

# Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: Effects of ACEI and ARB

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## **Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: Effects of ACEI and ARB.**

**Background.** Angiotensin II (Ang II) can up-regulate nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase, whose product superoxide anion ( $O_2^-$ ) can interact with nitric oxide (NO) to form peroxynitrite ( $ONOO^-$ ). We tested the hypothesis that Ang II subtype 1 ( $AT_1$ ) receptor activation enhances oxidative stress and nitrotyrosine deposition in the kidneys of rats with diabetes mellitus (DM).

**Methods.** After two weeks of streptozotocin-induced DM, rats received either no treatment, an angiotensin-converting enzyme inhibitor (ACEI) or an angiotensin receptor blocker (ARB) for two weeks. At four weeks, renal expression of the p47phox component of NAD(P)H oxidase, endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and nitrotyrosine were evaluated by Western blot and immunohistochemistry and related to plasma lipid peroxidation products (LPO), hydrogen peroxide production in the kidney and 24-hour protein excretion.

**Results.** Immunoreactive expression of p47phox and eNOS were increased in DM with an increase in plasma LPO, renal hydrogen peroxide production and nitrotyrosine deposition. Expression of nNOS was unaltered. Treatment with either ACEI or ARB prevented all these findings and also prevented significant microalbuminuria. The treatments did not affect the elevated blood sugar, nor did DM or its treatment affect the blood pressure or the creatinine clearance.

**Conclusion.** Early proteinuric diabetic nephropathy increases renal expression of the p47phox component of NAD(P)H oxidase and eNOS with increased indices of systemic and renal oxidative/nitrosative stress. An ACEI or an ARB prevents these changes and prevents the development of proteinuria, independent of blood pressure or blood sugar. This finding indicates a pathogenic role for  $AT_1$  receptors in the development of oxidative damage in the kidneys during early DM.

**Key words:** nitric oxide, reactive oxygen species, superoxide anion, nitrotyrosine, proteinuria,  $AT_1$  receptor.

Received for publication April 25, 2001

and in revised form August 2, 2001

Accepted for publication August 31, 2001

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An increased production of reactive oxygen species (ROS), including superoxide anion ( $O_2^-$ ) may contribute to diabetic complications [1–9] although the source of  $O_2^-$  remains unclear. The phagocyte contains a potent ROS-generating nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase that is composed of five major subunits. There are two membrane-associated proteins, p22phox and gp91phox, and three major cytosolic components, p47phox, p40phox, and p67phox [10]. While endothelial cells express gp91phox, rodent vascular smooth muscle cells express a different isoform termed MOX-1 [11] and, in the kidney, the gp91phox is replaced in certain tubule cells with another isoform termed Renox [12].  $O_2^-$  reacts with NO with great affinity to produce peroxynitrite ( $ONOO^-$ ) [13], which is a weak agonist for activation of cGMP [14]. Therefore,  $O_2^-$  effectively inactivates nitric oxide (NO). However,  $ONOO^-$  is a potent oxidant that can modify proteins. Nitrated tyrosine epitopes provide a marker for oxidative/nitrosative stress induced by the interaction of  $O_2^-$  and NO, and may be pathogenic by changing protein structure and function [15, 16].

An analogous NAD(P)H oxidase system has been reported in kidney [12, 17–21]. The p47phox is extensively regulated. During acute activation, the enzyme is phosphorylated and dephosphorylated in parallel with activation and inactivation of oxidase activity [22]. Up-regulation of p47phox is seen in the kidneys of spontaneously hypertensive rat (SHR), which is a model of angiotensin II subtype 1 ( $AT_1$ ) receptor-dependent oxidative stress (abstract; Chabrashvili et al, *J Am Soc Nephrol* 10:343A, 1999) [23]. In vascular smooth muscle cells p47phox is linked to Ang II-dependent JAK/STAT signaling events that underlie proliferation [24]. Therefore, our initial studies have focused on the p47phox component, although in vasculature angiotensin II (Ang II) also up-regulates p22phox [25] and p67phox [26]. The activation of NAD(P)H oxidase in diabetes mellitus (DM) is postulated to suppress the action of NO [3, 7, 8], to increase the expression of the mRNA for transforming growth factor- $\beta$ 1

(TGF- $\beta$ 1) and fibronectin in the glomerulus [4, 5], to decrease the expression of matrix metalloproteinases, and to increase the expression of the tissue inhibitor of metalloproteinases in the kidney [2, 6]. These diverse effects could contribute to the pathophysiology of diabetic nephropathy (DN) [7, 8]. We recently have shown that acetylcholine-induced endothelium-dependent relaxation factor (EDRF) in the rabbit isolated perfused afferent arteriole can be ascribed to NO [9]. This EDRF response is absent in arterioles microdissected from kidneys of rabbits with early streptozotocin-induced DM, but a partial response can be restored by a membrane-permeable nitroxide, tempol, that acts as a superoxide dismutase (SOD) mimetic [9]. While these data implicate the potential importance of oxidative stress in functional NO deficiency in diabetic nephropathy, the sources of  $O_2^-$  remain unclear since other oxidases, including constitutive nitric oxide synthase (NOS), lipooxygenase, and xanthine oxidase all can contribute to oxidative stress in other models of hypertension or renal pathology [27–29].

Angiotensin-converting enzyme inhibitors (ACEIs) [30–32] and angiotensin receptor blockers (ARBs) [33, 34] have beneficial effects on proteinuria in human diabetic nephropathy. However, it is not clear whether these drugs can alter oxidative stress in diabetic kidneys.

The first aim of this study was to evaluate the expression of the p47phox component of NAD(P)H oxidase and constitutive NOS in the kidneys of rats during early streptozotocin-induced experimental diabetes mellitus, and to relate these changes in indices of oxidative and nitrosative stress and glomerular damage. The second aim was to contrast the effects of an ACEI (quinapril; QUI) and an ARB (candesartan; CAN) on these parameters.

## METHODS

### Animal preparation

Female Sprague-Dawley rats weighing 180 to 240 g were purchased from Charles River Laboratories (Shizuoka, Japan). The animals were housed in a temperature- and humidity-controlled room with 12-hour light/dark cycles, and they were fed standard laboratory animal chow. Except for the quinapril-treated group, rats had free access to tap water. Diabetes was induced by a single tail vein injection of streptozotocin (60 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, USA) diluted in citrate buffer, pH 4.5. Nondiabetic control rats ( $N = 11$ ) were injected with an equal volume of citrate buffer. After three days, the induction of diabetes was confirmed by measurement of blood glucose concentration.

Two weeks after streptozotocin injection, the diabetic rats were randomly divided into three groups matched for body weight and blood glucose: diabetes mellitus alone group (DM,  $N = 11$ ); quinapril-treated group

(DM + QUI;  $N = 7$ ), composed of animals treated with the ACEI quinapril (Yoshitomi Pharmaceutical Industries, Fukuoka, Japan;  $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  in drinking water changed daily to quantify the volume intake) and candesartan-treated group (DM + CAN;  $N = 8$ ), composed of rats treated with a continuous subcutaneous infusion of candesartan (Takeda Chemical Industries, Tokyo, Japan;  $0.05 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from an osmotic minipump, Alzet Pharmaceuticals, Palo Alto, CA, USA). Twenty-four-hour urine was collected using metabolic cages at 0, 2 and 4 weeks after STZ injection.

Four weeks after induction of diabetes mellitus, animals were anesthetized with pentobarbital (50 mg/kg body weight). The abdominal aorta was cannulated and blood pressure was measured by a pressure transducer (Nihon Kohden, Tokyo, Japan), blood was collected and the kidneys were perfused retrogradely with ice-cold phosphate-buffered saline (PBS) followed by perfusion with periodate-lysine-paraformaldehyde (PLP) solution. Kidney slices for immunohistochemical study were immersed in PLP solution overnight at 4°C and embedded in wax (polyethylene glycol 400 distearate; Polysciences Inc., Warrington, PA, USA). These methods have been described in detail previously [35, 36].

### Measurement of lipid peroxidation, hydrogen peroxide, creatinine, albumin and glucose

The lipid peroxidation products in plasma were measured by thiobarbituric acid method [37]. After precipitation of proteins with 10% trichloroacetic acid, each 100  $\mu\text{L}$  of sample was mixed with 100  $\mu\text{L}$  of 4% sodium dodecyl sulfate (SDS), 400  $\mu\text{L}$  of 20% acetic acid at pH 3.5 and 400  $\mu\text{L}$  of 0.8% 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA; Wako Pure Chemical Industries LTD., Osaka, Japan). After a 60-minute incubation at 95°C, the malondialdehyde (MDA) formed was measured by fluorophotometry (Hitachi H-2000, Tokyo, Japan) with an excitation/emission wavelength at 515/553 nm.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production in kidney homogenates was measured using the 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) method as described previously [38]. Briefly, 100  $\mu\text{g}$  of protein of kidney homogenate was incubated with  $16 \mu\text{g} \times \text{mL}^{-1}$  final concentration of DCFH-DA (Molecular Probes, Eugene, OR, USA) for 20 minutes at 37°C. DCFH-DA is oxidized to the highly fluorescent compound, 2',7'-dichlorofluorescein (DCF) in the presence of  $\text{H}_2\text{O}_2$ . After centrifugation, the fluorescence of the supernatant was measured in a spectrofluorometer with excitation/emission wavelength at 485/535 nm.

Creatinine in urine and blood was measured by the Jaffe method using spectrophotometer. The albumin in urine was quantified by enzyme-linked immunosorbent assay (ELISA) kit (Panaform Lab., Kumamoto, Japan).

**Table 1.** Effect of angiotensin-converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) treatment on physiological and biochemical parameters in diabetes mellitus

	Control (N = 11)	DM (N = 11)	DM + QUI (N = 7)	DM + CAN (N = 8)
Body weight g	288.5 ± 7.4	237.0 ± 6.4 <sup>b</sup>	186.4 ± 10.8 <sup>cd</sup>	228.1 ± 12.1 <sup>ce</sup>
Blood glucose mg/dL	132.2 ± 8.5	389.1 ± 26.7 <sup>c</sup>	415.3 ± 64.9 <sup>c</sup>	447.0 ± 40.2 <sup>c</sup>
Mean blood pressure mm Hg	79.7 ± 4.7	85.6 ± 6.0	85.2 ± 2.9	81.0 ± 5.1
Creatinine clearance mL/min/100 g body weight	0.53 ± 0.05	0.59 ± 0.06	0.53 ± 0.11	0.63 ± 0.08
Urinary albumin mg/day	0.12 ± 0.02	1.23 ± 0.39 <sup>b</sup>	0.29 ± 0.09 <sup>d</sup>	0.51 ± 0.16 <sup>d</sup>
Blood LPO nmol/L/mL	0.35 ± 0.11	0.69 ± 0.10 <sup>a</sup>	0.33 ± 0.08 <sup>d</sup>	0.34 ± 0.07 <sup>d</sup>
DCF generation from DCFH-DA fluorescence unit/μg protein	3.78 ± 0.13	4.62 ± 0.20 <sup>a</sup>	3.85 ± 0.22 <sup>d</sup>	3.72 ± 0.33 <sup>d</sup>

Abbreviations are: DM, diabetes mellitus; QUI, quinapril; CAN, candesartan; LPO, lipoprotein oxide; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescein-diacetate.

<sup>a</sup>  $P < 0.05$ , <sup>b</sup>  $P < 0.005$ , <sup>c</sup>  $P < 0.001$  vs. control

<sup>d</sup>  $P < 0.05$  vs. DM

<sup>e</sup>  $P < 0.05$  vs. DM + QUI

Blood glucose was measured by Glutest E II (Kyoto Dai-iti Kagaku, Kyoto, Japan).

### Western blotting

As described in detail previously [35, 36], the right kidneys were removed immediately after perfusion with PBS. They were homogenized on ice with a Teflon-glass tissue homogenizer (Iwaki, Chiba, Japan), in 4 mL of buffer containing 20 mmol/L Tris, at pH 7.2, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 20 μmol/L leupeptin, 10 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride and 0.1 mmol/L Pefabloc SC. Homogenates were centrifuged at 4°C and 12,000 rpm for 15 minutes. The supernatants were diluted in the same volume of sodium dodecyl sulfate (SDS) sample buffer (0.125 mol/L Tris-HCl, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue in final concentration). The amount of protein was measured by the Bradford method (Bio-Rad, Richmond, CA, USA). Samples containing 25 μg of protein were applied to 4 to 20% gradient gel (Daichi Pure Chemicals Co., Tokyo, Japan) and electroblotted to nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris buffered saline containing 0.1% Tween 20 (TBST) for 30 minutes, following overnight incubation with monoclonal antibody for p47phox, endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS; Transduction Laboratories, Lexington, KY, USA) and nitrotyrosine (Upstate Technology, Lake Placid, NY, USA) in a 1:1000 dilution. After rinsing in TBST, membranes were incubated for two hours with a horseradish peroxidase (HRP)-conjugated secondary antibody against mouse IgG (Dako, Glostrup, Denmark) at a 1:1000 dilution, and rinsed with TBST followed by 0.8 mmol/L diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) with 0.01% H<sub>2</sub>O<sub>2</sub> and 3 mmol/L NiCl<sub>2</sub> for the detection of blots.

### Immunohistochemistry

Kidney slices were processed for immunohistochemistry using the labeled streptavidin biotin method as described previously [35, 36]. Wax sections (2 μm) were dewaxed, incubated first with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes to eliminate endogenous peroxidase activity and thereafter with a monoclonal antibody directed against p47phox, eNOS and nNOS (Transduction Laboratories) at 1:100 dilution for four hours after exposure to blocking serum. The sections were rinsed with TBST and biotinylated secondary antibody against mouse IgG (Dako) for one hour. After rinsing with TBST, the sections were incubated for one hour with HRP-conjugated streptavidin solution (Dako). HRP labeling was detected using a peroxide substrate solution with 0.8 mmol/L DAB and 0.01% H<sub>2</sub>O<sub>2</sub>. The sections were counterstained with hematoxylin before being examined under a light microscope.

### Statistical analysis

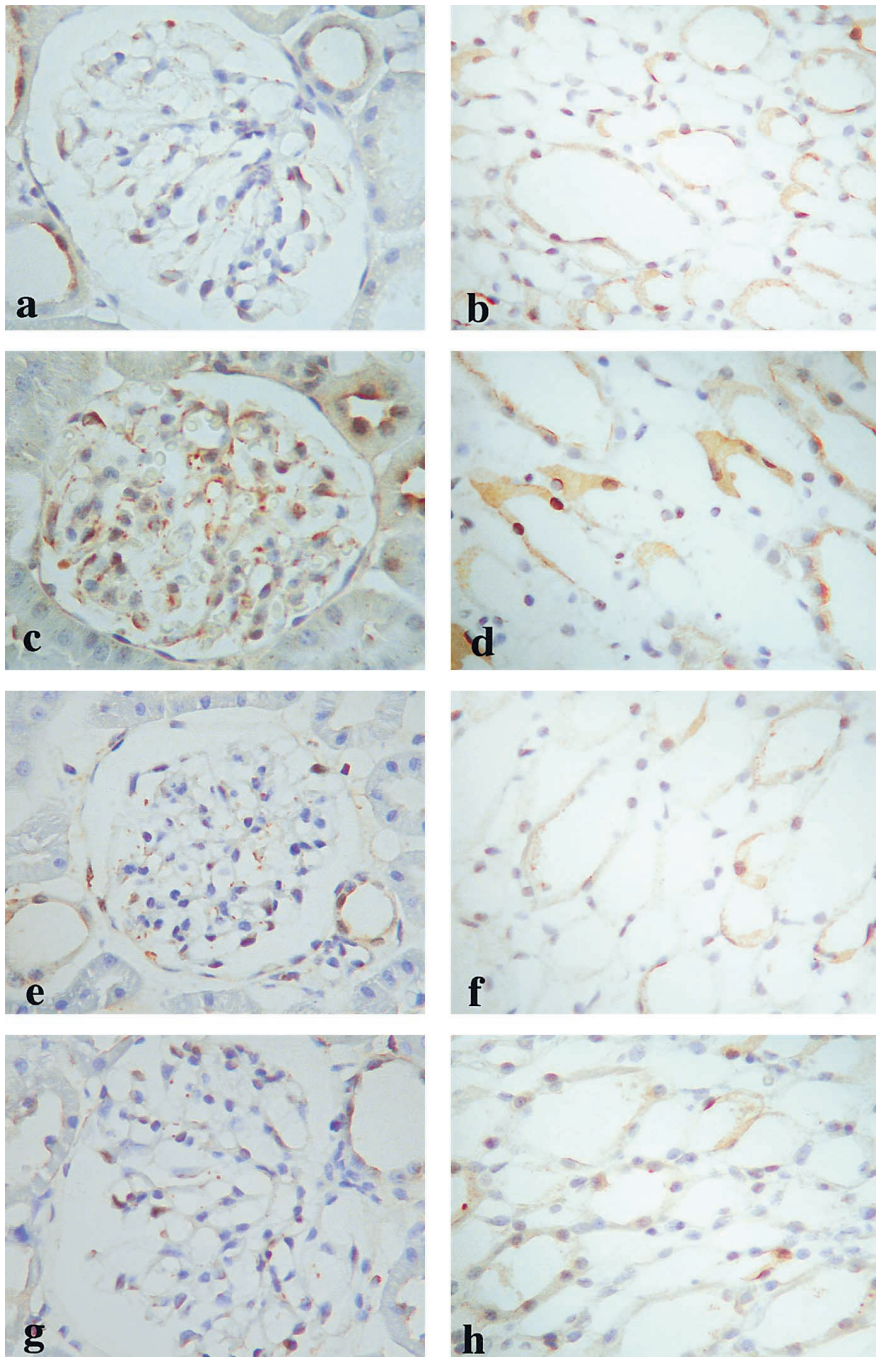
Data are presented as mean ± SEM value. The difference among the four groups was evaluated by one-way ANOVA followed by the Bonferroni method where appropriate. Comparisons of urinary and plasma LPO between groups were made by Mann-Whitney nonparametric test since the values were not normally distributed.

## RESULTS

### Physiological data

Blood glucose concentration of rats with DM was considerably higher than that of the controls but was not affected by ACEI or ARB (Table 1). The body weights were significantly lower in rats with DM than in controls during the experimental period. This effect was enhanced significantly in rats given the ACEI. The mean blood pressure (measured under anesthesia) and the creatinine clearance (measured from 24-hour urine collections) were similar in the four groups. Diabetic animals have a substantial increase in renal albumin excretion,





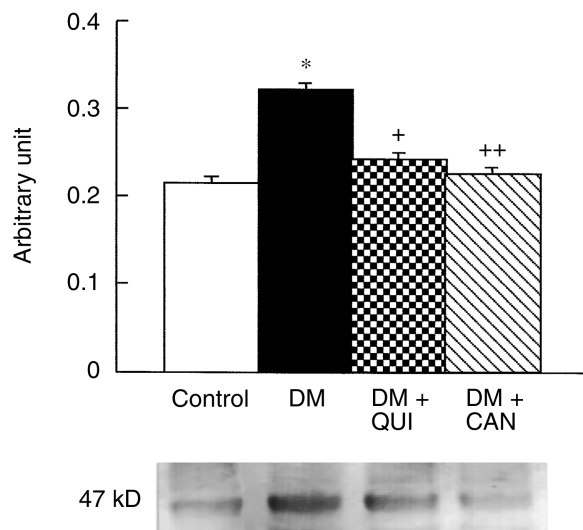
**Fig. 1.** Light micrographs illustrating immunostaining for NAD(P)H oxidase p47phox in glomerulus (a, c, e, g) and inner medulla (b, d, f, h). (a, b) control rat; (c, d) diabetic rat; (e, f) diabetic treated with quinapril; (g, h) diabetic treated with candesartan. Magnification  $\times 280$ .

which was reduced to values not significantly different from control animals by the ACEI and the ARB.

#### Expression of p47phox

The p47phox cytosolic component of NAD(P)H oxidase was expressed in control rats in the glomeruli, the distal tubules in the cortex and thin limbs of the loops of Henle, and the medullary collecting ducts in medulla. Its expression at these sites appeared enhanced in rats with DM (Fig. 1). Both the ACEI and the ARB appeared

to normalize the immunostaining. The quantitative analysis of p47phox protein in the kidney by Western blot showed a specific band corresponding to a molecular weight of 47 kD. Densitometry of this band confirmed an increase in protein amount in rats with DM compared to control ( $0.32 \pm 0.02$  vs.  $0.21 \pm 0.01$  arbitrary units;  $P < 0.0005$ ). This was suppressed significantly by both ACEI ( $0.24 \pm 0.02$ ,  $P < 0.005$  vs. DM) and ARB ( $0.23 \pm 0.01$ ;  $P < 0.002$  vs. DM) to values not significantly different from control rats (Fig. 2).



**Fig. 2.** Western blot for p47phox using whole kidney homogenate analyzed by densitometry (mean  $\pm$  SEM from 6 rats in each group). A prominent band for p47phox is observed at 47 kD. Abbreviations are: DM, streptozotocin-induced diabetic rats; DM + QUI, diabetic rats given quinapril; DM + CAN, diabetic rats given candesartan. \* $P$  < 0.0005 vs. Control; + $P$  < 0.005, ++ $P$  < 0.002 vs. DM.

### Plasma LPO and hydrogen peroxide production in the kidney

Plasma lipid peroxidation products (LPO) were increased in rats with DM. This was reduced significantly in rats with DM treated with ACEI and ARB to levels of control rats (Table 1). The  $H_2O_2$  production in the kidney measured by DCF generation was significantly increased in DM. Both ACEI and ARB treatments reduced the hydrogen peroxide production significantly to the control level (Table 1).

### Expression of NOS isoforms and nitrosylation of proteins

Immunohistochemical studies showed expression of type I or nNOS in the macula densa, whose expression seemed to be slightly increased in DM but did not change by each treatment (Fig. 3). Western blot analysis of whole kidney homogenates showed a band of 160 kD corresponding to nNOS whose densitometry showed no differences between the four groups (control  $0.27 \pm 0.02$ , DM  $0.28 \pm 0.03$ , DM + QUI  $0.23 \pm 0.03$ , DM + CAN  $0.22 \pm 0.04$  arbitrary units; NS; Fig. 4).

The immunohistochemical study showed a prominent expression of type III or eNOS in the endothelium of glomeruli, renal arteries and arterioles that appeared more prominent in those with DM, but was reduced in those given an ACEI or an ARB (Fig. 3). Western blot analysis (Fig. 5) confirmed a modest increase in eNOS expression in rats with DM ( $0.29 \pm 0.01$  vs.  $0.24 \pm 0.01$  arbitrary units,  $P$  < 0.05), which was not different from

control in diabetic rats treated with ACEI ( $0.25 \pm 0.01$ ) or ARB ( $0.21 \pm 0.02$  arbitrary units).

The production of oxidative products of NO in the kidneys was evaluated by measurement of nitrosylated proteins by Western blot. As in previous studies [39], several bands were detected in the kidney, as would be anticipated from the pleomorphic nature of proteins with nitrosylated tyrosine residues. The strongest band had a molecular weight about 60 kD. Densitometry of this band showed a modest increase in nitrosylated protein density in rats with DM ( $0.29 \pm 0.02$  vs.  $0.24 \pm 0.05$  arbitrary units,  $P$  < 0.05). This was reduced to values not different from controls by the ACEI ( $0.24 \pm 0.01$ ) and the ARB ( $0.23 \pm 0.02$ , Fig. 6).

### Renal albumin excretion

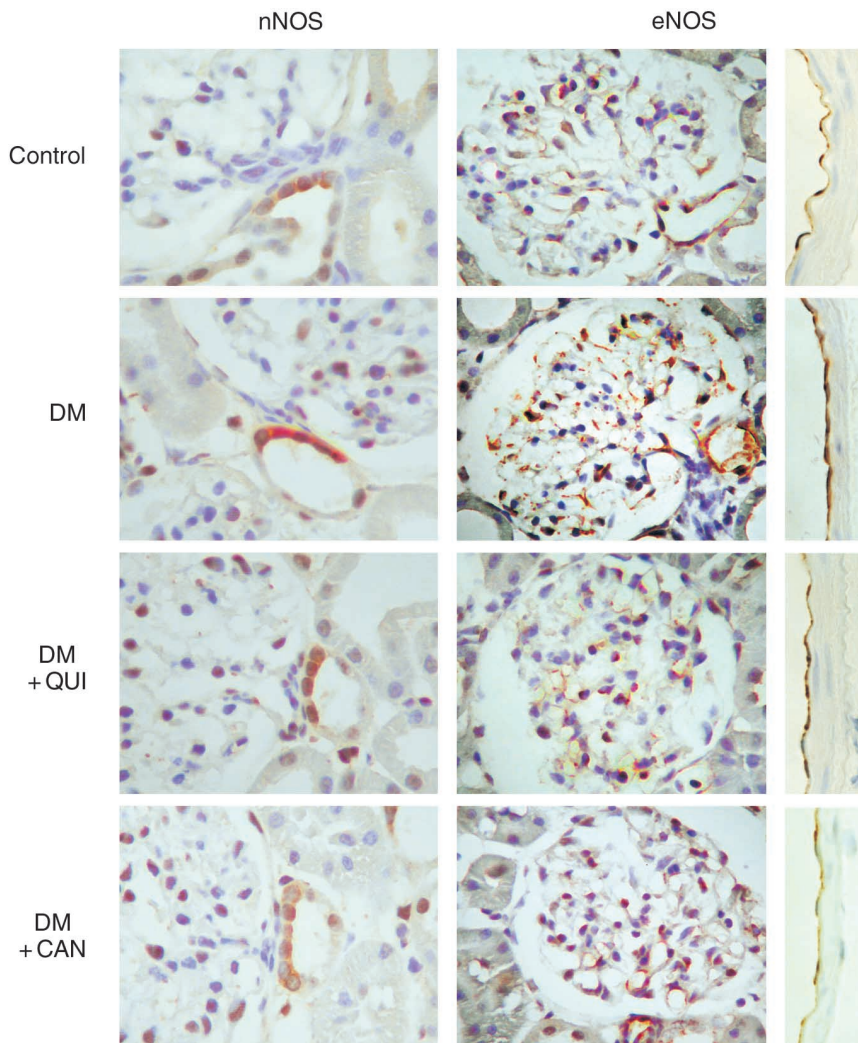
Renal albumin excretion was used as a marker for early diabetic nephropathy. Albumin excretion was elevated significantly in rats with DM at four weeks after induction of diabetes (Table 1). Both the ACEI and the ARB reduced renal albumin excretion significantly in DM. Although the final values for proteinuria were not different in these two groups, quinapril appeared somewhat more effective than candesartan (Fig. 7).

## DISCUSSION

The main new findings of this study are that rats with early proteinuric diabetes mellitus have evidence of oxidative stress associated with enhanced renal expression of p47phox and eNOS and nitrotyrosine. All of these changes are prevented in rats given an ACEI or an ARB for two weeks before the study.

### NAD(P)H oxidase expression and its oxidative products

The pathophysiology of diabetic nephropathy has been related to oxidative stress leading to an enhanced expression of TGF- $\beta$ 1 and fibronectin [4, 40, 41]. However, the source of ROS in the kidney remains undefined. Studies in models of hypertension or atherosclerosis have linked oxidative stress in the blood vessel to  $O_2^-$  generated by NAD(P)H oxidase in endothelial and vascular smooth muscle cells [42]. Hyperglycemia can increase the expression of p47phox in leukocytes [43]. In this study, p47phox expression indeed was enhanced in the kidneys of hyperglycemic, diabetic rats with prominent expression in renal vasculature, the thin limb of the loop of Henle, macula densa cells, distal convoluted tubules and collecting ducts. Although not studied quantitatively by Western analysis, these immunohistochemical findings suggest that there may be enhanced expression of p47phox in the cortex and the medulla. However, our data dissociate the activation of p47phox and eNOS, and the development of oxidative stress in DM from hyperglycemia itself,



**Fig. 3. Light micrographs illustrating immunostaining for neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS).** Immunoreactivity for nNOS is observed in macula densa and that for eNOS is in endothelial cells in glomerulus and renal artery. Magnification  $\times 280$  for nNOS,  $\times 240$  for eNOS in glomerulus and  $\times 180$  for eNOS in renal artery.

since the administration of an ACEI or an ARB failed to modify the hyperglycemia yet fully prevented the increased expression of p47phox, eNOS and their products including nitrotyrosine, plasma LPO and renal  $H_2O_2$  in diabetic rats. Recently, enhanced gp91phox NADPH oxidase and eNOS expression in aorta have been demonstrated in the early stage of streptozotocin-induced diabetic rats [44], supporting our findings of oxidative stress in the kidney of diabetic rats.

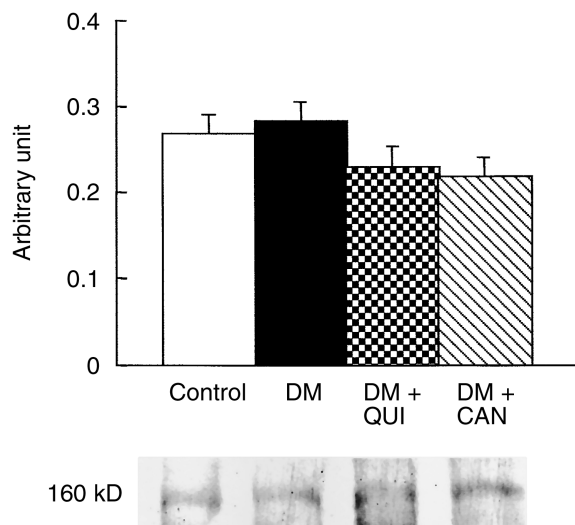
Angiotensin II stimulated NAD(P)H oxidase activity and expression of p22phox and p67phox in VSMCs [25, 26, 42]. Both the ACEI and the ARB suppressed p47phox expression significantly in the kidney of diabetic rats. These results are consistent with a previous report of a reduction in the mRNA for NAD(P)H oxidase in vascular smooth muscle cells of rats with DM by an ARB [45] that was independent of blood sugar. We have found that two weeks of treatment with an ARB or non-specific antihypertensive therapy with hydralazine, hydrochlorothiazide and reserpine normalized blood pressure in the

spontaneously hypertensive rat (SHR), yet only the ARB corrected oxidative stress and restored nitric oxide signaling in the juxtaglomerular apparatus [23].

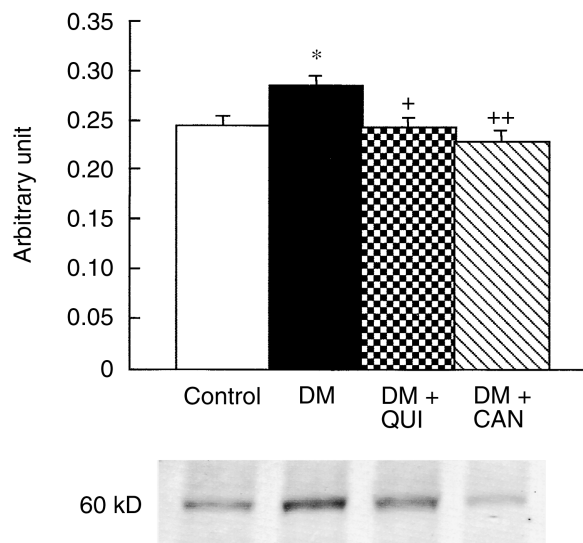
### NOS and nitrotyrosine expression in the kidney

Our findings address the controversy concerning the contribution of different NOS isoforms to intrarenal production of NO in diabetes. On the one hand, a stimulated renal NO system has been postulated to underlie the persistent hyperfiltration that precedes proliferative and sclerotic events in the glomeruli culminating in proteinuric azotemia [21, 46–48]. We detected an increased expression of eNOS in microvessels and kidneys of rats with DM. This confirms reports in rats with established DM [1, 21, 49]. In contrast, a study in rats with early DM detected no changes in renal eNOS protein expression [50]. This may relate to the time course of induction of diabetes [21]. eNOS is expressed significantly also in tubular epithelial cells, which may therefore have contributed to the increased eNOS protein expression de-

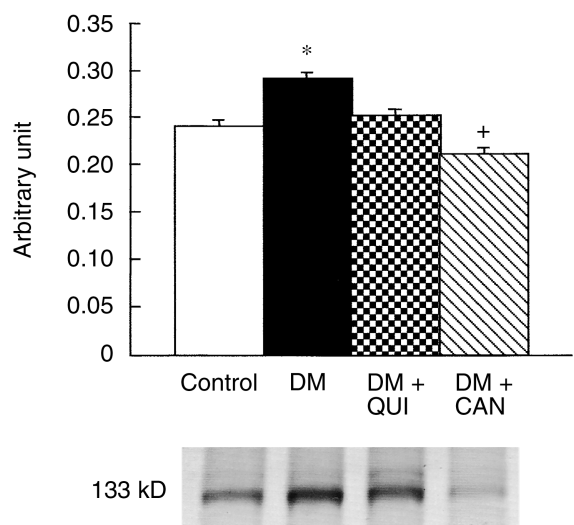




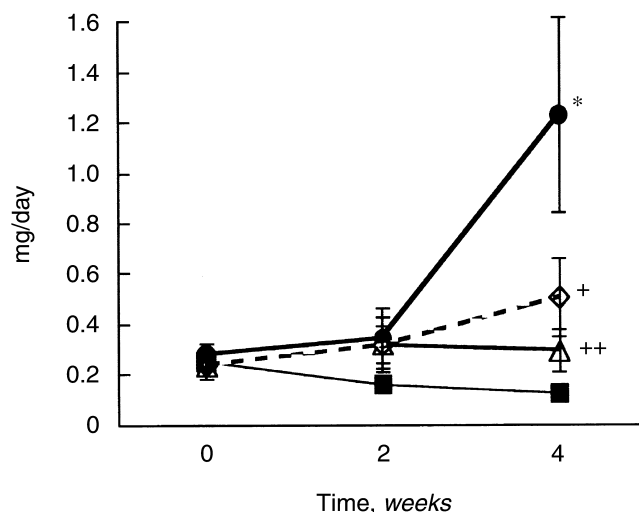
**Fig. 4.** Western blot studies of nNOS expression in the whole kidney homogenates showing a specific band at 160 kD. The bands were quantified by densitometry and expressed as mean  $\pm$  SEM values from six rats in each group.



**Fig. 6.** Western blot of nitrotyrosine in whole kidney homogenates quantified by densitometry (mean  $\pm$  SEM from 6 rats in each group). There was a proximate band at 60 kD. \* $P$  < 0.05 vs. Control;  $^+P$  < 0.05,  $^{++}P$  < 0.01 vs. DM.



**Fig. 5.** Western blot of eNOS expression in the whole kidney homogenates were quantified by densitometry (mean  $\pm$  SEM from 6 rats in each group). A specific band for eNOS was observed at 133 kD. \* $P$  < 0.05 vs. Control;  $^+P$  < 0.001 vs. DM.



**Fig. 7.** Albumin excretion in control rats and over four weeks of diabetes mellitus alone or with two weeks of quinapril or candesartan administration (weeks 2–4). Symbols are: (■) Control; (●) DM; (△) DM + QUI; (◇) DM + CAN; \* $P$  < 0.005 vs. Control;  $^+P$  < 0.05,  $^{++}P$  < 0.02 vs. DM.

tected by Western analysis in whole kidneys of diabetic rats. However, immunohistochemical studies do not detect prominent expression of eNOS protein in tubules [35]. Therefore, tubular expression of eNOS could not be assessed in this study. We could not confirm an increase in nNOS expression in the kidney of diabetic rats that others reported using immunohistochemistry [47, 48], Western analysis [1, 50] or reverse transcription-polymerase chain reaction (RT-PCR) analysis [50]. The prior increases in nNOS expression have been related

to the accompanying glomerular hyperfiltration. In our model of insulinogenic diabetes without insulin replacement, no increase in nNOS protein expression in the kidney was detected, nor was evidence of glomerular hyperfiltration as assessed indirectly from 24-hour creatinine clearance. It is possible that the difference between our study and those of others may relate to the severity of diabetes and the degree of accompanying hyperglycemia. Indeed, glucose concentrations above 350 to 500 mg/dL

bond covalently with NO, thereby curtailing NO bioactivity [51].

Studies in the peripheral circulation of patients with diabetes, and the renal vessels of animal models, have concluded that there is a defective EDRF response to endothelial agonists such as acetylcholine [52]. This could be explained if NO is scavenged in the diabetic kidney by over-production of  $O_2^-$ . Any ensuing increase in ONOO<sup>-</sup> production should enhance nitrotyrosine deposition. An example is the kidney of the SHR in which an increase in oxidative stress is accompanied by increased nitrotyrosine deposition [39]. Indeed, an increase in nitrotyrosine deposition has been detected in the placenta of diabetic patients where it has been related to vascular damage [15]. Moreover, immunocytochemical studies in human renal biopsies reveal an increase in nitrotyrosine immunoreactivity that is specific for diabetic patients [16]. Our study confirms an increase in renal nitrotyrosine expression in diabetes, although the change was rather modest. The source of the reactive oxygen species in the kidneys in our model is not established. Likely candidates include NAD(P)H oxidase or a dysfunction eNOS [44], since each of the systems was up-regulated in diabetes and normalized by ACEI or ARB therapy in parallel with nitrotyrosine expression and  $H_2O_2$  generation in the kidneys. Presently, it is unclear whether nitrotyrosine represents only a marker for oxidative stress from NO-induced oxidants or whether it alters protein structure sufficiently to cause abnormal enzyme, receptor, or signaling function. The administration of an ACEI or an ARB prevented the excessive nitrotyrosine deposition in diabetic kidneys, consistent with their effects in prevention of oxidative stress, as indexed by normalization of the renal expression of p47phox, renal hydrogen peroxide production and plasma LPO.

### Renal albumin excretion

Angiotensin converting enzyme inhibitors and ARBs reduce albumin excretion in patients with incipient or overt diabetic nephropathy [33, 53, 54]. This was confirmed in the present model of early DM where both the ACEI and the ARB reduced proteinuria substantially. The progression of nephropathy is correlated with the degree of albuminuria. Indeed, some authors postulate that the increased protein filtration and reabsorption may contribute to tubulointerstitial disease and progressive loss of renal function [55]. Our study suggests that this chain of events may originate from an AT<sub>1</sub>-receptor driven expression of oxidases in the diabetic kidney, since the ACEI and the ARB prevented the development of both oxidative stress and albuminuria. It is also possible that the effects of ACEIs and ARBs on albuminuria are secondary to changes in glomerular hemodynamics. However, our data did not show significant alteration in glomerular filtration by either treatment.

Proteinuric diabetic renal disease may be consequence

of AT<sub>1</sub>-receptor induced expression of a range of pathologic process in the kidney. Prominent among these may be p47phox-induced activation of NAD(P)H oxidase-dependent  $O_2^-$  generation and eNOS-induced NO generation leading to the formation of oxidative and nitrosative products including ONOO<sup>-</sup>. Intense interest is focused on the cellular events linking AT<sub>1</sub> receptor activation to expression of cytokines and growth factors such as TGF- $\beta$  in the kidneys of diabetics that predispose them to the development of nephropathy. The present findings suggest that co-expression of NAD(P)H oxidase and eNOS, leading to increased nitro-oxidative stress, could be one such factor. Intervention studies to block NAD(P)H oxidase or eNOS are needed to address this hypothesis directly.

### ACKNOWLEDGMENTS

This work was supported by a grant to Dr. Tojo (Grant no. C2-12671025) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by grants from the NIDDK to Dr. Wilcox (RO1-DK-49870 and RO1-DK-36079) and funds from the George E. Schreiner Chair of Nephrology.

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