NADPH Oxidase and PKC Contribute to Increased Na Transport by the Thick Ascending Limb During Type 1 Diabetes

Jing Yang, Jennifer S. Pollock, Pamela K. Carmines

Abstract—Type 1 diabetes triggers protein kinase C (PKC)-dependent NADPH oxidase activation in the renal medullary thick ascending limb (mTAL), resulting in accelerated superoxide production. As acute exposure to superoxide stimulates NaCl transport by the mTAL, we hypothesized that diabetes increases mTAL Na⁺ transport through PKC-dependent and NADPH oxidase–dependent mechanisms. An O₂-sensitive fluoroprobe was used to measure O₂ consumption by mTALs from rats with streptozotocin-induced diabetes and sham rats. In sham mTALs, total O₂ consumption was evident as a 0.34±0.03 U change in normalized relative fluorescence (ΔNRF)/min per mg protein. Ouabain (2 mmol/L) reduced O₂ consumption by 69±4% and 500 μmol/L furosemide reduced O₂ consumption by 58±8%. Total O₂ consumption was accelerated in mTAL from diabetic rats (0.74±0.07 ΔNRF/min/mg protein; P < 0.05 versus sham), reflecting increases in ouabain- and furosemide-sensitive O₂ consumption. NADPH oxidase inhibition (100 μmol/L apocynin) reduced furosemide-sensitive O₂ consumption by mTAL from diabetic rats to values not different from sham. The PKC inhibitor calphostin C (1 μmol/L) or the PKCa/β inhibitor G06976 (1 μmol/L) decreased furosemide-sensitive O₂ consumption in both groups, achieving values that did not differ between sham and diabetic. PKCβ inhibition had no effect in either group. Similar inhibitory patterns were evident with regard to ouabain-sensitive O₂ consumption. We conclude that NADPH oxidase and PKC (primarily PKCa) contribute to an increase in O₂ consumption by the mTAL during type 1 diabetes through effects on the ouabain-sensitive Na⁺-K⁺-ATPase and furosemide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter that are primarily responsible for active transport Na⁺ reabsorption by this nephron segment. (Hypertension. 2012;59[part 2]:431-436.) ● Online Data Supplement

Key Words: protein kinase C ▪ NADPH oxidase ▪ sodium reabsorption ▪ thick ascending limb ▪ type 1 diabetes ▪ oxygen consumption
ide also indirectly promotes Na⁺ transport by decreasing the bioavailability of NO, an inhibitor of NaCl reabsorption by the mTAL. The stimulatory impact of superoxide on NaCl transport by the mTAL is dependent on PKC, multiple isoforms of which are expressed by the mTAL. Accordingly, we hypothesized that T1D provokes an increase in Na⁺ reabsorption by the mTAL through PKC- and NOX-dependent mechanisms.

**Methods**

**Chemicals and Reagents**

The NOX inhibitor apocynin, the PKC inhibitor calphostin C, the PKCa/β inhibitor Gö6976, and the PKCβ inhibitor 3-(1-{3-\(\text{imidazol-1-ylpropyl}-1\text{H-indol-3-yl})-4\text{-amino-1H-pyrole}-2\text{-5-dione (indolylmaleimide-1) were purchased from Calbiochem/EMD (Madison, WI). Furosemide (10 mg/mL) was from Hospira (Lake Forest, IL). Linplant sustained-release insulin pellets and microcrystallized palmitic acid vehicle pellets were purchased from LinShin Canada (Scarborough, Ontario). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

**Induction of T1D**

All animal procedures were approved by the University of Nebraska Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These experiments used male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-300 g. STZ-injected rats with partial insulin replacement were used as a positive control indicating the maximum fluorescence emitted by the fluoroprobe in an oxygen-depleted environment, 1 well of each OBS microplate contained 100 mmol/L sodium sulfite (2Na₂SO₃ + O₂ → 2Na₂SO₄). As a negative control, three wells were left empty to allow the fluoroprobe-containing matrix to equilibrate with ambient air (maximum quenching of fluorescence). The sample-containing microplates were maintained at 37°C and fluorescence intensity (485 nm excitation; 630 nm emission) was quantified in the bottom-reading mode at 2 minutes intervals for 90 minutes using an Infinite M200 microplate reader (Tecan US). A 2-step normalization to ambient air was applied to the fluorescence intensity data for each sample, correcting for the baseline signal of each well before sample loading (factoring out slight well-to-well differences in measured fluor content), as well as the negative control for time point (factoring for slight fluctuations in temperature or other machine drift during the reading). The resulting dimensionless normalized relative fluorescence (NRF) values were used to quantify Qo₂, based on the linear change in NRF evident during the 30-50 minute time frame, expressed as ΔNRF/min per mg protein. This resulted in an assessment of the effects of various pharmacological agents on Qo₂, after a 30-minute pretreatment period, in accord with protocols used in our previous studies of mTAL function during T1D. Ouabain- and furosemide-sensitive Qo₂ were calculated as reflections of NKA- and NKCC2-dependent Na⁺ transport, respectively.

**Preparation of Fresh Rat Medullary TAL Suspensions**

Medullary TAL segments were obtained from rats in each group according to the methods previously described, and suspended in cold HBSS containing either 5.5 or 20 mmol/L d-glucose (for sham and STZ rats, respectively) to maintain the chronic in vivo glycemic environment of the donor rats. These suspensions were maintained on ice until used for assay of Qo₂.

**Oxygen Consumption Assay**

Qo₂ was quantified using the BD Oxygen Biosensor System (OBS; BD Biosciences), which incorporates an oxygen-sensitive fluorophore into a gas-permeable silicone matrix at the bottom of each well of a microplate. Oxygen reversibly quenches the fluorophore, so emitted fluorescence varies inversely with oxygen concentration. This system was used to quantify mTAL Qo₂ according to manufacturer instructions with some modifications as detailed in the literature. Briefly, after determining the protein concentration of the mTAL suspension, the mTAL were aliquoted into wells of an OBS microplate (15 µg protein per well) containing various pharmacological agents with or without 2 mmol/L ouabain or 500 µmol/L furosemide in HBSS (all treatments in triplicate). We previously reported that the PKC inhibitors used in this study do not exert cytotoxic effects on rat mTALs. For agents not soluble in HBSS, a stock solution in DMSO was diluted in HBSS to achieve the desired concentration of inhibitor in 0.05% DMSO. We confirmed that exposure to the DMSO vehicle did not alter mTAL Qo₂ (data not shown). In addition, preliminary experiments verified that none of the pharmacological agents used in this study interfered with the OBS fluorophore responsiveness to varied oxygen levels. As a positive control indicating the maximum fluorescence emitted by the fluoroprobe in an oxygen-depleted environment, 1 well of each OBS microplate contained 100 mmol/L sodium sulfite (2Na₂SO₃ + O₂ → 2Na₂SO₄). As a negative control, three wells were left empty to allow the fluoroprobe-containing matrix to equilibrate with ambient air (maximum quenching of fluorescence). The sample-containing microplates were maintained at 37°C and fluorescence intensity (485 nm excitation; 630 nm emission) was quantified in the bottom-reading mode at 2 minutes intervals for 90 minutes using an Infinite M200 microplate reader (Tecan US). A 2-step normalization to ambient air was applied to the fluorescence intensity data for each sample, correcting for the baseline signal of each well before sample loading (factoring out slight well-to-well differences in measured fluor content), as well as the negative control for time point (factoring for slight fluctuations in temperature or other machine drift during the reading). The resulting dimensionless normalized relative fluorescence (NRF) values were used to quantify Qo₂, based on the linear change in NRF evident during the 30-50 minute time frame, expressed as ΔNRF/min per mg protein. This resulted in an assessment of the effects of various pharmacological agents on Qo₂, after a 30-minute pretreatment period, in accord with protocols used in our previous studies of mTAL function during T1D. Ouabain- and furosemide-sensitive Qo₂ were calculated as reflections of NKA- and NKCC2-dependent Na⁺ transport, respectively.

**Statistics**

All values are expressed as mean±SEM (n=number of rats). Simple between-group comparisons (blood glucose and body weight) were done by unpaired Student t test. Qo₂ data were analyzed by 2-way repeated-measures ANOVA followed by post hoc comparison using the Holm-Sidak method. Probability values ≤0.05 were accepted as significant.

**Results**

Figure 1 summarizes the total, ouabain-sensitive, ouabain-insensitive, and furosemide-sensitive Qo₂ consumption measured in mTAL suspensions prepared from sham rats (n=6) and streptozotocin (STZ)-treated rats (n=6). NRF indicates normalized relative fluorescence. *P<0.05 versus sham.
Apocynin similarly influenced ouabain-sensitive Qo2, a l-
3.5% of untreated).

without effect on sham mTALs (99.7

values remaining

only partially reversed the increase in total and ouabain-

ullary thick ascending limbs (mTALs) from sham and streptozotocin (STZ)-treated rats. Shown are effects of 30-minute pretreatment

with 100

mol/L apocynin (NOX inhibitor), 1

mol/L calphostin C (broad-spectrum PKC inhibitor), 1

mol/L Gö6976 (PKCa/β inhibitor), and 50 nmol/L indolylmaleimide-1 (PKCβ inhibitor). NRF indicates normalized relative fluorescence. Two-way repeated-measured

ANOVA results are provided in each panel, with post hoc results indicated as *P<0.05 versus sham, †P<0.05 versus untreated, and ¶P<0.05 apocynin versus Gö6976.

Figure 2. Effects of NADPH oxidase (NOX) inhibition and protein kinase C (PKC) inhibition on components of O2 consumption by med-
ulary thick ascending limbs (mTALs) from sham and streptozotocin (STZ)-treated rats. Shown are effects of 30-minute pretreatment

with 100

mol/L apocynin (NOX inhibitor), 1

mol/L calphostin C (broad-spectrum PKC inhibitor), 1

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ANOVA results are provided in each panel, with post hoc results indicated as *P<0.05 versus sham, †P<0.05 versus untreated, and ¶P<0.05 apocynin versus Gö6976.

increases in ouabain- and furosemide-sensitive Qo22, with ouabain-insensitive Qo2 not significantly differing between mTAL from sham and STZ rats. The effects of T1D and ouabain on mTAL Qo2 were confirmed using the Clark electrode method (see Figure S1, online Data Supplement, available at http://hyper.ahajournals.org). These observations demonstrate that T1D increases Na+
transport-related (NKA-
and NKCC2-dependent) Qo2 by the rat mTAL.

The impact of NOX inhibition on mTAL Qo2 was assessed based on the response to 100

mol/L apocynin. The efficacy and specificity of this concentration of apocynin as a NOX inhibitor in our studies has been addressed previously.20 As shown in Figure 2, apocynin treatment of STZ mTAL decreased total Qo2 to 72.6±4.2% of untreated (P<0.05) without effect on sham mTALs (99.7±3.5% of untreated). Apocynin similarly influenced ouabain-sensitive Qo2, although the effect on STZ mTAL did not achieve statistical significance (P=0.058 versus untreated). Thus, apocynin only partially reversed the increase in total and ouabain-sensitive Qo2 evident STZ mTAL, with values remaining significantly greater than sham mTAL. However, apocynin significantly decreased furosemide-sensitive Qo2 by STZ mTAL to achieve values that did not differ from sham. Two-way repeated-measures ANOVA revealed no significant interaction (group

treatment) in terms of ouabain-insensitive Qo2 not significantly differing between

animal group (sham versus STZ). These data indicate that PKC activity contributes to Na+
transport-related Qo2 by the mTAL, and that this contribution is exaggerated in mTAL from STZ rats.

To determine which PKC isoform is involved in the increased mTAL Qo2 induced by T1D, we used PKC inhibitors with relative isoform specificity. At a concentration of 1

mol/L, Gö6976 abolishes the enzymatic activity of both PKCα and PKCβ (IC50=1.3–6.0 nmol/L) without effect on PKCδ, PKCɛ, or PKCζ.31 As shown in Figure 2, the effects of Gö6976 on total, ouabain- and furosemide-sensitive Qo2 mimic those of the broad-spectrum PKC inhibitor calphostin C. In addition, ouabain-insensitive Qo2 exhibited a small but statistically significant effect treatment of calphostin C that was independent of animal group (sham versus STZ). These data indicate that PKC activity contributes to Na+
transport-related Qo2 by the mTAL, and that this contribution is exaggerated in mTAL from STZ rats.

The role of PKC in determining Qo2 by sham and STZ mTAL was assessed based on responses to 1

mol/L calphostin C (IC50=0.05

mol/L).30 As shown in Figure 2, calphostin C treatment significantly decreased Qo2 by mTAL from both sham and STZ rats. In sham mTAL, calphostin C reduced total, ouabain- and furosemide-sensitive Qo2 values by ∼40% compared with untreated. Calphostin C exerted a greater impact on Qo2 by STZ mTAL, reducing values by ∼65% compared with untreated, such that final values that did not differ from those evident in calphostin C-treated sham mTAL. The impact of calphostin C on total and ouabain-sensitive Qo2 by STZ mTAL significantly exceeded the effect of apocynin, but this trend did not achieve statistical significance for furosemide-sensitive Qo2 (P=0.058). Ouabain-insensitive Qo2 exhibited a small but statistically significant effect treatment of calphostin C that was independent of animal group (sham versus STZ). These data indicate that PKC activity contributes to Na+
transport-related Qo2 by the mTAL, and that this contribution is exaggerated in mTAL from STZ rats.

Active transcellular Na+
reabsorption by the thick ascending limb requires Na+
entry into the cell through the apical NKCC2 or Na+/H+
exchanger (NHE3), which increases intracellular [Na+] and leads to Na+
extrusion across the
basolateral membrane through the NKA.\textsuperscript{33} Active transport Na\textsuperscript{+} reabsorption is the primary oxygen-consuming process in the kidney and, in the thick ascending limb, oxidative phosphorylation and NKA activity are stoichiometrically coupled.\textsuperscript{34} Accordingly, the effect of the NKA inhibitor ouabain on Qo\textsubscript{2} (ouabain-sensitive Qo\textsubscript{2}) is largely reflective of active transport Na\textsuperscript{+} reabsorption. However, as NKA activity also supports other cellular processes (maintenance of intracellular [K\textsuperscript{+}], etc), ouabain-sensitive Qo\textsubscript{2} is not purely indicative of active Na\textsuperscript{+} reabsorption. The effect of the NKCC2 inhibitor furosemide on Qo\textsubscript{2} (furosemide-sensitive Qo\textsubscript{2}) provides an alternative index of Na\textsuperscript{+} transport that specifically involves the NKCC2 transporter, which is the primary apical Na\textsuperscript{+} influx pathway in the mTAL. Previous reports indicate that ouabain reduces mTAL Qo\textsubscript{2} by 40–50%,\textsuperscript{35,36} with furosemide-sensitive Qo\textsubscript{2} representing 20–40% of total Qo\textsubscript{2}.\textsuperscript{25,36} In the present study, we found that the ouabain- and furosemide-sensitive components of Qo\textsubscript{2} represented \~70% and 60% of total Qo\textsubscript{2}, respectively. The quantitative disparity of these proportions compared with previous reports likely reflects, at least in part, the fact that we analyzed Qo\textsubscript{2} in mTAL suspensions equilibrated with ambient air (21% O\textsubscript{2}), rather than the 100% O\textsubscript{2} equilibration used in the previous studies.\textsuperscript{25,36} Similar to our results, Palm et al\textsuperscript{37,38} found that ouabain-sensitive Qo\textsubscript{2} by renal outer medullary cells in buffer equilibrated with ambient air is about 70% of total Qo\textsubscript{2}. Thus, under the conditions of our assay, Na\textsuperscript{+} transport-related Qo\textsubscript{2} represents the bulk of total Qo\textsubscript{2} by mTALs. Consistent with reports that Qo\textsubscript{2} is augmented in the mTAL of STZ rats indicates that T1D provokes an increase in Na\textsuperscript{+} transport-related Qo\textsubscript{2} that involves NKCC2 and NKA.\textsuperscript{33} The results of the present study reveal that T1D-stimulated Na\textsuperscript{+} reabsorption through NKCC2 and NKA pathways. The ability of apocynin to reduce furosemide-sensitive Qo\textsubscript{2} by STZ mTALs was greater than its effect on ouabain-sensitive Qo\textsubscript{2}, suggesting that a component of T1D-stimulated Na\textsuperscript{+} reabsorption is NOX-independent. For example, superoxide-independent mechanisms provoked by T1D (ie, increased PKC and SGK1 activation\textsuperscript{19,42}) may stimulate NHE3 or NKA, thereby contributing to the apocynin-resistant component of ouabain-sensitive Qo\textsubscript{2} in the STZ mTAL. Nevertheless, the ability of acute apocynin treatment to attenuate the T1D-induced increase in Qo\textsubscript{2} by the mTAL is in accord with the ability of chronic apocynin treatment to prevent the increase in ouabain-sensitive Qo\textsubscript{2} evident in proximal tubule cells from diabetic rats,\textsuperscript{43} thus reinforcing the concept that NOX-dependent oxidative stress disrupts renal oxygen metabolism in T1D through effects on electrolyte transport.

Some evidence indicates that PKC mediates superoxide-stimulated acceleration of Na\textsuperscript{+} reabsorption by rat mTAL,\textsuperscript{21,24} and our previous studies show that NOX-derived superoxide production by the mTAL during T1D is dependent on PKC.\textsuperscript{20} The results of the present study reveal that calphostin C reverses the increased Na\textsuperscript{+} transport-related Qo\textsubscript{2} by STZ mTALs, indicating that PKC mediates the T1D-induced stimulation of Na\textsuperscript{+} reabsorption by the mTAL. In addition, calphostin C treatment significantly reduced Na\textsuperscript{+} transport-related Qo\textsubscript{2} by sham mTALs, indicating that constitutively active PKC regulates Na\textsuperscript{+} reabsorption through NKCC2 and NKA pathways in the normal mTAL. These effects of the broad-spectrum PKC inhibitor were mirrored by the PKC\textalpha/\beta inhibitor G06976 but not by inhibition of PKC\textbeta alone. Thus, PKC\textalpha activity is implicated in promoting Na\textsuperscript{+} reabsorption by the mTAL under normal conditions, as well as the increased Na\textsuperscript{+} transport activity accompanying T1D.

In angiotensin II–dependent hypertension, Na\textsuperscript{+} transport-related Qo\textsubscript{2} is increased through a mechanism involving superoxide production and PKC\textalpha activity.\textsuperscript{25} The results of the present study indicate that a similar scenario arises during
T1D. This situation likely reflects the effect of T1D to increase PKC activity in the mTAL, a phenomenon associated with increased expression of PKCo and PKCδ.19 Our previous studies have revealed that both PKCo and PKCδ contribute to superoxide production by the mTAL during T1D.19 Moreover, NOX activity is increased (in concert with increased expression of Nox2, Nox4, and p47phox) in the mTAL and contributes to the PKC-dependent increase in superoxide production under these conditions.20 Interestingly, calphostin C is more effective than apocynin in reducing effect on Na+/H+ transport by the mTAL. For example, evidence indicates that PKC stimulates NKA in the proximal convoluted tubule,44–46 and PKCα-mediated phosphorylation of the NKA α-subunit has been reported to occur in the mTAL in response to C-peptide.47 PKC-dependent mechanisms evoking membrane translocation of NKA and NKCC2 have been reported in some cell types.48,49 In addition, NKCC1 is activated by Ste-20–related, proline-alanine–rich kinase (SPAK), and oxidative stress response kinase (OSR1).50–52 In human airway epithelial cells, hyperosmotic stress activates PKCδ, which phosphorylates SPAK, resulting in NKCC1 phosphorylation.53 As NKCC1 and NKCC2 are thought to be regulated by similar mechanisms, it is possible that PKCδ activates SPAK which, in turn, activates NKCC2 in the mTAL during T1D. However, we were unable to explore a potential role of PKCδ in the T1D-induced increase in Na+ transport-related Qo2 due to interference of the PKCδ inhibitor rottlerin with the OBS fluorophore (data not shown). Any or all of these events may contribute to PKC- and superoxide-dependent increases in Na+ transport that arise in the mTAL during T1D.

Previous work has detected no change or increased NKCC2 expression in uncontrolled STZ-induced T1D,54,55 whereas increases in mTAL NKA activity and α1-subunit mRNA expression have been reported 8 days after onset of STZ-induced T1D.56 These changes have been attributed to compensatory responses to the increased filtered load and delivery of Na+ to the mTAL during T1D, helping to limit dissipation of medullary interstitial osmolarity in the face of a prolonged osmotic diuresis. However, a change in NKCC2 or NKA expression probably does not underlie the rapidly reversible changes in Na+ transport-related Qo2 evident in the present study. Rather, the accumulating data suggest that altered posttranslational regulation of the NKCC2/NKA system (through PKCo- and NOX-dependent superoxide production) underlies the increased Na+ transport-related Qo2 evident in mTALs from diabetic rats.

Perspectives

Na+ retention occurs in the early stage of T1D, and individuals with T1D have increased risk to develop hypertension when they lose the ability to compensate for the early changes of Na+ handling.57 It is easy to envision how increased Na+ reabsorption by the mTAL would contribute to the Na+ retention accompanying T1D, although it remains difficult to resolve the quantitative relevance of this phenomenon in relation to the increase in NaCl delivery to this nephron segment and the osmotic diuresis accompanying T1D. The increase in NOX-dependent, Na+ transport-related Qo2 by the mTAL undoubtedly contributes to the antioxidant-sensitive outer medullary hypoxia that arises during T1D.57,58 As chronic hypoxia has been proposed to play a dominant role in provoking tubulointerstitial injury, thereby initiating the pathogenesis of diabetic nephropathy,59 increased Na+ transport-related Qo2 by the mTAL is poised to play an important role in that process. Thus, studies revealing mechanisms underlying increased Na+ reabsorption by the mTAL during T1D may allow development of new therapeutic strategies to reduce the prevalence of hypertension and prevent development of nephropathy in diabetic patients.

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Disclosures

None.

References


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NADPH oxidase and PKC contribute to increased Na transport by the thick ascending limb during type 1 diabetes

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Figure S1. Comparison of \( Q_{O_2} \) data measured in mTAL suspensions using the closed-chamber Clark electrode method (left panel) and preliminary experiments using the microplate-based OBS method (right panel). The Clark electrode-based method employed the YSI model 5300A Oxygen Monitor system with 5304 Micro Adaptor kit, according to manufacturer instructions. The electrode was calibrated using solutions equilibrated with 100% N\(_2\) and ambient air (21% O\(_2\)), after which mTAL in 1 ml HBSS were added to the closed chamber and \( O_2 \) concentration was recorded continuously at 37°C. Once an initial constant slope (change in \( O_2 \) concentration per unit time) was established, ouabain was added to the chamber (2 mmol/L) and the resulting slope was recorded. The OBS method is described in the main body of the paper. Both methods yield the same pattern of increased total and ouabain-sensitive \( Q_{O_2} \) in mTAL from STZ rats, compared with normal/sham rats, with no significant difference in ouabain-insensitive \( Q_{O_2} \) between groups. *\( P<0.05 \) vs. Normal/Sham.