

Hypertension Increases Pro-Oxidant Generation and Decreases Antioxidant Defense in the Kidney in Early Diabetes

Subrata K. Biswas Elisa B. Peixoto Denise S. Souza Jose B. Lopes de Faria

Renal Pathophysiology Laboratory, Division of Nephrology, Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas, Campinas, São Paulo, Brazil

Key Words

Diabetes · Hypertension · Nephropathy · Pro-oxidants · Antioxidants · Oxidative stress

Abstract

Aims: The combination of hypertension and diabetes exacerbates renal oxidative stress. The aim of the present study was therefore to evaluate the pro-oxidant and antioxidant mechanisms responsible for the induction of renal oxidative stress in the presence of hypertension and diabetes mellitus. **Methods:** Diabetes was induced in spontaneously hypertensive rats (SHR) and their genetically normotensive control Wistar-Kyoto (WKY) rats by streptozotocin at 12 weeks of age. After 10 days, pro-oxidant, antioxidant and oxidative stress parameters were evaluated in the renal tissue. **Results:** NADPH oxidase-dependent superoxide generation in the renal cortex was significantly elevated in WKY and SHR diabetic (D) groups compared to the respective control (C) groups ($p < 0.005$, $n = 5$). However, the highest level of superoxide generation was observed in the SHR-D group compared to all other groups. The expression of the gp91phox subunit of NADPH oxidase was significantly elevated in the

SHR-D ($p < 0.05$, $n = 5$), but not in the WKY-D group, compared to the respective control groups. The renal cortical extracellular-superoxide dismutase level was found to be markedly decreased in the SHR groups compared to the WKY groups ($p < 0.05$, $n = 5$). The antioxidant glutathione level was found to be lower in the SHR-D ($p = 0.03$, $n = 15$), but not in the WKY-D group, compared to the respective control groups. Finally, nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, markers of oxidative stress, were found to be similar in the kidneys of WKY-C and WKY-D, but were elevated in the SHR-D compared to the SHR-C group. **Conclusion:** We therefore conclude that hypertension increases pro-oxidant generation and decreases antioxidant defense, and thereby induces renal oxidative stress in early diabetes.

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Introduction

Diabetes mellitus (DM) and hypertension are the most important causes of nephropathy and end-stage renal disease (ESRD). A recent report shows that DM constitutes about half and hypertension constitutes about one third of the new cases of ESRD [1]. However, the underlying pathophysiological mechanism in the development of nephropathy and ESRD resulting from DM or hypertension are not clear. Genetic susceptibility and a complex interaction among a number of metabolic and hemody-

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Dr. Jose B. Lopes de Faria, MD, PhD
Renal Pathophysiology Laboratory, Division of Nephrology
Department of Internal Medicine, Faculty of Medical Sciences
State University of Campinas, 13084-971 Campinas, São Paulo (Brazil)
Tel. +55 19 3521 7499, Fax +55 19 3521 7366, E-Mail jblfaria@fcm.unicamp.br

namic factors, including reactive oxygen species (ROS), advanced glycation end-products, polyols, protein kinase C (PKC) isoforms, systemic and intraglomerular hypertension, and various vasoactive hormones are involved in the pathogenesis of nephropathy and its progression to ESRD [for review see 2–4]. However, recent studies strongly implicate the contribution of renal oxidative stress to the pathogenesis of nephropathy and ESRD [5–7]. In fact, oxidative stress has been demonstrated in the kidney in almost all types of nephropathy whatever the primary disease of its origin might be [6, 7].

Hyperglycemia is well known to induce oxidative stress through exaggerated generation of ROS from the mitochondrial electron transport chain [8]. Mitochondrial superoxide can inhibit the glycolytic enzyme glyceral-3-phosphate dehydrogenase and thereby can activate several pathways of hyperglycemic damage, including advanced glycation end-products, PKC, polyol, hexosamine and nuclear factor- κ B pathways [8, 9]. PKC-induced NADPH oxidase activation further enhances pro-oxidant generation and oxidative stress in hyperglycemia [9, 10]. Moreover, NADPH oxidase Nox4 is also directly involved in enhanced superoxide generation in the kidney in early diabetes [11]. Furthermore, neutralization of ROS or alleviation of renal oxidative stress in diabetes has been found to improve the functional and structural features of diabetic nephropathy, including albuminuria, renal hypertrophy, mesangial expansion and glomerular accumulation of fibronectin and collagen IV [11–14]. As in the case of DM, hypertension also induces oxidative stress in the kidney. In fact, exaggerated oxidative stress in the kidney has been shown in almost all animal models of hypertension, including spontaneously hypertensive rats (SHR), a rat model of human essential hypertension [15, 16]. Therefore, DM- or hypertension-induced oxidative stress in the kidney may participate in the pathogenesis of nephropathy and ESRD.

DM and hypertension frequently coexist in humans [17] and their combination increases the frequency and severity of nephropathy, sometimes in an additive manner [18, 19]. However, the underlying mechanistic basis of how the combination of diabetes and hypertension aggravates renal disease is not clear. In a recent study, we demonstrated that the presence of hypertension increases oxidative stress in the kidney in the early stage of experimental diabetes [20]. We have extended our findings in the present study with the aim of identifying specific abnormalities by which hypertension leads to early and enhanced oxidative stress in the kidneys of DM subjects.

Subjects and Methods

Animals and Experimental Protocol

The protocol for this study complied with the guidelines established by the Brazilian College of Animal Experimentation and was approved by the Institutional Ethical Committee. All reagents were purchased from Sigma, St Louis, Mo., USA, unless stated otherwise. The SHR and their genetically normotensive control Wistar-Kyoto (WKY) rats, derived from animals supplied by Taconic (Germantown, N.Y., USA) and bred in our animal facility, were used in this study. Rats were housed in a room maintained at around 22°C, exposed to a 12-hour dark/light cycle, and allowed free access to food and tap water. Diabetes was induced in 12-week-old hypertensive male SHR and their normotensive counterparts, age- and sex-matched WKY rats, by a single injection of streptozotocin (STZ, 50 mg/kg) dissolved in sodium citrate buffer (pH 4.5) via the tail vein after an overnight fast. Control groups received only vehicle (citrate buffer). Plasma glucose levels were measured using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) 72 h after the injection of STZ or citrate buffer. Plasma glucose concentrations of >15 mM/l were considered diabetic for these experiments.

Control and diabetic rats from each group were sacrificed using CO₂ gas 10 days after induction of diabetes. The abdomen was opened via a midline incision and the right kidney was immediately removed, decapsulated, weighed and further processed for homogenization of the cortical tissue. The left kidney was similarly removed and cut longitudinally into 2 halves. Part of the cortical tissue of one half was frozen in liquid nitrogen and preserved at –80°C, and the other half was fixed by immersion in a solution of methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid). One day before sacrifice, urine samples were collected by placing each rat in individual metabolic cages for 24 h. Urine samples were analyzed by single radial immunodiffusion to determine the albumin excretion rate (AER) as described previously [20].

Blood Pressure Determinations

Systolic blood pressure was obtained by tail-cuff plethysmography (3–5 determinations per rat) in unanesthetized rats using an MK III physiograph (Narco Bio-System, Houston, Tex., USA) before the day of diabetes induction and on the 9th day after induction. Rats were habituated to the procedure before taking blood pressure readings.

Preparation of Renal Cortical Extract

A renal cortical extract was prepared in tissue homogenization buffer (30 mM Tris-HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM DTT and 250 mM sucrose) supplemented with a cocktail of protease inhibitors as described previously [21].

NADPH Oxidase Activity

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as previously described by Gorin et al. [11] with a few modifications. A small piece (around 50 mg) of frozen kidney cortex was homogenized in 1 ml lysis buffer (20 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and a cocktail of protease inhibitors 40 μ l/ml) using a Dounce homogenizer (100 strokes on ice). Homogenates were subjected to low speed centrifugation at

800 g for 10 min at 4°C to remove unbroken cells and debris, and aliquots were used immediately. To start the assay, 50 µl of homogenates were added to 450 µl reaction buffer (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 luminescence units (RLU) was measured every 20 s for 10 min in a Turner TD-20e luminometer. There was no measurable activity in the absence of NADPH or tissue homogenate. To identify which pathway contributes superoxide production, tissue homogenates were pre-incubated on ice with diphenyleneiodonium and rotenone, at 20 and 100 µM final concentration for 10 min. Superoxide production was expressed as RLU/20 s/mg protein. Protein concentration was measured using the Bradford method and BSA as standard.

Western Blotting

Renal cortical homogenate was used for quantification of gp91phox, Nox4, extracellular superoxide dismutase (EC-SOD), heme oxygenase-2 (HO-2) and nitrotyrosine by Western blot analysis. Molecular weight markers (PageRuler™, Fermentas Life Sciences) were used as standards. To block nonspecific binding and antibody incubation, 1–5% non-fat milk was used in phosphate-buffered saline containing 0.1% Tween-20 (PBST). Fifty grams of cortical protein was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, Calif., USA). After blocking nonspecific binding, the membranes were incubated with a rabbit polyclonal anti-gp91phox IgG (1:750; Upstate, Lake Placid, N.Y., USA), a rabbit polyclonal anti-Nox4 (1:2,000; a gift from Dr. Karen Block), a rabbit polyclonal anti-EC-SOD (1:2,000; SOD-105, Stressgen Bioreagents Corp., Victoria, B.C., Canada), a rabbit polyclonal anti-HO-2 (1:3,000; Stressgen), or a mouse monoclonal anti-nitrotyrosine antibody (1:2,000; clone 1A6, Upstate). After washing with PBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, Ill., USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were re-probed for actin (goat polyclonal anti-actin antibody, 1:1,000, Santa Cruz). Exposed films were scanned with a laser densitometer (Bio-Rad) and were analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

Immunohistochemistry

To detect oxidative stress-induced DNA base modification, immunohistochemistry was done for 8-hydroxy-2'-deoxyguanosine (8-OHdG, a DNA base-modified product) in methacarn-fixed paraffin-embedded renal tissue sections (4 µm). After microwave exposure and blocking of endogenous peroxidase with 3% H₂O₂, slides were incubated with a 1:50 dilution of a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan), and subsequently a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, Calif., USA). After incubation with avidin-biotin complex reagent (Dako, Glostrup, Denmark), slides were developed in diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For negative controls, staining was performed omit-

ting the primary antibody or by using an irrelevant immunoglobulin. Tubulointerstitial cells containing 8-OHdG-positive nuclei were counted in 50 sequential high power microscopic fields (×400).

Determination of Reduced Glutathione Concentration

Renal cortical glutathione (GSH) level was measured by the method of Beutler et al [22] as described previously [21]. Briefly, a small piece of frozen kidney cortex was weighed and directly homogenized in cold 10% trichloroacetic acid on ice. Homogenate was centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was reacted with 0.3 M phosphate buffer and 0.04% 5,5'-dithiobis-(2-nitrobenzoic acid). Absorbance was read at 412 nm and the GSH concentration was expressed as micromoles of GSH per gram of frozen tissue from a standard curve. GSH (reduced form) was used as an external standard for preparation of a standard curve.

Statistical Analysis

The results are expressed as means ± SD. Comparisons between groups were done with ANOVA followed by Bonferroni corrected t test.

Nonparametric data are expressed as median (range), and are analyzed by Mann-Whitney U test (for 2 groups) and Kruskal-Wallis test (for multiple groups). Statistical significance was set at $p < 0.05$. All analyses were performed using statistical software StatView (SAS Institute Inc., Cary, N.C., USA).

Results

Physical and Metabolic Parameters

WKY rats and SHR were used in this study as normotensive and hypertensive animal models, respectively. Diabetes was induced for 10 days in 12-week-old WKY and SHR. Body weights of the diabetic rats were significantly reduced compared to the control rats in both rat strains (table 1). The SHR were hypertensive, and the systolic blood pressure of SHR was significantly higher than that of WKY ($p < 0.001$; table 1). However, STZ-induced diabetes of 10 days duration had no effect on systolic blood pressure in either WKY or SHR. Fasting plasma glucose levels were significantly elevated ($p < 0.001$; $n = 15$ in each group) in the WKY-diabetic (WKY-D) and SHR-diabetic (SHR-D) groups compared to the respective control (C) groups (table 1). However, there was no difference in fasting plasma glucose levels between the WKY-D and SHR-D groups. Diabetic rats showed hypertrophy of the kidney as evidenced by significantly higher kidney weight ($p < 0.02$; $n = 15$) and kidney weight/body weight ratio ($p < 0.001$; $n = 15$) in the diabetic groups than in the respective control groups (table 1). In addition, urinary AER was similarly elevated in both rat strains after 10 days of experimental diabetes ($p < 0.02$).

Table 1. Physical and metabolic parameters of the experimental groups at 10 days of diabetes mellitus

Group	Body weight g	Systolic BP mm Hg	Plasma glucose mM	Kidney weight g	KW:BW ratio, %	AER mg/day
WKY-C	415 ± 30	131 ± 9	8.7 ± 0.80	1.37 ± 0.16	0.33 ± 0.03	0.42 (0.30–0.91)
WKY-D	318 ± 30 ^a	130 ± 11	24.9 ± 2.19 ^a	1.65 ± 0.18 ^a	0.50 ± 0.05 ^a	1.15 (0.89–2.09) ^a
SHR-C	254 ± 37 ^b	187 ± 15 ^d	8.0 ± 0.95	0.90 ± 0.10	0.36 ± 0.08	0.41 (0.34–0.61)
SHR-D	201 ± 26 ^{a-c}	188 ± 16 ^d	25.69 ± 1.42 ^a	1.05 ± 0.12 ^a	0.51 ± 0.03 ^a	1.30 (0.74–2.23) ^a

Data are means ± SD and n = 15 per group. The 24-hour urinary albumin excretion rate (AER) is expressed as the median (range) and was analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test.

KW = Kidney weight; BW = body weight; C = control; D = diabetic.

^a p < 0.02 vs. the respective control group; ^b p < 0.001 vs. WKY-C; ^c p < 0.001 vs. WKY-D; ^d p < 0.001 vs. WKY-C/D.

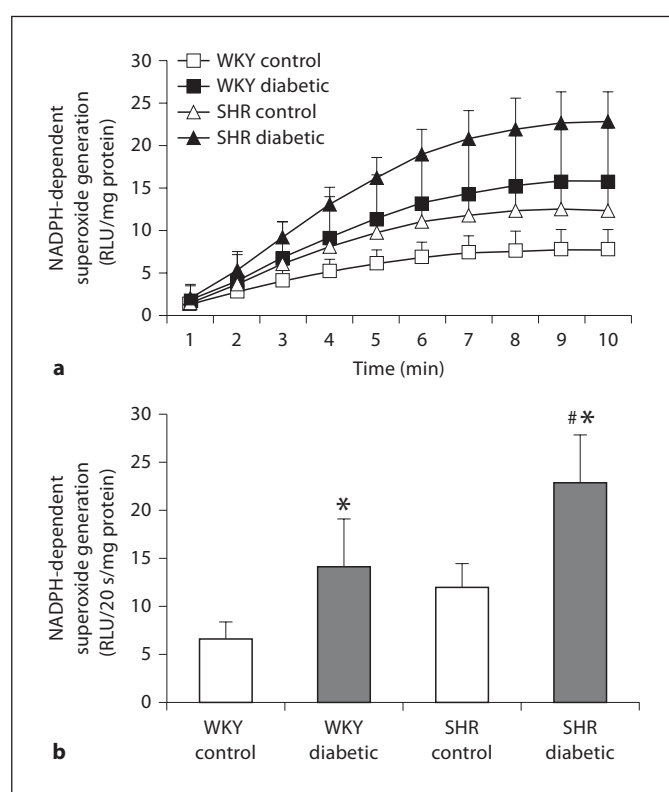


Fig. 1. NADPH oxidase activity in renal cortical homogenates. **a** Superoxide anion generation was determined by the lucigenin-enhanced chemiluminescence method and photoemission was measured every 20 s for 10 min. The peak level of superoxide generation was observed around 10 min after addition of NADPH to the reaction mixture containing renal cortical homogenates from different groups of rats. **b** Average NADPH oxidase activities during the last 5 min was used for comparison among the groups. NADPH-dependent superoxide generation was expressed as relative luminescence units (RLU)/20 s/mg protein. Bars represent the means ± SD of the activities from the kidney cortex of more than 5 rats per group. * p < 0.005 versus the respective control group; # p = 0.002 versus the WKY diabetic group.

Pro-Oxidant Parameters

NADPH oxidase is the major source of pro-oxidant superoxide in the vascular tissue [23]. We evaluated NADPH oxidase activity in the kidney cortex using the lucigenin-enhanced chemiluminescence method. Figure 1a shows the changes in the rate of reaction over 10 min after the addition of NADPH into the reaction mixture containing renal cortical homogenate, and figure 1b shows the average NADPH oxidase activity during the last 5 min of the measuring time. NADPH-dependent superoxide production was significantly increased in the renal cortical homogenates of diabetic animals compared with controls (WKY-C 6.54 ± 1.90 vs. WKY-D 14.05 ± 5.02 RLU/20 s/mg protein, $p = 0.001$; SHR-C 11.90 ± 2.56 vs. SHR-D 22.90 ± 5.04 , $p < 0.001$; $n = 5$ per group; fig. 1b). However, the highest level of superoxide generation was observed in the SHR-D group, which was significantly higher than the WKY-D group ($p = 0.002$). To identify the source of superoxide production, we used diphenyleneiodonium, an inhibitor of flavin-containing oxidases, and rotenone, an inhibitor of complex I of mitochondrial respiratory chain. Pre-incubation of the renal cortical homogenates with diphenyleneiodonium ($20 \mu\text{M}$ final concentration) completely blocked NADPH-induced superoxide production, while pre-incubation with rotenone (20 and $100 \mu\text{M}$ final concentration) did not produce any effect on superoxide production, indicating that the NADPH oxidase is the most likely source of the superoxide we detected.

To establish the involvement of the NADPH oxidase system in the exaggerated production of renal cortical superoxide in diabetes, we also evaluated the expression of NADPH oxidase subunit gp91phox in the renal cortex. We observed that the gp91phox expression did not alter in WKY groups after induction of diabetes (WKY-C 0.74

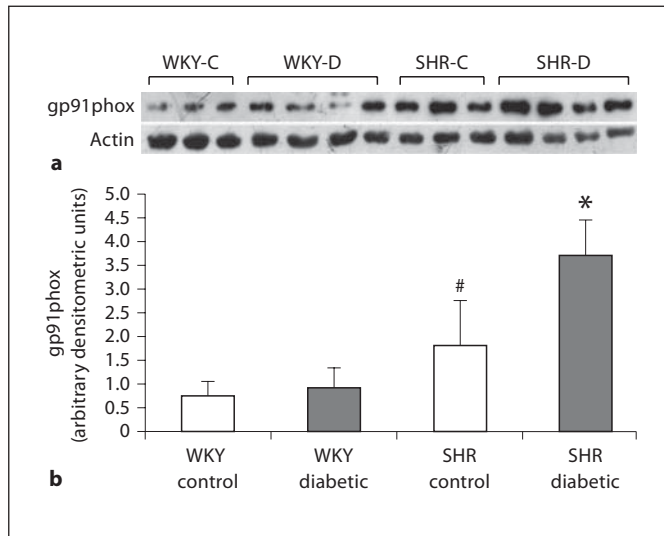


Fig. 2. a Western blot analysis of renal cortical gp91phox. Expression of the NADPH oxidase subunit gp91phox was determined by Western blot analysis of renal cortical homogenate. Representative Western blots from different groups of rats. Actin was used as a control of protein loading. **b** Densitometric analysis of the gp91phox/actin ratio in different groups of rats. Bars represent the means \pm SD of at least 3 independent experiments; $n = 5$ in each group. * $p < 0.05$ versus all other groups; # $p < 0.05$ versus WKY control. C = Control; D = diabetic.

± 0.31 vs. WKY-D 0.93 ± 0.41 expressed as a ratio of gp91phox/actin in densitometric units; fig. 2). However, the expression of gp91phox was significantly increased ($p < 0.05$; $n = 5$) in the SHR-D group (3.70 ± 0.76) compared with the SHR-C group (1.82 ± 0.95). In fact, the highest level of the expression of gp91phox was observed in the SHR-D group compared with all other groups ($p < 0.05$; $n = 5$). We also noticed that the expression of gp91phox was significantly higher in the SHR-C than the WKY-C group ($p < 0.05$; $n = 5$; fig. 2).

We also evaluated Nox4, a homologue of gp91phox, which has recently been implicated in the generation of superoxide and induction of oxidative stress in the kidney in early diabetes [11]. However, in our situation, Nox4 protein expression in the renal cortex was found unaltered in both the WKY (WKY-C 1 ± 0.18 vs. WKY-D 0.89 ± 0.26 densitometric units; $n = 5$) and SHR (SHR-C 1 ± 0.30 vs. SHR-D 1.25 ± 0.55 ; $n = 5$) groups after induction of diabetes for 10 days.

Antioxidant Parameters

As a measure of the protection against pro-oxidant stress in diabetes, we evaluated the antioxidant system in

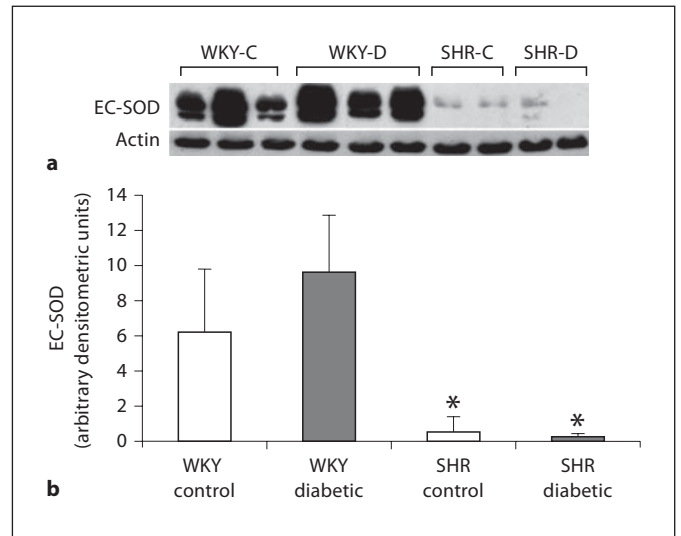


Fig. 3. a Western blot analysis of renal cortical extracellular superoxide dismutase (EC-SOD). Representative Western blots from different groups of rats. EC-SOD protein appeared as a double-band at the 34- and 32-kDa positions. Actin was used as control of protein loading. **b** Densitometric analysis of both bands of EC-SOD/actin ratio in different groups of rats. Bars represent the means \pm SD of at least 3 independent experiments; $n = 5$ in each group. * $p < 0.05$ versus WKY control/diabetic. C = Control; D = diabetic.

the renal cortical tissue. EC-SOD, one of the most important enzymatic antioxidants that provides defense against superoxide, was measured by Western blotting. As was demonstrated previously [24], two bands at around the 34- and 32-kDa positions were identified for EC-SOD in the renal cortical tissue. In spite of large intra-group variation in EC-SOD protein expression, we observed that the expression of EC-SOD in the renal cortex was extremely low in the SHR groups compared with the WKY groups ($p < 0.05$; $n = 5$ in each group; fig. 3). However, the induction of diabetes for 10 days did not significantly modify EC-SOD expression in either the WKY group (WKY-C 6.21 ± 3.60 vs. WKY-D 9.65 ± 3.19 expressed as a ratio of EC-SOD/actin in densitometric units) or the SHR group (SHR-C 0.56 ± 0.85 vs. SHR-D 0.27 ± 0.14).

We also assessed the non-enzymatic antioxidant GSH (reduced form), which provides important protection against the pro-oxidant molecule hydrogen peroxide. As we demonstrated previously [20], the GSH level did not show any significant difference between the WKY-C and SHR-C groups. However, the induction of diabetes for 10 days significantly decreased the GSH level in the SHR

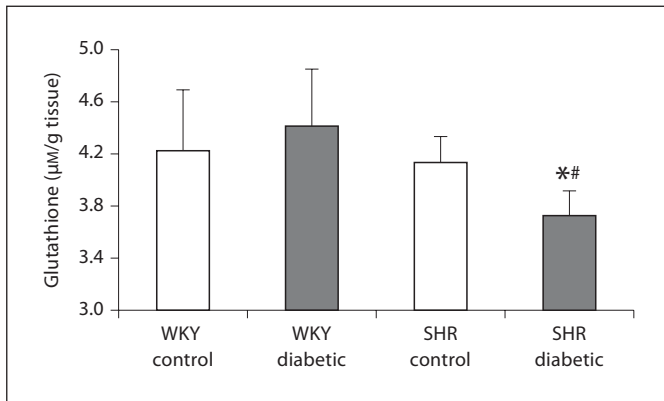


Fig. 4. Renal cortical reduced glutathione (GSH) level. The GSH concentration is expressed in $\mu\text{M/g}$ frozen tissue. Data are means \pm SD. * $p = 0.03$ versus SHR-C; # $p < 0.05$ versus all other groups. $n = 15$ in each group.

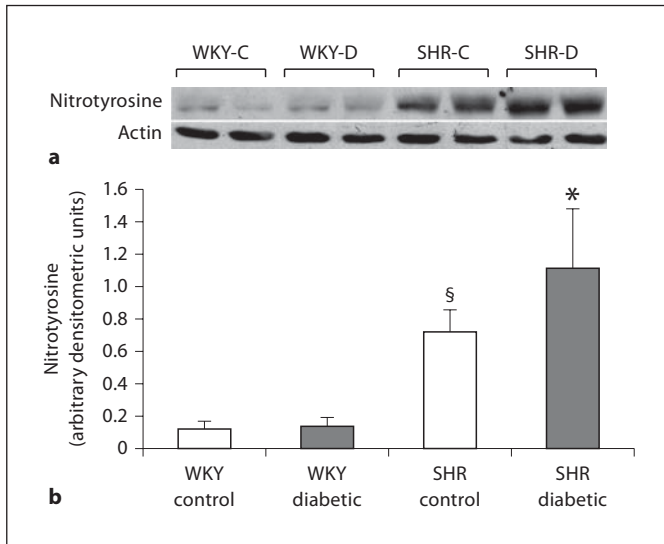


Fig. 5. a Western blot analysis of renal cortical nitrotyrosine. Representative Western blots from different groups of rats. Actin was used as a control of protein loading. **b** Densitometric analysis of nitrotyrosine/actin ratio in different groups of rats. Bars represent means \pm SD of at least 3 independent experiments; $n = 5$ in each group. * $p = 0.042$ versus SHR control; [§] $p = 0.001$ versus WKY control. C = Control; D = diabetic.

group (SHR-C 4.13 ± 0.20 vs. SHR-D 3.73 ± 0.19 $\mu\text{M/g}$ tissue; $p = 0.03$; $n = 15$), but not in the WKY group (WKY-C 4.22 ± 0.47 vs. WKY-D 4.41 ± 0.44 ; $n = 15$; fig. 4). Consequently, the lowest level of GSH was observed in the SHR-D group compared with all other groups ($p < 0.05$).

Another antioxidant, HO-2, is involved in bilirubin metabolism and provides protection against pro-oxidant stress particularly in the context of kidney tissue [25]. However, we did not observe any alteration in HO-2 expression in the kidney tissue after induction of diabetes for 10 days in either the WKY (WKY-C 0.56 ± 0.53 vs. WKY-D 0.82 ± 0.67 expressed as a ratio of HO-2/actin in densitometric units; $n = 5$) or the SHR (SHR-C 0.93 ± 0.53 vs. SHR-D 0.48 ± 0.40) groups.

Oxidative Stress Parameters

To identify whether the altered balance between pro-oxidant stress and antioxidant defense after a short-term of experimental diabetes culminates in oxidative tissue injury, we measured nitrotyrosine, a marker of oxidative/nitrosative stress-induced protein modification, and 8-OHdG, a marker of oxidative stress-induced DNA base modification, in renal cortical tissue. The nitrotyrosine level was found to be significantly elevated in the SHR group (SHR-C 0.72 ± 0.14 vs. SHR-D 1.11 ± 0.37 expressed as a ratio of nitrotyrosine/actin in densitometric units; $p = 0.042$, $n = 5$), but not in the WKY group (WKY-C 0.12 ± 0.05 vs. WKY-D 0.14 ± 0.05 ; $n = 5$), after the induction of diabetes for 10 days (fig. 5). We also noticed a markedly elevated level of renal cortical nitrotyrosine expression in the SHR-C group compared with the WKY-C group ($p = 0.001$; $n = 5$). In case of an oxidative stress-induced DNA base modification, we detected renal cortical cells containing the modified base 8-OHdG in the nucleus involving mainly the tubular cells of inner cortical region, as described previously [20]. The number of renal cortical cells containing 8-OHdG-positive nuclei (per 50 high power fields) was found to be elevated ($p = 0.001$; $n = 6$) in the SHR-D group (median 335 range 295–445) compared with the SHR-C group (median 155, range 31–308). However, there was no difference in the number of cells containing 8-OHdG-positive nuclei between the WKY-C (median 85, range 26–297) and the WKY-D (median 97, range 39–178) groups ($n = 6$; fig. 6).

Discussion

In the present study, we found that the presence of hypertension increases renal oxidative stress by increasing pro-oxidant generation and decreasing antioxidant defense in the early stage of experimental diabetes. It is known that both hypertension and diabetes individually increase oxidative stress [8, 9, 11–16]. However, identification of their individual contribution to oxidative stress

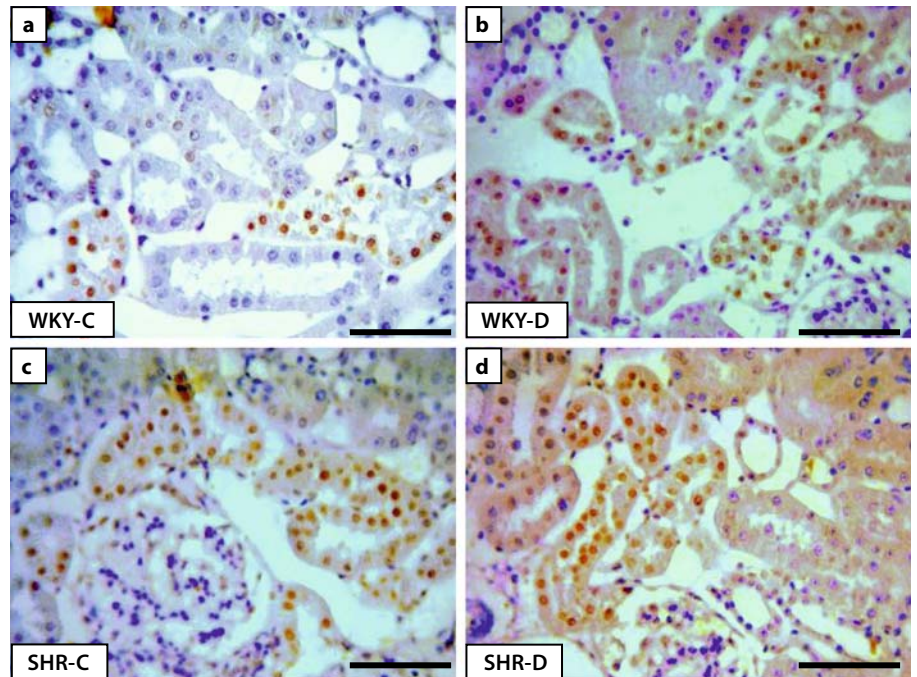
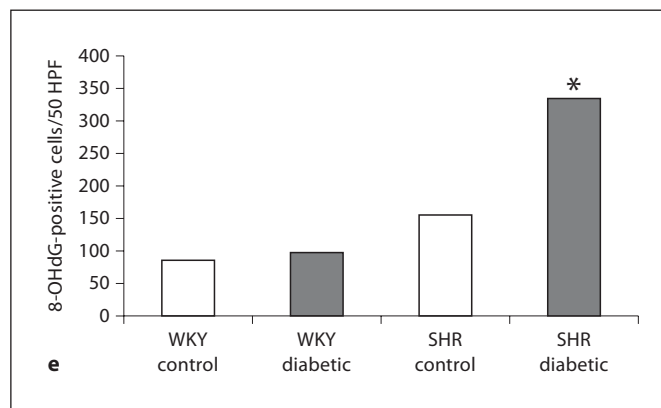


Fig. 6. Immunohistochemical detection of oxidative stress-induced DNA damage. **a–d** Photomicrographs of the immunohistochemical identification of 8-OHdG-containing cells in the kidney cortex. Tubulointerstitial cells containing 8-OHdG were identified by their brown-colored (darker) nuclei. Original magnification $\times 400$. Counterstained with hematoxylin. Scale bar = 50 μm . **e** The median number of tubulointerstitial 8-OHdG-containing cells per 50 HPF. At least 3 sections were stained and evaluated for each rat. * $p = 0.001$ versus SHR-C. $n = 6$ in each group. Data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test. HPF = High-power field; C = control; D = diabetic.



is difficult when both hypertension and diabetes exist concomitantly. Here, we circumvent this problem by using a duration factor, because we demonstrated previously that oxidative stress does not increase in the kidney with a short duration of diabetes of 10 days, but it increases in the presence of hypertension with the same duration of diabetes [20]. Therefore, in the present study, we tried to identify the influence of hypertension on pro-oxidant generation and antioxidant defense in the kidney 10 days after induction of experimental diabetes.

The free radical superoxide is a primary reactive species that generates several other reactive species (or pro-oxidants) of physiological significance as products of the downstream reaction cascade [26]. The superoxide is

produced by the mitochondrial respiratory chain and by NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxygenase, nitric oxide synthase and cytochrome p450 [27]. In the present study, however, we did not evaluate mitochondrial superoxide generation despite its well-documented involvement in the pathogenesis of diabetic complications [8, 9]. Elegant studies performed by Nishikawa et al. [8] showed that a high level of glucose rapidly increases superoxide generation in bovine aortic endothelial cells through the mitochondrial respiratory chain. We therefore hypothesized that a similar level of plasma glucose, as observed in our WKY-D and SHR-D groups, would produce a similar extent of superoxide through the mitochondrial respiratory chain. As the main focus of the

present study was to identify the contribution of hypertension to renal oxidative stress in the presence of hyperglycemia, we rather evaluated the NADPH oxidase system. Because NADPH oxidase-mediated renal oxidative stress has been demonstrated in hypertension [28], and high blood pressure has been shown to promote the expression of NADPH oxidase subunits in the arterial wall [29]. Furthermore, all or most of the components of the NADPH oxidase system have been reported to be present in different cell types in the kidney [30]. Therefore, we considered that the NADPH oxidase system might be more pertinent to the question we intended to answer in the present study.

Our present finding clearly demonstrates that the NADPH-induced superoxide generation increases in the kidney in the early stage of diabetes. This finding, therefore, supports many previous data showing that hyperglycemia in animals, or high glucose in cell culture media, rapidly induces pro-oxidant generation [8, 11–13]. However, a more important part of our data is that the hypertensive diabetic rats showed the highest level of superoxide production compared with normotensive diabetic rats or non-diabetic hypertensive rats. This finding is supported by the expression of gp91phox, the catalytic subunit of NADPH oxidase, the expression of which was found to be highest in the hypertensive diabetic group compared with all other groups. However, the expression of gp91phox increased in hypertensive SHR, but not in normotensive WKY, after the induction of diabetes. Apparently this finding does not correlate with NADPH-induced superoxide generation which was increased in both normotensive and hypertensive diabetic rats. It seems that the NADPH oxidase activity could be increased up to a certain level without an increase in gp91phox expression; however, for a greater increase in this enzyme activity, as seen in hypertensive diabetic groups, a significant elevation in the expression of gp91phox is needed. In fact, the NADPH oxidase enzyme complex is composed of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox and p40phox). Upon activation, cytosolic subunits are phosphorylated and translocated to the membrane and participate in superoxide generation [31]. Thus, the enzymatic activity of NADPH oxidase depends on the coordinated participation of several subunits, rather than on the expression of a particular subunit.

Among the other sources of pro-oxidant generation in diabetes, Gorin et al. [11] demonstrated that the Nox4, a homologue of the gp91phox subunit of NADPH oxidase, is a major source of renal cortical superoxide production

in early diabetes. They observed an overexpression of Nox4, and Nox4-mediated exaggerated renal cortical superoxide production in Sprague-Dawley rats 14 days after the induction of diabetes [11]. However, in the present study, we found that the renal cortical Nox4 expression did not alter after 10 days of induction of diabetes, either in WKY or in SHR. This discrepant finding could, at least partly, be explained by rat strain and duration of diabetes.

SODs are very important enzymatic antioxidants that rapidly catalyze the dismutation of superoxide. In case of a deficiency in SOD (or increased production of superoxide), the superoxide preferentially reacts with nitric oxide and produces peroxynitrite, a powerful oxidizing and nitrating agent that can directly damage proteins, lipids, and DNA [32]. Among the three mammalian isozymes of SOD, EC-SOD (the other two are Cu,Zn-containing intracellular SOD and Mn-containing mitochondrial SOD) constitutes as much as 70% of the total SOD activity in human and baboon aorta [33], and high levels of EC-SOD expression have been observed in the lungs and kidneys [34]. Recent studies indicate that renal cortical EC-SOD expression is around 50% lower in hypertensive SHR compared with normotensive WKY [35], and the gene transfer of EC-SOD can reduce blood pressure in SHR [36]. We therefore considered that the EC-SOD imbalance might be an important mechanistic aspect of hypertension-induced renal oxidative stress in early diabetes.

In the present study, we observed an abundant expression of renal cortical EC-SOD in normotensive WKY, which was found markedly reduced in hypertensive SHR. In addition, the non-enzymatic antioxidant GSH level was significantly reduced in the SHR group, but not in the WKY group, after 10 days of experimental diabetes. Therefore, taking together the results of pro-oxidant, antioxidant and oxidative stress markers, we could summarize that the reduced level of antioxidant EC-SOD and GSH in SHR, particularly in diabetic SHR, in the face of exaggerated superoxide generation rapidly induces oxidative damage to proteins and DNA, as evidenced by increased protein nitration (nitrotyrosine) and DNA base modification (8-OHdG), respectively. On the other hand, normotensive WKY group showed a relatively lower level of superoxide generation as well as higher levels of antioxidants, the levels of which were maintained in diabetes. Consequently, no evidence of oxidative protein or DNA damage was detected in the renal cortical tissue in WKY group after 10 days of experimental diabetes. We consider that this finding is of profound clinical importance. Because diabetes and hypertension frequently co-

exist in humans, and the combination of diabetes and hypertension increases the frequency and severity of nephropathy [17–19]. Thus, the findings of the present study suggest that the increased susceptibility to renal damage in the presence of hypertension and diabetes could be mediated through enhanced oxidative stress in the kidney.

In the present study, however, urinary AER similarly increased in both normotensive and hypertensive rats indicating that the hypertension or hypertension-induced renal oxidative stress does not affect urinary albumin excretion in the very early stage of diabetes [20]. This early finding, of course, does not exclude the important contribution of hypertension and/or oxidative stress in the pathogenesis of diabetic nephropathy [1, 5–7]. In fact, in a previous paper, we showed that long-term diabetes along with hypertension increased albuminuria and renal fibronectin expression, which was completely prevented by normalization of blood pressure [37]. Future studies with specific objectives are definitely needed to identify the contribution of hypertension-induced oxidative stress to the structural and functional renal damage in long-term diabetes.

We consider that the findings obtained in the present study could not be attributed to differences in metabolic control, because the levels of glycemia were similar between the WKY-D and SHR-D groups. Similarly, the oxidative imbalance observed in the SHR-D group could not be attributed to STZ toxicity, because the same dosage of STZ was used in the WKY and SHR groups to induce diabetes. However, we are aware that the control groups used in the present study were not truly comparable, because renal oxidative stress was already elevated in the hypertensive control group compared with the normotensive control. We do admit that this difference might

have partly confounded our findings, but we did not avail any truly comparable model since hypertension is almost invariably associated with renal oxidative stress [15, 16]. However, comparing all 4 groups together with appropriate statistical techniques, we observed that the hypertensive, but not normotensive, diabetic group exhibits the highest level of pro-oxidant generation with impaired antioxidant protection leading to increased renal oxidative stress. Furthermore, pair-wise comparison between normotensive control and diabetic groups, and then between hypertensive control and diabetic groups provided similar conclusions. Therefore, despite the limitation of the model used in the present study, our data strongly suggest that the presence of hypertension disrupts renal oxidative balance in the very early stage of experimental diabetes.

We conclude that the presence of hypertension increases pro-oxidant generation and decreases antioxidant defense, and thereby induces oxidative stress in the kidney in the early stage of diabetes. This finding strengthens the concept that hypertension contributes to diabetic nephropathy, and highlights the importance of blood pressure control to prevent and treat renal complications in diabetes.

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