Ethyl pyruvate ameliorates albuminuria and glomerular injury in the animal model of diabetic nephropathy

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Ju KD, Shin EK, Cho EJ, Yoon HB, Kim HS, Kim H, Yang J, Hwang YH, Ahn C, Oh KH. Ethyl pyruvate ameliorates albuminuria and glomerular injury in the animal model of diabetic nephropathy. Am J Physiol Renal Physiol 302: F606–F613, 2012. First published November 30, 2011; doi:10.1152/ajprenal.00415.2011.—Pyruvate is an endogenous antioxidant and anti-inflammatory substance. The present study was implemented to investigate the protective effect of ethyl pyruvate (EP) against the development and progression of diabetic nephropathy in an in vivo and in vitro model. Diabetic rats were prepared by injecting streptozotocin (65 mg/kg). Those that developed diabetes after 72 h were treated with EP (40 mg/kg) intraperitoneally. Diabetic rats without pyruvate treatment and nondiabetic rats were used for control. As an in vitro experiment, rat mesangial cells cultured primarily from Sprague-Dawley rats were treated in high-glucose (HG; 50 mM) or normal-glucose (NG; 5 mM) conditions and with or without pyruvate. Pyruvate-treated diabetic rats exhibited decreased albuminuria and attenuated NADPH-dependent reactive oxygen species generation. Immunohistochemistry showed reduced laminin, type IV collagen, and fibronectin deposition in the glomeruli compared with nontreated diabetic rats. Parallel changes were shown in tissue mRNA and protein expression levels of monocytic chemoattractant protein-1, transforming growth factor-β1, laminin, fibronectin, and type IV collagen in the kidney. Concordantly, protective effects were also exhibited in the mesangial cell culture system. These findings suggest that pyruvate protects against kidney injury via NADPH oxidase inhibition. The present study established that activation of NADPH oxidase plays a crucial role in diabetes-induced oxidative stress, glomerular hypertrophy, and ECM molecule expression. Pyruvate exhibited a renoprotective effect in the progression of experimental diabetic nephropathy. Future research is warranted to investigate the protective mechanism of pyruvate more specifically in relation to NADPH oxidase in diabetic nephropathy. Recently, numerous studies suggested that oxidative stress is involved in the pathogenesis of diabetic complications (2, 14, 24, 26). Indeed, a number of experimental and clinical studies have shown that oxidative stress plays an important role in the development and progression of DN (10). In addition, pathological changes in the glomeruli and an increase in urinary albumin excretion in diabetic rats were ameliorated by treatment with antioxidants such as vitamin E, taurine, or lipoic acid, supporting the role of oxidative stress in the pathogenesis of DN (6, 20, 29). Renal hypertrophy and ECM accumulation are early features of DN (3, 37, 40, 41). Elevated ROS levels contribute to the development of diabetic vascular complications, such as atherosclerosis and DN. In experimental and human diabetes, ROS generation is known to be increased via multiple pathways, including glucose autooxidation, increased mitochondrial superoxide production, PKC-dependent activation of NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS) activity, formation of advanced glycation end products (AGEs), and stimulation of cellular ROS production by extracellular AGEs through their receptors (5, 10). Among these, the activation of NADPH oxidase and uncoupling of eNOS mainly contribute to the increase in glomerular superoxide production in experimental diabetic rats (41). Recent studies indicate that a multicomponent phagocyte-like NADPH oxidase is a major source of ROS in many nonphagocytic cells, including renal cells such as tubular epithelial cells and glomerular mesangial cells (8, 11, 17, 25). Under physiological conditions, NADPH oxidases have a very low constitutive activity that can be upregulated in response to various stimuli (1, 16, 18, 19).

Pyruvate, a key intermediate metabolite of glucose and a potent antioxidant and free radical scavenger, has antioxidant and anti-inflammatory effects in an in vivo and in vitro model (31). However, the instability of pyruvate limits its usefulness as a therapeutic agent (31). Ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvic acid, is safer and more stable than pyruvate. In addition, in vitro EP was found to suppress the expression of several proinflammatory mediators (7, 28). Based on these observations, it was suggested that EP has a potential role as an anti-inflammatory agent, although its mode of action was not elucidated.

To date, however, the effect of pyruvate on diabetic kidney disease has not been explored yet. The following were the objectives of the present study: 1) to determine whether NADPH oxidase mediates ROS generation induced by experimental diabetes in vivo and by HG in cultured mesangial cells; 2) to explore whether pyruvate has a protective effect in the development and progression of...
experimental DN; and 3) to investigate whether its protective effect is mediated by the inhibition of NADPH oxidase and downstream proinflammatory and profibrotic molecules.

MATERIALS AND METHODS

Animals and Treatments

All animal studies were carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital, Male Sprague-Dawley rats (Koatech), weighing between 200 and 225 g, were divided into 4 groups of 12 rats/group. Rats were injected intraaperitoneally (ip) with either 65 mg/kg body wt streptozotocin (STZ, Sigma, St. Louis, MO) in sodium citrate buffer (0.01 M, pH 4.5, Sigma) to induce diabetes or injected with vehicle only as a non-diabetic control. Blood glucose concentration (LifeScan One Touch glucometer, Johnson&Johnson) was monitored periodically before and after the injection. Diabetic rats with blood glucose above 300 mg/dl were randomly divided into a pyruvate-treated group (DM+PY) and an untreated diabetes group (DM).

Seventy-two hours after STZ injection, rats in the DM+PY group were injected with EP (40 mg/kg ip, Sigma) every other day. All the diabetic rats were treated with 2 U of regular insulin (ip, Sigma) every other day. Rats injected with vehicle alone were further subdivided into a nondiabetic untreated control (Con) and nondiabetic pyruvate-treated control group (C+PY). All rats had unrestricted access to food and water. Body weights, serum glucose, blood urea nitrogen, serum creatinine, and 24-h urine albumin were measured every 4 wk. Glycated hemoglobin (HbA1c) was measured at the time of death. Rats were maintained in accordance with IACUC procedures. At week 12, all rats were euthanized, and the kidneys were weighed at the time of death. A slice of whole kidney at the pole was embedded in paraffin for light microscopy and image analyses or snap-frozen in liquid nitrogen. In addition, cortical tissue was used for RNA isolation from the glomeruli by differential sieving as described (1), and samples of cortical tissue were frozen for biochemical analyses. NADPH oxidase activity measurements were performed on freshly obtained tissue.

Urinary Albumin Measurement

The amount of albuminuria was determined with a Neprat II ELISA kit (Exocell, Philadelphia, PA) according to the manufacturer’s instructions.

Isolation and Culture of Primary Rat Mesangial Cells

Rat mesangial cells were isolated and cultured by employing a standard collagenase digestion method as previously described. Briefly, male Sprague-Dawley rats, weighing 180–200 g, were anesthetized, and their kidneys were removed. Renal cortical tissues were separated from the medulla and minced in D-Hanks’ balanced buffer through 250-, 200-, 125-, and then 75-μm stainless steel sieve filters and subsequently digested in 0.1% collagenase (type IV) solution at 37°C for 40 min. After centrifuging at 1,500 rpm for 10 min at room temperature, pellets were resuspended with 5 mmol/l glucose RPMI supplemented with 15% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The dispersed glomeruli were placed in 100-mm plastic dishes with the same culture medium and incubated in a humidified incubator at 37°C under 95% air-5% CO2. The culture medium was changed every 3 days. The cells from passages 3–5 were used for the present study. Primary cultured mesangial cells were seeded in six wells or 100-mm plastic dishes in RPMI under normal glucose (NG; 5 mmol/l glucose) or HG (50 mmol/l glucose) conditions. The cultured mesangial cells at ~70–80% confluence were cultured in serum-free RPMI with NG for 24 h to synchronize cell growth. After that, the media was replaced with fresh serum-free media containing NG, HG alone, or NG/HG plus various levels of EP. Treatment with 5 mmol/l glucose plus 45 mmol/l mannitol was employed for osmotic control.

RT-PCR

Gene expressions of monocyte chemoattractant protein (MCP)-1, TGF-β1, laminin, fibronectin, and type IV collagen mRNA were assessed using RT-PCR standardized by coamplifying with the housekeeping gene GAPDH, which served as an internal control. Total RNA was isolated from the glomeruli and mesangial cells with TRIzol (Invitrogen Japan, Tokyo, Japan) by the guanidine thiocyanate extraction method. Total RNA was reverse transcribed into DNA and used for PCR with rat-specific primers for MCP-1, TGF-β1, laminin, type IV collagen, and fibronectin. The promoter-specific primers used for 447-bp fragments of rat MCP-1 were 5’-ATGCCAGGTCCTGTCAGC-3’ (forward primer) and 5’-CTAGTGTCTCTGTCATACT-3’ (reverse primer), and for 154-bp fragments of TGF-β1 primers were 5’-TGAGTGGCTGTCTTGTGAGC-3’ (forward primer) and 5’-TGGACTGATCCCATATTGATT-3’ (reverse primer). The primers for lamminin corresponded to 5’-GCTTAAAGCCGCTCTGTCAG-3’ (forward primer) and 5’-GCTTTAACGCGCTCTGTC-3’ (reverse primer). The final PCR product was 275 bp in size. The primers for type IV collagen corresponded to 5’-CAGGGTGTGCGGTGTGTGAAG-3’, (forward primer) and 5’-AGCTCCCCCTGCCTTCAAGGT-3’ (reverse primer). The final PCR product was 328 bp in size. The primers for fibronectin corresponded to 5’-GGCCGAGCCCTCACTTCTC-3’ (forward primer) and 5’-AGTCCTTTAGGCGGCCT-3’ (reverse primer). The final PCR product was 213 bp in size. Sequences of GAPDH primers corresponded to 5’-AATCGATCCCGCTACCAACCA-3’ (forward primer) and 5’-GAGCCCTATATTCATTGCTCATA-3’ (reverse primer), giving a 515-bp PCR product.

Western Blot Analysis

The whole cell extracts (50 μg of protein/lane) were loaded, separated by 8–12% SDS-PAGE under reducing conditions, and transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL) by electroblotting. The transfer of the protein and the equalization of the loading in the lanes were verified using reversible staining with Ponceau S. The membranes were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.15% Tween 20) for 2 h at room temperature. The proteins were detected by antibodies for MCP-1, TGF-β1, laminin (Santa Cruz Biotechnology, Santa Cruz, CA), type IV collagen, and fibronectin (Abcam, Cambridge, UK) diluted in TBS-T containing 5% dry milk, and incubated overnight at 4°C in a 1:1,000 dilution of polyclonal antibody. After washing in TBS-T, the immunoreactive proteins were visualized using horseradish peroxidase-linked goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG (Santa Cruz Biotechnology) at 1:1,000 dilution, which was followed by enhanced chemiluminescence (Amersham). Exactly equal amounts of protein, determined by the Bradford method, were loaded in each lane.

ELISA

Levels of MCP-1 (Invitrogen, Camarillo, CA) and TGF-β1 in the culture medium were determined by ELISA kits (R&D System, Minneapolis, MN) according to the manufacturer’s instructions.

NADPH Oxidase Assay

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method.

Kidney cortex and glomeruli. Homogenates from the renal cortex or isolated glomeruli were prepared in 1 ml and 500 μl, respectively, of lysis buffer (20 mM KH2PO4, pH 7.0, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotonin, and 0.5 μg/ml leupeptin) by using a Dounce homogenizer (100 strokes on ice). Homogenates were subjected to

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low-speed centrifugation at 800 g, 4°C, for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100 μl of homogenates were added to 900 μl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Photon emission in terms of relative light units (RLU) was measured every 20 or 30 s for 10 min in a luminometer. There was no measurable activity in the absence of NADPH. A buffer blank (<5% of the cell signal) was subtracted from each reading. Superoxide production was expressed as RLU per milligram protein. Protein content was measured using the Bio-Rad protein assay reagent.

**Cultured mesangial cells.** NADPH oxidase activity in cells was measured as described previously (12). Briefly, mesangial cells grown in serum-free medium containing 5 or 50 mM glucose were washed five times in ice-cold PBS and scraped from the plate in the same solution followed by centrifugation at 800 g, 4°C, for 10 min. The cell pellets were resuspended in lysis buffer. Cell suspensions were homogenized with homogenizer on ice. Aliquots of the homogenates were used immediately to measure NADPH-dependent superoxide generation as above.

**Determination of Glomerular Cross-Sectional Area**

Light microscopy of serial sections from the different treatment groups was used for histomorphometric studies. The area (μm²) of a minimum of 20 glomerular sections from each animal was determined in digital images using Image-Pro Plus 4.5 software (Media Cybernetics). Glomerular cross-sectional area was measured in captured digital images by tracing around the perimeter of the glomerular capillary tuft using the polygram tool. The analysis software was calibrated to a stage micrometer.

**Immunohistochemistry**

Localization of cellular fibronectin was assessed by immunoperoxidase histochemistry using polyclonal antibodies or mouse monoclonal antibodies. Frozen cortical sections (6 μm thick) were fixed and permeabilized in acetone for 10 min and then rehydrated in PBS-0.1% BSA for 15 min. Sections were incubated with 0.6% hydrogen peroxide in methanol to block nonspecific peroxidase activity and 0.01% avidin, 0.001% biotin to block localization of endogenous species (ROS) generation. The sections were then dehydrated and mounted with Permount (Sigma) and viewed by brightfield microscopy.

**Statistical Analysis**

All values are expressed as means ± SE. Statistical analyses were performed using the statistical package SPSS for Windows version 11.0 (SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed post hoc by the Mann-Whitney U-test. Values <0.05 were considered statistically significant.

**RESULTS**

**Animal Experiments**

Briefly, male Sprague-Dawley rats were divided into four groups. At 12 wk, rats were euthanized. At the time of death, the ratios of kidney weight to body weight were significantly higher in DM and DM+PY rats than in Con rats (P < 0.05). The mean blood glucose levels at 12 wk were significantly elevated in both diabetic groups (DM and DM+PY). However, no differences were observed in blood glucose, HbAlc, and systolic blood pressure between the DM and DM+PY groups. The mean serum creatinine was higher in DM (0.99 ± 0.12 mg/dl) and DM+PY rats (0.97 ± 0.04 mg/dl) compared with Con rats (0.65 ± 0.02 mg/dl) (P < 0.05). However, the difference in serum creatinine concentration between DM and DM+PY rats did not reach statistical significance. The mean blood urea nitrogen was significantly higher in DM and DM+PY rats relative to Con rats but did not reach statistical significance between DM and DM+PY. Compared with the Con group (0.24 ± 0.01 mg/day), 24-h urinary albumin excretion was significantly higher in the DM group (5.13 ± 0.38 mg/day, P < 0.05), and pyruvate treatment remarkably re-

**Table 1. Parameters of experimental animals at euthanasia**

<table>
<thead>
<tr>
<th></th>
<th>Con (n = 12)</th>
<th>DM (n = 12)</th>
<th>C+PY (n = 12)</th>
<th>DM + PY (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>438 ± 12</td>
<td>239 ± 8*</td>
<td>439 ± 12</td>
<td>239 ± 12*</td>
</tr>
<tr>
<td>Kidney wt/body wt, %</td>
<td>0.62 ± 0.01</td>
<td>1.18 ± 0.08*</td>
<td>0.47 ± 0.14</td>
<td>1.19 ± 0.05*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>102.3 ± 1.6</td>
<td>608.6 ± 54.7*</td>
<td>104.2 ± 2.3</td>
<td>615.3 ± 29.3*</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>14.0 ± 0.3</td>
<td>35.3 ± 3.7*</td>
<td>13.9 ± 2.8</td>
<td>30.2 ± 1.3*</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.65 ± 0.02</td>
<td>0.99 ± 0.12*</td>
<td>0.74 ± 0.13</td>
<td>0.97 ± 0.04*</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>108.3 ± 1.3</td>
<td>103.7 ± 0.8*</td>
<td>106.8 ± 1.1</td>
<td>105.8 ± 1.1</td>
</tr>
<tr>
<td>HbAlc, %</td>
<td>4.22 ± 0.05</td>
<td>8.06 ± 0.41*</td>
<td>4.17 ± 0.02</td>
<td>8.18 ± 0.30*</td>
</tr>
<tr>
<td>Albuminuria, mg/day</td>
<td>0.24 ± 0.01</td>
<td>5.13 ± 0.38*</td>
<td>0.29 ± 0.01</td>
<td>1.96 ± 0.13*</td>
</tr>
</tbody>
</table>

Values are means ± SE of the values from the glomeruli of 6 animals/group. Con, untreated nondiabetic control group; DM, untreated diabetic group; C+PY, pyruvate-treated nondiabetic group; DM+PY, pyruvate-treated diabetic group. *P < 0.05 vs. Con. #P < 0.05 DM+PY vs. DM.

Fig. 1. Effects of pyruvate treatment on diabetes-induced reactive oxygen species (ROS) generation. A: NADPH oxidase activity in glomerular homogenates. NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence and expressed as relative light units (RLU)/mg protein. B: hydrogen peroxide generation was detected by STA-347. Values are means ± SE of the activities from the glomeruli of 6 animals/group. Con, untreated nondiabetic group; DM, untreated diabetic group; C+PY, pyruvate-treated nondiabetic group; DM+PY, pyruvate-treated diabetic group. *P < 0.05 vs. Con. #P < 0.05 DM+PY vs. DM.
Effect of Pyruvate on Glomerular NADPH Oxidase Activity and ROS Generation

Glomerular NADPH oxidase activity was detected by the lucigenin-enhanced chemiluminescence method. Compared with Con rats, glomerular NADPH oxidase activity was significantly increased in DM rats by 3.6-fold. Pyruvate treatment significantly ameliorated glomerular NADPH oxidase activity in DM+PY (P < 0.05 vs. DM; Fig. 1A). Glomerular ROS production represented by H2O2 was determined by a STA-347 OxiSelect ROS assay kit (Cell Biolabs). H2O2 generation was increased 6.2-fold in DM relative to Con, and this increase was significantly attenuated by pyruvate treatment (P < 0.05 vs. DM; Fig. 1B). These results demonstrate that pyruvate inhibits NADPH oxidase activity and ROS generation induced by diabetes.

Effect of Pyruvate on MCP-1 and TGF-β1 Expression in Glomeruli

The effect of pyruvate on mRNA and protein expression levels of MCP-1 and TGF-β1 were measured by RT-PCR and Western blot analysis in glomeruli. mRNA expression levels of MCP-1 and TGF-β1 were increased in glomeruli of diabetic rats (DM group), and these increases were significantly reduced by pyruvate treatment (Fig. 2A). As shown the Fig. 2B, pyruvate markedly ameliorated MCP-1 and TGF-β1 protein expression in glomeruli. Taken together, our data suggest that pyruvate led to a significant reduction of MCP-1 and TGF-β1 in glomeruli.

Effect of Pyruvate on Laminin, Type IV Collagen, and Fibronectin Deposition in Glomeruli

mRNA expressions of type IV collagen and fibronectin were increased in DM rats compared with Con rats, and these increases were attenuated by treatment with pyruvate (Fig. 2A). Similar to the mRNA results, glomerular type IV collagen and fibronectin protein expression determined by Western blot were significantly increased in DM compared with Con rats, and these increases were significantly ameliorated by pyruvate treatment (Fig. 2B). Immunohistochemistry revealed a strong expression of laminin, type IV collagen, and fibronectin in the renal cortex of the diabetic rats (DM group), and the expression of the above ECM proteins were remarkably reduced in DM+PY rats (Fig. 3A), which were consistent with the changes exhibited by RT-PCR and Western blotting. Collectively, these data indicated that pyruvate treatment attenuates the glomerular expression of ECM molecules in DN.

Effect of Pyruvate on Glomerular Diameter and Cross-Sectional Area

Glomerular diameter and glomerular cross-sectional area were quantified in the histological sections of the kidneys removed from the rats. Figure 3B shows that the glomeruli of diabetic rats (DM group) are significantly larger, in terms of both the diameter and cross-sectional area, compared with the controls (Con). Pyruvate treatment resulted in a decrease in glomerular size. Therefore, these results demonstrate that pyruvate treatment ameliorates glomerular hypertrophy in diabetic rats.
Effect of HG on NADPH Oxidase Activity and ROS Generation in Cultured Mesangial Cells

NADPH oxidase activity was determined by the lucigenin-enhanced chemiluminescence method. Compared with NG-treated cells, NADPH oxidase activity in HG-treated mesangial cells was significantly increased by 8.3-fold and EP treatment significantly ameliorated NADPH oxidase activity to 2.2-fold in HG-stimulated mesangial cells (P < 0.05, HG vs. HG EP; Fig. 4A). H2O2 production was examined with a STA-347 OxiSelect ROS assay kit (Cell Biolabs). Relative to NG-treated rat mesangial cells, H2O2 generation in HG-treated mesangial cells was increased 16-fold (P < 0.05, HG vs. NG; Fig. 4B), and this was significantly attenuated with HG + EP treatment (P < 0.05, HG vs. HG + EP; Fig. 4B). These results suggested that EP inhibits NADPH oxidase activity and ROS generation induced by HG in a diabetic condition.

Effect of EP on HG-Induced MCP-1 and TGF-β1 in Cultured Mesangial Cells

mRNA and protein expression levels of MCP-1 and TGF-β1 were detected by RT-PCR and ELISA. mRNA expression levels of MCP-1 and TGF-β1 were increased in HG-treated mesangial cells in a time-dependent manner, and these increases were significantly attenuated by EP treatment in a dose-dependent manner (Figs. 5 and 6). In Figure 7, osmotic controls were included to determine whether the effects of HG might be due to hyperosmolality. Thus treatment with 5 mmol/l glucose plus 45 mmol/l mannitol (NG + mannitol for osmotic control) had no effect on the expressions of MCP-1 and TGF-β1 compared with NG.

Effect of EP on HG-Induced Laminin, Type IV Collagen, and Fibronectin Expression in Cultured Mesangial Cells

The effect of HG and EP on the mRNA and protein expression levels of the ECM molecules were determined by RT-PCR and Western blot analysis. Figure 8 shows increased expression of laminin, type IV collagen, and fibronectin in a time- and dose-dependent manner in both mRNA and protein expression levels (Fig. 8, A and B). EP treatment markedly suppressed HG-induced laminin, type IV collagen, and fibronectin expression (Fig. 8, C and D). Taken together, our data suggest that EP treatment led to the significant suppressions in the mesangial cells of MCP-1,
TGF-β1, laminin, type IV collagen, and fibronectin, which were upregulated in the diabetic condition.

DISCUSSION

This study shows that NADPH oxidase activity and ROS are upregulated both by experimental diabetes in vivo and by HG in vitro. It was evident in the present study that upregulations of NADPH oxidase activity and the ROS system induced by experimental diabetes and HG were significantly ameliorated by pyruvate treatment. Pyruvate exhibited a protective effect on the development and progression of DN in the animal experiment. We proved the protective effects of pyruvate in a number of different ways: significant reduction of albuminuria and amelioration of tissue expression of inflammatory, profibrotic, and ECM molecules. Such protective effects of pyruvate were also demonstrated in a mesangial cell culture system under HG.

Oxidative stress has been implicated in the pathogenesis of diabetic complications (2, 14, 16, 19, 24, 26). However, the mechanisms of ROS generation in diabetes are not fully understood. In this study, we demonstrated that NADPH oxidase is one of the major sources of ROS overproduction in DN and that NADPH-derived ROS mediates glomerular hypertrophy and ECM expression. Several studies reported that elevated ROS levels contribute to the development of diabetic complications such as atherosclerosis and DN (2, 14, 16, 18, 19, 24, 26). NADPH oxidase was originally found in neutrophils and is composed of the catalytic subunit gp91 phox together with the regulatory subunits p22 phox , p47 phox , and p67 phox and the small GTPase Rac (11, 18, 32). Electrons from NADPH are transferred through the enzyme to molecular oxygen to generate superoxide and subsequently other ROS such as hydrogen peroxide. In this study, we showed increased expression of NADPH oxidase activity in the kidneys of diabetic rats that is associated with an increased in NADPH-dependent ROS generation in the renal cortex and isolated glomeruli. We have also shown that NADPH oxidase is involved in the upregulation of MCP-1, TGF-β1, and various ECM molecules in experimental DN. Effective inhibition of mRNA and protein upregulations of the above molecules in mesangial cells cultured under HG at pyruvate doses inhibiting NADPH oxidase activation are the supportive evidences for the role of NADPH oxidase in DN. In a future study, it is warranted to investigate the activation of each NADPH oxidase subunit using immunostaining and Western blot analysis. A more complete explanation will await a better understanding of the mechanism of NADPH oxidase activation and its precise subunit requirement.

Pyruvate is an intermediate metabolite in glucose metabolism and an effective scavenger of ROS. The redox imbalance induced by elevated glucose levels in the diabetic milieu results in increased oxidation of sorbitol to fructose, which is coupled to reduction of NAD(P)⁺ to NAD(P)H. High intracellular concentration of glucose, both by glycolysis and activating polyol pathway, leads to the increased intracellular NAD(P)H/NAD(P)⁺ ratio. Such an increase in the cytosolic NAD(P)H/NAD(P)⁺ ratio, described as diabetic pseudohypoxia, plays an important role in mediating various complications associated with diabetes. It was shown that EP could be converted into lactate with coproduction of NAD(P)⁺ from NAD(P)H, thus resulting in the normalization of the NAD(P)H/NAD(P)⁺ ratio. Pyruvate could be an escape route to reduce the potential toxicity associated with HG in diabetes (21, 23, 35, 36). Strong antioxidant and anti-inflammatory effects of pyruvate have been implicated in an animal model of endotoxemia, wherein pyruvate treatment suppressed the production of TNF-α, the NF-κB signaling pathway, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and IL-6 mRNA (28, 30, 38, 39). However, no one has yet shown the beneficial effect of pyruvate on DN.

The present study is the first report elucidating the renoprotective effect of pyruvate in experimental DN. Based on our results, we evaluated the underlying mechanism of the renoprotective effect of pyruvate. Pyruvate-treated diabetic animals exhibited a significantly ameliorated albuminuria, glomerular hypertrophy, and tissue expression of MCP-1, TGF-β1, and ECM molecules. This protective effect of pyruvate was independent of blood glucose concentration and blood pressure, since the pyruvate-treated rats were similar to the untreated diabetic rats with respect to the above parameters. Pyruvate-treated rats exhibited significantly reduced glomerular NADPH oxidase activity and ROS generation, compared with diabetic control. The protective effect of pyruvate on oxidative stress and various inflammatory and profibrotic molecules were also shown in the mesangial cells cultured under HG. Unlike other exogenous antioxidants or anti-inflammatory agents that have been suggested to exhibit a protective effect on DN, pyruvate is an endogenous molecule and constitutes the physiological antioxidant system of the human body. Therefore, it is likely that pyruvate will be established as a safer therapeutic agent in DN.
In conclusion, the present study established that activation of NADPH oxidase plays a crucial role in diabetes-induced oxidative stress, glomerular hypertrophy, and ECM molecule expression. Pyruvate exhibited a renoprotective effect on the progression of experimental DN. Future research is warranted to investigate the protective mechanism of pyruvate more specifically in relation to NADPH oxidase in DN.

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