Kidney expression of glutathione peroxidase-1 is not protective against streptozotocin-induced diabetic nephropathy

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De Haan, Judy B., Nada Stefanovic, David Nikolic-Paterson, Lyndee L. Scurr, Kevin D. Croft, Trevor A. Mori, Paul Hertzog, Ismail Kola, Robert C. Atkins, and Gregory H. Tesch. Kidney expression of glutathione peroxidase-1 is not protective against streptozotocin-induced diabetic nephropathy. Am J Physiol Renal Physiol 289: F544–F551, 2005. First published April 12, 2005; doi:10.1152/ajprenal.00088.2005.—In many diseases, including progressive renal disorders, tissue injury and pathological intra-cellular signaling events are dependent on oxidative stress. Glutathione peroxidase-1 (Gpx1) is an antioxidant enzyme that is highly expressed in the kidney and removes peroxides and peroxynitrite that can cause renal damage. Therefore, we examined whether this abundant renal antioxidant enzyme limits renal damage during the development of type 1 diabetic nephropathy. Wild-type (Gpx1+/+) and deficient (Gpx1−/−) mice were made diabetic by intraperitoneal injection of streptozotocin (100 mg/kg) on 2 consecutive days. Diabetic Gpx1+/+ and −/− mice with equivalent blood glucose levels (23 ± 4 mM) were selected and examined after 4 mo of diabetes. Compared with normal mice, diabetic Gpx1+/+ and −/− mice had a two- to threefold increase in urine albumin excretion at 2 and 4 mo of diabetes. At 4 mo, diabetic Gpx1+/+ and −/− mice had equivalent levels of oxidative renal injury (increased kidney reactive oxygen species, kidney lipid peroxidation, urine isoprostanes, kidney deposition of advanced glycoxidation, and nitrosylation end products) and a similar degree of glomerular damage (hypertrophy, hypercellularity, sclerosis), tubular injury (apoptosis and vimentin expression), and renal fibrosis (myofibroblasts, collagen, TGF-β excretion). A lack of Gpx1 was not compensated for by increased levels of catalase or other Gpx isoforms in diabetic kidneys. Contrary to expectations, this study showed that the high level of Gpx1 expressed in the kidney is not protective against the development of renal oxidative stress and nephropathy in a model of type 1 diabetes.

A gene deficiency; oxidative stress; renal injury

ANTIOXIDANT ENZYMES PROTECT cells and tissues from oxidative injury. In addition, antioxidant enzymes regulate redox signaling within cells mediated by reactive oxygen species (ROS) and can thereby control gene expression (21). Therefore, an imbalance between production of ROS and antioxidant enzymes may be critical in influencing tissue damage.

Oxidative stress contributes to the progression of diabetic tissue injury (13). During diabetes, hyperglycemia promotes the overproduction of superoxide by the mitochondrial electron-transport chain, which increases formation of secondary ROS molecules (hydrogen and organic peroxides) and activation of multiple pathways of hyperglycemic damage (polyol, hexosamine, PKC, and AGE pathways) (3). In addition, advanced glycation end products (AGE), ANG II, and cytokines can increase intracellular ROS in multiple cell types (17, 22, 36, 39). ROS react with other macromolecules causing destruction of protein function and peroxidation of lipids, leading to progressive tissue injury. ROS can also promote disease by activating signaling pathways (PKC, MAPK, JAK/STAT) and transcription factors (NF-κB, AP-1) (21, 24), stimulating epithelial to myofibroblast transdifferentiation and inducing expression of chemokines, PAI-1 and extracellular matrix (14, 15, 30). However, therapeutic suppression of ROS accumulation can inhibit this damage. Injury to diabetic rodent kidneys has been reduced by overexpression of the antioxidant enzyme superoxide dismutase (SOD) (6) and by treatment with antioxidants such as vitamin C and E (5, 20) or the superoxide scavenger α-lipoic acid (26), suggesting that such therapies have clinical potential.

In streptozotocin (STZ)-treated diabetic rats, renal oxidative stress and kidney damage are increased by a dietary deficiency of the essential trace element selenium (34). In contrast, selenium supplementation to rats or humans with diabetes reduces renal injury (9, 18). For normal animals, a deficiency of selenium causes a profound reduction in the activity of glutathione peroxidases (Gpx) in the kidney and liver (37). Selenium is an integral component of the catalytic sites of most glutathione peroxidases (Gpx1–4), promoting their full catalytic activity (1). These Gpx enzymes protect cells against oxidative damage by reducing hydrogen peroxide and organic peroxides with reduced glutathione (8). Therefore, the increased diabetic renal injury associated with selenium deficiency may be due to decreased expression and activation of kidney Gpx.

Gpx is found in all mammalian organs; however, the level of expression varies according to isofrom and tissue. High amounts of Gpx are detected in kidney proximal and distal tubules and the smooth muscle cells of renal arteries (29). Of the known Gpx isoforms, Gpx1 and Gpx4 are readily found in kidney tubular epithelial cells, Gpx3 is weakly detected in kidney proximal tubules, and Gpx2 and 5 have not been...
detected within the kidney. Gpx1 is the major isoform of Gpx expressed in normal kidney, accounting for 96% of kidney Gpx activity (7). Therefore, manipulation of kidney Gpx1 expression and activity is postulated to play a central role in the ability of the kidney to cope with oxidative stress.

Studies with Gpx1-deficient mice have demonstrated the importance of Gpx1 in regulating acute oxidative stress. Although Gpx1 knockout mice appear to be phenotypically normal, they are highly susceptible to injury induced by paraquat (a superoxide generator), cerebral ischemia-reperfusion (stroke), and cold-induced head trauma (8). In addition, Gpx1-deficient fibroblasts show enhanced sensitivity to oxidant-induced apoptosis (8) and Gpx1-deficient macrophages produce more nitric oxide on activation (12).

Given the ability of hyperglycemia to induce oxidative stress and renal injury, our current study explored whether Gpx1 was important in regulating acute oxidative stress. Although Gpx1 knockout mice appear to be phenotypically normal, they are highly susceptible to injury induced by paraquat (a superoxide generator), cerebral ischemia-reperfusion (stroke), and cold-induced head trauma (8). In addition, Gpx1-deficient fibroblasts show enhanced sensitivity to oxidant-induced apoptosis (8) and Gpx1-deficient macrophages produce more nitric oxide on activation (12).

Given the ability of hyperglycemia to induce oxidative stress and renal injury, our current study explored whether Gpx1 was protective against diabetic renal injury by examining the effect of Gpx1 deficiency on the development of renal oxidative stress and diabetic nephropathy in mice with STZ-induced diabetes.

MATERIALS AND METHODS

Materials. STZ was purchased from Sigma. ELISA kits were used to detect mouse urine albumin (Bethyl Laboratories, Montgomery, TX) and transforming growth factor (TGF)-β1 (Promega, Madison, WI). The commercial antibodies used were fluorescein-conjugated anti-α-smooth muscle actin (1A4, Sigma), rabbit anti-mouse collagen IV (Collaborative Biomedical Products, Bedford, MA), anti-activated caspase-3 (Asp175, Cell Signaling Technology, Beverly, MA), antivimentin (V9, Dako, Carpinteria, CA), rabbit anti-heme-oxygenase-1 (HO-1; Stressgen Biotechnologies, Victoria, BC, Canada), and rabbit anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY). Anti-mouse CD45 mAb (M1/9.3.4) was produced by cell culture of a hybridoma obtained from the American Type Culture Collection. Rabbit antibody that detects carboxymethyllysine (CML)-modified proteins was kindly provided by Dr. J. Forbes (Baker Medical Research Institute, Prahran, VIC, Australia) (11). Purified normal rabbit and rat IgG were used as negative control antibodies.

Animal model. Glutathione peroxidase-1-deficient (Gpx1−/−) mice were created at the Centre for Functional Genomics and Human Disease at the Monash Institute of Reproduction and Development, Monash University, Australia (7). Gpx1-intact (+/+ and -/−) littermates were bred from Gpx1+/+ mice with a C57BL/6 background (9 backcrosses). These mice, at 2 mo of age, were made diabetic by intraperitoneal injection of STZ (100 mg/kg) on 2 consecutive days. One week after STZ administration, groups of eight Gpx1+/+ and −/− mice with equivalent blood glucose levels (23 ± 4 mM) were selected and then killed after 4 mo of diabetes. These diabetic mice did not require insulin treatment because they had stable blood glucose levels and did not develop ketonuria or show significant weight loss. Groups of four age-matched normal Gpx1−/− mice were used as controls. Approval for these animal studies was obtained from the Monash Medical Centre Animal Ethics Committee in accordance with the Australian code of practice for the care and use of animals for scientific purposes (7th Edition, 2004).

Biochemical analysis. Glucose levels in fresh blood obtained from the tail vein of conscious mice were measured every 2 wk (between 9 and 10 AM) in nonfasted mice by glucometer (Medisense, Abbot Laboratories, Bedford, MA). Urine was collected at 2 and 4 mo from mice housed in metabolic cages for 18 h (4 PM to 10 AM collections). ELISA kits were used to measure urine levels of albumin (Bethyl Laboratories) and TGF-β1 (Promega). Urine total protein (picric acid method) and urine creatinine (creatininase method) were determined by the Department of Biochemistry at the Monash Medical Centre.

Antioxidant enzyme activity. Kidneys were washed in ice-cold normal saline and homogenized for 10 s in ice-cold homogenization buffer (100 mg tissue/ml, 50 mM potassium phosphate, 0.1% Triton X-100, pH 7.0). The homogenate was centrifuged at 18,000 g and 4°C for 30 min. The supernatant was separated and analyzed for total protein and catalase and glutathione peroxidase activity (32).

For assessment of catalase activity, homogenates (100 μl) were added to 800 μl of reaction mixture (50 mM potassium phosphate, pH 7.0) and allowed to incubate at room temperature for 5 min before the initiation of the reaction by the addition of 100 μl of 100 mM H2O2 solution. The activity was calculated at a velocity constant, K, which was determined from the absorption of the reaction at 240 nm measured at 10 and 25 s, where K = 2.3I/logA0/A (t = 15, A0/A = absorbances at 10 and 25 s, respectively). Activity units were recorded as K per milligram of protein.

Gpx1 activity was measured using an assay in which oxidation of glutathione and NADPH are coupled in the presence of glutathione reductase (7). A reaction mixture was prepared containing 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM NaN3, 0.2 mM NADPH (1 mg/ml), 1 U/ml glutathione reductase, and 1 mM reduced glutathione. Homogenates (100 μl) were added to 800 μl of reaction mixture and allowed to incubate at 37°C for 5 min before the initiation of the reaction by the addition of 100 μl of 0.25 mM H2O2 solution. Absorbance at 340 nm was recorded every minute for 5 min and the activity was calculated from the slope of these lines as micromoles of NADPH oxidized per minute. Activity was expressed as micromoles of NADPH oxidized to NADP+ per minute per milligram of protein (U/mg) using an extinction coefficient (6.3 × 104) for NADPH.

ROS. Kidney ROS levels were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) as previously described (38). DCFH-DA is a nonfluorescent probe that is hydrolyzed by mitochondrial esterase to form DCFH, which is then oxidized by ROS to form the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Kidneys were homogenized (50 mg/ml) in 0.32 M sucrose in 50 mM potassium phosphate buffer pH 7.4 and centrifuged at 1,800 g for 10 min at 4°C. The supernatant was transferred to another tube and centrifuged again at 31,655 g for 15 min at 4°C to yield a crude mitochondrial pellet. The mitochondrial pellet was resuspended in three parts (wt/vol) HEPES-saline buffer, pH 7.4 (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.2 mM Na2HPO4, 5 mM NaHCO3, 6 mM glucose, 1 mM MgCl2, 2 mM CaCl2). For sample measurement, 140 μl of HEPES-saline buffer were added to each well of a fluorescence microtitre plate followed by 10 μl of sample and 50 μl of DCFH-DA (diluted 1:200 with HEPES-saline buffer from DCFH-DA stock of 5 mg/ml in ethanol). Plates were incubated at 37°C for 1 h to allow formation of DCF and then analyzed for fluorescence (excitation 485 nm/emission 520 nm) on a FLUOstar Optima reader (BMG Labtechnologies, Offenberg, Germany). Sample autofluorescence was determined using ethanol to replace DCFH-DA stock and subtracted.

Probes. cDNA fragments of mouse Gpx1 (437 bp), mouse catalase (1582 bp), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 358 bp) were amplified by reverse-transcription PCR and cloned into the pMOSBlue vector (Amersham Pharmacia Biotech, Sydney, Australia). Anti-sense riboprobes for Gpx1, catalase, and GAPDH were labeled with digoxigenin (DIG)-UTP using a T7 RNA polymerase kit (Roche Biochemicals, Mannheim, Germany) and used for Northern blotting.

Quantitative PCR was performed using a commercial VIC-labeled primer/probe combination for rodent GAPDH (Applied Biosystems) and Gpx1-4 primer/probes designed using Primer Express and labeled with FAM (Applied Biosystems).

Northern blotting. Total cellular RNA was extracted from whole kidneys using TRIzol (GIBCO BRL, Grand Island, NY), RNA samples (10 μg) were denatured with glyoxal and dimethylsulphoxide, size fractionated on 1.2% agarose gels, and capillary blotted onto...
positively charged nylon membranes (Roche). Membranes were hybridized overnight with DIG-labeled cRNA probes at 68°C in DIG Easy Hyb solution (Roche). Following hybridization, membranes were washed and incubated with sheep anti-DIG Ab Fab conjugated with alkaline phosphatase. Chemiluminescence substrate (CPD-star, Roche) was then incubated with the membrane and emissions were captured on Kodak XAR film. The exposed film was analyzed by densitometry using the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD).

**Real-time quantitative PCR.** cDNA from total kidney RNA was prepared by reverse transcription using random hexamers and SuperScript II (Invitrogen). Real-time PCRs of prepared cDNA were examined over 60 cycles on a ABI 7700 Sequence Detector (Perkin Elmer) using Platinum quantitative PCR supermix UDG (Invitrogen), VIC, or FAM-labeled primer/probe combinations and ROX dye (Applied Biosystems). A rodent GAPDH primer/probe (Applied Biosystems) was used as a loading reference. The Gpx primers and probes used were as follows: Gpx1 sense primer (CTC ACC CGC TCT TTA CCT TCC T), Gpx1 antisense primer (ACG CAG GAC ACC AAA TGA TGT ACT), Gpx1 probe (ACC CGC TCT CTC TTA CCT GGA ACA), Gpx2 sense primer (GTG CCG TCA CTC GGA ACA), Gpx2 antisense primer (CAG TTC TCC TGA TGT CCG AAC TG), Gpx2 probe (CTC GTT AGT TCT CGG TTT TGG CA), Gpx3 sense primer (CAT ACC GGT TAT GCG CTG GTA), Gpx3 antisense primer (CCT GCC GCC TCA TGT AAC AG), Gpx3 probe (GAC CGC ACC ACA GTC AGC AAC GTC), Gpx4 sense primer (TGA GCC AAA ACT GAC GTA AAC TAC A), Gpx4 antisense primer (GCT CCT GCC CCA AAT ACT G), and Gpx4 probe (TGG TTT ACG AAT CCT GCC CTT CCC CT). Data were analyzed using ABI Sequence Detection Systems software version 1.7 (Perkin Elmer) and transcript levels of Gpx1–4 isoforms were reported as Gpx/GAPDH ratios.

**Immunohistochemistry.** Immunoperoxidase staining for total leukocytes (CD45+ cells) and collagen IV was performed on 2% paraformaldehyde-lysine-periodate (PLP) fixed kidney cryostat sections (5 μm). Immunoperoxidase staining for myofibroblasts (α-smooth muscle actin), vimentin, activated caspase-3, HO-1, CML, and nitrotyrosine was performed on formalin-fixed, paraffin-embedded sections (4 μm). PLP-fixed tissue sections were incubated for 20 min each with 0.6% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA) and 20% normal sheep serum (NShS) to prevent nonspecific detection. Sections were then incubated overnight at 4°C with 5 μg/ml of primary antibody in 1% BSA. After being washed in PBS, sections were incubated with biotinylated secondary antibodies (goat anti-rat IgG 1:200, goat anti-rabbit IgG 1:200, Vector) for 1 h followed by ABC solution (ABC Kit, Vector) for 1 h and developed with 3,3-diaminobenzidine (DAB; Sigma) to produce a brown color.

To detect myofibroblasts, formalin-fixed sections were incubated sequentially in 20% NShS (30 min), 5 μg/ml of fluorescein-conjugated 1A4 mAb in 1% BSA (overnight at 4°C), 0.6% hydrogen peroxide (20 min), peroxidase-conjugated sheep anti-fluorescein F(ab) fragments (1:300, 1 h, Roche), and then developed with DAB.

To detect vimentin and activated caspase-3, formalin-fixed sections were microwave treated in 400 ml of 0.1 M sodium citrate buffer (pH 6) for 12 min to facilitate antigen retrieval and prevent antibody cross-reactivity (25). Sections were then incubated sequentially with 20% NShS (30 min), 5 μg/ml of primary mAb in 1% BSA (overnight at 4°C), 0.6% hydrogen peroxide (20 min), peroxidase-conjugated goat anti-mouse IgG (1:50, 45 min, Dako), mouse peroxidase-anti-peroxidase complexes (mouse PAP, 1:50, 45 min, Dako), and then developed with DAB.

To detect HO-1, formalin-fixed sections were microwave treated in 300 ml of antigen retrieval buffer (Dako) for 10 min, washed in PBS, and incubated sequentially with 0.6% hydrogen peroxide, 5% BSA (60 min), 20% NShS (30 min), rabbit anti-HO-1 Ab (1:1,000) in 2% BSA (overnight at 4°C), avidin (20 min, Vector), biotin (20 min, Vector), biotin-conjugated sheep anti-rabbit IgG (1:250, 30 min, Zymed), ABC reagent (30 min, Vector), and then developed with DAB. The presence of CML and nitrotyrosine was identified using the same immunostaining procedure without microwave treatment and with primary antibodies at dilutions of 1:500 (anti-CML) and 1:120 (anti-nitrotyrosine). For development of CML and nitrotyrosine immunostaining, the DAB solution contained 0.0025% cobalt chloride and 0.0025% ammonium nickel sulphate.

**Table 1. Kidney antioxidant enzyme activity**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpx1+/+</td>
<td>10.2±3.3</td>
<td>0.2±0.3</td>
</tr>
<tr>
<td>Gpx1+/-</td>
<td>5.6±2.2</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.8±0.8</td>
<td>1.7±0.5</td>
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</tbody>
</table>

Values are means ± SD. *P < 0.05 vs. normal of same strain. Gpx1+/+ and Gpx1+/−, glutathione peroxidase-1 wild-type and deficient mice, respectively.
Blood glucose, mM 8.9

Biochemical analysis of diabetic renal injury

Quantitative PCR analysis for Gpx 1–4 was performed on RNA extracted from normal and diabetic kidneys obtained from Gpx1-intact mice. Graphs show the kidney mRNA expression relative to GAPDH for Gpx1 (A), Gpx2 (B), Gpx3 (C), and Gpx4 (D). Values are means ± SD, n = 4. **P < 0.01.

Fig. 2. Effect of Gpx1 deficiency and diabetes on kidney Gpx isoforms. Quantitative PCR analysis for Gpx 1–4 was performed on RNA extracted from normal and diabetic kidneys obtained from Gpx1-intact and -deficient mice. Graphs show the kidney mRNA expression relative to GAPDH for Gpx1 (A), Gpx2 (B), Gpx3 (C), and Gpx4 (D). Values are means ± SD, n = 4. **P < 0.01.

Renal pathology. Formalin-fixed kidney sections (2 μm) were stained with periodic acid Schiff’s (PAS) reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of glomeruli were obtained from microscopy at high power (×400). Glomerular cellularity was determined by counting the number of nuclei in 20 hilar glomerular tuft cross-sections (gcs) per animal. Glomerular volume was assessed by measuring the glomerular tuft area with computer image analysis. Mesangial matrix deposits were analyzed by determining the percent area of PAS material or immunostained collagen IV within the glomerular tuft, scoring 20 gc±s/animal. Tubular injury was assessed by counting the percent of cortical tubules containing vimentin (4) or apoptotic cells expressing activated caspase-3, as determined by immunostaining. Kidney leukocytes detected by CD45 immunostaining were counted under high power (×400) in 20 gc±s and 25 consecutive interstitial fields. Interstitial leukocytes were expressed as cells per millimeter squared. Interstitial leukocytes were expressed as percent area immunostained within the cortical interstitium by computer image analysis. All scoring was performed on blinded slides.

Urine F2-isoprostanes. F2-isoprostanes were used to measure renal oxidative stress. F2-isoprostanes consist of a series of chemically stable prostanoid F2-like compounds that are generated during peroxidation of unsaturated fatty acids (primarily arachidonic acid) in membrane phospholipids (28). Increased urine F2-isoprostanes are a specific response to renal oxidative stress (25) and a proven marker of oxidative stress within diabetic kidneys (27). To detect F2-isoprostanes, urine samples were thawed, and 500-μl aliquots were removed. After addition of the internal standard, d4–8-iso-PGF2a (5 ng), samples were acidified and subjected to solid-phase extraction, HPLC purification, and quantitation using negative ion gas chromatography mass spectrometry as previously described (28).

Kidney lipid peroxides. Levels of lipid peroxide (malondialdehyde content) were assessed in snap-frozen kidneys by lipid extraction and spectrophotometric measurement of thiobarbituric acid-reactive substances (TBARS), using 1,2,3,3-tetramethoxypropane as the standard (6). Results were expressed as nanomoles per milligram of protein.

Statistical analysis. Statistical differences between two groups were analyzed by the unpaired Student’s t-test and differences between multiple groups of data were assessed by one-way ANOVA with Bonferroni’s multiple comparison test. Data were recorded as means ± SD, and values of P < 0.05 were considered significant. All analyses were performed using GraphPad Prism 3.0 (GraphPad Software).

RESULTS

Expression of Gpx isoforms and catalase in normal and diabetic kidneys. Gpx1 and catalase mRNA levels were abundant in normal wild-type mouse kidney (Fig. 1). Similar levels of catalase mRNA were identified in the kidneys of Gpx1+/− mice, which expressed no Gpx1 mRNA. After 4 mo of STZ-induced diabetes, the mRNA levels of Gpx1 in wild-type diabetic kidneys remained similar to normal kidneys. In con-

Table 2. Biochemical analysis of diabetic renal injury

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic 2 mo</th>
<th>Diabetic 4 mo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Gpx1+/+</td>
<td>Gpx1−/−</td>
<td>Gpx1+/+</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>8.9±0.9</td>
<td>10.0±1.5</td>
<td>27.7±3.9*</td>
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<tr>
<td>Proteinuria, mg/mg creatinine</td>
<td>6.4±1.2</td>
<td>7.1±1.0</td>
<td>13.9±3.2*</td>
</tr>
<tr>
<td>Albuminuria, μg/mg creatinine</td>
<td>48±9</td>
<td>45±6</td>
<td>90±17*</td>
</tr>
<tr>
<td>Urine TGF-β1, pg/mg creatinine</td>
<td>28±13</td>
<td>41±6</td>
<td>474±264*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.001 vs. normal of same strain. Normals were age matched to mice that had been diabetic for 4 mo. TGF-β1, transforming growth factor-β1.
Fig. 3. Renal pathology in diabetic kidneys. PAS and hematoxylin staining shows the histology of a normal mouse glomerulus (A) compared with the similarly damaged glomeruli of a diabetic Gpx1+/+ (B) and a diabetic Gpx1−/− mouse (C). Collagen IV was identified in a normal mouse glomerulus (D) by immunostaining and was increased equally in the sclerosed diabetic glomeruli of a Gpx1+/+ (E) and a Gpx1−/− mouse (F). Immunostaining did not detect vimentin in normal mouse tubules (G); however, a similar pattern of tubular vimentin expression was seen in the diabetic tubules of a Gpx1+/+ (H) and Gpx1−/− mouse (I). Apoptotic cells expressing activated caspase-3 (arrows) are rarely detected in normal mouse tubules (J) but are observed at a similar higher frequency in the dilated cortical tubules of diabetic kidneys in a Gpx1+/+ (K) and a Gpx1−/− mouse (L). Expression of α-smooth muscle actin is only seen within vessels of a normal mouse kidney (M). In contrast, equivalent numbers of interstitial myofibroblasts are seen to express α-smooth muscle actin in the diabetic kidneys of a Gpx1+/+ (N) and a Gpx1−/− mouse (O). Magnification A-F: ×1,000; G-L: ×400; M-O: ×250.

Table 3. Analysis of renal pathology in diabetic mice

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
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<tbody>
<tr>
<td></td>
<td>Gpx1+/+</td>
<td>Gpx1−/−</td>
</tr>
<tr>
<td>Glomerular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume, μm² × 10⁴</td>
<td>146±8</td>
<td>149±13</td>
</tr>
<tr>
<td>Cellularity, cells/gcs</td>
<td>34.8±1.4</td>
<td>34.1±1.3</td>
</tr>
<tr>
<td>Matrix fraction, %</td>
<td>15.3±1.2</td>
<td>16.7±1.3</td>
</tr>
<tr>
<td>Collagen IV, %</td>
<td>14.0±0.7</td>
<td>14.1±1.0</td>
</tr>
<tr>
<td>Leukocytes, cells/gcs</td>
<td>0.9±0.4</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Tubules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin+, %</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Apoptotic, %</td>
<td>0.15±0.17</td>
<td>0.13±0.05</td>
</tr>
<tr>
<td>Interstitial</td>
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<tr>
<td>α-SMA+ % area</td>
<td>1.7±0.4</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Leukocytes, cell/mm²</td>
<td>128±15</td>
<td>130±10</td>
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Values are means ± SD. α-SMA, α-smooth muscle actin; gcs, glomerular tuft cross-sections. *P < 0.05, †P < 0.01, ‡P < 0.0005 vs. normal of same strain.
with normal kidneys of Gpx1+/+ mice but was undetectable in both normal and diabetic Gpx1+/− mice.

Diabetic renal damage is unchanged by the absence of Gpx1. The development of type 1 diabetes in mice resulted in a two- to threefold increase in blood glucose and albuminuria at 2 and 4 mo after STZ treatment (Table 2). This was accompanied by a marked increase in urine TGF-β1 excretion, suggesting that the kidney was producing greater amounts of this cytokine. The urine levels of albumin and TGF-β1 were similar in diabetic Gpx1+/+ and Gpx1+/− mice.

Histological analysis of kidney sections stained with PAS and hematoxylin identified significant damage to diabetic kidneys including glomerular hypertrophy, glomerular hypercellularity, increased mesangial matrix (PAS deposits, collagen IV deposition), interstitial cell infiltrate, and tubular dilatation and atrophy (Fig. 3 and Table 3), which is consistent with the early pathological lesions of diabetic nephropathy. Immunostaining of diabetic kidneys demonstrated significant tubular injury (apoptosis and vimentin expression), increased numbers of CD45+ leukocytes in the glomeruli and interstitium, and accumulation of interstitial myofibroblasts expressing α-smooth muscle actin (Fig. 3 and Table 3). Assessment of the histological damage and immunostaining showed no difference between Gpx1+/+ and Gpx1+/− diabetic kidneys.

Renal oxidative stress is unaltered by the absence of Gpx1. Fluorescence detection of DCF identified a similar increase in ROS in the kidneys of Gpx1+/+ and Gpx1+/− mice after 4 mo of diabetes, compared with normal animals (Fig. 4A). The values are means ± SD, n = 8. **P < 0.01. ***P < 0.001.

Fig. 4. Assessment of kidney oxidative stress in diabetic mice. A: kidney levels of reactive oxygen species (ROS) were determined by measuring the oxidation of 2′,7′-dichlorodihydrofluorescein to the fluorescence compound 2′,7′-dichlorofluorescein. ROS levels were significantly greater in diabetic compared with nondiabetic kidneys but were not different between diabetic Gpx1+/+ and Gpx1+/− mice. B: quantitation of urine F2-isoprostanes identified a progressive increase in this marker of lipid peroxidation at 2 and 4 mo of diabetes compared with normal animals, which was similar between Gpx1+/+ and Gpx1+/− mice. C: assessment of kidney malondialdehyde content by measurement of thiobarbituric acid-reactive substances found a 3- to 4-fold increase in diabetic compared with normal kidneys, which was not different between Gpx1+/+ and Gpx1+/− mice.

Fig. 5. Localization of oxidative stress in diabetic kidneys. Immunostaining for heme oxygenase-1 found no expression in normal mouse renal cortex (A) but identified distinct expression in dilated tubules and occasional podocytes from diabetic mouse kidneys, which was similar in Gpx1+/+ (B) and Gpx1+/− mice (C). Immunostaining for carboxymethyllysine-modified proteins was weak in the glomeruli and cortical tubules of a normal mouse kidney (D) but was significantly increased in diabetic kidneys, particularly in dilated tubules, with a similar pattern observed in Gpx1+/+ (E) and Gpx1+/− mice (F). Likewise, weak immunostaining for nitrotyrosine was detected in the glomeruli and cortical tubules of a normal mouse kidney (G) but was increased in the glomeruli and dilated tubules of diabetic kidneys with similar expression observed in Gpx1+/+ (H) and Gpx1+/− mice (I). Magnification A−I: ×400.
development of renal oxidative stress in diabetic mice was supported by the progressive rise in urine F2-isoprostane levels at 2 and 4 mo of diabetes compared with normal animals, which was similar in Gpx1+/+ and Gpx1−/− mice after 4 mo of diabetes (Fig. 4B). In addition, assessment of 4-mo diabetic kidneys showed a significant increase in malondialdehyde (Fig. 4C) and immunostaining of oxidative stress markers (HO-1, CML, nitrosyrosine; Fig. 5) compared with normal kidneys, which was not different between Gpx1+/+ and Gpx1−/− mice. HO-1 is an antioxidant enzyme that is released by the kidney in response to oxidative stress (19). CML- and nitrosyrosine-modified proteins are formed as a result of protein glycoxidation and nitrosation during the development of diabetes (10). Immunostaining of diabetic kidneys found that HO-1, CML, and nitrosyrosine were mainly localized in dilated cortical tubules (Fig. 5) with a similar pattern of expression in Gpx1+/+ and Gpx1−/− mice.

DISCUSSION

Diabetic renal injury, although associated with oxidative stress, appears to be independent of Gpx1 in STZ-treated mice, as the absence of Gpx1 did not exacerbate the level of damage seen in diabetic kidneys over 4 mo of disease. Using known markers of oxidative damage in diabetic kidneys (ROS, urine F2-isoprostanes, kidney malondialdehyde, and expression of HO-1, CML, and nitrosyrosine) (10, 16, 19, 25, 27, 34, 35, 38), we could detect no differences in the localization or levels of kidney oxidative stress between Gpx1-intact and -deficient diabetic mice. In addition, no alteration in the pathological development of diabetic nephropathy (albuminuria, glomerular hypertrophy, glomerular hypercellularity, glomerular matrix accumulation, leukocyte infiltration, tubular apoptosis, and interstitial myofibroblast accumulation) was detected in the absence of Gpx1. These results suggest that Gpx1 is not a crucial regulator of oxidative stress, oxidative injury, or redox-mediated gene activation in the type 1 diabetic kidney.

The Gpx1 isoform is expressed in most kidney cells (29). This study demonstrated that the Gpx1 isoform accounts for almost all (>96%) Gpx activity in both normal and diabetic kidneys. Because Gpx1 is known to be protective against some types of injury caused by oxidative stress (7), we hypothesized that the induction of renal oxidative stress during diabetes would promote increased expression and activity of this antioxidant enzyme as a protective mechanism. Surprisingly, we found that kidney Gpx1 mRNA and activity were not increased for this molecule to regulate oxidative stress in the diabetic kidney. Because Gpx1 is known to be protective against some types of injury caused by oxidative stress (7), we hypothesized that the induction of renal oxidative stress during diabetes would promote increased expression and activity of this antioxidant enzyme as a protective mechanism. Surprisingly, we found that kidney Gpx1 mRNA and activity were not increased for this molecule to regulate oxidative stress in the diabetic kidney.

In contrast to our hypothesis, this study identified a high level of redundancy for Gpx1 as a regulator of oxidative stress in the diabetic kidney. Because kidneys also express catalase and other Gpx isoforms (Gpx1−4) that can eliminate peroxides (7), we determined whether any of these enzymes were upregulated within diabetic kidneys to compensate for the absence of Gpx1. Kidney mRNA levels of catalase and Gpx2−4 were not increased in diabetic relative to normal kidneys and were not affected by the absence of Gpx1. In contrast, levels of catalase mRNA were reduced in Gpx1−/− compared with Gpx1+/+ diabetic kidneys, suggesting that Gpx1 may play a role in the induction of catalase gene activity; however, the mechanisms responsible were not investigated in this study. Previous work showed that both catalase inhibition and selenium deficiency enhance oxidative renal injury induced by puromycin aminonucleoside (2, 32). Hence, we might expect that a reduction in catalase in addition to the absence of Gpx1 in diabetic kidneys would exacerbate oxidative stress and renal injury. However, we found that the reduced levels of catalase mRNA detected in diabetic Gpx1−/− kidneys did not diminish the catalase activity in those kidneys. Therefore, because the development of oxidative stress and injury during diabetic nephropathy is unaffected by Gpx1 deficiency, it is possible that either catalase takes over the antioxidant role of kidney Gpx1 and its reduction in Gpx1−/− mice is not enough to significantly increase oxidative stress or the kidney has other important antioxidant mechanisms that protect it from hyperglycemia-induced oxidative stress. Indeed, recent studies have identified other renal antioxidant enzymes, including thioredoxin and peroxiredoxins (31), which are effective at eliminating intracellular peroxides (33) and may thereby regulate oxidative stress in the diabetic kidney.

In conclusion, our study shows that, despite being highly expressed and responsible for almost all Gpx activity in diabetic kidneys, the antioxidant enzyme Gpx1 is not protective against oxidative renal injury during the early development of diabetic nephropathy. This finding provides important insight into the mechanisms regulating oxidative stress in diabetic kidneys.

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REFERENCES

Glutathione peroxidase-1 in diabetic nephropathy


