Effects of Antioxidants in Diabetes-Induced Oxidative Stress in the Glomeruli of Diabetic Rats

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Abstract. Numerous reports have demonstrated that oxidative stress induced by diabetes plays an important role in the development and progression of diabetic vascular complications including nephropathy. Indeed, there is emerging evidence that the formation of reactive oxygen species (ROS) is a direct consequence of hyperglycemia. Biomarkers for oxidative damage to DNA, lipids, and proteins are also supporting the concept of increased oxidative stress in diabetes and diabetic nephropathy. However, there is an unanswered question: When does oxidative stress as a pathogenetic event occur in the process of diabetic nephropathy? To answer this question, glomerular ROS was imaged with the use of 2', 7'-dichlorofluorescein diacetate (DCFH-DA). The image of DCF fluorescence was strong in glomeruli from diabetic rats as compared with that of glomeruli from nondiabetic control rats. mRNA expression of antioxidant enzymes such as catalase, glutathione peroxidase, Cu/Zn superoxide dismutase, and heme oxygenase-1 (HO-1) was also determined because oxidative stress definitely refers to the situation of an imbalance between the production of ROS and antioxidant defense. The mRNA expression of catalase, glutathione peroxidase, and Cu/Zn superoxide dismutase 2 wk after the induction of diabetes was not significantly different from that in control rats. Alternatively, mRNA and protein expression of HO-1 was strongly induced by 16-fold in diabetic glomeruli after the induction of diabetes. Antioxidant treatment with either vitamin E or probucol almost completely normalized HO-1 overexpression in diabetic glomeruli, supporting the existence of oxidative stress in the glomeruli of early diabetes. Furthermore, it has reported that antioxidant treatment with vitamin E, probucol, α-lipoic acid, or taurine normalized diabetes-induced not only renal dysfunction such as albuminuria and glomerular hypertension but also glomerular pathologies. In summary, oxidative stress by diabetes could play a crucial role in the development and progression of diabetic nephropathy, and antioxidant treatment could be a potential therapeutic procedure for diabetic nephropathy.

Diabetic nephropathy is a leading cause of end-stage renal failure, accounting for 35 to 40% of all new cases that require dialysis therapy worldwide. Recent clinical studies clearly demonstrated that hyperglycemia is an important causal factor in mediating the development and progression of diabetic kidney disease (1–3). However, a growing number of patients still have diabetic kidney disease, although diabetic patients with renal complications have been treated with intensive insulin treatment (1–3) and antihypertensive therapy with angiotensin-converting enzyme inhibitors and/or angiotensin II receptor antagonists (4–6). Therefore, an understanding of the molecular mechanisms by which prolonged exposure to hyperglycemia induces a large number of alterations in renal vasculature has become urgent to provide new insights into therapeutic strategies for diabetic kidney disease. Multiple biochemical mechanisms have emerged to explain the adverse effect of hyperglycemia, including protein kinase C (PKC), mitogen-activated protein kinase (MAPK) (7–9), polyl pathway (10,11), advanced glycation end products (AGE) (12), and oxidative stress (13).

Among these, oxidative stress has been suggested extensively as a potential mechanism for diabetic kidney disease because oxidative stress promotes the formation of AGE as well as PKC-MAPK activation (14,15). Indeed, involvement of oxidative stress has been indicated by the presence of lipid peroxidation products and 8-hydroxydeoxyguanosine in the kidney from streptozotocin (STZ)-induced diabetic rats (15,17). Regarding the defense system to oxidative stress, antioxidant enzymes such as catalase and Cu/Zn-superoxide dismutase (Cu/Zn-SOD) were found to be enhanced in kidneys of STZ-induced diabetic rats (18). However, it remains unknown whether these findings reflect a common consequence of the tissue damage in diabetic kidney or oxidative stress has a primary role in the pathogenesis of diabetic kidney disease. Our work shows that oxidative stress occurs in the glomeruli at the early stage of diabetes and induces enhancement of an antioxidant enzyme, heme oxygenase (HO)-1. We also show that these alterations are normalized by the treatment with antioxidants such as vitamin E and probucol (19).

Methods

Experimental Protocol

Male Sprague-Dawley (SD) rats weighing 180 to 200 g, purchased from Japan SLC (Shizuoka, Japan), were randomly separated into
control and diabetic rats with or without either vitamin E (40 mg/kg body wt every other day intraperitoneal injection; Eisai Co. Ltd., Tokyo, Japan) or probucol (given food mixed at the concentrations of 1%; Daiichi Pharmaceutical, Tokyo, Japan). Diabetic rats were made by intravenous injection of STZ (Sigma, St. Louis, MO) of 50 mg/kg body wt in 0.05 mmol/L citrate buffer (pH 4.5), and control rats received intravenous citrate buffer. These rats were maintained on laboratory diet and water *ad libitum* for 2 wk. All experiments were approved by Shiga University of Medical Science Animal Care Committees.

**Imaging of Reactive Oxygen Species**

Glomeruli isolated from control rats, diabetic rats, and diabetic rats treated with either vitamin E or probucol for 2 wk were incubated in Krebs-Henseleit bicarbonate buffered solution containing 2,7-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) at a final concentration of 5.0 μmol/L for 10 min. After washing twice with Krebs’ solution, glomeruli were placed on a slide glass. Images were collected with a confocal laser scanning microscopy (MRC600-UV; Bio-Rad Laboratories, Richmond, CA). The reactive oxygen species (ROS) generation was evaluated as the fluorescence intensity (an excitation wavelength 488 nm, an emission wavelength 515 nm) of DCF.

**Northern Blot Analysis**

Northern blot analysis was performed as described previously (20). In brief, a total RNA (12 μg per each lane) of isolated glomeruli using a commercial preparation (TriZol Reagent; Life Technologies BRL, Grand Island, NY) was electrophoretically separated on formaldehyde 1.0% agarose gel and transferred onto a nylon membrane (NYTRAN; Schleicher & Schuell, Dassel, Germany). After immobilizing, hybridization was performed in a buffer (0.5 mol/L sodium phosphate [pH 7.0], 1% BSA, 7% SDS, 1 mmol/L EDTA) containing cDNA labeled with α-[32P]CTP (New England Nuclear, Boston, MA) by a random primer method (BcaBEST; TAKARA, Shiga, Japan) at 65°C for 16 h. The hybridized filter was washed in a buffer (30 mmol/L sodium chloride, 3 mmol/L sodium citrate, and 0.1% SDS) at 65°C and autoradiographed with a Kodak XAR film. A radioactivity of the corresponding bands was measured quantitatively by a phosphoimager analyzer (Molecular Analyst, Bio-Rad Laboratories, Hercules, CA). After radioactive probes were stripped off the membrane, it was rehybridized with a radioactive probe of acidic ribosomal phosphoprotein PO (36B4) as an internal standard (21). Rat catalase cDNA was provided by Drs. T. Hashimoto and S. Yoshida (Shinshuu University, Matsumoto, Japan) (22). cDNA for rat Cu/Zn-SOD and glutathione peroxidase (GPx) was cloned by RT-PCR from total RNA isolated from rat hepatoma cell line (23,24). cDNA for rat HO-1 was cloned by RT-PCR from total RNA isolated from rat kidney (25).

**Western Blot Analysis**

Glomeruli were homogenized in 0.5 ml of ice-cold buffer (20 mmol/L Tris-hydrogen chloride [pH 7.4], 150 mmol/L sodium chloride, 2 mmol/L EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L sodium fluoride, 1 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, 10 μg/ml aprotinin, 1 mmol/L PMSF, and 1 μg/ml leupeptin). After sonication at 4°C for 10 s, glomerular homogenates were centrifuged at 12,000 × g at 4°C for 20 min and supernatants were used. After boiling for 5 min, samples (20 μg protein/lane measured by a Bio-Rad protein assay kit; Hercules, CA) were electrophoresed on 12% SDS–polyacrylamide gels and transferred to a polyvinylidene difluoride filter (Immobilon; Millipore, Bedford, MA) for 1 h at 100 mA using HORIZBLOT AE6677P (ATTO, Tokyo, Japan). For blocking, the filter was incubated in 5% nonfat milk in buffer containing 10 mmol/L Tris-hydrogen chloride (pH 7.6), 150 mmol/L sodium chloride, and 0.1% Tween-20 (TBS-T) at 4°C overnight. The filter was then washed several times over 30 min in TBS-T at room temperature and incubated with rabbit polyclonal antibodies against rat HO-1 (Stress-Gen, Victoria, BC, Canada) for 1 h at room temperature at dilution of 1:1000 in TBS-T with 5% milk. After multiple washes in TBS-T, the filter was incubated with a horseradish peroxidase–conjugated donkey anti-rabbit IgG secondary antibody for 1 h at 1:1000 dilutions in TBS-T with 5% milk. After washing the filter several times with TBS-T, the specific proteins were detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

**Results**

**Animal Characteristics**

The blood glucose levels in diabetic rats were significantly higher than in control rats. Body mass was smaller and kidney mass was heavier in diabetic rats than in control rats. The treatment of antioxidants with either probucol or vitamin E did not affect blood glucose levels, body weights, and kidney weights in both control and diabetic rats.

**Imaging of ROS**

The image of DCF fluorescence was strong in glomeruli from diabetic rats as compared with that of glomeruli from control rats (Figure 1). This increased DCF fluorescence in

**Control**

- - -

**Vitamin E**

- + -

**Probucol**

- - +

**Diabetic**

- - +

**Vitamin E**

- + -

**Probucol**

- - +

*Figure 1*. Representative images of ROS in isolated glomeruli from control rats, control rats treated with or without vitamin E or probucol, diabetic rats, and diabetic rats treated with or without vitamin E and probucol. The isolated glomeruli from three rats of each group were incubated in Krebs-Henseleit bicarbonate buffered solution containing DCFH-DA at a final concentration of 5.0 μmol/L for 10 min. Images were collected with a confocal laser scanning microscopy. The ROS generation was evaluated as the fluorescent intensity of DCF. A representative was shown from three independent experiments. Magnification, ×200.
glomeruli of diabetic rats was normalized by the treatment with either vitamin E or probucol (Figure 1).

**mRNA Expression of Antioxidant Enzymes**

The mRNA expression of catalase and GPx was slightly increased in glomeruli of diabetic rats but not significantly different from mRNA expression in control rats (data not shown). The mRNA expression of Cu/Zn SOD did not differ between control and diabetic rats (data not shown). However, mRNA expression of HO-1 was significantly enhanced in the glomeruli of diabetic rats as compared with that in control rats (Figure 2). Moreover, protein expression of HO-1 was also significantly increased in the glomeruli of diabetic rats as compared with that in control rats (Figure 2). Increased mRNA and protein HO-1 expression in glomeruli of diabetic rats was completely normalized by the treatment with either probucol or vitamin E (Figure 2), similar to the results of ROS images.

**Discussion**

In the present study, we clearly demonstrated that excessive oxidative stress occurs in vivo in glomeruli of diabetic rats, resulting in an enhancement of glomerular expression of HO-1 without altering the expression of constitutive antioxidant enzymes such as catalase, GPx, and Cu/Zn SOD. The enhancement of HO-1 expression by oxidative stress was further confirmed by normalization of mRNA and protein overexpression of HO-1 in diabetic rats treated with antioxidants in parallel with the decrease in oxidative stress in glomeruli.

Increased formation of ROS may occur in diabetes for reasons possibly related to an increase in glucose concentrations in plasma and tissues (14,15) and may have a role in the pathogenesis of diabetic nephropathy. However, the functional and pathophysiologic role of excessive oxidative stress in diabetic kidney disease was indicated merely by the presence of increased levels of lipid peroxides and 8-hydroxydeoxyguanosine in the kidney of STZ-induced diabetic rats (16,17). These observations are lacking in the direct evidence for the presence of oxidative stress in glomeruli and might reflect a common consequence of diabetic kidney damage. Here, we provide the evidence for the presence of excessive oxidative stress in glomeruli of diabetic rats using a fluorescent dye, DCFH-DA, which has been used to estimate the formation of ROS or the oxidative metabolites (26). Moreover, we also found that vitamin E, a widely known antioxidant, was able to reverse intense ROS production in glomeruli of diabetic rats. Our findings at an early stage in diabetes do not reflect a common consequence of diabetes-induced glomerular damage but could support the primary role of oxidative stress in the pathogenesis of diabetic nephropathy.

In response to oxidative stress, antioxidant enzymes are induced to protect cellular and tissue injury (18,19,27). Although Cu/Zn SOD and catalase mRNA levels have been shown to be significantly induced in the total kidney of untreated diabetic rats (18), we were not able to find any change in mRNA content of Cu/Zn SOD, GPX, and catalase in glomeruli of diabetic rats (19). Because HO-1, a stress response protein, is highly induced in response to various agents that cause oxidative stress (28,29), we thus examined the expression of mRNA and protein of HO-1 in glomeruli of diabetic rats. Both mRNA expression and protein expression of HO-1 were significantly increased in glomeruli of diabetic rats, whereas control rats exhibited only a faint expression of HO-1. These findings are the first demonstration of HO-1 induction in the glomeruli of diabetic rats. We also found that increased HO-1 mRNA and protein content in glomeruli of diabetic rats was completely normalized by the treatment with either vitamin E or probucol. Because antioxidant treatment affected neither blood glucose level nor the body weight of the diabetic rats, the inhibitory effect of antioxidants on diabetes-induced overexpression of HO-1 seems to be mediated possibly through scavenging oxidative stress.

The biologic significance of glomerular HO-1 induced by diabetes, however, is still unknown in the present study. Recently, emerging attention has focused on the beneficial role of HO-1 in protecting a variety of tissues from oxidative and inflammatory injury (30–32). Therefore, glomerular HO-1 induction by diabetes may have protective roles in the development of diabetic nephropathy. Further study is necessary to clarify the precise function of HO-1 in diabetic kidney disease.

In conclusion, we provided evidence that oxidative stress occurs in vivo in glomeruli of early diabetic rats and that antioxidant treatment can reverse oxidative stress, suggesting the beneficial effect of antioxidant treatment in diabetic kidney disease.
References


