PP2B-dependent NO production in the medullary thick ascending limb during diabetes

Jan M. Foster,1 Pamela K. Carmines,2 and Jennifer S. Pollock1

1Vascular Biology Center, Medical College of Georgia, Augusta, Georgia; and 2Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

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Foster JM, Carmines PK, Pollock JS. PP2B-dependent NO production in the medullary thick ascending limb during diabetes. Am J Physiol Renal Physiol 297: F471–F480, 2009. First published May 20, 2009; doi:10.1152/ajprenal.90760.2008.—Calcineurin (PP2B) has recently been shown to be upregulated in the medullary thick ascending limb (mTAL) during diabetes. The mTAL expresses all three isoforms of nitric oxide synthase (NOS), which are subject to phosphoregulation and represent substrates for PP2B. Therefore, we hypothesized that diabetes induces PP2B-dependent upregulation of NOS activity and NO production in the mTAL. Three weeks after injection of streptozotocin (STZ) rats or vehicle (sham rats), mTAL suspensions were prepared for use in functional and biochemical assays. PP2B activity and expression were increased in mTALs from STZ rats compared with sham. Nitrite production was significantly reduced in mTALs from STZ rats compared with sham. However, incubation with the free radical scavenger, tempol, unmasked a significant increase in nitrite production by mTALs from STZ rats. Inhibition of PP2B attenuated the increase in nitrite production and NO activity evident in mTALs from STZ rats. Analysis of specific NOS isoform activity revealed increased NOS1 and NOS2 activities in mTALs from STZ rats. All three NOS isoform activities were regulated in a PP2B-dependent manner. Western blot analysis detected no differences in NOS isoform expression, although phosphorylation of pThr495-NOS3 was significantly reduced in mTALs from STZ rats. Phosphorylation of pSer1177-NOS3 and pSer633-NOS3, and pSer1177-NOS3 was similar in mTALs from STZ and sham rats. Inhibition of PP2B did not alter the phosphorylation of NOS1 or NOS3 at known sites. In conclusion, while NO bioavailability in mTALs is reduced during diabetes, free radical scavenging with tempol unmasked increased NO production that involves PP2B-dependent activation of NOS1 and NOS2.

Address for reprint requests and other correspondence: J. S. Pollock, Vascular Biology Center, Medical College of Georgia, 1459 Laney Walker Blvd., Augusta, GA 30912 (e-mail: jpollock@mcg.edu).


GOOCH ET AL. (11) reported increased expression and activity of the serine/threonine protein phosphatase 2B (PP2B; calcineurin) in the renal cortex and medulla of rats with streptozotocin (STZ)-induced diabetes mellitus (STZ rats; 2 wk after onset). The most prominent increase in PP2B immunostaining in kidneys from STZ rats is evident in the medullary thick ascending limb (mTAL) (12). STZ rats treated chronically with the selective PP2B inhibitor, cyclosporin A (CsA), exhibit increased transforming growth factor (TGF)-β and fibronectin mRNA levels and extracellular matrix accumulation in the renal tubulointerstitium, as well as exaggerated diabetic hyperfiltration relative to untreated STZ rats (11). Thus, upregulation of PP2B may represent a protective mechanism to limit renal damage during diabetes.

Among the myriad substrates of PP2B, nitric oxide synthases (NOS) are widely expressed in the kidney and changes in their phosphorylation status represent a major mechanism controlling NO production. There are known serine/threonine phosphorylation sites on NOS synthase 1 (NOS1; neuronal NOS) and NO synthase 3 (NOS3; endothelial NOS). PP2B has been shown to dephosphorylate pSer852-NOS1 in hypothalamic neurons (50), as well as pSer1177-NOS3 and pThr633-NOS3 in endothelial cells (15). Dephosphorylation of the NOS1 site and the threonine NOS3 site results in increased NOS activity and NO production. Thus, increased PP2B activity in the mTAL during diabetes may promote increased NOS activity and NO production. Although limited investigation focused on the NO pathway in the renal medulla during diabetes, our laboratory documented increased NOS3 activity without concomitant changes in NOS3 protein levels in renal medullary homogenates from STZ rats (22). Shin et al. (42) found increased NOS1 and NOS3 mRNA expression in the outer medulla of STZ rats, but no change in NO synthase 2 (NOS2; inducible NOS) mRNA, with the greatest increase in immunostaining intensity for NOS1 and NOS3 located in the proximal straight tubule and the mTAL. Interestingly, very little is known regarding the regulation of NOS2 phosphorylation. Currently, there is no evidence in the literature suggesting that PP2B may regulate NOS2 phosphorylation.

Despite immunohistochemical evidence suggesting that both PP2B and NOS protein levels may be upregulated in the mTAL during diabetes, there is no specific information available regarding the impact of diabetes on the activities of these enzymes in the mTAL or the role of PP2B in regulating NOS activity and NO production by the mTAL under these conditions. The overall hypothesis guiding our study states that diabetes induces PP2B-dependent NO production and NOS activity. Accordingly, the aims of this study were 1) to verify the upregulation of PP2B activity and expression in mTAL suspensions from diabetic rats, 2) to test the hypothesis that NO production and NOS activity are increased in mTALs from diabetic rats and to determine which NOS isoform(s) are involved, and 3) to test the hypothesis that increased activity of one or more NOS isoforms in the mTAL during diabetes is PP2B dependent.

METHODS

Animal model. All animal studies were approved by the Medical College of Georgia Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments utilized male Sprague-Dawley rats (250 g body wt, Harlan Laboratories). On the first day of the study, each rat was randomly divided into two groups: sham rats (vehicle treatment) and STZ rats (STZ-induced diabetes). The rats were weighed and initial blood
glucose measurements were obtained (Accu-Check III model 766; Boehringer Mannheim, Indianapolis, IN). Under isoflurane anesthesia, STZ (65 mg/kg; Sigma, St. Louis, MO) or vehicle (saline) was injected intravenously. Upon recovery from anesthesia, the rats were returned to the animal facility and provided free access to food and water. The following day, a 2.3 × 2.0-mm sustained-release insulin implant (Linplant; Linshin Canada, Scarborough, Ontario) was inserted subcutaneously in STZ rats to maintain afternoon blood glucose levels within 400–500 mg/dl range. Sham rats received a vehicle (palmitic acid) implant. Blood glucose and body weights were measured approximately every 3 days. Terminal studies were performed 3 wk after STZ or vehicle injection.

**mTAL suspensions and incubations.** mTAL suspensions were prepared following the method originally described by Chamberlin et al. (3), with slight modifications. Briefly, under pentobarbital sodium anesthesia (50 mg/kg ip), the kidneys were flushed via retroperitoneal perfusion from the aorta between the renal arteries with 20 ml of 95% O2-5% CO2-equilibrated HBSS (MediaTech, Manassas, VA) containing 1 mg/ml collagenase (Sigma), protease inhibitors (1 μM aprotinin, 2 μM leupeptin, 1 μM pepstatin A, 1 mM PMSF; Sigma), a panel of phosphatase inhibitors targeting acid and alkaline phosphatases and protein tyrosine phosphatases but not serine/threonine phosphatases (2 mM imidazole, 1 mM NaF, 1.15 mM Na3VO4, 1 mM Na2VO4, 4 mM Na2NH3C2H4O2·H2O; from Calbiochem Phosphatase Inhibitor Cocktail Set II) and either 5.5 mM glucose (sham rats) or 20 mM glucose (STZ rats). The inner stripe of the outer medulla was dissected from coronal slices of the kidneys and minced into ~1-mm3 pieces using a McIlwain tissue chopper (Warner Instruments, Hamden, CT). Tissue pieces were digested in HBSS/collagenase with protease and phosphatase inhibitors at 37°C for 30 min while bubbling with 95% O2-5% CO2, with gentle agitation every 5 min. After centrifugation, pellets were resuspended in ice-cold HBSS without collagenase with protease and phosphatase inhibitors and continuously agitated on ice for 20 min. The neprhin segments were filtered through a 250-μm nylon mesh and washed twice with ice-cold HBSS. For most experiments, the resulting mTAL suspensions were incubated at 37°C for 30 min in the presence of one or more of the following: 250 μM l-arginine (NOS substrate; Sigma), 10 mM 4-hydroxy-TEMPO (tempol; free radical scavenger; Sigma), 200 μM PEG-SOD (polyethylene glycol-superoxide dismutase; cell-permeable superoxide scavenger; Sigma), 100 ng/ml CsA (PP2B inhibitor; Sigma), 100 μM calcineurin auto-inhibitory peptide (CAIP; PP2B inhibitor, Calbiochem/EMD, Gibbstown, NJ), 100 μM scrambled peptide (SP; GenScript, Piscataway, NJ), 100 μM poly-arginine peptide (GenScript), 1 μM Nω-(1-imino-3-butenyl)-l-ornithine (VNIO; NOS1 inhibitor; Alexis/Enzo, Plymouth Meeting, PA), 100 mM 1400W (dihydrochloride; 1400W; NOS2 inhibitor; Cayman, Ann Arbor, MI), or vehicle (HBSS). Following this incubation, mTAL suspensions were pelleted by centrifugation. The incubation buffer and mTAL pellets were flash-frozen separately in liquid nitrogen for storage at ~80°C. Nitrite levels were measured in the incubation buffer; the mTAL pellets were utilized for Western blot analysis, as well as measurement of PP2B activity and NOS activity.


**PP2B activity.** PP2B activity was measured using a commercially available, colorimetric Calcineurin Activity Kit (Calbiochem) that utilizes the RII phosphopeptide substrate. Briefly, mTALs were lysed with manufacturer’s supplied lysis buffer and centrifuged at 150,000 g. The soluble fraction was desalted to remove excess phosphate. To discriminate between the activity of PP2B and other phosphatases, activity was measured in the presence and absence of EGTA (Ca2+ chelator) and okadaic acid (PP1 and PP2A inhibitor). Free phosphate released was detected using the malachite green reagent, with absorbance read at 620 nm. Protein concentration was determined using the standard Bradford assay. Activity is reported as nanomoles of phosphate per milligram of protein per 30 min.

**NOS activity.** NOS activity was determined by the conversion of [3H]arginine to [3H]citrulline, as previously described (22). Briefly, mTALs incubated in the presence/absence of CsA, but in the absence of exogenous l-arginine, were lysed by sonication in homogenization buffer (39) containing protease and phosphatase inhibitors. Total NOS activity was defined as the [3H]arginine to [3H]citrulline conversion inhibited by the nonselective NOS inhibitor Nω-nitro-l-arginine (l-NNA; 1 mM). The activity of each NOS isoform was determined based on the impact of isoform-selective inhibitors of NOS1 (1 μM VNIO) and NOS2 (100 nM 1400W). NOS3 activity was calculated as total NOS activity minus NOS1 and NOS2 activities. Results were normalized to protein concentration determined by the Bradford method and expressed as picomoles per nanogram of protein per 30 min.

**Nitrite production.** After incubation of mTAL suspensions for 30 min in the presence of exogenous l-arginine and in the absence/ presence of various inhibitors, the incubation buffer was collected for determining nitrite production. The concentration of nitrite, a metabolite of NO, in the incubation buffer was measured using a sensitive HPLC system (ENO-20; EiCom, Kyoto, Japan), according to the manufacturer’s protocol. Briefly, the nitrite present in 80 μl of mTAL incubation buffer was separated from other substances on a unique separation column and mobile phase. Nitrite was reacted with Griess reagent postcolumn to generate diazo compounds that were measured by absorbance at 540 nm with an in-line detection system. The sensitivity limit of this assay is 0.1 pmol nitrite and sample quantification was achieved using a nitrite standard curve. Nitrite was normalized to protein concentration determined by the Bradford method (Bio-Rad).

**Western blot analysis.** Western blot analysis was performed as described previously (39) with modifications. Briefly, mTALs were lysed by sonication in homogenization buffer (39) containing protease and phosphatase inhibitors. Proteins (80 μg per lane) were separated by SDS-PAGE and transferred to PVDF. Membranes were blocked with Blocking One-P (Piscataway, NJ), pSer633NOS3 (1:500; Upstate), pSer 617NOS3 (1:500; Upstate), NOS1 (1:250; BD Transduction Laboratories, San Jose, CA), NOS2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), NOS3 (1:500; BD Transduction Laboratories), and β-actin (1:10,000, Sigma). Some assays employed phosphorylation site-specific primary antibodies targeting pSer1177NOS3 (1:500; BD Transduction Laboratories) and pSer442NOS3 (1:500; Upstate), pThr495NOS3 (1:500; Upstate), pSer1163NOS3 (1:500; Upstate), or pSer852NOS1 (1:100; Santa Cruz Biotechnology). After incubation with secondary antibody (IRDye 800-conjugated affinity-purified anti-mouse IgG, 1:2,000 or AlexaFluor 680-conjugated affinity-purified anti-rabbit IgG, 1:2,000), the membrane was scanned and the intensity of specific bands was quantified using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE).

**Live/dead assay.** The Molecular Probes LIVE/DEAD Viability/ Cytotoxicity Kit (Invitrogen, Carlsbad, CA) was used to measure cell viability, according to the manufacturer’s standard protocol. Briefly, mTALs were incubated for 30 min in the presence of l-arginine (250 μM), tempol (10 mM), and in the presence/absence of CsA (100 ng/ml) or CAIP (100 μM). Following incubation, 100 μl of mTAL suspensions were loaded onto a 96-well plate in triplicate. Calcein AM fluorescent dye (2 μM) was added to each well for 30 min. Fluorescence was determined using an automated plate reader at the
RESULTS

During the 3-wk period between injection of STZ (or vehicle) and the terminal study, blood glucose values were significantly greater in STZ rats (473 ± 15 mg/dl; n = 22) than in sham rats (94 ± 3 mg/dl; n = 20; P < 0.05). Rats in both groups gained weight during this period, although the STZ rats did so at a slower rate. Accordingly, at the time of the terminal study, body weight of sham rats (320 ± 7 g; n = 20) significantly exceeded that of STZ rats (284 ± 4 g; n = 22; P < 0.05).

**PP2B expression and activity.** PP2B protein levels were measured by Western blot analysis of the regulatory (PP2B-B) and catalytic (PP2B-Aβ) subunits in mTAL homogenates from sham and STZ rats. As shown in Fig. 1A, both subunits of PP2B were significantly upregulated in mTALs from STZ rats compared with sham. In addition, PP2B activity was increased by ~40% in mTALs from STZ rats compared with sham (Fig. 1B). Furthermore, 30-min incubation of mTALs with 100 ng/ml CsA significantly reduced PP2B activity by ~90% (P < 0.05 vs. untreated) in both sham and STZ groups, thus validating the efficacy of the acute CsA treatment as a PP2B inhibitor in our experimental setting.

**NOS activity.** Total NOS activity was significantly greater in mTAL homogenates from STZ rats than in homogenates prepared from sham rats (Fig. 2A). Moreover, incubation of the mTALs with CsA substantially reduced total NOS activity in mTALs from STZ rats, but had no significant effect on total NOS activity in mTALs from sham rats (Fig. 2A). NOS1 and NOS2 activities were found to be increased in mTALs from STZ rats, compared with sham (Fig. 2B, C, and D). Incubation in the presence of CsA reduced both NOS1 and NOS2 activities to levels below detection in mTALs from STZ rats, but did not significantly alter NOS1 or NOS2 activities in mTALs from sham rats. NOS3 activity in mTAL homogenates did not differ between STZ and sham rats (Fig. 2D); however, CsA treatment significantly reduced NOS3 activity in STZ rats (P < 0.05 vs. untreated). Thus, although NOS3 activity in the mTAL is PP2B dependent, it is not increased during diabetes. Rather, NOS1 and NOS2 activities are significantly increased in the mTAL during diabetes in a PP2B-dependent manner.

**Nitrite production.** Nitrite production by mTALs was measured based on accumulation of nitrite, an NO metabolite, in the buffer during a 30-min incubation period. Results were normalized to sham control (256 ± 74 pmol nitrite-mg protein⁻¹·30 min⁻¹; n = 9). In preliminary experiments, nitrite production was not affected by the presence or absence of L-arginine in the incubation media (data not shown); nevertheless, because NO production can be substrate limited in mTALs studied in vitro (37), the nitrite production experiments presented here were all conducted in the presence of 250 μM L-arginine. Under these conditions and in the absence of pharmacological agents, nitrite production by mTALs from STZ rats was ~50% less than that of mTALs from sham rats (P < 0.05; Fig. 3A). As the mTAL is a primary renal site of superoxide anion (O₂⁻) production (23, 53), and because of the ability of O₂⁻ to rapidly react with NO to form peroxynitrite (which is not subsequently metabolized to nitrite), we also measured nitrite production by mTALs incubated in the presence of 10 mM tempol (free radical scavenger) or 200 U/ml PEG-SOD (superoxide scavenger). Nitrite production by mTALs from STZ rats was increased more than sevenfold in the presence of tempol compared with that evident in the absence of tempol (Fig. 3A; P <
Moreover, in the presence of tempol, nitrite production by mTALs from STZ rats was three times higher than that of mTALs from sham rats (Fig. 3A; \(P < 0.05\)). Similarly, nitrite production by mTALs from STZ rats incubated in the presence of PEG-SOD was 3.3 \(\pm\) 0.4 (n = 6) times higher than mTALs from sham rats \((P < 0.05)\). Nitrite production by mTALs from sham rats was unaltered by tempol (Fig. 3A; \(P < 0.05\)). Based on these observations, nitrite production measured in the presence of tempol is considered to represent a reasonable index of NO production.

To determine the impact of PP2B on NO production during diabetes, we measured nitrite production by mTALs from sham and STZ rats incubated with tempol in the presence or absence of CsA (100 ng/ml), CAIP (100 \(\mu\)M), SP (100 \(\mu\)M), or poly-arginine peptide (100 \(\mu\)M). As shown in Fig. 3B, there was no significant effect of CsA on mTALs from sham rats; however, incubation with CsA reduced nitrite production under these conditions by \(~44\%\) in mTALs from STZ rats \((P < 0.05\) vs. STZ untreated/vehicle), achieving values that did not differ significantly from sham. As shown in Fig. 3C, CAIP reduced nitrite production by \(~59\%\) in mTALs from STZ rats \((P < 0.05\) vs. STZ untreated/vehicle), while the SP (a control for CAIP) did not change nitrite production. The 11 amino acid poly-arginine peptide also failed to alter nitrite production (data not shown).

Additional experiments were performed to determine which NOS isoform(s) represent the enzymatic source of accelerated NO production by mTALs during diabetes. Nitrite production by mTALs from STZ rats, measured in the presence of tempol, was found to be significantly reduced by isoform-selective NOS1 and NOS2 inhibitors (VNIO and 1400W, respectively; Fig. 3D). These inhibitors did not alter nitrite production by mTALs from sham rats measured under identical conditions. Thus, the increased NO production by mTALs from STZ rats is NOS1 and NOS2 dependent.

**NOS protein expression.** Protein levels for each NOS isoform were measured by Western blot analysis and normalized to \(\beta\)-actin. As shown in Fig. 4A, we detected NOS1-immunoreactive bands at 155 and 130 kDa. NOS1 (155 kDa) is the predominant NOS1 isoform expressed in all organisms. The other band likely represents NOS1 (130-kDa protein). We did not observe any difference in the expression of either NOS1 variant in mTALs from sham vs. STZ rats. We were also able to detect NOS2 in mTALs from both sham and STZ rats (Fig. 4B), but no difference was apparent between groups. The specificity of the NOS2 antibody was confirmed by antigen blockade with the specific NOS2 peptide antigen (Santa Cruz Biotechnology; data not shown). Moreover, as shown in Fig. 4C, NOS3 protein levels were similar in mTALs from sham and STZ rats. Thus, there were no significant differences in the expression of any NOS isoform in mTAL suspensions from sham vs. STZ rats.

**Phospho-specific NOS protein expression.** We examined phosphorylation of NOS1 and NOS3 at known serine and threonine regulatory sites, quantified based on the intensity of the phospho-specific band normalized to the NOS isoform detected with an antibody to a nonphospho-specific site. Two-color detect-
tion with the phospho-specific and nonphospho-specific immuno-
blots viewed as a merged image confirmed the specificity of each
phosphorylation site within NOS1 and NOS3.

There is one known serine phosphorylation site on NOS1,
Ser852, but our data failed to reveal any difference between
mTALs from sham vs. STZ rats with regard to pSer852NOS1/
NOS1 (Fig. 5A). This finding holds true for both NOS1/H9251
and NOS1/H9252. We also probed the phosphorylation sites on NOS3:
Ser116, Thr495, Ser617, Ser633, and Ser1177. As shown in Fig. 5B,
pThr495NOS3/NOS3 in homogenates of mTALs from STZ rats
was reduced by more than 40% compared with that detected in
sham rats (P/H11006 0.05). In contrast, mTAL homogenates from
sham and STZ rats did not differ with regard to pSer633NOS3/
NOS3 and pSer1177NOS3/NOS3 levels. We were unable to
detect pSer116NOS3 and pSer617NOS3 in mTALs from either
group (data not shown), despite the ability of the phospho-
specific primary antibodies to detect phosphorylation at these
sites in endothelial cells.

Finally, we determined whether PP2B inhibition alters the
phosphorylation status of NOS1 and NOS3 in mTALs from STZ
rats. As shown in Fig. 6, Western blot analysis revealed no
effect of CsA on Ser852NOS1/NOS1, pThr495NOS3/NOS3,
pSer633NOS3/NOS3, or pSer1177NOS3/NOS3 in mTALs
from either sham or STZ rats. These results indicate that
PP2B does not directly alter NOS1 or NOS3 phosphoryla-
tion at these known regulatory sites.

Fig. 3. Nitrite production by mTALs from STZ and sham rats. A: effect of 10 mM tempol on nitrite production. B: effect of PP2B inhibition (100 ng/ml CsA)
on nitrite production measured in the presence of 10 mM tempol. C: effect of PP2B inhibition [100 μM calcineurin auto-inhibitory peptide (CAIP)] and scrambled peptide (100 μM SP) on nitrite production measured in the presence of 10 mM tempol. D: effect of inhibiting NOS1 (1 μM VNIO) or NOS2 (100 nM 1400W)
on nitrite production measured in the presence of 10 mM tempol. Values are normalized to sham control (256 ± 74 pmol nitrite/mg protein 1-30 min-1), expressed as means ± SE (n = 6–14 rats). *P < 0.05 vs. sham. †P < 0.05 vs. untreated.

**DISCUSSION**

In type 1 diabetes mellitus, net sodium retention results in a
positive sodium balance (32, 43). DiPetrillo et al. (7) found that
diabetic rats were not able to excrete sodium to the level
needed to match the rise in dietary sodium intake due to
hyperphagia. While the mTAL is an important region of the
nephron for maintenance of sodium homeostasis, normally
reabsorbing ~20% of the filtered sodium, the mechanisms
regulating sodium reabsorption by the mTAL during diabetes
remain unclear. NO directly inhibits sodium reabsorption at
various sites along the nephron (10, 13, 25, 51), including the
mTAL (37), under normal physiological conditions. The

Effect of CsA and CAIP on cell viability. The concentration of
CsA (100 ng/ml) used in all experiments was based on the
literature demonstrating that this concentration was not cytotoxic
in murine cultured mTAL cells (47, 49). However, to ensure that
the changes in NO production induced by CsA and CAIP were not
the result of cytotoxicity, cell viability was measured in mTAL
suspensions incubated for 30 min in the presence of L-arginine
(25 μM), tempol (10 mM), and CsA (100 ng/ml) or CAIP (100
μM). There were no significant differences between the percent-
age of viable cells between vehicle control, CsA-, or CAIP-treated
 tubules (vehicle control: 97 ± 2%, CsA: 96 ± 3%, CAIP: 108 ± 5%, n = 6 for all groups).

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mTAL expresses all three isoforms of NOS; however, few studies examined the regulation of the NO/NOS pathway in the mTAL under diabetic conditions. The activity of each NOS isoform is regulated, in part, by alterations in phosphorylation status. PP2B has been postulated to be upregulated as a compensatory mechanism in the kidney during diabetes and is one of the phosphatases that can act on NOS. The major findings of the present study are 1) PP2B catalytic and regulatory subunit protein expression and PP2B activity are increased in mTALs from diabetic rats, 2) increased NOS activity and nitrite production in mTALs from diabetic rats are attributable to NOS1 and NOS2 activities, although diabetes does not alter protein expression of either NOS isoform in the mTAL, 3) diabetes increases PP2B-dependent nitrite production and NOS activity in the mTAL; however, net NO bioavailability is reduced, 4) phosphorylation of NOS3 at Thr^495 is reduced in mTALs from diabetic rats while phosphorylation at other known regulatory sites on NOS1 and NOS3 is unchanged, and 5) PP2B regulation of NOS activity does not appear to directly alter the phosphorylation status of known NOS1 or NOS3 sites.

It is well-known that diabetes reflects a state of oxidative stress characterized by accumulation of reactive oxygen species, specifically O$_2^•^-$. Reaction of O$_2^•-$ with NO results in a decrease in NO bioavailability, which has been documented following exposure to high glucose in human aortic endothelial
cells (6) and human glomerular endothelial cells (19). Indeed, reduced NO bioavailability has been shown to be the primary mechanism involved in endothelial dysfunction in the vasculature in diabetes (17). As the mTAL is a prominent source of renal O$_2^•$ production (23, 53), which can be accelerated by acute exposure to high glucose levels (30), we reasoned that mTALs may produce sufficient O$_2^•$ to reduce NO bioavailability. (Examination of the regulation of O$_2^•$ production by mTALs under diabetic conditions is the focus of another study in our laboratory.) Indeed, incubation in the presence of tempol, a free radical scavenger, or PEG-SOD, superoxide scavenger, unmasked a significant increase in nitrite production by mTALs from diabetic rats that was not observed in sham rats. This observation is interpreted as evidence of reduced NO bioavailability in mTALs from STZ rats. We postulate that the increase in NO generation is an adaptive mechanism in the mTAL to compensate for the activation of reactive oxygen species production during diabetes, blunting the magnitude of the decrease in NO bioavailability.

A previous study from our laboratory demonstrated an increase in renal medullary NOS3 activity in STZ rats compared with sham (22). The present study utilized mTAL suspensions from a similar model of diabetes, yet we found NOS1 and NOS2 activities were increased during diabetes, with no increase in NOS3-specific activity. The obvious difference in these two studies from our laboratory is the tissue source that was evaluated. Lee et al. (22) examined homogenates from whole medullary tissue containing various nephron segments and vascular structures with several cell types, while the present study focused solely on the mTAL. We also previously reported similar NOS3 protein levels in renal medullary homogenates from sham and STZ rats, yet we demonstrated reduced pThr$^{495}$NOS3 staining in the mTAL from STZ rats by immunohistochemical analysis (22). Indeed, the present study confirms these phenomena in mTAL suspensions.

Results of the present study revealed increased NOS1 and NOS2 activities in mTALs from diabetic rats, although we did not detect any change in NOS1 or NOS2 protein levels following 3 wk of diabetes. NOS1 is regulated by alternative splicing and expression of these splice variants has been shown in the kidney (33). NOS1 splicing results in proteins with NH$_2$-terminal variants; thus, we utilized a COOH-terminal-specific NOS1 primary antibody to detect expression of all possible NOS1 variants by Western blot. NOS1-specific bands were detected at 155 kDa (NOS1$^{155}$) and 130 kDa (likely NOS1$^{130}$). NOS1$^{155}$ is reported to have 80% of the enzymatic activity of NOS1$^{130}$. Neither NOS1 variant exhibited altered protein levels in mTALs from STZ rats. While Shin et al. (42) found increased immunostaining for NOS1 in the mTAL following 6 wk of diabetes, it is not known which NOS1 splice variants were detected in their report. Possibly, a more prolonged diabetic state (6 wk) induces NOS1 protein expression, as well as the increased NOS1 activity that was evident in the present study (3 wk after onset).

NOS2 is known to be transcriptionally upregulated through activation of cytokine pathways under proinflammatory conditions. There is consistent evidence of increased NOS2 mRNA and protein expression by whole kidney, macrophages, mesangial cells, or glomeruli under proinflammatory conditions such as ischemia-reperfusion injury (21, 36) or lipopolysac-
in the kidney, primarily in the proximal tubules, while a previous study (2) demonstrated PP2B-A subunit (PP2B-A) has three related isoforms: α, β, and γ. A previous study (2) demonstrated PP2B-Aα mRNA expression in the kidney, primarily in the proximal tubules, while PP2B-Aβ mRNA was primarily in the medulla, with highest expression in the mTAL. Immunohistochemical analysis indicates that PP2B-Aβ protein expression is upregulated specifically in the mTAL following 2 wk of STZ-induced diabetes (12). This agrees with our findings that protein levels of PP2B-Aβ, as well as the regulatory subunit PP2B-B, are increased in mTAL suspensions from diabetic rats. Our results also extend previous observations by revealing that PP2B activity is increased in mTAL lysates from STZ rats.

NOS isoform protein expression is unchanged while NOS activity is increased in mTALs during diabetes. Thus, we focused on posttranslational phosphorylation mechanisms of NOS isoform activation. We hypothesized that the phosphatase, PP2B, activates NO production and NOS activity in the mTAL. To examine this hypothesis, we primarily utilized the PP2B inhibitor, CsA. Notably, CsA is one of the most potent, specific, and well-known inhibitors of PP2B (4, 14, 27, 34, 38, 41). Furthermore, CsA does not inhibit members of the PP1, PP2A, or PP2C classes of serine/threonine phosphatases and PP2B is the exclusive cellular target of CsA (26). We also utilized the mechanistically distinct PP2B inhibitor, CAIP, in some of our studies (45), yielding effects similar to those evoked by CsA. The data reveal that NOS activity and NO production (nitrite production measured in the presence of tempol) are increased in mTALs from diabetic rats in a PP2B-dependent manner. In contrast, PP2B inhibition does not affect NOS activity or nitrite production by mTALs under normal physiological conditions, despite the fact that CsA blocked PP2B activity in mTALs from both sham and STZ rats. Thus, while constitutive PP2B activity has little impact on NOS activity in the mTAL under normal conditions, it is likely that the increase in PP2B activity that accompanies diabetes is responsible for the increase in NOS activity and NO production evident in mTALs from diabetic rats.

To our knowledge, no other study showed a role for PP2B regulation of NOS1 activity in diabetes. In a recent study, PP2B was reported to dephosphorylate NOS1 at Ser852 in primary rat hypothalamic neurons (50). However, we found no effect of CsA on phosphorylation of Ser852NOS1, suggesting that the PP2B-dependent NOS1 activation in the mTAL during diabetes is not the result of direct PP2B effects at this NOS1 site and possibly arises via an indirect mechanism.

The specific mechanisms regarding PP2B regulation of NOS2 are poorly understood. It has been demonstrated previously that PP2B inhibition led to reduced NOS activity and NO production in cultured rat macrophages (5). Reports showed that PP2B inhibition by CsA inhibits NOS2 gene transcription and associated NO production in porcine proximal tubule cells (18) and vascular smooth muscle cells (28). Interestingly, CsA has been shown to reduce NOS2 mRNA and NO production in cultured mouse mTAL cells (48). However, none of these studies assessed the effects of CsA and NOS2 under diabetic conditions. Posttranslational regulation of NOS2 activity by phosphorylation has been reported (52); however, as specific phosphorylation sites have not been identified, we did not explore NOS2 phosphorylation in this study.

Phosphorylation of NOS3 has been examined primarily in cultured endothelial cells under conditions of shear stress (8) or in mTALs under nondiabetic conditions such as increased flow (35) or a high-salt diet (16). In cultured endothelial cells, Thr495NOS3 is a negative regulatory site, such that reduced

fig. 6. Effect of PP2B inhibition (100 ng/ml CsA) on NOS3 phosphorylation in mTAL homogenates from STZ rats. Top: representative Western blots of pThr495NOS3, pSer633NOS3, pSer1177NOS3, and total NOS3. Bottom: summary of NOS3 phosphorylation data showing pThr495NOS3, pSer633NOS3, and pSer1177NOS3 (each normalized to total NOS3). Values are means ± SE (n = 5–7 rats).
phosphorylation indicates increased NO production. Furthermore, pThr⁴⁹⁵NOS3/NOS3 levels were reduced in mTALs from diabetic rats [in accord with our previously published immunohistochemical observations (22)], suggesting that NOS3-derived NO production may be increased. Surprisingly, NOS3 activity was found to be similar in mTALs from STZ and sham rats. Further research is needed to resolve this finding. The inability to detect pSer¹¹¹NOS3 and pSer⁶¹⁷NOS3 in mTALs, despite the fact that NOS3 is highly expressed in this nephron segment, would suggest that the Ser¹¹¹ and Ser⁶¹⁷ sites on NOS3 are phosphorylated at very low levels in mTALs, both in normal rats and during diabetes. In cultured bovine aortic endothelial cells, PP2B dephosphorylates the Thr⁴⁹⁵ residue on NOS3 resulting in increased bradykinin stimulation of NO release (15). In porcine aortic endothelial cells, CsA fails to affect phosphorylation of NOS3 at the Thr⁴⁹⁵ and Ser¹¹⁷ sites (9). In the present study, however, CsA did not affect any of the detectable phosphorylation sites, including pThr⁴⁹⁵NOS3, in the mTAL under diabetic conditions.

Our study reveals that diabetes induces PP2B-dependent activation of NO production, without demonstrating a direct regulation of known phosphorylation sites on NOS isoforms. Thus, we suspect that PP2B may regulate NO production indirectly via a NOS-interacting protein and/or by regulating the production of a NOS cofactor. Two possible candidates that are regulated by phosphorylation/dephosphorylation pathways are caveolin and GTP cyclohydrolase. A protein constituent of caveolae, caveolin has been shown to interact with and function as a negative regulator of NOS activity in endothelial cells (20). Caveolin is also known to be regulated by phosphorylation (24), although no information is available with regards to caveolin regulation of NOS activity in the mTAL. GTP cyclohydrolase is the rate-limiting enzyme in the production of tetrahydrobiopterin (BH₄), a critical cofactor necessary for NOS activity. In endothelial cells, shear stress activates phosphorylation of GTP cyclohydrolase, increasing production of BH₄ and subsequent NO production (46). Future studies will address whether these processes underline the PP2B-dependent increase in NOS activity and NO production in the mTAL during diabetes.

In summary, while NO bioavailability in mTALs is reduced during diabetes, free radical scavenging with tempol unmasks an increase in NO production involving PP2B-dependent activation of NOS1 and NOS2. In addition, PP2B regulates NOS3 activity in the mTAL under both normal and diabetic conditions. We propose that the upregulation of PP2B and NOS activity in the mTAL during diabetes is an adaptive compensatory mechanism. Under diabetic conditions, the balance of NO and O₂⁻ is altered, with a dominance of O₂⁻ production. O₂⁻ has been shown to promote sodium reabsorption in the mTAL, while NO inhibits sodium reabsorption in the mTAL. We propose that the increased NO production may be an adaptive mechanism allowing the rat to remain normotensive with a positive sodium balance under the high O₂⁻ conditions. Of further interest are the clinical implications of the use of CsA in transplant patients. CsA is a potent and widely used immunosuppressant and its use is complicated by the development of nephropathy, nephrotoxicity, and substantial hypertension (1, 44). We propose that CsA reduces the PP2B-dependent increase in NOS activity and NO production resulting in enhanced sodium reabsorption in the mTAL contributing to the development of hypertension. Further work is needed to address this hypothesis.

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