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VASCULAR BIOLOGY – HEMODYNAMICS – HYPERTENSION

Effects of NADPH oxidase inhibitor in diabetic nephropathy

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Effects of NADPH oxidase inhibitor in diabetic nephropathy.

Background. We used apocynin to test the hypothesis that superoxide anion (O_2^-) from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase underlies the development of diabetic nephropathy in the rat.

Methods. Rats received apocynin (16 mg/kg/day) from 2 to 8 weeks after inducing diabetes mellitus (DM) with streptozotocin.

Results. DM increased excretion of hydrogen peroxide (H_2O_2), lipid peroxidation products (LPO), nitric oxide products (NOx), and protein. The kidneys of rats with DM had increased expression of p47phox and gp91phox and endothelial nitric oxide synthase (eNOS), and increased mesangial matrix with expression of fibronectin and collagen I. Apocynin prevented the increase in excretion of H_2O_2, LPO, and protein in diabetic rats, increased renal NOx generation, and prevented the increased renal expression of gp91phox and the membrane fraction of p47phox, and reverted the mesangial matrix expansion.

Conclusion. Activation of NADPH oxidase with translocation of p47phox to the membrane underlies the oxidative stress and limited NO generation, despite enhanced eNOS expression in a model of diabetic nephropathy. Apocynin prevents these changes and the associated proteinuria.

The balance between the production of reactive oxygen species (ROS), notably superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and the antioxidant defense system that includes superoxide dismutase (SOD) and peroxidases, determines the degree of oxidative stress. An increased production and/or decreased metabolism of ROS have been implicated in the pathogenesis of renal injury in diabetes mellitus (DM) [1, 2].

An important source of ROS production is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme generates O_2^- during the respiratory burst in phagocytes. The phagocyte NADPH oxidase consists of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p40phox, p67phox, and Rac) [3]. When activated, p47phox is phosphorylated, and the cytosolic components translocate to the membrane, where they form a molecular cluster of the catalytically active oxidase. The mechanism is somewhat different in other cells. For example, NADPH oxidase is assembled primarily on intracellular organelles in endothelial cells [4]. Moreover, there are homologues of the gp91phox subunit that include Nox1, which is expressed in vascular smooth muscle cells (VSMCs), and Nox4, which is expressed in the kidney and blood vessels [5]. In the kidney, NADPH oxidase components are expressed abundantly in the renal vessels and in the glomerular mesangial and podocyte cells, the macula densa, and the thick ascending limb, distal tubule, and collecting ducts [6]. The renal expression of NADPH oxidase is enhanced in rat diabetic nephropathy. In this model, an angiotensin-converting enzyme inhibitor (ACEI) or an angiotensin receptor blocker (ARB) suppresses renal NADPH oxidase, renal ROS generation, and microalbuminuria [2]. Thus, we tested the hypothesis that administration of the NADPH oxidase inhibitor, apocynin, to diabetic rats will reduce renal O_2^- production and prevent proteinuria.

Apocynin is a methoxy-substituted catechol from the medicinal herb *Picroria kurroa*. It inhibits NADPH oxidase by impeding the assembly of p47phox and p67phox subunits within the membrane NADPH oxidase complex [7, 8]. Administration of apocynin to rats with collagen-induced arthritis prevents joint destruction [9]. Likewise, apocynin administration to a rat model of Crohn's disease reduces small intestinal inflammation [10]. It has also been proposed as a therapeutic agent for atherosclerotic disease [8], but its effect in diabetic nephropathy has not been studied.

We evaluated the effect of apocynin on NADPH oxidase expression and examined its effect on ROS generation and proteinuria in a rat model of insulinopenic diabetic nephropathy.

Key words: NADPH oxidase, nitric oxide synthase, proteinuria, diabetic nephropathy, apocynin.

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METHODS

Animal preparation

Female Sprague-Dawley rats weighing 180 to 250 g were housed in cages and fed standard pellet diet (Na^+ content 0.21 g/100 g) and tap water ad libitum. Diabetes was induced by a single tail vein injection of streptozotocin (STZ, 60 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, USA) diluted in citrate buffer, pH 4.5.

Two weeks later, the diabetic rats were randomly divided into 2 groups matched for body weight and blood glucose: DM group without treatment (DM, $N = 12$) and DM given apocynin (DM + apocynin, $N = 12$) composed of rats treated with apocynin (16 mg/kg/day in drinking water; Avocado Research Chemicals, Heysham, England). This dose of apocynin (4-hydroxy-3-methoxyacetophenone) was based on in vivo studies of prolonged dosing in which apocynin (4 to 100 mg/kg/day) prevented NADPH oxidase activation, vascular superoxide (O_2^-) production and remodeling, hypertension, inflammation, and/or organ dysfunction in a number of models of chronic oxidative stress [10–15]. Since a daily dose of 100 mg/kg was no more effective than 15 mg/kg in preventing neuropathy and reduced nerve blood flow in diabetic rats [12], a dose of 16 mg/kg was selected for this study. Age-matched rats without STZ injection served as controls ($N = 12$). Twenty-four–hour urine was collected using metabolic cages at 4 and 8 weeks after STZ injection.

At these time points, 6 animals from each group were anesthetized with pentobarbital (50 mg/kg body weight, IP). The abdominal aorta was cannulated, and mean blood pressure (MBP) was measured by a pressure transducer (Nihon Koden, Tokyo, Japan). Blood was collected, and the kidneys were perfused retrogradely with ice-cold phosphate-buffered saline (PBS). The right kidney was removed and cut into half for Western blotting ($N = 6$), cryosection ($N = 3$), or glomerular isolation ($N = 3$). The left kidney was perfused with periodate-lysine-paraformaldehyde (PLP) solution. Kidney slices for immunohistochemical analysis were immersed in PLP solution overnight at 4°C and embedded in wax (polyethylene glycol 400 distearate; Polysciences, Inc., Warrington, PA, USA).

Measurement of lipid peroxidation, H_2O_2, nitrite, creatinine, protein, and glucose

The lipid peroxidation products in urine were measured by the thiobarbituric acid method [16]. Samples (100 μL) were mixed with 100 μL of 8% sodium dodecyl sulfate (SDS), 400 μL of 20% acetic acid at pH 3.5, and 400 μL 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 F-2000, Tokyo, Japan) with excitation/emission wavelengths of 515/553 nm.

Production of peroxides, including H_2O_2 and peroxynitrite, was measured in urine and isolated glomeruli obtained by graded sieving using the 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) method, as described previously [17]. Briefly, samples were incubated with DCFH-DA (16 μg/mL final concentration; Molecular Probes, Eugene, OR, USA) for 20 minutes at 37°C. DCFH-DA is oxidized by peroxides to the highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF), which was measured with a spectrofluorometer using excitation/emission wavelengths of 485/535 nm [18].

Nitrite production in kidney homogenates was measured by the Griess method. Protein was precipitated by adding equal volumes of 0.3N NaOH and 5% $ZnSO_4$. The supernatants were incubated with Griess solution, and nitrite was measured with a spectrophotometer at 540 nm [19]. To confirm the specificity of DCF signal for hydrogen peroxide production, isolated glomeruli from DM rat were preincubated with 5000 U/mL of catalase (Wako) for 20 minutes, and then incubated with DCFH-DA reagent with catalase for another 20 minutes.

Urine protein was measured by the Bradford method (Bio-Rad, Hercules, CA, USA), and corrected by urinary creatinine as described previously [2]. Blood glucose was measured by Glutest E II (Kyoto Daiichi Kagaku, Kyoto, Japan). HbA_{1c} was measured with the DCA 2000 plus system (Bayer Medical, Tokyo, Japan).

Western blotting

As described in detail previously [20], the right kidney was removed immediately after perfusion with PBS and homogenized in 4 mL of ice-cold 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. Homogenates were centrifuged at 5000*g* for 15 minutes at 4°C. The supernatants were centrifuged at 48,000*g* for 60 minutes at 4°C to isolate cytosolic and membrane fractions. The resulting supernatant was removed (cytosol fraction), and the pellet was resuspended in buffer containing 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, and 250 mmol/L sucrose (membrane fraction) [21].

Samples were diluted in an equal volume of SDS sample buffer. Aliquots (25 μg of protein/lane) were applied to 4 to 20% polyacrylamide gradient gels (Daiichi Pure Chemicals Co., Tokyo, Japan) and electrophoresed, followed by electroblotting on to nitrocellulose membranes. The membranes were blocked for 30 minutes with 5% nonfat dried milk in Tris-buffered saline containing 0.1%

Tween 20 (TBST), followed by overnight incubation with monoclonal antibodies recognizing p47phox, gp91phox, and p22phox [6] at 1:1000 dilutions, and a polyclonal antibody recognizing endothelial nitric oxide synthase (eNOS; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:100 dilution. After washing with TBST, membranes were incubated for 4 hours with horseradish peroxidase (HRP)-conjugated antimouse IgG or antirabbit IgG secondary antibodies (1:1000 dilutions; Dako, Glostrup, Denmark) to detect p47phox, gp91phox, and p22phox or eNOS, respectively. Blots were washed again with TBST, followed by developing solution containing 0.8 mmol/L diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan), 0.01% H_2O_2, and 3 mmol/L $NiCl_2$. The density of the bands was analyzed using NIH Image software.

Immunohistochemistry

Kidney slices were processed for immunohistochemistry using the labeled streptavidin biotin method, as described previously [20]. Wax sections (2 μm) were dewaxed, incubated first with 3% H_2O_2 for 10 minutes to eliminate endogenous peroxidase activity, and thereafter with blocking serum for 15 minutes. They were incubated overnight with polyclonal antibodies recognizing p47phox, gp91phox [6], and eNOS (Santa Cruz Biotechnology), type I collagen, and fibronectin (Chemicon International, Inc., Temecula, CA, USA) at 1:100 dilutions. The sections were rinsed with TBST and incubated for 1 hour with biotinylated antirabbit IgG secondary antibody at a 1:400 dilution. After rinsing with TBST, the sections were incubated for 1 hour with HRP-conjugated streptavidin solution (Dako). HRP labeling was detected using a peroxide substrate solution containing 0.8 mmol/L DAB and 0.01% H_2O_2. The sections were counterstained with hematoxylin before being examined under a light microscope.

NO production detected by NADPH diaphorase histochemistry and DAF-2

Kidneys were embedded in OCT compound and frozen. Cryosections (5 μm) were cut, rinsed in PBS, and incubated with 1 mmol/L reduced β-NADPH (Sigma Chemical Co.), 0.2 mmol/L nitro blue tetrazolium (Wako), and 0.2% Triton X-100 for 40 minutes at 37°C. Sections were rinsed with 0.1 mol/L Tris-HCl buffer, and photographed under a light microscope [22].

Cryosections were incubated with 10 μmol/L diaminofluorescein-2 diacetate (DAF-2 DA, Daiichi Pure Chemicals Co.) for 1 hour. This probe is converted covalently to a fluorescent compound on interaction with NO. It has been validated as a measure of NO activity in isolated vasa recta [18, 23]. The NO production was detected using a fluorescence microscope with excitation/emission wavelengths of 495/515 nm. To confirm the specificity of NO production, samples from DM rats were preincubated with 1 mmol/L Nω-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co.) for 60 minutes, and then NADPH diaphorase or DAF-2DA reagents were added with 1 mmol/L L-NAME.

Morphologic study

Wax sections (2 μm) were stained with periodic acid–Schiff (PAS) for light microscopic morphologic study. The degree of mesangial matrix expansion was assessed by an experienced observer who was blinded as to the sample origin, using a semiquantitative scoring method: grade 0, normal glomeruli; grade 1, mesangial expansion area up to 25% (minimal); grade 2, 25% to 50% (moderate); grade 3, 50% to 75% (moderate-severe); grade 4, 75% to 100% (severe). The glomerular matrix expansion index (GMI) was calculated as the average of 100 glomeruli in each kidney slice [24, 25]. The data were assessed from 5 rats in each group.

Statistical analysis

All data are expressed as mean ± SE. The mean values were compared among the 3 groups using analysis of variance (ANOVA), followed, where appropriate, by Fisher protected least significant difference test. Probability values less than 0.05 were required for statistical significance.

RESULTS

Physiologic data

As shown in Table 1, blood glucose and HbA_{1c} were significantly higher in diabetic rats than in controls. Diabetic rats administered apocynin had higher values for HbA_{1c} compared to those with DM at 4 weeks, but there was no difference at 8 weeks. Mean blood pressure was comparable between DM and control, but it was reduced by apocynin at 4 and 8 weeks.

Expression of NADPH oxidase subunits

The p47phox component of NADPH oxidase was expressed in glomerular cells and distal tubules in kidneys of control rats. Its expression was enhanced in kidneys of DM rats, especially in the podocytes. Apocynin treatment reduced p47phox staining in diabetic rats (Fig. 1). Western blotting confirmed that p47phox protein was increased significantly in DM rats at 8 weeks in both membrane and cytosolic fractions of the kidney [membrane fraction: 0.33 ± 0.04 vs. 0.20 ± 0.03 arbitrary units (au), $P < 0.05$; cytosolic fraction: 0.29 ± 0.03 vs. 0.18 ± 0.03 au, $P < 0.05$, Fig. 2]. Apocynin significantly reduced p47phox expression in the membrane fractions of the

Table 1. Physiologic data

	Control (4 week, $N = 12$) (8 week, $N = 6$)	DM (4 week, $N = 12$) (8 week, $N = 6$)	DM + apocynin (4 week, $N = 12$) (8 week, $N = 6$)
Body weight *g*	281 ± 7	245 ± 8^{b}	233 ± 11^{c}
	323 ± 10	288 ± 16	276 ± 18^{a}
Blood glucose *mg/dL*	141 ± 6	437 ± 38^{c}	491 ± 37^{c}
	175 ± 12	365 ± 27^{c}	407 ± 24^{c}
HbA_{1c}%	2.63 ± 0.04	7.03 ± 0.55^{c}	$8.38 \pm 0.24^{c,d}$
	2.75 ± 0.10	9.30 ± 0.63^{c}	8.80 ± 0.45^{c}
Mean blood pressure *mm Hg*	99 ± 6	84 ± 7	77 ± 4^{a}
	99 ± 6	89 ± 5	79 ± 6^{a}
Urinary LPO *nmol/mg Cr*	7.8 ± 1.0	44.2 ± 5.4^{c}	39.7 ± 3.8^{c}
	6.5 ± 0.7	98.1 ± 7.6^{c}	$66.0 \pm 10.7^{c,d}$
Urinary H_2O_2 *FI unit/mg Cr*	7.2 ± 0.4	11.4 ± 1.0^{c}	7.4 ± 0.6^{f}
	11.4 ± 1.2	26.9 ± 6.3^{a}	10.1 ± 1.3^{e}
Urinary protein *mg/mg Cr*	0.37 ± 0.03	0.61 ± 0.07^{b}	0.42 ± 0.03^{d}
	0.31 ± 0.03	0.55 ± 0.13^{a}	0.27 ± 0.02^{d}

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs. control; [d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ vs. DM.

kidney (0.21 ± 0.03, $P < 0.05$ vs. DM, Fig. 2) but not in the cytosolic fraction (0.25 ± 0.02, NS vs. DM, Fig. 2). The ratio of p47phox expression in membranes:cytosol was 1.10 ± 0.07 in control rats. This ratio was unchanged in DM rats (1.13 ± 0.05), but was reduced significantly in DM rats given apocynin (0.85 ± 0.06; $P < 0.05$ vs. DM or vs. control). This indicates that apocynin inhibited membrane translocation of p47phox.

The gp91phox was expressed in glomerular cells and distal tubules. Its expression was enhanced in DM rats at 8 weeks. Apocynin reduced the expression of gp91phox in the glomeruli (Fig. 1). Western blot analysis of the membrane fraction of the kidney showed a specific band for gp91phox that was increased in DM rats (0.30 ± 0.02 vs. 0.20 ± 0.02 au, $P < 0.01$, Fig. 3). Apocynin prevented the significant increase in the membrane fraction of gp91phox protein expression of DM rats (0.25 ± 0.02, NS vs. control, Fig. 3).

The expression of p22phox in the membrane fraction was unchanged in DM rats compared to control (0.27 ± 0.01 vs. 0.24 ± 0.01 au, NS, Fig. 3), and was not modified by apocynin (0.24 ± 0.01, NS vs. DM, Fig. 3).

Glomerular and urinary oxidative products

Associated with the increased NADPH oxidase components in DM rats, their isolated glomeruli showed increased peroxide production, which was reduced by apocynin treatment (Fig. 4). The DCF signal in the glomerulus from DM rat was reduced by catalase pretreatment, indicating that the signals represent hydrogen peroxide production. Renal excretion of LPO was increased in DM rats at both 4 and 8 weeks. This was reduced at 8 weeks by apocynin. Renal excretion of peroxide was increased in DM rats, and was reduced by apocynin at both 4 and 8 weeks (Table 1).

Expression of eNOS and nitrite production in the kidney

Immunohistochemical analysis showed that the expression of eNOS in endothelial cells of the renal artery was increased in DM rats (Fig. 1), confirming our previous study [2]. Western blot analysis confirmed an increase in eNOS expression in DM rats (0.28 ± 0.01 vs. 0.19 ± 0.01 au, $P < 0.001$, Fig. 5). Treatment with apocynin did not change eNOS expression in DM rats (0.32 ± 0.01, NS vs. DM, Fig. 5).

NADPH diaphorase histochemistry was used as a descriptive method to detect NOS expression [22, 26, 27], and DAF-2 fluorescence to detect NO activity [18, 23]. These methods indicated that NO production from NOS in proximal tubules and endothelial cells of the renal vasculature was increased in DM rats, and that was suppressed by L-NAME. NO production was increased further by apocynin treatment (Fig. 6). NO production, assessed quantitatively by NOx generated in the kidney homogenates, was increased in DM rats (71.0 ± 4.7 vs. 32.2 ± 3.0 μmol/mL, $P < 0.01$, Fig. 7) and increased further by apocynin (100.2 ± 11.6, $P < 0.05$ vs. DM, Fig. 7).

Urinary protein excretion

Urinary protein excretion was increased in DM rats. Apocynin reduced urinary protein excretion at 4 and 8 weeks (Table 1).

Mesangial matrix expansion

DM rats had increased mesangial matrix expansion (GMI: 1.36 ± 0.16 vs. 0.27 ± 0.05, $P < 0.001$, Fig. 1), which was ameliorated by apocynin (0.38 ± 0.03, $P < 0.001$ vs. DM, Fig. 1). Immunohistochemistry demonstrated increased mesangial expression of fibronectin and type I collagen in DM rat mesangial cells. Apocynin suppressed these changes (Fig. 1).

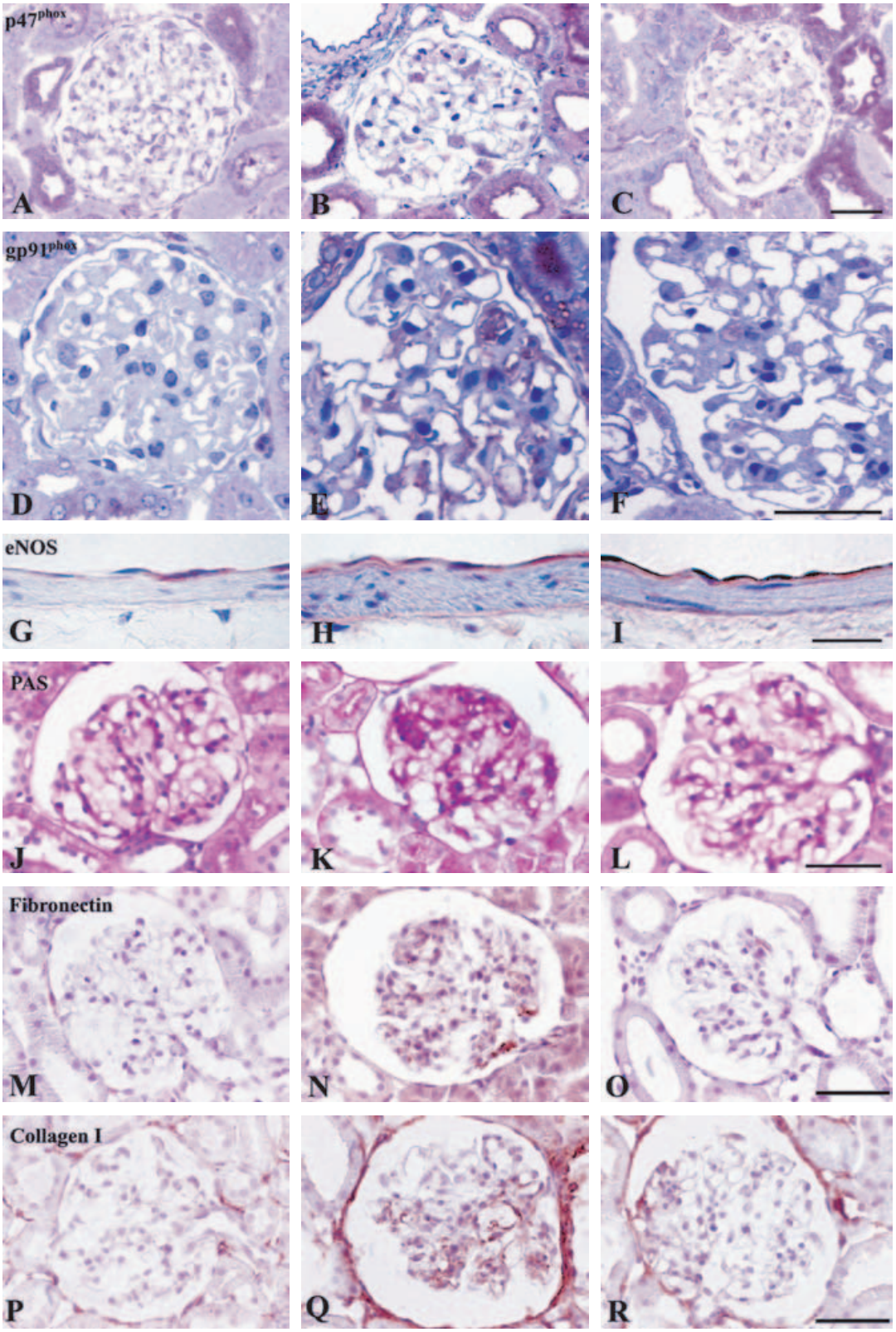

Fig. 1. Light micrographs illustrating immunostaining for NADPH oxidase p47phox (*A* to *C*) and gp91phox (*D* to *F*) in the kidney, immunostaining for eNOS in the renal artery (*G* to *I*), periodic acid–Schiff (PAS)-stained sections of the kidney (*J* to *L*), and immunostaining for fibronectin (*M* to *O*) and type I collagen (*P* to *R*). Control (A, D, G, J, M, and P), DM rat at 8 weeks (B, E, H, K, N, and Q), and DM rat treated with apocynin (C, F, I, L, O, and R). Each bar indicates 50 μm. Magnification, (A to C) ×240, (J to R) ×300, (D to F) ×360, and (G to I) ×400.

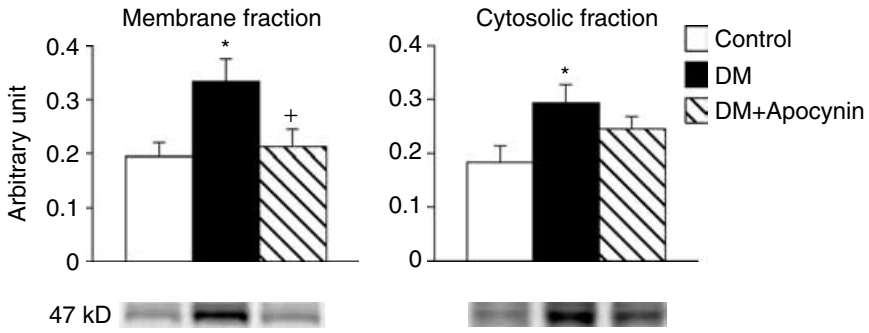

Fig. 2. Western blot analysis of NADPH oxidase p47phox in membrane and cytosolic fractions at 8 weeks. The band at the molecular weight of 47 kD was quantified by densitometry from 6 rats in each group. $^{*}P < 0.05$ vs. control; $^{+}P < 0.05$ vs. DM.

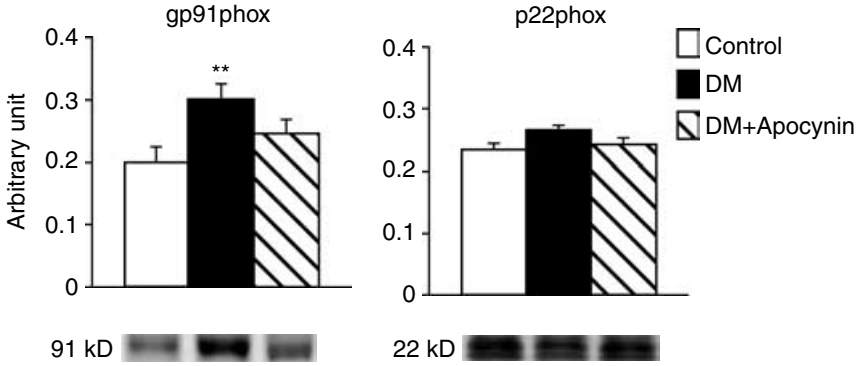

Fig. 3. Western blot analysis of NADPH oxidase gp91phox and p22phox in the membrane fraction at 8 weeks. The band at the molecular weight of 91 kD and 22 kD was quantified by densitometry from 6 rats in each group. $^{**}P < 0.01$ vs. control.

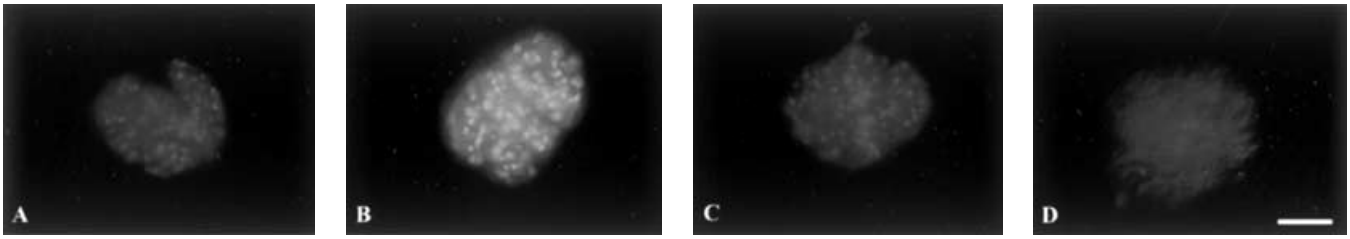

Fig. 4. Peroxide production in isolated glomeruli. Control (*A*), DM rat at 8 weeks (*B*), DM rat treated with apocynin (*C*), and an isolated glomerulus from DM rat pretreated with catalase (*D*). Representative pictures from 3 rats in each group. Bar indicates 50 μm. Magnification, ×240.

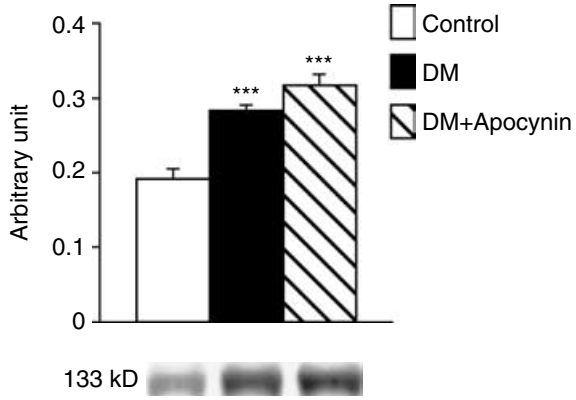

Fig. 5. Western blot analysis of eNOS in whole kidney homogenate at 8 weeks. The band at the molecular weight of 133 kD was quantified by densitometry from 6 rats in each group. $^{***}P < 0.001$ vs. control.

DISCUSSION

The main new findings of this study are that blockade of NADPH oxidase with apocynin in diabetic rats prevents the membrane translocation of p47phox and the increased expression of gp91phox in the kidney, and prevents oxidative stress, proteinuria, and glomerulopathy, while enhancing NO generation. These effects are independent of changes in blood sugar, but are accompanied by a reduction in blood pressure.

The activity of NADPH oxidase is regulated both by expression of the subunits and by phosphorylation of p47phox following stimulation by agents such as angiotensin II, IL-1β, and TNF-α [28–30]. We showed by Western blotting and immunohistochemistry that the renal expression of p47phox and gp91phox were enhanced in diabetic rats. This is consistent with recent reports that p47phox and gp91phox are up-regulated in the kidneys

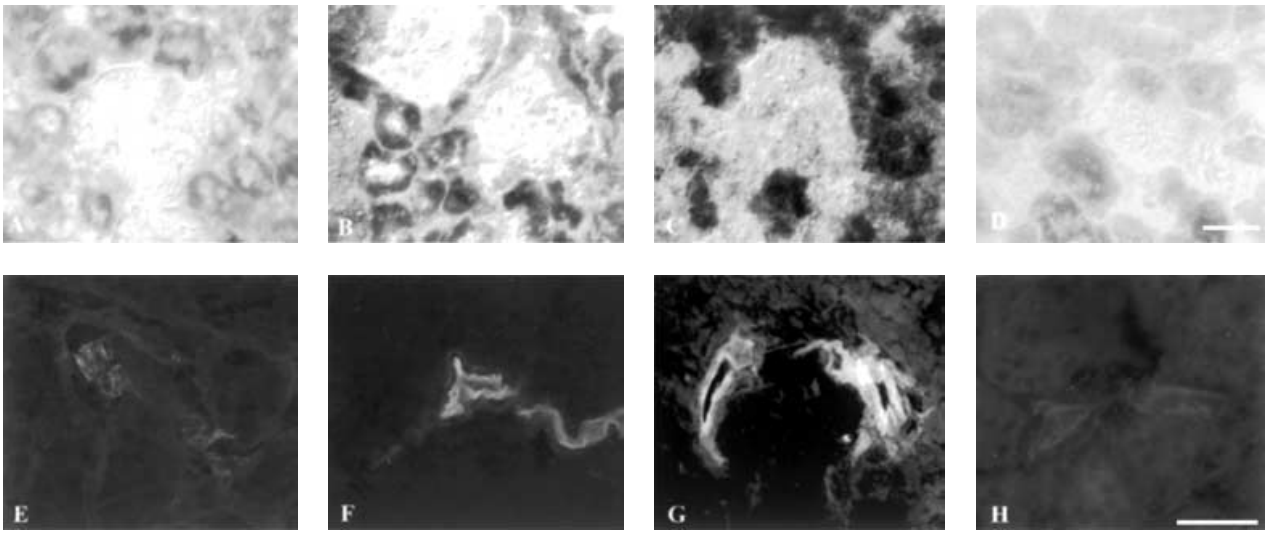

Fig. 6. NO production detected by NADPH diaphorase histochemistry (*A* to *D*) and DAF-2 (*E* to *H*). Control (A, E), DM rat at 8 weeks (B, F), DM rat treated with apocynin (C, G), and DM rat pretreated with 1 mmol/L L-NAME (D, H). Representative pictures from 3 rats in each group. Each bar indicates 50 μm. Magnification, ×240.

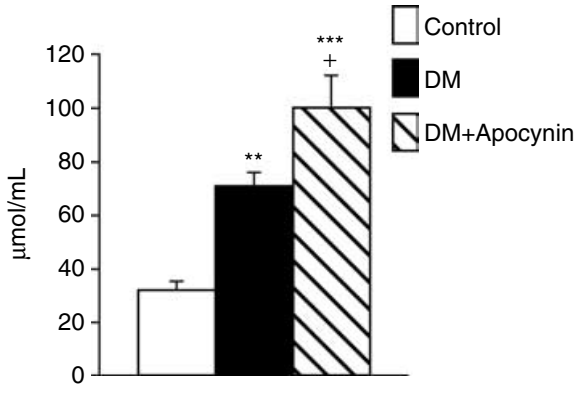

Fig. 7. Nitrite production in whole kidney homogenate at 8 weeks. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{+}P < 0.05$ vs. DM.

of salt loaded rats [31] since hyperglycemic rats have an expanded extracellular fluid volume. Apocynin reduced the expression of p47phox in the membrane, but not in the cytosolic fraction, indicating that it inhibited translocation of the p47phox subunit to the membrane fraction. The expression of another membrane-bound subunit, gp91phox, was increased in the kidney of DM rats, and this increase also was prevented by apocynin. Another critical membrane subunit, p22phox, did not change significantly with diabetes, and was not altered by apocynin. Thus, a mechanism of NADPH oxidase inhibition by apocynin may be suppression of translocation of the cytosolic p47phox component to the membrane fraction, with associated suppression of its anchor, gp91phox, where it forms a molecular cluster to activate NADPH oxidase. It is not clear from this study whether this represents translocation to the cell membrane, or to associated intracellular organelles that would also be separated with the membrane components. These effects of apocynin in the kidney are consistent with its known actions. Apocynin, after cellular uptake and peroxidation, prevents serine phosphorylation of p47phox, and blocks its association with gp91phox. This blunts NADPH oxidase activation, as shown in studies of neutrophils [32] and angiotensin II (Ang II)-stimulated VSMCs from human resistance arteries [33]. Apocynin is excreted in active form through the kidneys [34]. Apocynin pretreatment prevents the generation of oxidative stress in the heart [35] and diaphragm [15] after lipopolysaccharide, in the lung after ischemia-reperfusion [36], in the intestine after iodoacetamide treatment [10], in arteries after endothelial injury [13], and in the blood vessels after mineralocorticoid/salt-induced hypertension [11, 14]. While the primary established target for apocynin is to block assembly and activation of NADPH oxidase, there are secondary consequences of this in endothelial cells where xanthine oxidase expression and O_2^- generation from this enzyme also is inhibited [37].

NO generation in the kidneys, as assessed in this study by NOx generation by kidney tissue, is increased in early streptozotocin-induced DM [38]. Increased NO has been assigned a key role in the accompanying glomerular hyperfiltration [38–40] and prevention of hypertension [41]. Increased NO has been ascribed both to an increased expression of eNOS [2, 38, 40], which was confirmed in this study, and to activation of neuronal NOS expressed in the macula densa [40]. Fluorescent NO-sensitive dye and diaphorase histochemical methods were used to locate the source of NOS and the activity of NO in this study. They located increased NO in renal proximal tubule cells and vascular endothelial cells in the DM rats. While

apocynin did not further modify eNOS expression, it enhanced renal NOx generation, and enhanced NO bioactivity, as assessed from DAF-2 fluorescence in vascular endothelial cells. This indicates that apocynin reduced NO bioinactivation by O_2^- in diabetes. It may have protected eNOS from the effects of oxidation of tetrahydrobiopterin, which can uncouple eNOS from NO synthesis and perpetuate O_2^- generation [42–45]. This is consistent with the enhanced renal nitrotyrosine deposition in the kidneys in this DM model, which implies an enhanced interaction of O_2^- with NO [2].

Proteinuria in DM is considered to have both a hemodynamic (glomerular capillary hypertension and hyperfiltration) and a structural/cellular basis (alterations in basement membrane, mesangial cell matrix, and podocyte function) [38]. As in a previous study, diabetic rats developed modest proteinuria by 4 weeks [2], but this was prevented by apocynin. Apocynin has an organ-protective effect in models of rheumatoid arthritis, inflammatory bowel disease, ischemic reperfusion lung injury, and sepsis [9, 10, 15, 36, 46], where it inhibits the neutrophil oxidative burst and generation of cytokines, including TNF-α, IL-1β, IFN-γ, IL-4, and IL-10 [9, 36, 46]. The mechanism of the antiproteinuric effect of apocynin is not clear. Apocynin did prevent the DM-induced increases in renal expression of gp91phox, membrane-associated p47phox, H_2O_2 excretion, glomerular mesangial expansion, and mesangial expression of fibronectin and collagen I and expression of p47phox in podocytes, and of H_2O_2 generation by isolated glomeruli. Apocynin may have prevented deterioration in glomerular mesangial function or in glomerular protein permeability in DM. For example, preventing activation of p47phox in podocytes may limit local oxidative stress. While this remains conjectural, oxidative stress is known to impair the integrity of tight junctions between cells [47]. Our data are not a direct evidence of correlation between proteinuria and H_2O_2 excretion, and apocynin prevented the increase in protein excretion of DM rats at both 4 and 8 weeks, yet reduced (and did not prevent) the increase in LPO excretion only at 8 weeks. However, the relationship between H_2O_2 generation in glomeruli and LPO or H_2O_2 excretion in the urine is presently unclear.

To our knowledge, this is the first report of an effect of apocynin to limit proteinuria and development of glomerulopathy in early diabetic nephropathy. While it requires further study to determine its exact mechanism of action, it extends previous studies of the actions of apocynin in diabetes or hyperglycemia. Thus, apocynin blocks the effect of increased glucose concentration to activate protein kinase C (PKC)-induced NADPH oxidase, and fibronectin secretion by peritoneal cells [48] to cause endothelial dysfunction in isolated vessels [49], and to impair contractility in cardiac myocytes [50]. Apocynin blocks the increase in PKC, O_2^- generation and proliferation during incubation of mesangial cells with glycated albumin [51]. Administration of apocynin to rats with DM reduces vascular O_2^- (assessed by electron paramagnetic resonance [52]), and prevents diabetic neuropathy and reduced nerve blood flow [12]. Apocynin protects against atherosclerosis in several models [8]. Therefore, apocynin could have benefits beyond prevention of diabetic nephropathy.

CONCLUSION

Activation of NADPH oxidase in a model of diabetic nephropathy with translocation of p47phox to the membrane underlies the generation of peroxides and limits NO generation. Apocynin effectively prevents these changes, and prevents the associated proteinuria and glomerular matrix expansion.

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