacting with ROS. NADPH is an essential electron donor for the regeneration of GSH by glutathione reductase and for the activity of the NADPH-dependent thioredoxin system (Kirsch and de Groot, 2001), both of which are important cellular antioxidant processes. NADPH is also required for the formation of active catalase tetramers (Kirkman and Gaetani, 1999); therefore, the capacity of the kidney to respond to oxidative injury induced by hyperglycemia may be ultimately related to the supply of NADPH.

The isocitrate dehydrogenases (ICDHs, EC 1.1.1.41 and EC 1.1.1.42) use NAD^+ or $NADP^+$ to catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate and produce NADH or NADPH, respectively (Jo et al., 2001; Kim et al., 1999; Koh et al., 2004; Lee et al., 2002). The two eukaryotic NADP$^+$-dependent ICDHs, mitochondrial ICDH (IDPm) and cytosolic ICDH (IDPc), are homodimers and expressed in a tissue-specific manner (Jo et al., 2001; Koh et al., 2004; Lee et al., 2002).

We previously reported that IDPm and IDPc play key roles in cellular defense against oxidative damage by providing the NADPH needed for mitochondrial and cytosolic GSH production, respectively (Jo et al., 2001; Lee et al., 2002). While a

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considerable level of IDPc mRNA transcript was expressed in the kidney (Koh et al., 2004; Lee et al., 2002), the biological significance of IDPc in the kidney was poorly understood. In this report, we provide evidence that IDPc is induced by hyperglycemia in the kidney to prevent ROS-induced cellular damage and fibrosis accompanied by diabetic nephropathy.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (150-170 g) were used for the experiments. The animals were allowed to acclimatize in the local vivarium for 7 d, housed at 22-24°C with a 12 h light-dark cycle and fed a standard diet (Purina rat chow) with tap drinking water *ad libitum*. Diabetes mellitus was induced by intraperitoneal injection of freshly prepared streptozotocin (STZ, 60 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5). The control group ($n = 8$) received an injection of citrate buffer alone. Blood glucose levels were determined 6 days after a single STZ injection, and the rats with blood glucose levels higher than 200 mg/dl were considered diabetic rats ($n = 8$). Blood from a tail vein was taken once a week and used to determine the fasting glucose level with reactive strips and a glucometer (GlucocardII, Arkray, Japan).

Cell culture

Human embryonic kidney cells (HEK293), murine proximal tubular cells (OK), human proximal tubular cells (HK2) and canine distal collecting duct cells (MDCK) were purchased from the American Type Cell Collection. Cells were grown in Minimum Essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Immunoblot analyses

For detection of IDPc protein, a peptide consisting of the C-terminal 13 amino acids (HYRMYQKGQETST, corresponding to amino acid residues 316-327) of mouse IDPc (Lee et al., 2002) was synthesized and used to prepare polyclonal anti-IDPc antibodies in rabbits. The cytosolic homogenates from cultured cells were subjected to immunoblot analyses as previously described (Lee et al., 2002).

Other experimental methods

Isolation of cytosolic fractions, enzyme assay, measurement of intracellular ROS, lipid peroxidation assay, protein oxidation assay, and measurement of proline incorporation, cellular NADPH and GSH levels were described in Supplementary Materials and Methods.

Statistical analyses

All results are represented as means ± S.E. Data were subjected to variance analysis followed by Student's *t*-test for multiple comparisons. *p* values of less than 0.05 were considered significant.

RESULTS

IDPc is induced in rat kidneys with diabetic nephropathy

In normal rat kidneys, IDPc was highly expressed in the proximal convoluted tubule and distal convoluted tubule in the cortex, but was not detectable in the glomerulus (Supplementary Figs. 1A and 1B). In contrast to the cortex, IDPc was ubiquitously expressed in the medullar at a low level (data not shown).

To analyze the changes in renal IDPc expression upon diabetic nephropathy, diabetes was induced in rats by streptozotocin (STZ) treatment (Liu et al., 2008). As seen in Supplementary Table 1, the blood glucose levels and kidney/body weight ratios in STZ-induced rats were highly elevated compared to those of control rats. In the STZ-induced diabetic rat kidneys, glomerular sclerosis was observed with thickening and widening of the proximal tubule (Supplementary Fig. 1), indicating the occurrence of diabetic nephropathy (Wang et al., 2003). In the proximal tubule of the diabetic kidneys, IDPc protein expression was higher compared to normal rats (Supplementary Fig. 1). Similarly, the IDPc protein level was also elevated in the kidneys of type 2 diabetic $Lepr^{db}$ (*db/db*) mice compared to non-diabetic C57BL/6J mice (Supplementary Fig. 2), suggesting that IDPc might be involved in renal defense against diabetic nephropathy.

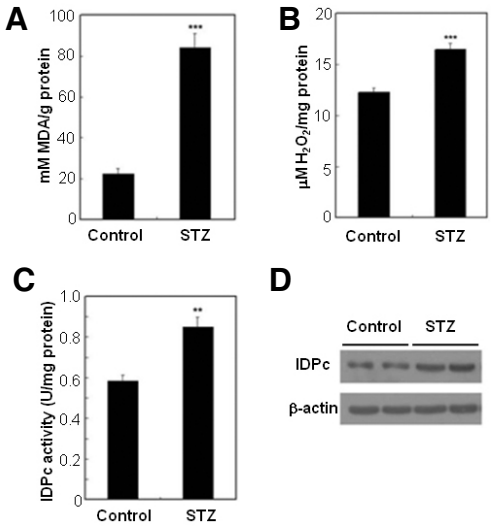

Fig. 1. Oxidative damage and IDPc induction in STZ-induced diabetic rat kidneys. (A) Lipid peroxidation levels as determined by malondialdehyde (MDA) production, (B) peroxide generation and (C) IDPc activity assays of extracts from rat renal cortexes. (D) Immunoblot analysis of IDPc protein expression in homogenates (30 μg of protein) of renal cortexes. Each value represents the mean ± S.E. from three separate experiments. ** $p < 0.01$ and *** $p < 0.001$ compared with the untreated control group.

In STZ-induced rats with diabetic nephropathy, the levels of malondialdehyde (MDA) and peroxides in the renal cortex were elevated by 3.82- and 1.36-fold, respectively, compared to those of the control group (Figs. 1A and 1B), indicating the occurrence of ROS-induced renal injury in rats with diabetic nephropathy. Similarly to the histology data (Supplementary Figs. 1 and 2), IDPc activity in the renal cortex of diabetic rats was also 40% higher than the control (Fig. 1C). In addition, expression of IDPc protein was increased in the renal cortex of diabetic rats (Fig. 1D). These results together suggest the possibility that IDPc is induced to protect against ROS-induced renal injury caused the diabetes.

Increased ROS generation by hyperglycemia induces IDPc expression in cultured renal proximal tubular cells

To further investigate the role of renal IDPc in ROS-induced oxidative stress in diabetic nephropathy *in vitro*, we analyzed IDPc expression in various kidney cell lines, including human embryonic kidney cells (HEK293), murine proximal tubular cells (OK), human proximal tubular cells (HK2) and canine distal collecting duct cells (MDCK). OK cells showed the highest IDPc

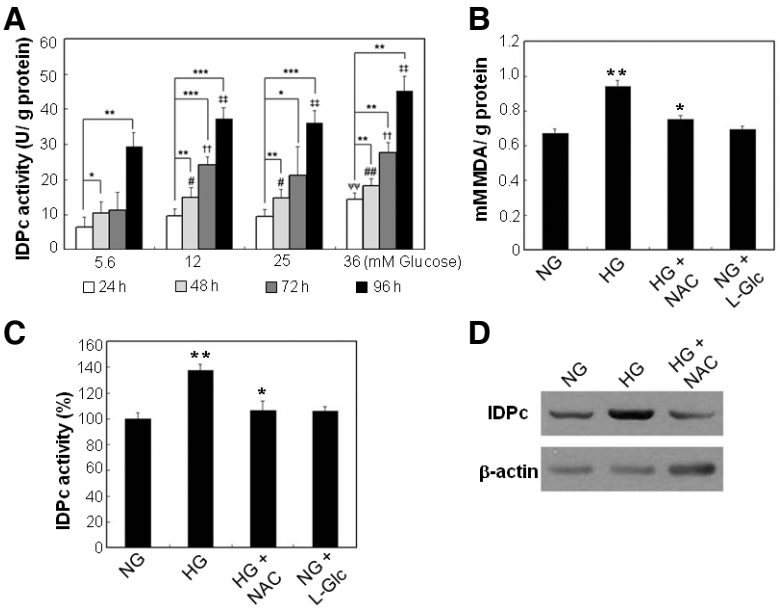

Fig. 2. Effect of glucose stress and the antioxidant NAC on the levels of IDPc enzyme activity and expression in OK cells. (A) OK cells were treated with various concentrations of glucose for different amounts of time. IDPc activity increased in an incubation time- and glucose concentration-dependent manner. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with 24 h exposure to each glucose concentration; ψψ $p < 0.01$ compared with exposure to 5.6 mM glucose for 24 h; $p < 0.05$ and ## $p < 0.01$ compared with exposure to 5.6 mM glucose for 48 h; †† $p < 0.01$ compared with exposure to 5.6 mM glucose for 72 h; ‡‡ $p < 0.01$ compared with exposure to 5.6 mM glucose for 96 h. (B) Effect of the antioxidant NAC on glucose stress-induced lipid peroxidation in OK cells. Cells were cultured with NG or HG for 48 h in the absence or presence of the antioxidant NAC. Each bar represents the mean ± S.E of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared with the NG control group. (C) Effect of the antioxidant NAC on glucose stress-mediated increase in IDPc activity in OK cells. Cells were cultured for 48 h in the absence or presence of the antioxidant NAC. Each bar represents the mean ± S.E of three independent experiments. $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$* compared with the NG control group. (D) Immunoblot analysis of IDPc protein levels in OK cells cultured with NG or HG for 48 h in the absence or presence of the antioxidant NAC. NG, normal glucose concentration (5.6 mM D-glucose); HG, high glucose concentration (25 mM D-glucose); NAC, N-acetyl-cysteine (2.5 mM); L-Glc, L-glucose (25 mM).

protein level among the tested kidney cells (data not shown). Therefore, OK cells were exposed to various concentrations of D-glucose with different incubation times, and IDPc activity in OK cells increased with D-glucose in a dose- and incubation time-dependent manner (Fig. 2A).

To verify that IDPc induction by high glucose concentrations is a biological defense mechanism against oxidative stress, OK cells were treated with physiologically normal (5.6 mM, NG) and high (25 mM, HG) D-glucose concentrations in the absence or presence of the well-characterized antioxidant N-Acetyl-Cysteine (NAC) (Han et al., 2008). The MDA level in OK cells exposed to HG was 40% higher than that in cells exposed to NG. However, the increased MDA level was significantly reduced by NAC (Fig. 2B). In contrast to D-glucose, the MDA level was not increased by treatment with a high concentration of L-glucose (25 mM) (Fig. 2B). Similarly, the enzyme activity and protein level of IDPc were increased by HG treatment, whereas these effects were abrogated by co-treatment with NAC (Figs. 2C and 2D). These results together indicate that IDPc induction in renal cells by the presence of high concentrations of D-glucose is a direct consequence of increased ROS generation.

Stable transfection of IDPc constructs in renal proximal tubular cells

To further confirm the direct role of IDPc in high glucose-mediated ROS production, two different lines of OK cells for each recombinant IDPc construct were isolated after stable transfection of the sense IDPc (S1, S2), antisense IDPc (AS1, AS2) or LNCX-vector alone (Vector) as previously described (Koh et al., 2004). The S1 and S2 cells exhibited 1.8- and 1.7-fold higher IDPc activity, respectively, than the control cells with the vector alone (Supplementary Fig. 3A). In contrast, the AS1 and AS2 cells exhibited 28% and 58% lower IDPc activities, respectively, compared the control (Supplementary Fig. 3A). Immunoblot analysis using an anti-IDPc antibody further confirmed the increased expression of IDPc in S1 and S2 cells compared to the control cells and that the AS1 and AS2 cells expressed significantly less IDPc protein (Supplementary Fig. 3B). To demonstrate any differences in hyperglycemia-induced oxidative damage between cells transfected with sense or antisense IDPc, we compared the S1 and AS2 cells.

When exposed to NG, the activities of the major antioxidant enzymes, including superoxide dismutase, catalase and glutathione reductase, were similar in the S1 and AS2 cells and the control cells (Supplementary Table 2). In addition, these antioxidant enzyme activities were not significantly increased by HG treatment, suggesting that transfection of the IDPc cDNAs did not affect the activities of the antioxidant enzymes, even in the presence of HG.

IDPc protects against oxidative stress induced by glucose stress in renal cells

To investigate the role of IDPc in defense against high glucose concentration-induced oxidative stress in renal cells, we determined the level of ROS generation in the different transfected cells. When cells were exposed to NG, ROS production, as determined by DCF fluorescence, was slightly increased in AS2 cells and substantially decreased in S1 cells compared to the control cells (Fig. 3A). After the HG treatment, ROS generation was markedly increased in AS2 and control cells, whereas it remained the same in S1 cells (Fig. 3A). Similar inverse relationships between IDPc activity and levels of protein oxidation and lipid peroxidation were observed when the S1 and AS2 cells were exposed to HG (Figs. 3B and 3C). These results indicate that IDPc plays an important role in protection against renal cell injury by reducing hyperglycemia-induced oxidative stress.

Since ECM accumulation is induced in diabetic nephropathy (Ha et al., 2008; Kanwar et al., 2008), we studied the effect of IDPc on high glucose concentration-induced collagen synthesis by measuring [^{3}H]-proline incorporation in IDPc sense and antisense transfected OK cells. As shown in Fig. 3D, the incorpo-

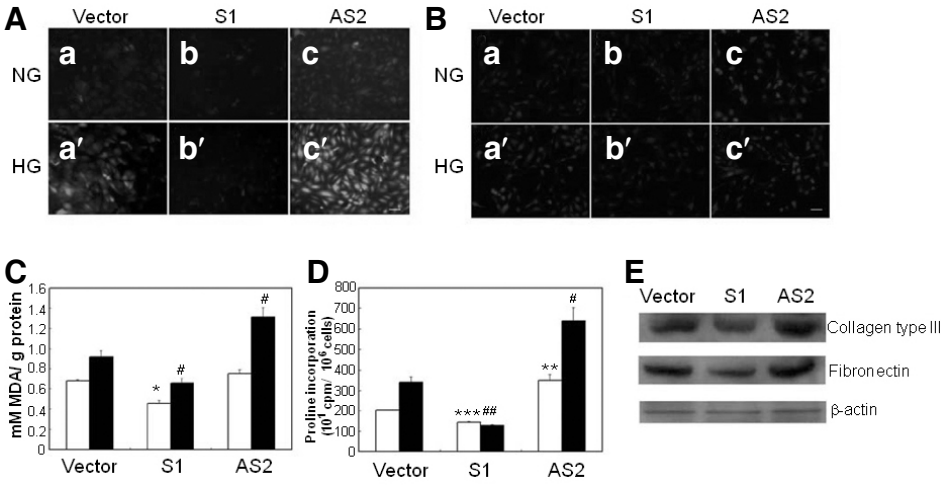

Fig. 3. Effect of transfected IDPc on oxidative damage induced by glucose stress in OK cells. (A) Effect of IDPc on production of total peroxides. Typical patterns of DCF fluorescence are presented for IDPc transfected cells cultured with NG or HG for 24 h. Fluorescence images were obtained under a fluorescence microscopy from three separate experiments. (B) Effect of IDPc on protein oxidation. IDPc transfected cells cultured with NG or HG for 24 h and protein carbonyl contents were visualized by immunostaining with anti-DNP antibodies and anti-rabbit-FITC antibodies. (C) Effect of IDPc on lipid peroxidation. Levels of malondialdehyde (MDA) production in transfected cells cultured with NG (open bars) or HG (shaded bars) for 24 h were determined in triplicate. Each bar represents the mean ± S.E. * $p < 0.05$ compared with the control vector cells cultured in NG. # $p < 0.05$ compared with control vector cells cultured in HG. (D) Effects of IDPc on proline incorporation. Transfected cells were grown in serum-free medium containing NG (open bars) or HG (shaded bars) for 48 h. The amount of proline incorporation in cells was measured in a liquid scintillation counter. Each bar represents the mean ± S.E. of three separate experiments. ** $p < 0.01$ and *** $p < 0.001$ compared with control vector cells cultured in NG. # $p < 0.05$ and ## $p < 0.01$ compared with control vector cells cultured in HG. (E) Effects of IDPc expression on ECM protein accumulation in transfected cells. Whole-cell lysates (50 μg of protein) from transfected cells cultured in HG for 48 h were analyzed by immunoblotting.

ration rate of proline was decreased in S1 cells and increased in AS2 cells in the presence of NG when compared to the control vector cells. In addition, proline incorporation was further stimulated in control and AS2 cells by treatment with HG, but incorporation did not increase in S1 cells. Similarly, expression of collagen type III and fibronectin proteins were also increased in AS2 cells and clearly reduced in S1 cells compared to the control cells after exposure to HG (Fig. 3E). These results strongly suggest that IDPc can inhibit hyperglycemia-induced ECM accumulation in renal cells.

IDPc provides reducing equivalents required for antioxidation in renal cells during hyperglycemia

NADPH plays a critical role in the antioxidative system and is required for the regeneration of GSH by glutathione reductase. To further verify that the decreased production of ROS and ECM in S1 cells upon exposure to HG (Fig. 3) was due to the increased requirement for the reducing equivalent NADPH, we analyzed changes in NADPH and GSH production in IDPc transfected renal cells. Upon exposure to NG, NADPH production was increased and slightly decreased in the S1 and AS2 cells, respectively, when compared with the control vector cells (Fig. 4A). In the presence of HG, the level of NADPH production in S1 cells was much higher than that in the control or AS2 cells. Furthermore, the level of NADPH production in S1 cells with HG was even slightly higher compared to control cells with NG (Fig. 4A). The cellular GSH level was also measured by the GSSG/(GSSG + GSH) ratio. As expected, even under NG and HG conditions, S1 cells showed the lowest GSSG/(GSSG + GSH) ratio (Fig. 4B). These results together indicate that the level of IDPc expression is positively correlated with the level of NADPH, which is needed for regeneration of reduced glutathione in renal cells.

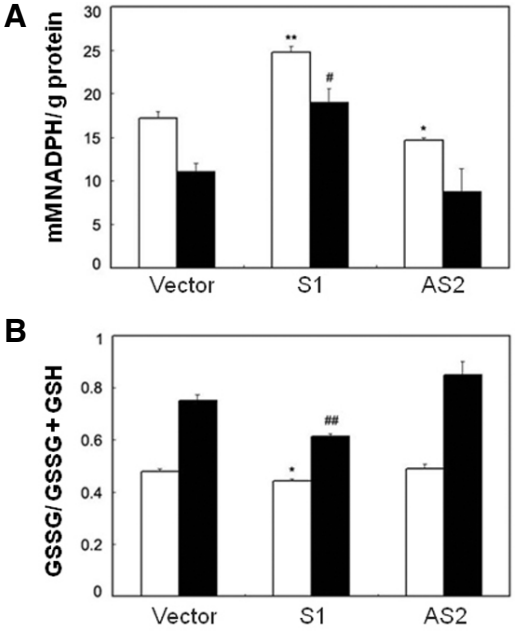

Fig. 4. Effect of transfected IDPc on cellular redox status changes induced by glucose stress. (A) Effects of IDPc on the levels of cytosolic NADPH production in transfected cells cultured in NG or HG are represented as open bars and shaded bars, respectively. Each value represents the mean ± S.E. from three separate experiments. * $p < 0.05$ and ** $p < 0.01$ compared with control vector cells cultured in NG. # $p < 0.05$ compared with control cells cultured in HG. (B) Effect of IDPc on the levels of cytosolic GSSG *versus* the total GSH pool. Open and shaded bars represent the GSSG ratios in the cytosol of transfected cells cultured in NG and HG, respectively. Each value represents the mean ± S.E. from three separate experiments. * $p < 0.05$ compared with control vector cells cultured in NG. ## $p < 0.01$ compared with control vector cells cultured in HG.

DISCUSSION

The kidney has a high rate of aerobic metabolism, particularly

in the proximal tubules, and is potentially exposed to high concentrations of oxidants and reactive electrophiles. Therefore, the kidney requires an adequate supply of the reducing equivalent GSH to maintain normal function (Lash, 2005). All forms of diabetes are characterized by chronic hyperglycemia and the development of microvascular pathology in the retina, renal glomerulus and peripheral nerves. As a consequence of the microvascular pathology, diabetes is a leading cause of end-stage renal disease, blindness and a variety of debilitating neuropathies (Brownlee, 2001). In the diabetic state, hyperglycemia directly increases ROS production in renal cells and stimulates the progression of diabetic nephropathy by increasing cellular damage and accumulation of ECM (Forbes et al., 2008; Ha et al., 2008; Kanwar et al., 2008). Therefore, defense against ROS-induced oxidative stress in renal cells might play an important role in protection from or development of diabetic nephropathy.

In aerobic organisms, a supply of NADPH is a prerequisite for regeneration of biological antioxidant substances, including GSH and the low molecular weight thioredoxins, glutaredoxins and peroxiredoxins proteins, which protect cells against oxidative stress in the cytoplasm and/or nucleus (Kemp et al., 2008). In this context, the major NADPH producer might contribute to defense against ROS-mediated cellular damage. In fact, we previously demonstrated that IDPc plays essential roles in protection against oxidative cellular damage induced by xenobiotics, UV radiation, γ-rays and lipopolysaccharides (Jo et al., 2002; Lee et al., 2002; 2004; Maeng et al., 2004).

Glucose-6-phosphate dehydrogenase (G6PDH) is considered a major NADPH producer in cells and its role in cellular protection from oxidative stress is well established (Pandolfi et al., 1995; Salvemini et al., 1995; Scott et al., 1991). However, a growing body of evidence has indicated that IDPc is the major enzyme that produces cytosolic NADPH in cells (Jo et al., 2002; Koh et al., 2004; Lee et al., 2002; 2004; Maeng et al., 2004). In addition, an earlier study also indicated that IDPc in the rat liver was 16- and 18-fold higher than G6PDH and malic enzyme, respectively (Veech et al., 1969), further suggesting that IDPc plays a pivotal role in the production of cytoplasmic NADPH in the liver and kidney.

In this report, we demonstrated that the expression and enzymatic activity of IDPc in diabetic rat kidneys and renal cells exposed to hyperglycemia were significantly increased. However, this increase in IDPc in renal cells was abrogated by treatment with the antioxidant NAC, indicating that ROS induced by hyperglycemia directly stimulates IDPc expression. In cultured renal cells, overexpression of IDPc significantly reduced hyperglycemia-induced ROS generation, lipid peroxidation and ECM accumulation with increasing NADPH and GSH levels in S1 cells, while those effects were all reversed in AS2 cells by a reduction in IDPc expression.

Interestingly, ischemia-perfusion, which is also known to generate ROS in various organs (Cuzzocrea et al., 2001; Kaminski et al., 2002), significantly decreased the expression and enzymatic activity of IDPc in mouse kidneys (Kim et al., 2009), thus suggesting differential roles of hyperglycemia-induced ROS and ischemia-reperfusion-induced ROS in the regulation of IDPc expression in renal cells. In contrast to IDPc induction by hyperglycemia, the expression and enzyme activity of G6PDH were decreased in STZ-induced diabetic rat kidney cortexes (Xu et al., 2005), suggesting that the contribution of G6PDH to defense against hyperglycemia-medicated oxidative stress in renal cells might be less important than IDPc. In agreement with this hypothesis, IDPc activity was 10- to 30-fold higher than G6PDH activity in various kidney tissues (Kim et al., 2009).

In conclusion, our data indicate that hyperglycemia-mediated ROS generation leads to IDPc induction, which increases the cytoplasmic supply of NADPH required for protection against oxidative stress in renal cells. Therefore, it will be of interest to investigate the effect of IDPc agonists on protection from or improvement of diabetic nephropathy in diabetes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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