Protein Kinase C-Dependent NAD(P)H Oxidase Activation Induced by Type 1 Diabetes in Renal Medullary Thick Ascending Limb

Jing Yang, Pascale H. Lane, Jennifer S. Pollock, Pamela K. Carmines

Abstract—Type 1 diabetes provokes a protein kinase C (PKC)-dependent accumulation of superoxide anion in the renal medullary thick ascending limb (mTAL). We hypothesized that this phenomenon involves PKC-dependent NAD(P)H oxidase activation. The validity of this hypothesis was explored using mTAL suspensions prepared from rats with streptozotocin-induced diabetes and from sham (vehicle-treated) rats. Superoxide production was 5-fold higher in mTAL suspensions from diabetic rats compared with suspensions from sham rats. The NAD(P)H oxidase inhibitor apocynin caused an 80% decrease in superoxide production by mTAL from diabetic rats (P<0.05 vs untreated) without altering superoxide production by sham mTAL. NAD(P)H oxidase activity was >2-fold higher in mTAL from diabetic rats than in sham mTAL (P<0.05). Pretreatment with calphostin C (broad-spectrum PKC inhibitor) or rottlerin (PKCδ inhibitor) reduced NAD(P)H oxidase activity by ≈80% in both groups; however, PKCa/β or PKCβ inhibition did not alter NAD(P)H oxidase activity in either group. Protein levels of Nox2, Nox4, and p47phox were significantly higher in diabetic mTAL than in mTAL from sham rats. In summary, elevated superoxide production by mTAL from diabetic rats was normalized by NAD(P)H oxidase inhibition. PKC-dependent, PKCδ-dependent, and total NAD(P)H oxidase activity was greater in mTAL from diabetic rats compared with sham. Protein levels of Nox2, Nox4, and p47phox were increased in mTAL from diabetic rats. We conclude that increased superoxide production by the mTAL during diabetes involves a PKCδ-dependent increase in NAD(P)H oxidase activity in concert with increased protein levels of catalytic and regulatory subunits of the enzyme. (Hypertension. 2010;55[part 2]:468-473.)

Key Words: NAD(P)H oxidase ■ protein kinase C ■ thick ascending limb ■ type 1 diabetes

Oxidative stress is involved in several diseases, including hypertension, atherosclerosis, heart failure, and diabetes. During type 1 diabetes (T1D), oxidative stress is evident in endothelial cells, fibroblasts, vascular smooth muscle cells, renal mesangial cells, and renal tubular epithelial cells.1 Each of these cell types can be found in the kidney and oxidative stress is a pathogenic cofactor in the development of the renal complications of T1D, contributing to the glomerulosclerosis and tubulointerstitial fibrosis that ultimately lead to diabetic nephropathy.2 Although oxidative stress can result from a decrease in local antioxidant capacity,3 it seems that the more typical scenario involves excess production of superoxide anion (O2−), which initiates a series of reactions that generate other reactive oxygen species, including peroxynitrite, hydrogen peroxide, hydroxyl radical, and hypochlorous acid. The primary site of O2− production within the normal rat kidney is the thick ascending limb,4 and we recently provided evidence that T1D causes a protein kinase C (PKC)-dependent increase in O2− production by the medullary thick ascending limb (mTAL);5 however, the mechanism underlying this phenomenon has not yet been determined.

The major renal sources of O2− are mitochondrial respiratory chain enzymes and nonphagocytic NAD(P)H oxidase.6,7 NAD(P)H oxidase is similar to the NADPH oxidase responsible for the neutrophil respiratory burst; however, it can use either NADH or NADPH as substrate and can generate O2− for longer periods but at rates somewhat lower than the phagocytic NADPH oxidase. NAD(P)H oxidase consists of multiple subunits: a Nox family member (the catalytic subunit), p22phox, p47phox, p67phox, p40phox, and rac1. Seven Nox family members (Nox1–5; Duox1–2) have been detected in nonphagocytic cells and have 27% to 58% sequence similarity to Nox2 (gp91phox; expressed in phagocytic cells and a variety of nonphagocytic cells).1,8 The Nox family member(s) expressed by specific cell types and the functional relevance of different Nox proteins are still being elaborated. It is at least clear that NAD(P)H oxidase activity can be regulated by expression of the subunits, as well as by p47phox phosphorylation (generally triggered by agonist stimulation). Evidence available to date implicates NAD(P)H oxidase as a source of excess renal and vascular
O$_2$\textsuperscript{−} production during T1D,\textsuperscript{1,8,9} a process that may result from increased expression of various NAD(P)H oxidase subunits\textsuperscript{9–11} or the ability of T1D to induce activation of various PKC isoforms.\textsuperscript{5,12–15} PKC activation is required for translocation of p47\textsuperscript{phox} and p67\textsuperscript{phox}, and subsequent O$_2$\textsuperscript{−} production by NAD(P)H oxidase in glomeruli from diabetic rats.\textsuperscript{10} However, at present, little is known with regard to TID-induced changes in the expression and activity of NAD(P)H oxidase in the mTAL or the role of this enzyme in the PKC-dependent acceleration of O$_2$\textsuperscript{−} production by this nephron segment during T1D. Therefore, we hypothesized that increased O$_2$\textsuperscript{−} production in the mTAL during T1D results from PKC-dependent NAD(P)H oxidase activation.

**Materials and Methods**

**Induction of T1D**

All animal procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing ∼300 grams (Harlan Laboratories) were anesthetized with methohexital sodium (50 mg/kg intraperitoneal) to facilitate intravenous injection of 65 mg/kg streptozotocin (STZ rats) or vehicle (ice-cold phosphate-buffered saline; pH 4; sham rats). The next day, the rats were anesthetized again for subcutaneous insertion of a 2.3 × 2.0 mm sustained-release insulin pellet (Linplant; STZ rats) or vehicle pellet ( sham rats) via a 16-gauge needle. The rats were provided ad libitum food and water for the ensuing 3 to 4 weeks while housed in a temperature-controlled room with a 12–12-hour light–dark cycle. During this period, STZ rats gained less weight (33 ± 10 grams) than sham rats (90 ± 5 grams). Blood glucose levels were measured with an Accu-Chek Advantage kit (Roche Diagnostics) before STZ or vehicle injection and twice weekly thereafter, with values averaging 21.0 ± 0.7 mmol/L in STZ rats (n = 24) and 5.0 ± 0.1 mmol/L in sham rats (n = 28).

**Preparation of mTAL Suspensions**

Fresh mTAL suspensions were prepared from the inner stripe of the outer medulla according to established methods\textsuperscript{16,17} with slight modification as previously described.\textsuperscript{5} The mTAL were suspended in cold HBSS containing either 5.5 or 20 mmol/L d-glucose (for sham or STZ rats, respectively, to maintain the chronic in vivo condition of the donor rats), equilibrated with 95% O$_2$–5% CO$_2$, and adjusted to pH 7.4. Suspensions were kept on ice until initiating any in vitro treatment (30-minute incubation at 37°C in the absence or presence of a pharmacological agent) or tissue homogenization for biochemical analysis.

**Measurement of O$_2$\textsuperscript{−} Production in mTAL Suspensions**

Polystyrene tubes containing aliquots of mTAL suspension were placed in the chamber of a Berthold Sirius tube luminometer to obtain a blank value. Lucigenin (final concentration = 5 μmol/L) was added to the sample and, after a 2-minute dark adaptation period, the luminescence signal (relative light units/sec [RLU/sec]) was averaged in 30-second blocks for the ensuing 5 minutes. Blank-corrected values obtained during the final 90 seconds of this period, normalized per mg protein, were used for comparisons. We previously demonstrated the temporal sensitivity of this parameter,\textsuperscript{5} thus indicating that the data primarily reflect O$_2$\textsuperscript{−} production.

**NAD(P)H Oxidase Activity Assay**

The mTAL suspensions were subjected to gentle centrifugation, the supernatant was decanted, and 100 μL homogenizing buffer was mixed with the pellet. The homogenizing buffer consisted of 20 mmol/L KH$_2$PO$_4$ and 1 mmol/L EGTA (pH 7.8) with EDTA-free protease inhibitor cocktail (1:100; Thermo Scientific). After homogenization on ice (50 strokes with a Kontes PELLET PESTLE Micro Grinder), homogenates were centrifuged at 1000g for 15 minutes to remove unbroken cells and debris. An aliquot of the supernatant (50 μg protein) was added to a polystyrene tube containing HBSS with lucigenin and NADPH provided to achieve final concentrations of 5 and 100 μmol/L, respectively. After a dark adaptation period, emitted light (RLU/sec) was detected in the luminometer, averaged during the final 90 seconds of a 5-minute measurement sequence. Lucigenin chemiluminescence in this assay required exogenous NADPH, with minimal signal evident if NADH was substituted for NADPH. Hence, we report NAD(P)H oxidase activity as RLU/sec/μg protein measured in the presence of 100 μmol/L NADPH.

**Western Blot**

Homogenates prepared from mTAL suspensions were prepared and utilized for Western blot analysis according to methods previously detailed,\textsuperscript{5} except that we used Immobilon-P PVDF transfer membranes (Millipore) for p47\textsuperscript{phox} detection and nitrocellulose membranes (Thermo/Fierce) for Nox2 and Nox4 detection. The following primary antibodies were utilized: 1:200 anti-Nox2/4 (961144; BD Transduction), 1:200 anti-Nox4 (sc-21860; Santa Cruz), 1:200 anti-p47\textsuperscript{phox} (sc-7660-R; Santa Cruz), and 1:5000 anti-β-actin (ab8226 or ab8227; Abcam). We used secondary antibodies conjugated to infrared dyes, allowing signal detection using a LI-COR Odyssey Imager. For quantification, band intensities were normalized to β-actin and expressed as a percent of averaged sham values evident on the same gel.

**Statistical Analysis**

All data are reported as means ±SEM, with n values representing the number of rats. Statistical comparisons utilized the unpaired t test or ANOVA with post hoc comparisons made using the Student-Newman-Keuls method. If the data were not normally distributed, then the Kruskal-Wallis ANOVA on Ranks was used, with post hoc comparisons utilizing the Holm-Sidak method. P < 0.05 was considered significant.

**Results**

**NAD(P)H Oxidase Inhibition Normalizes mTAL O$_2$\textsuperscript{−} Production During T1D**

Consistent with our previous study,\textsuperscript{5} O$_2$\textsuperscript{−} production measured by lucigenin chemiluminescence in mTAL suspensions from sham rats averaged 430 ± 31 RLU/sec/mg protein and was accelerated 5-fold in mTAL from STZ rats (P < 0.05; Figure 1). Preincubation with the NAD(P)H oxidase inhibitor apocynin (100 μmol/L; Calbiochem) decreased O$_2$\textsuperscript{−} production by 80% in STZ mTAL but had no effect on sham mTAL. Thus, NAD(P)H oxidase inhibition reversed the increase in O$_2$\textsuperscript{−} production otherwise apparent in mTAL suspensions from STZ rats.

**PKC-Dependent NAD(P)H Oxidase Activation in the mTAL During T1D**

As shown in Figure 2, NAD(P)H oxidase activity in STZ mTAL was >2-fold higher than that in sham mTAL (P < 0.05). Pre-treatment of mTAL suspensions with 1 μmol/L calphostin C (a broad-spectrum PKC inhibitor with an IC$_{50}$ of 0.05 μmol/L)\textsuperscript{18} before tissue homogenization reduced NAD(P)H oxidase by ∼80% in both groups (P < 0.05 vs untreated). Calphostin C-sensitive (PKC-dependent) NAD(P)H oxidase activity calculated from these data was significantly greater in STZ mTAL (45.8 ± 9.6 RLU/sec/μg protein) than in sham mTAL (19.0 ± 2.0 RLU/sec/μg protein). These data reveal that NAD(P)H oxidase...
activity is increased in the rat mTAL during T1D and this phenomenon is PKC-dependent.

To determine which PKC isoform is involved in the accelerated NAD(P)H oxidase activity accompanying T1D, NAD(P)H oxidase activity was assayed after pretreatment of mTAL suspensions with PKC inhibitors having relative isoform selectivity: 1 μmol/L Gö6976 (PKCε/β inhibitor, IC50 = 1.3–6 nmol/L),19 50 mmol/L indolylmaleimide-1 (PKCβ inhibitor marketed by Calbiochem; IC50 = 5–21 nmol/L),20 or 10 μmol/L rottlerin (PKCδ inhibitor; IC50 = 3.6 μmol/L).21 We previously confirmed that none of these agents adversely alter mTAL viability under these experimental conditions.5 As shown in Figure 2, Gö6976 and indolylmaleimide-1 had no effect on NAD(P)H oxidase activity in mTAL from sham or STZ rats. However, rottlerin significantly reduced NAD(P)H oxidase activity by ≈80% in both sham and STZ mTAL (P < 0.05 vs untreated for both groups). Rottlerin-sensitive (PKCδ-dependent) NAD(P)H oxidase activity calculated from these data was significantly higher in STZ mTAL (45.6±10.8 RLU/sec/µg protein) than in sham mTAL (18.1±2.1 RLU/sec/µg protein). Thus, the T1D-induced increase in PKCδ-dependent NAD(P)H oxidase activity is virtually identical in magnitude to the increase in PKC-dependent NAD(P)H oxidase activity and appears to underlie the increase in total NAD(P)H oxidase activity evident under these conditions.

**T1D Increases Nox2, Nox4, and p47phox Levels in the mTAL**

Western blot was used to detect NAD(P)H oxidase subunit protein levels in mTAL from sham and STZ rats. As shown in Figure 3, Nox2, Nox4, and p47phox protein levels were significantly increased in mTAL from STZ rats, achieving values averaging 161%±21%, 134%±14%, and 130%±9% of sham, respectively. Thus, T1D triggers upregulation of these NAD(P)H oxidase subunits in the rat mTAL.

**Discussion**

In humans, systemic oxidative stress is evident at onset of T1D in children and adolescents and is increased by early adulthood.22 Thus, the mechanisms underlying renal oxidative stress in the early stage of T1D likely have significant pathophysiological relevance. Accordingly, our study focused on events evident 3 to 4 weeks after induction of T1D in rats by STZ injection, with moderate hyperglycemia achieved by partial insulin replacement. These animals display renal hypertrophy and hyperfiltration, as well as increased urinary excretion of TGF-β (a profibrotic molecule), H2O2, thiobarbituric acid reactive substances, and 8-hydroxy-2′-deoxyguanosine (indicators of oxidative stress); however, only moderate microalbuminuria is evident at this time point.23 Insulin replacement or pancreatic beta cell transplantation to achieve euglycemia prevents the multiple renal functional changes evident in STZ rats,24,25 thus ruling out the possibility that these phenomena represent a direct renal toxic effect of STZ. We recently reported increased O2− production in the mTAL of STZ rats, and that this phenomenon is PKC-dependent.5 The increased O2− production seems to be initiated by the hyperglycemic milieu, because acute (30 minutes) exposure of mTAL from normal rat kidney to 20 mmol/L glucose is sufficient to accelerate O2− production 2-fold.5 The results of the present study reveal that apocynin reverses excess O2− production in mTAL suspensions from STZ rats. Moreover, increased expression of NAD(P)H oxidase subunits (Nox2, Nox4, and p47phox) was evident in STZ mTAL, in concert with increased NAD(P)H oxidase activity. Furthermore, PKC inhibition (either broad-spectrum or PKCδ-specific) abolished the increase in NAD(P)H oxidase activity. These data implicate PKC-dependent NAD(P)H oxidase activation, together with increased subunit expression, in the accelerated O2− production that arises in the mTAL during the early stage of T1D.

The present study used apocynin (100 μmol/L) to determine the contribution of NAD(P)H oxidase to O2− produc-
in mTAL under the conditions of our study. Although we cannot absolutely rule out the possibility that apocynin exerted nonspecific effects in our experimental setting, the ability of apocynin to normalize lucigenin chemiluminescence in mTAL suspensions from STZ rats supports the contention that NAD(P)H oxidase is a primary source of increased O$_2^-$ production by the mTAL during T1D.

In contrast with the effects observed in mTAL from STZ rats, apocynin had no effect on O$_2^-$ production in mTAL from sham rats. This observation differs somewhat from the results of Li et al.\(^4\) who concluded that NAD(P)H oxidase is a major enzyme responsible for O$_2^-$ production by the thick ascending limb from normal rat kidney. Their conclusion was based on dihydroethidium fluorescence responses to diphenyleneiodonium in the cortical thick ascending limb. It is possible that cortical and medullary portions of the thick ascending limb have different enzymatic sources of O$_2^-$, or that the effects of diphenyleneiodonium in this nephron segment reflect its ability to inhibit not only NAD(P)H oxidase but also other flavin-containing enzymes that also have the capacity to generate O$_2^-$ (ie, nitric oxide synthase). In contrast, apocynin acts by binding to p47phox, preventing the assembly of NAD(P)H subunits that is required for enzymatic activity. The lack of effect of apocynin on mTAL suspensions from sham rats suggests that enzymes other than NAD(P)H oxidase may be responsible for O$_2^-$ production in the absence of agonist stimulation or a pathophysiological process. It is also possible that O$_2^-$ production by mTAL from sham rats reflects the functional impact of Nox4, which is capable of constitutively generating O$_2^-$ in the absence of the various regulatory subunits of NAD(P)H oxidase\(^30\) and, thus, is unlikely to be influenced by apocynin.

NAD(P)H oxidase activation has been demonstrated in renal cortex\(^31,32\) and glomeruli of STZ rats. Consistent with these findings, we found that NAD(P)H oxidase activity was increased significantly in STZ mTAL. Depending on the Nox family member serving as the catalytic subunit, NAD(P)H oxidase activity can be regulated by subunit expression or cytosolic activator proteins. Both of these possibilities were explored in the present study. Because PKC activity in the mTAL is increased during T1D, and because PKC inhibition markedly attenuates O$_2^-$ production in mTAL suspensions from STZ rats,\(^5\) we assessed the involvement of PKC in the T1D-induced NAD(P)H oxidase activation using a pharmacological approach. The effect of calphostin C to markedly reduce NAD(P)H oxidase activity in mTAL from sham and STZ rats confirms the key involvement of PKC as a determinant of NAD(P)H oxidase activity in these cells. We also found that calphostin C-sensitive NAD(P)H oxidase activity was increased in STZ mTAL compared with sham, indicating a role for PKC in the NAD(P)H oxidase activation accompanying T1D. These observations are consistent with evidence that high glucose levels can stimulate reactive oxygen species production through PKC-dependent activation of NAD(P)H oxidase in vascular smooth muscle and endothelial cells.\(^33\)

Glucose-induced or diabetes-induced NAD(P)H oxidase activation has been shown to be reliant on PKC\(_\alpha\), PKC\(_\beta\), PKC\(_\delta\), or PKC\(_\zeta\), depending on the tissue studied.\(^8\) In the

### Figure 3. Effect of T1D on NAD(P)H oxidase subunit protein levels in the mTAL. Shown are representative Western blots and densitometric analysis of Nox2 (sham n=5; STZ n=7), Nox4 (n=8 per group), and p47phox (n=16 per group) levels in mTAL from sham and STZ rats. *P<0.05 vs sham.
mTAL, T1D increases expression of PKCα and PKCβ, and evidence suggests that both are activated in STZ rats. Moreover, PKCδ activity (based on cytosol vs membrane distribution) is substantial in the mTAL under normal and diabetic conditions. To determine which isofrom is involved in the NAD(P)H oxidase activation in STZ mTAL, we utilized PKC inhibitors with relative isofrom specificity. The results revealed the ability of PKCδ inhibition to decrease NAD(P)H oxidase activity in both STZ and sham mTAL, whereas inhibition of PKCα/β or PKCβ had no effect. Moreover, PKCδ-dependent NAD(P)H oxidase activity was much higher in STZ mTAL than in sham mTAL. These data indicate that PKCδ (but not PKCα or PKCβ) is critical for both basal and T1D-stimulated NAD(P)H oxidase activity in mTAL. Interestingly, our previous investigation provided evidence that increased O$_2^-$ production by intact STZ mTAL was dependent on PKCα and PKCδ; however, results of the present study implicate PKCδ (but not PKCα) in the T1D-induced NAD(P)H oxidase activation measured in mTAL homogenates. The O$_2^-$ production assay performed in intact cells relies solely on endogenous cofactors and substrate availability, whereas the NAD(P)H oxidase activity assay utilizes cell homogenates with the provision of excess substrate (NADPH), so it is difficult to make a direct comparison of the results. However, it seems that PKCα influences O$_2^-$ production during T1D through an NAD(P)H oxidase-independent mechanism, perhaps through effect on nitric oxide synthase, xanthine oxidase, or the mitochondrial respiratory chain enzymes.

Based on our accumulated observations, it is likely that the tonically high level of PKCδ activity in the mTAL contributes to O$_2^-$ production via effects on NAD(P)H oxidase activation. In the setting of T1D, in which there is no apparent change in PKCδ protein level or translocation (activity) in the mTAL, increased PKCδ-dependent NAD(P)H oxidase activity most likely results from an increase in the availability of PKCδ substrate. This scenario is consistent with our observation that mTAL from STZ rats express elevated protein levels of p47phox, a known substrate of PKCδ. Given that p47phox phosphorylation is key to subunit assembly, and that this process requires PKCδ, we postulate that the combination of high basal PKCδ activity and increased p47phox protein levels promotes increased NAD(P)H oxidase activation in the mTAL during T1D. The impact on enzyme activity is likely facilitated by the concomitant increase in protein levels of the catalytic subunit Nox2. The observation of increased mTAL expression of Nox2 and p47phox is consistent with reported changes evident at the whole kidney level that were prevented by chronic apocynin treatment. This observation suggests that oxidative stress in T1D provokes a positive-feedback increase in NAD(P)H oxidase subunit expression.

Interestingly, mTAL express at least 2 different Nox family members, Nox2 and Nox4, and protein levels of both are elevated in T1D. An increase in Nox4 protein levels in the mTAL during T1D is consistent with reports of similar changes in kidney cortex and isolated glomeruli. The T1D-induced increase in Nox4 protein levels should contribute to increased NAD(P)H oxidase activity and O$_2^-$ production, independent of changes in expression or phosphorylation of regulatory subunits. Nox4 expression is subject to regulation by PKCα, which is upregulated in the mTAL during diabetes. Thus, PKC-dependent regulation of NAD(P)H oxidase activity and O$_2^-$ production during diabetes may involve both pre-translational and post-translational mechanisms.

**Perspectives**

The initial renal structural alteration in T1D is glycogen deposition in the epithelial cells of the thick ascending loop of Henle, which leads to decreased production of Tamm-Horsfall protein by these cells. Recent evidence indicates that decreased urinary Tamm-Horsfall protein concentration in T1D is associated with an 8-fold increased risk of cardiovascular death and uremia. This observation underscores the importance of understanding the effects of T1D on mTAL function; however, surprisingly little investigation has focused on this topic. The product of NAD(P)H oxidase, O$_2^-$, can influence Na$^+$ transport by the normal mTAL, exerting a stimulatory effect that counteracts the inhibitory impact of nitric oxide. Our recent work indicates that nitric oxide bioavailability in the mTAL is reduced during T1D, and that O$_2^-$ scavenging with tempol unmasks increased nitric oxide production under these conditions. Therefore, it is likely that the mechanisms underlying NAD(P)H oxidase activation during T1D exert an impact on Na$^+$ balance and extracellular fluid volume homeostasis, possibly favoring volume retention with an attendant link to hypertension. Moreover, antioxidant therapy that minimizes the T1D-induced increase in NAD(P)H oxidase activity may curtail the development of tubulointerstitial fibrosis, which ultimately contributes substantially to diabetic nephropathy. Further investigation will be necessary to unveil the systemic and chronic consequences of NAD(P)H oxidase activation in the mTAL during diabetes.

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**Disclosures**

None.

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