

# Combined Antioxidant and COMT Inhibitor Treatment Reverses Renal Abnormalities in Diabetic Rats

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**The development and progression of diabetic nephropathy is dependent on glucose homeostasis and many other contributing factors. In the present study, we examined the effect of nitecapone, an inhibitor of the dopamine-metabolizing enzyme catechol-*O*-methyl transferase (COMT) and a potent antioxidant, on functional and cellular determinants of renal function in rats with streptozotocin-induced diabetes. Administration of nitecapone to diabetic rats normalized urinary sodium excretion in a manner consistent with the dopamine-dependent inhibition of proximal tubule Na,K-ATPase activity. Hyperfiltration, focal glomerulosclerosis, and albuminuria were also reversed by nitecapone, but in a manner that is more readily attributed to the antioxidant potential of the agent. A pattern of elevated oxidative stress, measured as CuZn superoxide dismutase gene expression and thiobarbituric acid-reactive substance content, was noted in diabetic rats, and both parameters were normalized by nitecapone treatment. In diabetic rats, activation of glomerular protein kinase C (PKC) was confirmed by isoform-specific translocation and Ser23 phosphorylation of the PKC substrate Na,K-ATPase. PKC-dependent changes in Na,K-ATPase phosphorylation were associated with decreased glomerular Na,K-ATPase activity. Nitecapone-treated diabetic rats were protected from these intracellular modifications. The combined results suggest that the COMT-inhibitory and antioxidant properties of nitecapone provide a protective therapy against the development of diabetic nephropathy. *Diabetes* 49:1381-1389, 2000**

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BSA, bovine serum albumin; COMT, catechol-*O*-methyl transferase; ECL, enhanced chemiluminescence; GFR, glomerular filtration rate; MAP, mean arterial pressure; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; PVDF, polyvinylidene difluoride; RT, reverse transcriptase; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substance; TGF, transforming growth factor.

**T**he outcome of the Diabetes Control and Complications Trial effectively established that a most successful strategy for preventing diabetic complications is intensive treatment of hyperglycemia (1). However, restoring blood glucose to near-normal values is not sufficient to provide complete protection from the development of retinopathy, neuropathy, and nephropathy in all individuals. Consequently, approaches aimed at modifying the influence of additional factors may prove useful in providing supplemental organ protection.

Diabetic nephropathy is commonly associated with systemic hypertension, and it is well established that hypertension increases the rate of progression of renal dysfunction (2-4). The etiology of hypertension is multifactorial, but sodium retention and salt sensitivity may be important primary causal factors (5,6). Since an expansion of total body exchangeable sodium is consistently found in diabetes, decreasing tubule sodium reabsorption may be a useful therapeutic strategy.

Vascular complications of diabetic nephropathy are readily observed in abnormalities of glomerular function occurring early after the onset of hyperglycemia. Experimental studies of cultured glomerular cells have clearly demonstrated that hyperglycemia itself can initiate a series of cellular changes that characterize progression to glomerulosclerosis (7-9); today, there are numerous hypotheses that may account for this phenomenon (10). Among currently debated theories, a potential role for oxidative stress has been identified as an early initiating mechanism by which the high-glucose environment may lead to cellular injury in diabetes (11,12).

Nitecapone is a specific inhibitor of the dopamine-metabolizing enzyme catechol-*O*-methyl transferase (COMT), and it also has the capacity to scavenge free radical species (13-15). Based on its ability to increase the availability of intrarenal dopamine, nitecapone increases sodium excretion (16). This natriuretic response occurs as a result of dopamine-dependent inhibition of renal tubule Na,K-ATPase, the enzyme responsible for active transport of sodium throughout the nephron (17). The antioxidant properties of nitecapone include the ability to effectively quench peroxy and hydroxyl radicals and superoxide (14,15). In vivo, nitecapone can protect against ischemia-reperfusion injury through its radical quenching and iron-chelating properties (18,19). Because sodium retention (20) and oxidative stress

may contribute to the development of diabetic complications (10), we set out to establish whether the dual properties of nitecapone provide the compound with a unique ability to ameliorate diabetic renal dysfunction.

## RESEARCH DESIGN AND METHODS

**Materials.** Nitecapone was kindly provided by Orion Pharma (Espoo, Finland). Liquid scintillation cocktail was purchased from LKB (Wallac, Sweden). Rabbit polyclonal protein kinase C (PKC) antibodies were obtained from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse Ig secondary antibodies, Hybond-P:polyvinylidene difluoride (PVDF) membranes, enhanced chemiluminescence (ECL) plus Western blotting detection system,  $\gamma$ [ $^{32}$ P]ATP,  $\alpha$ [ $^{32}$ P]dCTP, and  $^{86}$ RbCl were purchased from Amersham International (Buckinghamshire, U.K.). M-MLV-reverse transcriptase, RNasin, and Oligo dT were obtained from Promega (Madison, WI). Taq DNA polymerase was from Perkin Elmer (Foster City, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Animals.** Experiments were performed on adult male Sprague-Dawley (B&K Universal, Sollentuna, Sweden) and Dahl salt-sensitive rats (Möllegaards Breeding Center, Ejby, Denmark). Both rat strains were fed standard rodent diet (Beaky, Fixed Formula; Bantin & Kingman Ltd., U.K.) containing 18% protein and 0.22% sodium and were provided with water ad libitum. Diabetes was induced by a single tail-vein injection of streptozotocin (STZ) (Sigma; 70 mg/kg in NaCl, 0.09%). Blood glucose concentration, weight gain, and food intake were monitored regularly. Only those diabetic rats that exhibited a blood glucose concentration >17 mmol/l were included in the study. Rats receiving nitecapone were given the compound either as an aqueous solution administered via gavage at a dose of 30 mg/kg body weight twice a day or as a supplement in their regular drinking water at a final concentration of 25  $\mu$ g/ml. Similar renal protection occurred with both routes of administration. Nitecapone treatment was initiated immediately for those rats receiving STZ. Rats were sacrificed at different time points (2 days, 10 days, or 8 weeks of diabetes) depending on the individual experimental protocol. Nitecapone did not alter blood glucose and body weight of 2- or 10-day diabetic Sprague-Dawley rats or 8-week Dahl salt-sensitive diabetic rats (results not shown). The dopamine 1 receptor antagonist, SCH 23390, was given as an intravenous infusion at 30  $\mu$ g  $\cdot$  kg body wt $^{-1} \cdot$  h $^{-1}$ . Because we previously showed that glomerulosclerosis progresses rapidly in Dahl salt-sensitive diabetic rats (21), the effect of nitecapone on manifestations of diabetic nephropathy (albuminuria and renal morphology) was studied using this rat model. All other studies were performed with Sprague-Dawley rats.

**Renal function.** In Sprague-Dawley rats ( $n = 5$ ) diabetic for 10 days, glomerular filtration rate (GFR) was measured as the clearance of inulin, as previously described (21). Briefly, a 5% solution of Inutest (Laevosan; Gesellschaft, Linz, Austria) diluted in Ringer's solution was infused at a dose of 1% of body wt/h. This was preceded by a priming dose of the infusate at 1% of body weight. Urine collection was initiated for two 20-min collection periods after a 60-min equilibration. Plasma and urine were subsequently analyzed for inulin content. Sodium was analyzed with a flame photometer (Ependorph 6524, Hamburg, Germany).

**Isolation and preparation of proximal tubules.** Measurement of Na,K-ATPase activity was performed in Sprague-Dawley rats ( $n = 6$ ) 10 days after the induction of diabetes. Kidney perfusion and tubule dissection were performed as previously described (21). Briefly, the left kidney was perfused with a cold modified Hanks' buffer solution containing (in mmol/l): 137 NaCl, 5 KCl, 0.8 MgSO $_4$ , 0.33 Na $_2$ HPO $_4$ , 0.44 KH $_2$ PO $_4$ , 1 CaCl $_2$ , 1 MgCl $_2$ , 10 Tris-HCl (pH 7.4), 0.05% collagenase (Sigma), and 0.1% bovine serum albumin (BSA) (Behringwerke, Marburg, Germany). The kidney was removed and cut along its corticopapillary axis into small pieces, which were subsequently incubated at 35°C for 20 min in 10 ml perfusion solution (bubbled with oxygen) containing 10 $^{-3}$  mol/l butyrate. After incubation, the tissue pieces were rinsed and transferred to a similar solution supplemented with 0.25 mmol/l CaCl $_2$  but devoid of collagenase and BSA. Single proximal tubules were then manually dissected under a stereo microscope at 4°C and individually transferred to the concavity of a bacteriological slide, at which point they were photographed for length determination using an inverted microscope at 100 $\times$  magnification. Tubules were stored on ice until dissection was completed.

**Determination of Na,K-ATPase activity.** Na,K-ATPase activity was measured in single proximal tubules. Tubule segments were made permeable by hypotonic shock and rapid freeze/thaw treatment. Individual segments were incubated for 15 min at 37°C in a medium containing the following (in mmol/l): 50 NaCl, 5 KCl, 10 MgCl $_2$ , 1 EGTA, 100 Tris-HCl (pH 7.4), 10 Na $_2$ ATP (grade II; Sigma), and [ $\gamma$ - $^{32}$ P]ATP (New England Nuclear, Boston, MA) 2–5 Ci/mmol in tracer amounts (5 nCi/ml). For determination of ouabain-

insensitive ATPase activity, NaCl and KCl were omitted, Tris-HCl was raised to 150 mmol/l, and 2 mmol/l ouabain (Merck, Darmstadt, Germany) was added. The free  $^{32}$ P liberated by hydrolysis of [ $\gamma$ - $^{32}$ P]ATP was separated by filtration through a Millipore filter after the absorption of unhydrolyzed nucleotide on activated charcoal. Radioactivity was measured in a liquid scintillation spectrophotometer. In each study, total ATPase activity and ouabain-insensitive activity were measured in 6–8 segments. Na,K-ATPase activity was calculated as the difference between the mean value for total ATPase activity and ouabain-insensitive activity and is expressed as picomoles of  $^{32}$ P hydrolyzed per millimeter of tubule per hour.

**Albuminuria.** Dahl salt-sensitive rats ( $n = 6$ –10) were used to assess the effect of nitecapone on albuminuria following 8 weeks of diabetes. Urinary albumin concentration was determined on an MIRA autoanalyzer (Cobas, Japan) using immunoturbidometry (22).

**Renal morphology.** Morphometric analysis was performed 8 weeks after the induction of diabetes in Dahl salt-sensitive rats ( $n = 6$ –8), as previously described (21). In brief, the left kidney was immersion fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3 coronal blocks that were each 1.5 mm thick. The tissue blocks were serial sectioned at 3  $\mu$ m. Every third section was collected and stained with periodic acid Schiff. In the middle of each set of 30 sections per block, glomeruli were systematically and randomly sampled and examined completely throughout adjacent sections, up and down, to identify segmental sclerotic lesions.

The percentage of glomeruli showing segmental or global sclerosis was determined out of 150 glomeruli per animal from randomly selected sections. Segmental sclerosis was defined as segments of glomerular tuft demonstrating collapsed, obliterated capillaries with sparseness of normal cellular elements.

Using a point ocular grid with 19.87 mm between each point at tissue level ( $\times 420$ ), the mesangial volume fraction was estimated by calculating the ratio between the total number of points hitting the mesangial areas divided by the total number of points hitting the whole glomerular tuft. Twenty glomeruli per animal were analyzed. Only glomeruli free from sclerotic lesions were evaluated.

Mean glomerular volume was determined according to the point-counting method of Hirose et al. (23). Using the same point ocular grid and magnification described above, the number of points of each glomerular tuft were counted together with the number of sampled glomerular profiles. In a randomly selected section,  $\sim 75$  glomeruli per animal were counted.

**RNA isolation and reverse transcriptase.** Total RNA was extracted from renal cortices dissected from Sprague-Dawley rats using a commercially available kit (RNeasy KIT; Qiagen, Hilden, Germany). Quantification of RNA was performed by spectrophotometry. Rats used for the determination of CuZn superoxide dismutase (SOD) gene expression were killed after 2 days of diabetes ( $n = 3$ –6), whereas those rats used for transforming growth factor (TGF)- $\beta$ 1 gene expression were diabetic for 10 days ( $n = 3$ –7). Oligo dT primed cDNA synthesis was performed on total RNA (500 ng) reverse transcribed in a 10  $\mu$ l standard reaction mixture containing 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl $_2$ , 10 mmol/l dithiothreitol, 500 ng oligo dT, 1 mmol/l dNTP, 20 U ribonuclease inhibitor (RNasin) and 100 U reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min and then heated for 10 min at 95°C in a Gene Amp PCR system 9600 (Perkin Elmer).

**Polymerase chain reaction and quantification of CuZnSOD and TGF- $\beta$ 1.** Reaction mixtures for CuZnSOD PCR analysis were performed using 10  $\mu$ l of synthesized cDNA product in a buffer containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 2.5 mmol/l MgCl $_2$ , 0.2 mmol/l dNTP, 2.5 U Taq DNA polymerase, 0.5  $\mu$ l  $\alpha$ [ $^{32}$ P]dCTP, and 0.4  $\mu$ mol/l each of upstream and downstream primers in a final volume of 10  $\mu$ l. Reactions were carried out for 26 cycles according to the following sequence: denaturation (95°C for 30 s), primer annealing (58°C for 45 s), and extension (72°C for 90 s).  $\beta$ -actin was used as an internal control. The resultant polymerase chain reaction (PCR) products were separated by polyacrylamide gel electrophoresis and semiquantified using a phosphor-imager system (GS-250 Molecular Imager and Molecular Analyst Software; Bio-Rad, Richmond, CA). Determination of TGF- $\beta$ 1 abundance was performed in a manner similar to that described for CuZnSOD. PCR buffer was identical to that described above except that 0.8  $\mu$ mol/l each of TGF- $\beta$ 1-specific upstream and downstream primers were used. The conditions for amplification were as follows: 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. A final extension was performed at 72°C for 3 min. The total number of cycles was 32.

**Determination of renal thiobarbituric acid-reactive substances.** Analysis of renal lipid peroxide content was determined by measuring the abundance of thiobarbituric acid-reactive substance (TBARS) (24). Briefly, renal cortical tissue isolated from 10-day Sprague-Dawley diabetic rats was homogenized and sonicated in 1.15% KCl solution and then solubilized with an equal volume of 8% SDS. To this mixture, 20% acetic acid and 0.8% thiobarbituric acid was added. The complete mixture was heated to 95°C for 60 min, after which the chromogenic substrate was extracted into the organic phase with butanol/pyri-

dine (15:1). Absorbance of the organic layer was measured at 532 nm. A wavelength scan indicated that this was the predominant peak of absorbance. Absorbance was measured relative to a standard using 1,1,3,3-tetramethoxypropane.

**Isolation of glomeruli.** Kidneys were excised, capsules removed, and renal cortices separated from the medulla. Renal cortical tissue was finely chopped on ice and then passed through a series of nylon meshes of decreasing pore sizes (150, 105, and 77  $\mu\text{m}$ ) by applying gentle pressure and repeated washing with phosphate-buffered saline (PBS; pH 7.4). Glomeruli were then collected from the final 77  $\mu\text{m}$  collection sieve in PBS and kept on ice. Light microscopic examination of the glomerular suspension revealed few contaminating tubule fragments.

**Immunoblotting of PKC isozymes and detection of Na,K-ATPase phosphorylation.** For determination of PKC isozyme distribution, glomeruli from each experimental group of Sprague-Dawley rats (10 days of diabetes) were resuspended in buffer A containing 150 mmol/l sucrose, 20 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EGTA, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 20  $\mu\text{g/ml}$  leupeptin, and 100 IU aprotinin and disrupted by brief sonication. An aliquot of this lysate (total glomerular protein) was kept on ice, and the remainder was centrifuged at 100,000g for 60 min at 4°C. The resultant pellet was subsequently resuspended in buffer A supplemented with 1% (vol/vol) Triton X-100 and placed on ice for 1 h. This was followed by centrifugation at 100,000g for 60 min at 4°C. The resultant supernatant fraction (detergent-soluble proteins) and total protein aliquot were assessed for protein concentration by the Bio-Rad detergent-compatible protein assay with BSA as a standard and loaded onto a 8% SDS-PAGE gel. Gels were run for 1 h at 150 V, and proteins were transferred to PVDF membranes followed by blocking for 1 h in 0.1% Tween-20 PBS. Membranes were subsequently incubated with polyclonal antibodies to PKC- $\alpha$  (1:1,000) and PKC- $\beta$ 1 (1:2,500) for 1 h, rinsed, and exposed to secondary antibody (1:5,000) for 1 h. Immunosignals were visualized using Amersham's ECL plus reagents according to the manufacturer's specifications. PKC- $\beta$ 2 content was also analyzed by immunoblotting, but signal intensity was extremely faint and could not be quantified despite increasing the antibody concentration and film exposure time.

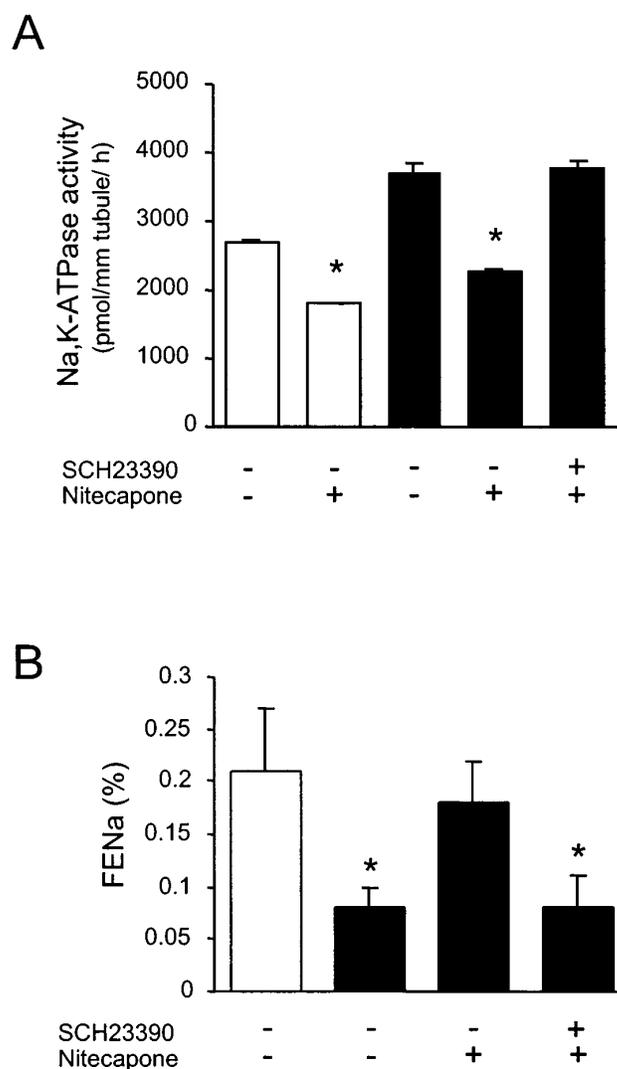
For determination of the PKC-mediated site-specific phosphorylation of Na,K-ATPase  $\alpha$ 1 at Ser23, freshly isolated glomeruli were resuspended in sample buffer (60 mmol/l Tris-HCl, 2% SDS, 2.5% [vol/vol]  $\beta$ -mercaptoethanol, and 10% glycerol), immediately loaded (~10  $\mu\text{g}$ ) onto an 8% SDS-PAGE gel, and run as described above. Immunodetection of Na,K-ATPase phosphorylation was revealed with Mck1, a Ser23 dephosphorylation-sensitive monoclonal antibody. This antibody specifically recognizes the Ser23 dephosphorylated form of rat Na,K-ATPase  $\alpha$ 1 and does not exhibit any binding affinity toward the phosphorylated form of the enzyme (25). Total Na,K-ATPase  $\alpha$ 1 protein abundance was determined with a mouse monoclonal antibody that is insensitive to changes in pump phosphorylation. To detect and correct for differences in protein loading between lanes within individual blots, membranes were stained with amido black dye following immunodetection.

**Measurement of glomerular  $^{86}\text{Rb}^+$  uptake.** Glomeruli freshly isolated from 10-day diabetic Sprague-Dawley rat kidneys were resuspended in Dulbecco's modified Eagle's medium supplemented with 24 mmol/l sodium bicarbonate and 20 mmol/l HEPES and incubated under humidified atmosphere with 5%  $\text{CO}_2/95\%$   $\text{O}_2$  at 37°C for 40 min in the presence or absence of 1 mmol/l ouabain. Preliminary experiments revealed that ouabain-mediated inhibition of  $^{86}\text{Rb}^+$  uptake had reached a plateau by 35 min and that this plateau was maintained for at least 50 min. After ouabain pretreatment,  $^{86}\text{Rb}^+$  uptake was initiated by the addition of 5  $\mu\text{Ci/ml}$   $^{86}\text{RbCl}$ . Incubations were carried out for 5 min (during which time  $^{86}\text{Rb}^+$  influx was linear) and then immediately arrested on ice with cold PBS containing 5 mmol/l  $\text{BaCl}_2$ , thereby preventing further intracellular accumulation of radioactive tracer. Extracellular radioactivity was removed from each individual glomerular treatment group by filtering through a cellulose membrane (pore size 0.22  $\mu\text{m}$ ) and repeated washes ( $3 \times 2$  ml) with PBS- $\text{BaCl}_2$ . Protein content of each glomerular sample was determined by the Bio-Rad protein assay method with BSA as a standard.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Differences between means were evaluated by Student's *t* test and analysis of variance. Statistical significance was accepted at  $P < 0.05$ .

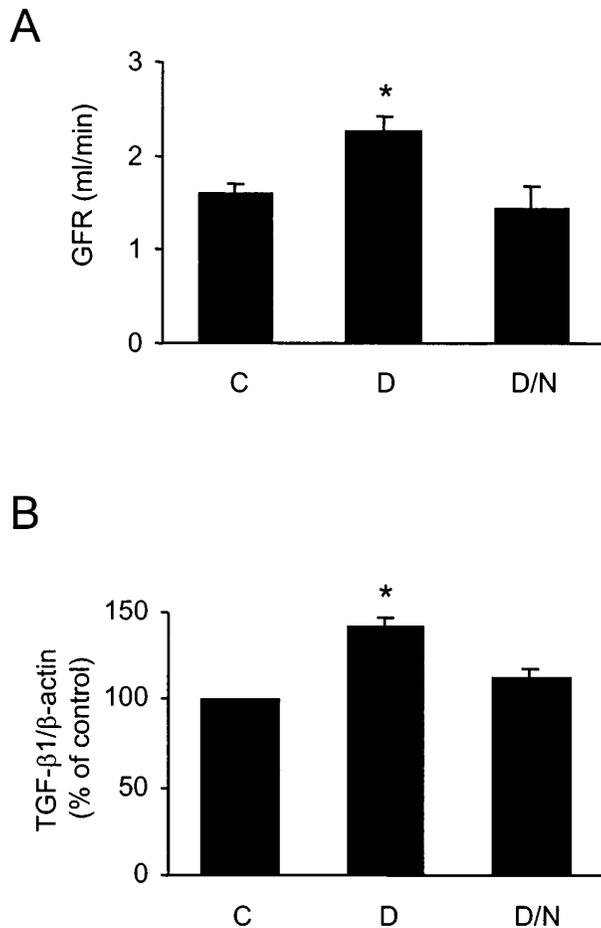
## RESULTS

**Proximal tubule Na,K-ATPase activity and sodium excretion.** Proximal tubule Na,K-ATPase activity was significantly higher in 10-day diabetic rats, and nitecapone treatment effectively blunted this increase (Fig. 1A). The nitecapone effect was likely mediated by increased activation of dopamine 1 receptors since SCH 23390, a specific antagonist



**FIG. 1.** Proximal tubule Na,K-ATPase activity (A) and fractional sodium excretion (FeNa) (B). A: Na,K-ATPase activity in single proximal tubules was measured as the rate of ouabain-sensitive ATP hydrolysis. Na,K-ATPase activity was elevated in 10-day diabetic rats. Nitecapone blocked this response, presumably via an increase in renal dopamine availability since SCH 23390 (dopamine 1 receptor antagonist) abolished the response to nitecapone. Values are means  $\pm$  SE,  $n = 6$  for each group.  $\square$ , control rats;  $\blacksquare$ , diabetic rats. \* $P < 0.001$  vs. respective bars for control and diabetic rats. B: Fractional sodium excretion was measured in control ( $\square$ ) and diabetic ( $\blacksquare$ ) rats treated with nitecapone and SCH23390 (means  $\pm$  SE,  $n = 5$ ). \* $P < 0.01$  vs. other groups.

of this receptor, completely prevented Na,K-ATPase normalization. Na,K-ATPase activity measured in normal rats treated with nitecapone was significantly lower than that of untreated rats, thereby suggesting a basal action of dopamine to blunt Na pump activity. Nitecapone-treated control and nitecapone-treated diabetic rats had similar proximal tubule Na,K-ATPase activities. We next examined whether changes in Na,K-ATPase activity were reflected by similar changes in urinary sodium excretion, as suggested by our earlier study (16). Results shown in Fig. 1B reveal a significant reduction in FeNa and a normalization toward control values in diabetic rats treated with nitecapone. This nitecapone-mediated response was prevented by dopamine 1 receptor blockade

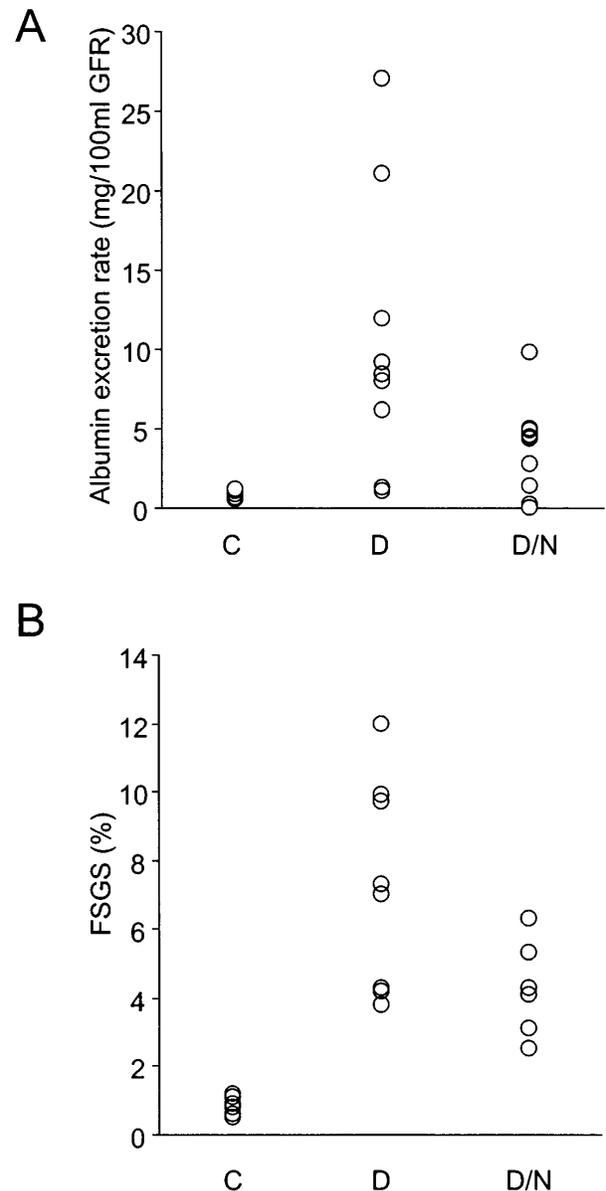


**FIG. 2.** Effect of nitecapone on glomerular filtration rate and TGF- $\beta$ 1 gene expression in control (C), diabetic (D), and nitecapone-treated diabetic (D/N) rats. GFR (A) and TGF- $\beta$ 1 (B) was measured using rats with 10 days' diabetes duration. A:  $n = 5$ ,  $*P < 0.001$  vs. other groups. B: Semiquantitative RT-PCR was used to measure mRNA levels of TGF- $\beta$ 1 in the renal cortex.  $\beta$ -actin was used to standardize quantification. Results are expressed as means  $\pm$  SE of duplicate measurements. Number of rats for C, D, and D/N groups are 7, 5, and 3, respectively.  $*P < 0.01$  vs. other groups.

and supports a role for proximal tubule Na,K-ATPase in determining diabetes-induced urinary sodium excretion.

**Glomerular filtration rate and renal cortical TGF- $\beta$ 1 mRNA expression.** Results presented in Fig. 2A show that rats rendered diabetic by STZ had a 43% increase in GFR. This response was completely abrogated by nitecapone administration. Nitecapone-treated control rats did not show any change in GFR (15). As an indicator of early diabetic glomerular alterations, semiquantitative reverse transcriptase (RT)-PCR techniques were used to examine renal cortical TGF- $\beta$ 1 gene expression in 10-day diabetic rats. TGF- $\beta$ 1 mRNA expression was elevated 42% in diabetic rats compared with control rats, and this elevation was reversed by nitecapone (Fig. 2B).

**Albumin excretion rate, glomerular morphology, and mean arterial pressure.** To establish the long-term efficacy of nitecapone in preventing the development and progression of diabetic nephropathy, Dahl salt-sensitive rats diabetic for 8 weeks were used. This particular rat strain was used because it more readily develops severe renal lesions char-



**FIG. 3.** Nitecapone prevents diabetes-induced changes in albuminuria and glomerulosclerosis. Albumin excretion rate (A) and focal segmental glomerulosclerosis (FSGS) (B) were measured in Dahl salt-sensitive rats following 8 weeks of diabetes. This rat strain was chosen because it is known to develop diabetic nephropathy early. A:  $n = 6$ , 9, and 10 for control (C), diabetic (D), and nitecapone-treated diabetic (D/N) rats, respectively. B:  $n = 6$ , 8, and 6 for C, D, and D/N, respectively.

acteristic of the advanced phase of diabetic nephropathy (21). According to this model, diabetic rats exhibited a pronounced increase in albumin excretion rate compared with nondiabetic rats. This response was markedly reduced by nitecapone (Fig. 3A). Figure 3B shows an increased prevalence of glomerulosclerosis in diabetic rats compared with both control and nitecapone-treated diabetic rats. Upon examination of nonsclerotic glomeruli exhibiting morphological changes compatible with those found in long-term human diabetes, we did not observe a significant increase in either glomerular volume or volume fraction of the mesangium (results not shown). Although mesangial expansion, in contrast to focal glomerulosclerosis, may be more relevant in extending our

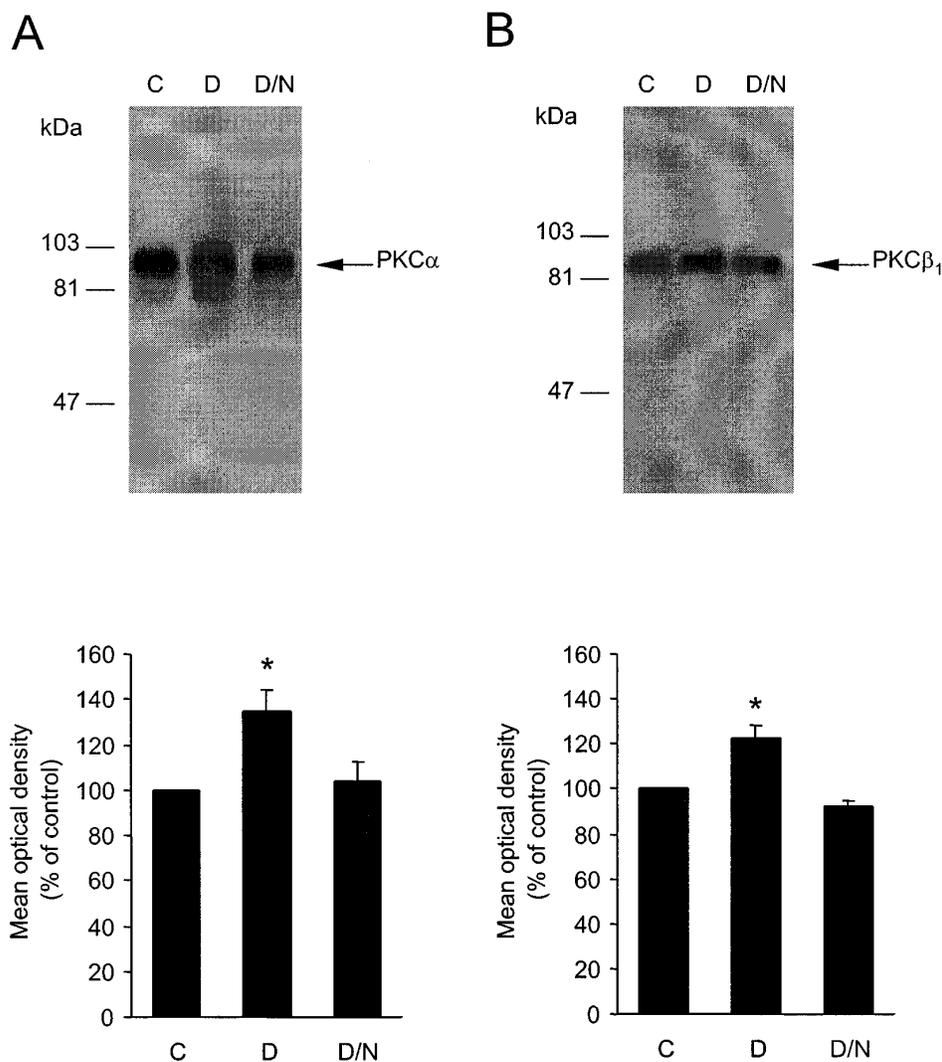
findings to human nephropathy, the relatively short duration of our experiments (compared with the time scale of human diabetic nephropathy) may have precluded the development of mesangial expansion in the diabetic rats. Extrapolation to humans of morphological changes present in diabetic rats is further complicated by differences in the development of glomerulopathy between rat and human. Such data on the efficacy of nitcapone in ameliorating human nephropathy are outside the scope of the present article, but may be obtained in clinical trials planned for the future.

Mean arterial pressure (MAP) of Dahl salt-sensitive rats used for long-term studies (8 weeks) was evaluated following anesthesia. Under these conditions, MAP levels obtained from control ( $n = 6$ ) and diabetic ( $n = 9$ ) rats were  $131 \pm 2.4$  and  $127 \pm 4.3$  mmHg, respectively. We did note a slight MAP decrease in nitcapone-treated diabetic rats ( $122 \pm 7.2$  mmHg,  $n = 10$ ; NS vs. control and diabetic).

**Glomerular PKC- $\alpha$  and PKC- $\beta$ 1.** PKC translocation to detergent-soluble membrane components is associated with activation of specific PKC isoforms. Such assays have implicated isoforms  $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\delta$ , and  $\epsilon$  in the development of diabetic complications (8,26,27). In the present study, PKC isozymes  $\alpha$  and  $\beta$ 1 were analyzed by immunoblotting of glomeruli isolated from 10-day diabetic Sprague-Dawley rats.

Total glomerular PKC- $\alpha$  and PKC- $\beta$ 1 protein expression was not significantly altered under each test condition (% control: PKC- $\alpha$ : diabetic  $118 \pm 19$ , diabetic/nitcapone  $122 \pm 23$ ,  $n = 4$ ; PKC- $\beta$ 1: diabetic  $114 \pm 17$ , diabetic/nitcapone  $108 \pm 26$ ,  $n = 4$ ), whereas the membrane-associated portion of each isoform was significantly enhanced in diabetic rats (Fig. 4). Nitcapone treatment normalized PKC- $\alpha$  and - $\beta$ 1 isoform membrane association.

**Glomerular Na,K-ATPase activity and state of phosphorylation.** The catalytic  $\alpha$ 1 subunit of rat renal Na,K-ATPase can be phosphorylated by PKC and protein kinase A, and phosphorylation is generally associated with diminished enzyme activity. The PKC phosphorylation site has been identified on the Ser23 residue in the NH<sub>2</sub>-terminal region of rat  $\alpha$ 1, whereas the PKA phosphorylation site is located in the fourth cytoplasmic loop. Here, we have probed glomerular Na,K-ATPase with an antibody (Mck1) that recognizes non-phosphorylated Ser23 in the NH<sub>2</sub>-terminal region and another antibody (6F) that recognizes the  $\alpha$ 1 subunit at another site, independent of any change in phosphorylation (25). To determine the degree of PKC-dependent Ser23 phosphorylation, glomerular proteins were transferred to PVDF membranes and immunoblotted with Mck1. Recognition of glomerular nonphosphorylated Na,K-ATPase  $\alpha$ 1 from STZ-diabetic rats



**FIG. 4.** Immunodetection of PKC- $\alpha$  and PKC- $\beta$ 1 isoforms from glomeruli of control (C), diabetic (D), and nitcapone-treated diabetic (D/N) rats. Representative blots depicting PKC isoforms  $\alpha$  (A) and  $\beta$ 1 (B) membrane expression are shown in the upper panels. No other significant bands were detected on the membranes. The results of densitometric analysis of blots from five 10-day diabetic rats within each group are presented in the lower panels. The position of molecular weight markers is indicated to the left of each blot. \* $P < 0.05$  vs. other groups.

was reduced to 63% of that observed in control rats. Nitecapone treatment reversed this response (Fig. 5A). Detection of total Na,K-ATPase abundance was carried out with 6F and—as depicted in Fig. 5B—Na,K-ATPase protein abundance remained similar in each experimental group.

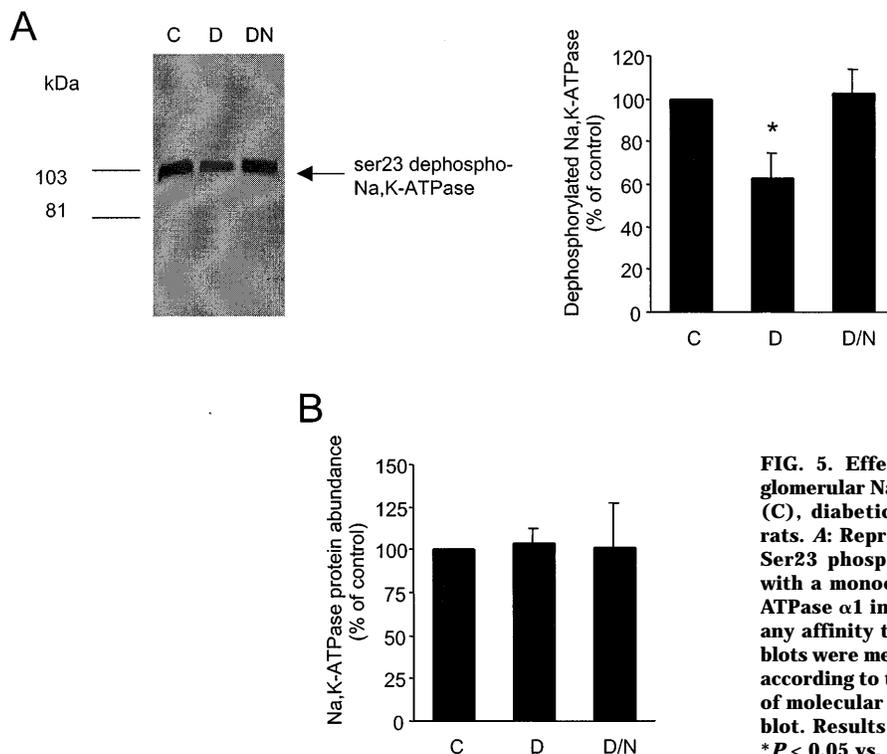
Ouabain-sensitive  $Rb^+$  uptake was used as an index of glomerular Na,K-ATPase activity.  $Rb^+$  uptake in diabetic glomeruli was significantly reduced compared with control (control  $76 \pm 6.9$  vs. diabetic  $55 \pm 4.6$  pmol  $\cdot$  mg $^{-1}$   $\cdot$  min $^{-1}$ ,  $P < 0.05$ ). The uptake of nitecapone-treated diabetic rats was similar that of control ( $78 \pm 8.4$  pmol  $\cdot$  mg $^{-1}$   $\cdot$  min $^{-1}$ ). Maintenance of diabetic rats on insulin therapy ameliorated the decrease in  $Rb^+$  uptake (result not shown).

**CuZnSOD mRNA expression and TBARS.** As an indicator of early altered oxidative status, renal cortical CuZnSOD gene expression was measured 2 days after the induction of diabetes. Results presented in Fig. 6A show that CuZnSOD mRNA levels were significantly elevated over control in diabetic rats and that nitecapone treatment completely prevented this increased gene expression. To establish whether hyperglycemia or a possible nephrotoxic effect of STZ was responsible for the observed changes in CuZnSOD gene expression at this early time point, 2-day STZ-diabetic rats were maintained normoglycemic by insulin administration. As shown in Fig. 6A, the prevention of hyperglycemia by insulin treatment abrogated the increase in CuZnSOD gene expression. This result strongly supports a direct role for hyperglycemia in mediating elevated CuZnSOD expression in diabetes and does not support a potential nonspecific effect of STZ. As another measure of modified oxidative homeostasis, analysis of renal cortical lipid peroxide content was determined by measuring TBARS levels in 10-day diabetic rats. Results presented in Fig. 6B show TBARS levels were elevated in diabetic rats and that nitecapone-treated diabetic rats had normalized levels.

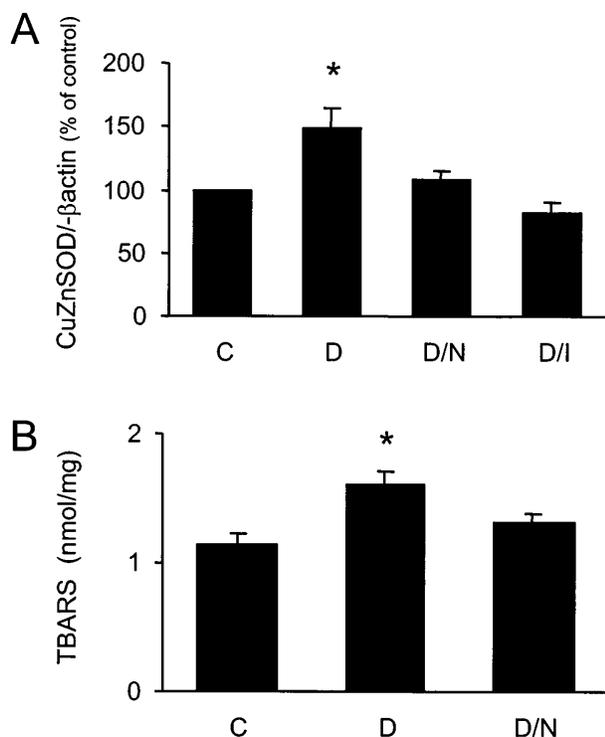
## DISCUSSION

Nitecapone [3-(3,4-dihydroxy-5-nitrophenyl)methylene-2,4-pentadione], a compound that is both an antioxidant and an inhibitor of the dopamine-metabolizing enzyme COMT, dramatically reversed many characteristic physiological and cellular manifestations of renal dysfunction in STZ-induced diabetic rats. Administration of nitecapone effectively abolished glomerular hyperfiltration, significantly attenuated albuminuria and the development of glomerulosclerosis, and normalized urinary sodium excretion in diabetic rats. At the biochemical level, early overexpression of the pro-sclerotic cytokine TGF- $\beta$ 1 was abolished, and glomerular derangements in PKC and Na,K-ATPase function were normalized. The results indicate that nitecapone can prevent many of the physiological and biochemical changes associated with diabetic renal dysfunction. In addition, as discussed below, it is likely that both the COMT inhibitory and antioxidant properties of the drug are responsible for its protective effects.

We suggest that the natriuretic effect of nitecapone in diabetic rats can be most readily attributed to its capacity to inhibit COMT and thereby increase the intrarenal availability of dopamine. Previously, we showed that acute administration of nitecapone induces dopamine-dependent proximal tubule Na,K-ATPase inhibition and associated natriuresis (16). In diabetes, proximal tubule Na,K-ATPase activity is increased and this may contribute to enhanced sodium retention (20). In the present series of experiments, nitecapone-treated diabetic rats exhibited elevated proximal tubule Na,K-ATPase activity and reduced urinary sodium excretion compared with untreated diabetic rats. Similar to the situation described above, we found that by blocking dopamine signaling with the dopamine 1 receptor antagonist, SCH 23390, we could also prevent the effect of nitecapone on natriuresis and proximal tubule Na,K-ATPase in diabetic rats. The com-



**FIG. 5.** Effect of nitecapone treatment on the state of glomerular Na,K-ATPase  $\alpha$ 1 Ser23 phosphorylation in control (C), diabetic (D), and nitecapone-treated diabetic (D/N) rats. **A:** Representative blot of glomerular Na,K-ATPase  $\alpha$ 1 Ser23 phosphorylation. Immunodetection was performed with a monoclonal antibody that specifically detects Na,K-ATPase  $\alpha$ 1 in its dephosphorylated state and does not show any affinity toward the phosphorylated form. Bands from 3 blots were measured densitometrically and were standardized according to total Na,K-ATPase expression (**B**). The position of molecular weight markers is indicated to the left of each blot. Results are means  $\pm$  SE;  $n = 3$  for each animal group. \* $P < 0.05$  vs. C and D/N rats.



**FIG. 6.** Effect of nitecapone on renal cortical CuZnSOD gene expression and TBARS content. **A:** CuZnSOD gene expression was measured in 2-day diabetic rats. Semiquantitative RT-PCR was used to measure mRNA levels of CuZnSOD.  $\beta$ -actin was used to standardize quantification.  $n = 6$  for control (C), diabetic (D), and nitecapone-treated diabetic (D/N) rats.  $n = 3$  for insulin-treated diabetic (D/I) rats. \* $P < 0.05$  vs. other groups. **B:** TBARS were measured in duplicate using renal cortices isolated from 10-day diabetic rats. Data are means  $\pm$  SE,  $n = 6$ . \* $P < 0.05$  vs. other groups.

bined effect of nitecapone on proximal tubule Na,K-ATPase activity and natriuresis is compatible with COMT inhibition and a concomitant increase in dopamine 1 receptor signaling.

A discussion of our MAP data is warranted in light of the sodium-wasting that occurs in response to nitecapone treatment. Several lines of evidence suggest that hyperglycemia leads to sodium retention and the development of hypertension, a common complication of type 2 diabetes (2). Hypertension aggravates diabetic nephropathy, and antihypertensive therapy is widely used to prevent disease progression. In Dahl salt-sensitive rats, we found that MAP was slightly lower in diabetic rats that received nitecapone than in those that did not. This may suggest that the natriuretic and related antihypertensive effect of nitecapone contributed to the attenuation of albuminuria and the reduced incidence of focal segmental glomerulosclerosis seen in these long-term studies. The MAP data should be interpreted with caution, however, since the measurements were performed in anesthetized rats. MAP of untreated diabetic rats was not significantly different from that of control rats. It is possible that the diabetic rats were, because of their rather pronounced hyperglycemia, somewhat more sensitive to the arterial pressure lowering effects of anesthesia than were the control rats. It will be an important topic for further study to directly address the influence of nitecapone on sodium metabolism, MAP, and other circulatory parameters.

Although hyperglycemia results in elevated renal proximal tubule Na,K-ATPase activity, this activity is paradoxically diminished in other tissues that are affected by diabetic complications, such as the nerves, retina, and glomeruli. In contrast to the ability of nitecapone to reduce diabetic proximal tubule Na,K-ATPase activity, we think it unlikely that COMT inhibition is responsible for the capacity of nitecapone to increase abnormal diabetic glomerular Na,K-ATPase activity. The reasons for this are 2-fold. First, we have not found any effect of dopamine on glomerular Na,K-ATPase activity when administered to normal nondiabetic rats (unpublished results). Second, dopamine has never been observed to have a stimulatory effect on Na,K-ATPase activity. The regulation of glomerular Na,K-ATPase activity is discussed later.

In contrast to the proximal tubule response, the effect of nitecapone on hyperfiltration and other signs of glomerular dysfunction are unlikely to be explained by COMT inhibition and increased renal dopamine availability. Dopamine is known to increase GFR in nondiabetic rats; therefore, it would be predicted that this effect might exacerbate glomerular dysfunction in diabetes. Accordingly, we predict that nitecapone-mediated amelioration of glomerular function may occur via a mechanism dependent on the antioxidant properties of the drug. This is supported by the apparent relationship between diabetes and elevated oxidative stress and by the reported ability of various agents possessing different antioxidant characteristics to ameliorate many of the glomerular abnormalities associated with diabetes (28–30). Altered levels of circulating antioxidants (28,31), intracellular scavenging enzymes (32,33), and increased amounts of oxidatively modified cellular components (12,30,31) have been used as indicators of an oxidative stress imbalance in diabetes. In the present protocol, we examined 2 indexes that have previously been used as measures of oxidative stress, namely CuZnSOD gene expression and TBARS content. Both markers were increased in diabetic rats and normalized by nitecapone treatment. This result is consistent with a diabetes-induced change in oxidative homeostasis and establishes the antioxidant potential of nitecapone in this model. The degree to which the antioxidant nature of nitecapone contributes directly to the prevention of diabetic nephropathy is not definitively established in the present study, but our results regarding the ability of nitecapone to normalize biochemical mediators (PKC, TGF- $\beta$ 1) critically associated with the development of late complications (albuminuria and glomerulosclerosis) establishes a potential mode of action.

According to one paradigm of hyperglycemia-mediated cellular dysfunction, PKC is a key early mediator (7,34). Strategies aimed at preventing PKC activation in diabetes-sensitive tissues, including glomeruli, have provided promising results suggesting that intervention at this level can protect against hyperglycemia-mediated abnormalities in glomerular filtration and albuminuria (8,26,29). Similar to findings reported by Koya et al. (8), we detected an increase in membrane-associated PKC- $\alpha$  and PKC- $\beta$ 1 glomerular proteins in glomeruli isolated from diabetic rats. Although additional PKC isoforms may be translocated and modulated independently of one another in this particular model (27), the significance of the various isozyme responses in mediating glomerular dysfunction are unknown. Only in terms of PKC- $\beta$ 1 and - $\beta$ 2 has evidence emerged to definitively impli-

cate these isozymes in initiating diabetic complications (8,26). With respect to nitecapone-treated diabetic rats, a normalization of glomerular PKC activation was noted, which is consistent with a potential antioxidant-dependent mechanism of PKC inhibition (9,29,35). The common pathway by which different antioxidants act to prevent PKC activation in diabetes has not been clearly defined, but it is speculated that they exert their neutralizing effect either by interfering with diacylglycerol metabolism (29) or by disrupting the ability of diacylglycerol to interact with and activate PKC (9). Interestingly, PKC can be activated by reactive oxygen species (36,37), and it is possible that antioxidant therapies exert their beneficial effects through an ability to directly quench the radicals that are generated during hyperglycemia. Such possible scenarios for nitecapone action need to be examined in future studies.

To confirm the likelihood that PKC translocation is also associated with PKC activation in diabetic glomeruli, we chose to examine the *in vivo* phosphorylation of Na,K-ATPase  $\alpha 1$ , an endogenous cellular substrate of PKC (25,38–41). By analyzing the change in PKC-dependent Na,K-ATPase  $\alpha 1$  phosphorylation, we have integrated the activation of PKC with the regulation of a physiologically relevant substrate whose activity is reduced in diabetic glomeruli (8,42). To assess whether diabetic glomeruli possess an increased kinase activity toward Na,K-ATPase, we used a monoclonal antibody that uniquely and specifically recognizes the Na,K-ATPase  $\alpha 1$  subunit based on its phosphorylation at Ser23 located within a PKC motif in the NH<sub>2</sub>-terminus (25). This site has recently been identified as the major PKC phosphorylation locus of rat Na,K-ATPase  $\alpha 1$ , and it is not phosphorylated by any other known kinases (39,41). As shown in Fig. 5, Na,K-ATPase  $\alpha 1$  Ser23 phosphorylation was increased in glomeruli isolated from diabetic rats (visualized as reduced recognition with the Ser23 dephospho-sensitive antibody) in the absence of any significant change in total Na,K-ATPase  $\alpha 1$  protein abundance. The observed increase in PKC-dependent Na,K-ATPase Ser23 phosphorylation reflects an increase in activated PKC and supports our translocation data.

Altered function of the electrogenic Na,K-ATPase is also a typical characteristic of diabetes-sensitive tissues such as the glomerulus, and elevated PKC activity has been coupled to this observation (8,43). Evidence in favor of a possible PKC-mediated decrease in glomerular Na,K-ATPase activity in diabetic glomeruli is provided by the observed nitecapone-dependent blockade of PKC activation and Na,K-ATPase  $\alpha 1$  ser 23 phosphorylation concomitant with the normalization of Na,K-ATPase activity. Since phosphorylation of rat  $\alpha 1$  Ser23 has been reported to confer a reduction in pump activity (41,44), it is not unlikely that prevention of PKC activation and Na,K-ATPase phosphorylation directly contributes to the observed normalization of pump activity produced by nitecapone. Although the combined observations strengthen the potential role of oxidative stress in regulating PKC-dependent activation of downstream Na,K-ATPase activity, it is also possible that increased hyperglycemia-mediated oxidative stress directly alters Na,K-ATPase function (45).

An interesting question in treating diabetes is whether intervention directed at inhibiting a single event can provide protection. Indeed, many alterations occur simultaneously and interdependently during the course of disease, and treatments cannot be considered separately. In addition to the

maintenance of normoglycemia, supplemental therapies can provide an added level of organ protection. In this framework, we observed that nitecapone, a dual natriuretic and antioxidant compound, can provide protection against many features of diabetes-induced renal dysfunction. The potential therapeutic use of nitecapone should be considered and examined in future clinical trials.

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