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\textsuperscript{+} transport through PKC-dependent and NADPH oxidase–dependent mechanisms. An O\textsubscript{2}-sensitive fluoroprobe was used to measure O\textsubscript{2} consumption by mTALs from rats with streptozotocin-induced diabetes and sham rats. In sham mTALs, total O\textsubscript{2} 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 O\textsubscript{2} consumption by 69±4% and 500 μmol/L furosemide reduced O\textsubscript{2} consumption by 58±8%. Total O\textsubscript{2} 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 O\textsubscript{2} consumption. NADPH oxidase inhibition (100 μmol/L apocynin) reduced furosemide-sensitive O\textsubscript{2} consumption by mTAL from diabetic rats to values not different from sham. The PKC inhibitor calphostin C (1 μmol/L) or the PKCα/β inhibitor Gö6976 (1 μmol/L) decreased furosemide-sensitive O\textsubscript{2} 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 O\textsubscript{2} consumption. We conclude that NADPH oxidase and PKC (primarily PKCα) contribute to an increase in O\textsubscript{2} consumption by the mTAL during type 1 diabetes through effects on the ouabain-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and furosemide-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter that are primarily responsible for active transport Na\textsuperscript{+} 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

There is an approximately 3-fold greater prevalence of hypertension in adults with type 1 diabetes (T1D), compared with age-matched individuals without diabetes.\textsuperscript{1,2} Increased nocturnal blood pressure and salt sensitivity of blood pressure have been reported in patients with T1D before the onset of microalbuminuria,\textsuperscript{3,4} suggesting that prohypertensive mechanisms arise before development of diabetic nephropathy. Indeed, when allowed unrestricted Na\textsuperscript{+} intake, patients with uncomplicated moderately hyperglycemic T1D display net Na\textsuperscript{+} retention, and increases in both extracellular fluid volume and exchangeable Na\textsuperscript{+}.\textsuperscript{5–9} These events are evident early after onset of T1D and may ultimately contribute to the eventual development of hypertension in susceptible individuals.

Rats with streptozotocin (STZ)-induced T1D also exhibit net Na\textsuperscript{+} retention,\textsuperscript{10,11} as well as increased renal Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter (NKCC2), leading to an increase in intracellular [Na\textsuperscript{+}] that stimulates the basolateral NKA.\textsuperscript{21} Extracellular superox-
ide also indirectly promotes $\text{Na}^+$ transport by decreasing the bioavailability of NO, an inhibitor of NaCl reabsorption by the mTAL. The stimulatory impact of superoxide on the bioavailability of NO, an inhibitor of NaCl reabsorption by the mTAL.

**Methods**

**Chemicals and Reagents**

The NOX inhibitor apocynin, the PKC inhibitor calphostin C, the PKCa/β inhibitor Go6976, and the PKCB inhibitor 3-[(3-imidazol-1-ylpropyl)-1H-indol-3-yl]-4-aminolo-1H-pyrole-2,5-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 micromer crystalized 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 ~300 g. STZ-injected rats with partial insulin replacement were used as a control. Sham rats received vehicle substitutes for STZ and insulin. The sham and STZ rats were provided ad libitum food and water for 19±1 day, after which kidneys were harvested under pentobarbital sodium anesthesia (50 mg/kg IP) for in vitro study of mTAL function. Blood glucose levels, which were measured twice weekly with an Accu-Chek Advantage meter (Roche Diagnostics, Indianapolis, IN), averaged 4.9±0.1 mmol/L in sham rats and 22.3±1.2 mmol/L in STZ rats.

**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 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 fluorophore 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 fluorophore-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 fluent content), as well as the negative control for each 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 O2 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.
Figure 2. Effects of NADPH oxidase (NOX) inhibition and protein kinase C (PKC) inhibition on components of O2 consumption by medullary thick ascending limbs (mTALs) from sham and streptozotocin (STZ)-treated rats. Shown are effects of 30-minute pretreatment with 100 μmol/L apocynin (NOX inhibitor), 1 μmol/L calphostin C (broad-spectrum PKC inhibitor), 1 μmol/L Gö6976 (PKCβ/δ inhibitor), and 50 nmol/L indolylmaleimide-1 (PKCγ 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.

Increases in ouabain- and furosemide-sensitive Qo2 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 μmol/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 in untreated 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, with no treatment effect of apocynin evident. These observations indicate that NOX activity contributes to the elevation in Na+ transport-related Qo2 by STZ mTAL, without apparent involvement in sham mTAL.

The role of PKC in determining Qo2 by sham and STZ mTAL was assessed based on responses to 1 μmol/L calphostin C (IC50=0.05 μmol/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 treatment effect 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.

To determine which PKC isozyme is involved in the increased mTAL Qo2 induced by T1D, we used PKC inhibitors with relative isozyme specificity. At a concentration of 1 μmol/L, Gö6976 abolishes the enzymatic activity of both PKCα and PKCβ (IC50=1.3–6.0 nmol/L) without effect on PKCδ, PKCζ, or PKCε.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 effect of Gö6976 that was independent of group (sham versus STZ). In contrast, exposure to 50 nmol/L indolylmaleimide-1 (PKCβ inhibitor; IC50=5–21 nmol/L, with ≥100 nmol/L influencing other PKC isozymes)32 had no effect on any component of Qo2 by sham or STZ mTAL. These data indicate that PKCα is a primary determinant of Na+ transport-related Qo2 by the mTAL, with an augmented influence in STZ mTAL.

Discussion

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

Table 1. Effects of NADPH oxidase (NOX) inhibition and protein kinase C (PKC) inhibition on components of O2 consumption by medullary thick ascending limbs (mTALs) from sham and streptozotocin (STZ)-treated rats. Shown are effects of 30-minute pretreatment with 100 μmol/L apocynin (NOX inhibitor), 1 μmol/L calphostin C (broad-spectrum PKC inhibitor), 1 μmol/L Gö6976 (PKCβ/δ inhibitor), and 50 nmol/L indolylmaleimide-1 (PKCγ 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.

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<th>Component</th>
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<td>Group</td>
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<td>Treatment</td>
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<td>Group x Treatment</td>
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<td>Ouabain-Sensitive Qo2</td>
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<td>Furosemide-Sensitive Qo2</td>
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<td>Ouabain-Insensitive Qo2</td>
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basolateral membrane through the NKA.\textsuperscript{33} Active transport \(Na^+\) 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 \(Q_o2\) (ouabain-sensitive \(Q_o2\)) is largely reflective of active transport \(Na^+\) reabsorption. However, as NKA activity also supports other cellular processes (maintenance of intracellular \([K^+]\), etc), ouabain-sensitive \(Q_o2\) is not purely indicative of active \(Na^+\) reabsorption. The effect of the NKKC2 inhibitor furosemide on \(Q_o2\) (furosemide-sensitive \(Q_o2\)) provides an alternative index of \(Na^+\) transport that specifically involves the NKKC2 transporter, which is the primary apical \(Na^+\) influx pathway in the mTAL. Previous reports indicate that ouabain reduces mTAL \(Q_o2\) by 40–50%,\textsuperscript{35,36} with furosemide-sensitive \(Q_o2\) representing 20–40% of total \(Q_o2\).\textsuperscript{25,36} In the present study, we found that the ouabain- and furosemide-sensitive components of \(Q_o2\) represented \(\approx 70\%\) and 60% of total \(Q_o2\), respectively. The quantitative disparity of these proportions compared with previous reports likely reflects, at least in part, the fact that we analyzed \(Q_o2\) in mTAL suspensions equilibrated with ambient air (21% \(O_2\)), rather than the 100% \(O_2\) equilibration used in the previous studies.\textsuperscript{25,36} Similar to our results, Palm et al.\textsuperscript{37,38} found that ouabain-sensitive \(Q_o2\) by renal outer medullary cells in buffer equilibrated with ambient air is about 70% of total \(Q_o2\). Thus, under the conditions of our assay, \(Na^+\) transport-related \(Q_o2\) represents the bulk of total \(Q_o2\) by mTALs. Consistent with reports that \(Q_o2\) is augmented in kidney of diabetic rats predominantly in the medullary region,\textsuperscript{37,38} we also found that T1D increases total and ouabain-sensitive \(Q_o2\) by mTALs. Increased ouabain-insensitive \(Q_o2\) has been also reported in medullary cells from diabetic rats\textsuperscript{39}; however, we failed to detect a significant impact of T1D on this parameter in the mTAL. This discrepancy may suggest that other cells (ie, from the S3 segment of the proximal tubule) are responsible for T1D-induced changes in ouabain-insensitive \(Q_o2\) in the renal medulla or, alternatively, that the OBS system used in the present study is relatively insensitive in distinguishing differences at low levels of \(Q_o2\). Nevertheless, our observation of markedly increased ouabain- and furosemide-sensitive \(Q_o2\) by the mTAL of STZ rats indicates that T1D provokes an increase in \(Na^+\) transport-related \(Q_o2\) that involves NKKC2 and NKA. This phenomenon is apparent in nonperfused mTALs, independent of the increased NaCl delivery to the loop of Henle that occurs in vivo during T1D.\textsuperscript{40} Increased mTAL \(Na^+\) transport probably contributes to the reduction in \(Na^+\) delivery to the macula densa, evident during T1D, and a consequent tubuloglomerular feedback-dependent increase in glomerular filtration rate.\textsuperscript{40}

Published reports have demonstrated that exogenously produced superoxide stimulates NaCl reabsorption by the TAL.\textsuperscript{21,24,25,41} Moreover, the ability of the superoxide scavenger tempol to decrease \(Cl^-\) reabsorption by the TAL from normal rat kidney indicates that superoxide tonically promotes NaCl reabsorption.\textsuperscript{42} Because our previous work revealed that T1D increases superoxide production by the rat mTAL,\textsuperscript{19} we reasoned that the accelerated superoxide production would contribute to increased \(Na^+\) reabsorption by this segment during T1D. Preliminary experiments revealed that tempol interferes with the fluorophore that allows monitoring oxygen levels using the OBS system, thereby precluding the use of this antioxidant to investigate the impact of superoxide on \(Q_o2\) by the mTAL. Rather, given evidence showing the key role of NOX in mediating the T1D-induced acceleration of superoxide production by mTAL,\textsuperscript{30} we evaluated the effect of apocynin (NOX inhibitor) on \(Q_o2\) by mTALs. In contrast with the ability of tempol to decrease \(Cl^-\) reabsorption by isolated perfused TAL,\textsuperscript{41} our data failed to reveal any effect of apocynin on \(Na^+\) transport-related \(Q_o2\) by sham mTALs. These observations suggest that the tonic impact of superoxide on NaCl transport by the mTAL from normal rat kidney is not the result of NOX activity but rather involves alternative sources of superoxide. However, apocynin pretreatment decreased total and \(Na^+\) transport-related \(Q_o2\) by mTALs from STZ rats. In particular, apocynin fully restored furosemide-sensitive \(Q_o2\) by STZ mTALs to a value not significantly different from that observed in sham mTALs. These observations indicate that NOX is involved in T1D-stimulated \(Na^+\) reabsorption through NKKC2 and NKA pathways. The ability of apocynin to reduce furosemide-sensitive \(Q_o2\) by STZ mTALs was greater than its effect on ouabain-sensitive \(Q_o2\), suggesting that a component of T1D-stimulated \(Na^+\) 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 \(Q_o2\) in the STZ mTAL. Nevertheless, the ability of acute apocynin treatment to attenuate the T1D-induced increase in \(Q_o2\) by the mTAL is in accord with the ability of chronic apocynin treatment to prevent the increase in ouabain-sensitive \(Q_o2\) 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 \(Na^+\) 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 \(Na^+\) transport-related \(Q_o2\) by STZ mTALs, indicating that PKC mediates the T1D-induced stimulation of \(Na^+\) reabsorption by the mTAL. In addition, calphostin C treatment significantly reduced \(Na^+\) transport-related \(Q_o2\) by sham mTALs, indicating that constitutively active PKC regulates \(Na^+\) reabsorption through NKKC2 and NKA pathways in the normal mTAL. These effects of the broad-spectrum PKC inhibitor were mirrored by the PKC\(\alpha/\beta\) inhibitor G69796 but not by inhibition of PKC\(\beta\) alone. Thus, PKC\(\alpha\) activity is implicated in promoting \(Na^+\) reabsorption by the mTAL under normal conditions, as well as the increased \(Na^+\) transport activity accompanying T1D.

In angiotensin II–dependent hypertension, \(Na^+\) transport-related \(Q_o2\) is increased through a mechanism involving superoxide production and PKC\(\alpha\) activity.\textsuperscript{25} The results of the present study indicate that a similar scenario arises during

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TID. This situation likely reflects the effect of TID to increase PKC activity in the mTAL, a phenomenon associated with increased expression of PKCα and PKCδ.19 Our previous studies have revealed that both PKCα and PKCδ contribute to superoxide production by the mTAL during TID.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 superoxide production (and its effect on Na+/H+ transport) in uncontrolled STZ-induced T1D.54,55

As NKCC1 and NKCC2 are involved in NKCC1 phosphorylation.53 As NKCC1 and NKCC2 are known to be 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 phosphorlates 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 TID. 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 TID.

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 increase 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 PKCα- 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 NaCI 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 antioxidase-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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Short Title: Thick ascending limb Na transport in 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.