NADPH oxidase inhibition reduces tubular sodium transport and improves kidney oxygenation in diabetes

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Persson P, Hansell P, Palm F. NADPH oxidase inhibition reduces tubular sodium transport and improves kidney oxygenation in diabetes. Am J Physiol Regul Integr Comp Physiol 302: R1443–R1449, 2012. First published May 2, 2012; doi:10.1152/ajpregu.00502.2011.—Sustained hyperglycemia is associated with increased oxidative stress resulting in decreased intrarenal oxygen tension (PO2) due to increased oxygen consumption (QO2). Chronic blockade of the main superoxide radicals producing system, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, normalizes QO2 by isolated proximal tubular cells (PTC) and reduces proteinuria in diabetes. The aim was to investigate the effects of acute NADPH oxidase inhibition on tubular Na+ transport and kidney PO2 in vivo. Glomerular filtration rate (GFR), renal blood flow (RBF), filtration fraction (FF), Na+ excretion, fractional Li+ excretion, and intrarenal PO2 were measured in control and streptozotocin-diabetic rats during baseline and after acute NADPH oxidase inhibition using apocynin. The effects on tubular transporters were investigated using freshly isolated PTC. GFR was increased in diabetics compared with controls (2.2 ± 0.3 vs. 1.4 ± 0.1 ml·min−1·kidney−1). RBF was similar in both groups, resulting in increased FF in diabetics. PO2 was reduced in cortex and medulla in diabetic kidneys compared with controls (34.4 ± 0.7 vs. 42.5 ± 1.2 mmHg and 15.7 ± 1.2 vs. 25.5 ± 2.3 mmHg, respectively). Na+ excretion was increased in diabetics compared with controls (24.0 ± 4.7 vs. 9.0 ± 2.0 µmol·min−1·kidney−1). In controls, all parameters were unaffected. However, apocynin increased Na+ excretion (+112%) and decreased fractional lithium reabsorption (−10%) in diabetics, resulting in improved cortical (+14%) and medullary (+28%) PO2. QO2 was higher in PTC isolated from diabetic rats compared with control. Apocynin, dimethylamiloride, and ouabain reduced QO2, but the effects of combining apocynin with either dimethylamiloride or ouabain were not additive. In conclusion, NADPH oxidase inhibition reduces tubular Na+ transport and improves intrarenal PO2 in diabetes.

Key Words: apocynin; dimethylamiloride; ouabain; oxygen tension; oxidative stress; proximal tubular cells; rat; streptozotocin

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bolus dose of 185 kBq (American Radiolabeled Chemicals, St. Louis, MO) and then continuously infused in Ringer solution (185 kBq/kg body wt.−1 h−1). The 3H-labeled activities in urine and plasma were measured using standard liquid scintillation technique, and GFR was calculated according to GFR = U/V/P, where U and P denote the activity of [3H]ulin in urine and plasma, respectively, and V denotes the urine flow (in ml/min). After a 45-min recovery period baseline data were obtained for 30 min followed by administration of apocynin (10 mg/kg bw bolus iv) or vehicle. Thereafter, all parameters were followed for an additional 20–30 min periods. Total renal blood flow (RBF) was measured using an ultrasound probe (Transonic Systems, Ithaca, NY) placed around the left renal artery. Li+ was administered as a 4-mg ip bolus of LiCl after completion of surgery followed by a continuous intravenous infusion (2.1 mg·h−1·rat−1) resulting in plasma concentration of 0.5–1.0 mM Li+. Kidneys were weighed at the end of each experiment. Urine flows were measured gravimetrically, and urinary and plasma Na+ and Li+ concentrations were determined by flame spectrophotometry (model IL543, Instrumentation Lab, Milan, Italy). Regional renal PO2 was measured using modified Clark-type microelectrodes (∼1 μm OD, Unisense, Aarhus, Denmark). Electrodes were two-point calibrated in water saturated with either Na2SO3 (zero) or air (147 mmHg). Nitrate/nitrite was analyzed using a commercially available kit according to manufacturer’s instruction (Cayman Chemicals, Ann Arbor, MI). Thiobarbituric acids reactive substances (TBARS) were measured fluorometrically (5). Briefly, 125 μl thiobarbituric acid (Mercck, Darmstadt, Germany) were added to 100 μl of diluted urine sample and heated to 97°C for 60 min. Thereafter, samples were cooled on ice, and a 150-μl mixture of methanol and 1 mol/l NaOH (91:9) were added followed by centrifugation (3,000 rpm, 5 min). Fluorescence was measured in the supernatant (ex. 532 nm, em. 553 nm; Safire2, Tecan, Männedorf, Switzerland). Standards were prepared from malondialdehyde-bis-(diethylacetae) (Merck-Schuchart, Schuchart, Germany).

Measurement of QO2 in vitro. PTC were isolated from normoglycemic controls and diabetic rats as previously described (5). Briefly, kidney cortex was minced through a metallic mesh strainer and injected into the chamber, and the rate of O2 disappearance was recorded. At the end of each experiment, 1 ml was removed from the chamber to determine protein concentration after centrifugation (15,000 g for 10 min) and resuspension in 200 μl dH2O, using DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). QO2 was calculated as the rate of O2 disappearance adjusted for protein concentration. Measurements were performed during baseline and after incubation with dimethylamiloride (DMA; 1 mmol/l) to inhibit the Na+/H+ exchanger (NHE) (4), apocynin (1 mmol/l) to inhibit the NADPH oxidase, and ouabain (2 mmol/l) to inhibit the Na+/K+-ATPase or apocynin in combination with DMA and ouabain. In all experiments samples were preincubated at 37°C for 10 min before being added to the Oxygraph-2k. The same concentration of each inhibitor as in the preincubation of the cells was present in the Oxygraph-2k before cells were added. It was also confirmed that none of the inhibitors interfered directly with technique used to measure QO2.

RESULTS

All diabetic animals developed hyperglycemia compared with controls (22.9 ± 0.7 vs. 5.6 ± 0.2 mM) and gained less weight compared with control animals (326 ± 7 vs. 357 ± 3 g). Food intake was higher in diabetic animals compared with controls (54 ± 3 vs. 26 ± 1 g/24 h). Left kidney weight was increased in diabetic animals compared with normoglycemic controls (1.85 ± 0.05 vs. 1.28 ± 0.03 g). Diabetic rats displayed 57% higher GFR than control, which was not affected by NADPH oxidase inhibition (Table 1). There was no difference in total RBF or MAP between the two groups, and filtration fraction was elevated in diabetic animals compared with normoglycemic controls (1.95% vs. 0.7% vs. 5.6% vs. 3.5% respectively) (Fig. 1, A and B). Furthermore, fractional Li+ reabsorption only decreased (−10%) in diabetic animals after NADPH oxidase inhibition (Fig. 2). PO2 was reduced in both kidney cortex (∼19%) and medulla (∼39%) of the diabetic rats compared with controls, but NADPH oxidase inhibition normalized PO2 in cortex.

Table 1. Mean arterial pressure, glomerular filtration rate, total renal blood flow, filtration fraction, urine flow, and hematocrit in normoglycemic control and hyperglycemic diabetic rats during baseline and after acute NADPH-oxidase inhibition by apocynin

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>GFR, ml·min−1·kidney−1</th>
<th>RBF, ml·min−1·kidney−1</th>
<th>FF</th>
<th>Vc, μl·min−1·kidney−1</th>
<th>Hct, %</th>
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<tbody>
<tr>
<td>Normoglycemic (n = 11)</td>
<td></td>
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<tr>
<td>Baseline</td>
<td>107 ± 4</td>
<td>1.4 ± 0.1</td>
<td>8.8 ± 1.0</td>
<td>0.31 ± 0.03</td>
<td>3.4 ± 0.5</td>
<td>46 ± 0.4</td>
</tr>
<tr>
<td>Apocynin</td>
<td>103 ± 4</td>
<td>1.2 ± 0.1</td>
<td>9.0 ± 1.0</td>
<td>0.27 ± 0.02</td>
<td>3.5 ± 0.4</td>
<td>46 ± 0.5</td>
</tr>
<tr>
<td>Diabetic (n = 13)</td>
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<tr>
<td>Baseline</td>
<td>104 ± 2</td>
<td>2.2 ± 0.3*</td>
<td>10.1 ± 0.9</td>
<td>0.45 ± 0.04*</td>
<td>24.9 ± 3.5*</td>
<td>48 ± 0.4*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>105 ± 3</td>
<td>2.4 ± 0.2*</td>
<td>11.1 ± 1.3</td>
<td>0.42 ± 0.03*</td>
<td>20.8 ± 3.2*</td>
<td>40 ± 0.7*</td>
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</table>

Values are means ± SE; n is the number of rats. MAP, mean arterial pressure; GFR, glomerular filtration rate; RBF, total renal blood flow; FF, filtration fraction; Vc, urine flow; Hct, hematocrit. *P < 0.05 compared with corresponding control.
DISCUSSION

The new findings from the present study are that diabetes-induced oxidative stress, originating from the NADPH oxidase, stimulates tubular Na\(^+\) transport and contributes to intrarenal hypoxia. This was evident as substantially decreased fractional reabsorption of Li\(^+\) (−10%) and reduced PTC transport-dependent QO\(_2\) (−32%) after NADPH oxidase inhibition. This indicates an effect of NADPH oxidase inhibition in the proximal tubule in diabetes. However, concomitant effects in more distal parts of the nephron are likely since it is well known that Li\(^+\) also is handled by mTALs and collecting ducts. This is further supported by the more pronounced alterations in total and fractional urinary Na\(^+\) excretion (+112% and +90%, respectively). The reduced tubular Na\(^+\) transport resulted in a concomitant increase in kidney Po\(_2\) in both cortex and medulla. Furthermore, NADPH oxidase inhibition reduced QO\(_2\) in control and diabetic PTC isolated from both control and diabetic rats, but the effect of NADPH oxidase inhibition was not additive to that of inhibiting either the Na\(^+\)-K\(^+\)-ATPase using ouabain or NHE3 using DMA. These results demonstrate that active tubular transport by the PTC is directly linked to NADPH oxidase activity. Kidney Po\(_2\) is determined by the delivery of oxygen by RBF, the extraction from hemoglobin, and QO\(_2\) related to basal metabolism and active tubular transport (31). Diabetes-induced glomerular hyperfiltration is present early in both patients and animal models of insulinopenic diabetes. Several theories have been suggested, but the underlying cause is still under debate (33). One implication for this phenomenon is that more electrolytes are filtered across the glomerular membrane into the primary urine, which requires increased tubular transport and increased QO\(_2\) to maintain Na\(^+\) balance. Previous studies have established that the diabetic kidney is working under reduced intrarenal Po\(_2\) (6, 29), and this study confirmed these observations. There are two possible explanations for the reduced kidney Po\(_2\) in diabetes; the first is related to increased tubular Na\(^+\) transport and the second is increased formation of ROS. However, the increased oxidative stress appears to be the most important factor since treatment...
with the antioxidant dl-α-tocopherol throughout the course of diabetes normalized renal P0₂ without affecting GFR (29). One explanation might be that ROS formation and tubular transport are closely connected since both exogenous and endogenous O₂⁻ stimulates NKCC and NHE isoform 3 in mTAL in isolated tubules (15, 16, 27). Furthermore, O₂⁻ interferes with cofactors required for NO synthesis by inhibiting dihydroptero-
dine reductase, the enzyme responsible for reduction of dihy-
drobiopterin (BH₂) to tetrahydrobiopterin (BH₄) (18). O₂⁻ causes depletion of BH₄ resulting in uncoupling of NOS, which further increases the O₂⁻ production (40). Reduced plasma concentrations of BH₄ have been demonstrated in models of type 2 diabetes (25). BH₄ supplementation improves vascular function in isolated aortic rings from diabetic rat (34) as well as endothelium-dependent vasodilation in type 2 diabetic patients (10), indicating that BH₄ levels are compromised in diabetes. Indeed, it has been shown that NO concentration is reduced in the cortex of diabetic rats (28) making it relevant since NO itself can directly inhibit tubular electrolyte transport mediated by NKCC2, NHE1, and NHE3 in mTAL (11). However, acute NADPH oxidase inhibition did not increase NO production, as estimated by urinary excretion of nitrate/nitrite. It is still possible that NO is involved since there is an important difference between NO production and NO bioavailability. The latter is highly influenced by oxidative stress, and acute NADPH oxidase inhibition did lower the urinary excretion of the lipid peroxidation marker TBARS in the diabetic animals. It should be noted that urinary TBARS has previously been demonstrated to correlate with the increased oxidative stress in diabetes measured by in vivo electron spin resonance (37).

Li⁺ clearance has been used as a marker of proximal tubular reabsorption (19), although Li⁺ also can be reabsorbed in the

Fig. 3. A: cortical oxygen tension in normoglycemic controls (n = 11) and hyperglycemic diabetic rats (n = 13) during baseline and after a bolus dose of apocynin. B: medullary oxygen tension in normoglycemic controls (n = 11) and hyperglycemic diabetic rats (n = 13) during baseline and after a bolus dose of apocynin.

Fig. 4. Urinary excretion of nitrate and nitrite in normoglycemic controls (n = 11) and hyperglycemic diabetic rats (n = 13) during baseline and after a bolus dose of apocynin.

Fig. 5. Urinary excretion of the lipid peroxidation marker thiobarbituric acids reactive substances (TBARS) in normoglycemic controls (n = 11) and hyperglycemic diabetic rats (n = 13) during baseline and after a bolus dose of apocynin.
mTAL and cortical collecting duct. In this study, NADPH oxidase inhibition decreased fractional reabsorption of Li\(^+\), indicating that O\(_2\)\(^{-}\) directly influences proximal tubular transport in vivo. A recent report demonstrates that diabetes induce increased transport-dependent QO\(_2\) in isolated mTAL via protein kinase C-\(\alpha\) and NADPH oxidase activation (41). Thus the reduced Na\(^+\) reabsorption after NADPH oxidase inhibition is likely to be influenced by altered transport also in this tubular segment. However, the involvement of the proximal tubule is verified by the in vitro experiments demonstrating reduced transport-dependent QO\(_2\) in the isolated PTC after NADPH oxidase inhibition. Inhibition of either NADPH oxidase or Na\(^+\)/K\(^+\)-ATPase reduced QO\(_2\), but the effects were not additive suggesting a direct effect on transport-dependent QO\(_2\) in the proximal tubule. QO\(_2\) was higher in PTC from diabetic rats during baseline but also after Na\(^+\)/K\(^+\)-ATPase inhibitor ouabain, and apocynin in combination with DMA or ouabain. *\(P < 0.05\) compared with baseline within the same group. 

Fig. 6. Oxygen consumption by isolated proximal tubular cells from normoglycemic controls (\(n = 7\)) and hyperglycemic diabetic rats (\(n = 8\)) after incubation with the NADPH oxidase inhibitor apocynin (APO), the Na\(^+\)/H\(^+\) exchanger (NHE3) inhibitor dimethylamiloride (DMA), the Na\(^-\)/K\(^+\)-ATPase inhibitor ouabain, and apocynin in combination with DMA or ouabain. 

\(\# P < 0.05\) compared with baseline within the same group.

Interestingly, reducing tubular Na\(^+\) excretion by inhibiting the NADPH oxidase did not alter GFR and RBF in the present study. This suggests that other mechanisms than altered tubular Na\(^+\) transport determine GFR in diabetes, or that acutely reduced oxidative stress activates compensatory mechanisms to maintain normal GFR and RBF in the diabetic kidney. Urine flow was unaltered despite increased natriuresis after NADPH oxidase inhibition, which may reflect differential regulation of tubular Na\(^+\) transport and water permeability. Potential mechanisms for such differential regulation are beyond the scope of this study.

Finally, this study confirms that increased tubular load of electrolytes due to the diabetes-induced glomerular hyperfiltration is not causing the reduction in kidney PO\(_2\) per se, since PO\(_2\) was improved in both the cortex and medulla by NADPH oxidase inhibition, despite unchanged GFR and RBF. Previous studies have demonstrated that chronic apocynin treatment during diabetes reduces proteinuria and prevents histological alterations, providing further evidence for oxidative stress being an important factor for the development of diabetic nephropathy (1).

In conclusion, an overactivated NADPH oxidase in diabetes results in increased transport-dependent QO\(_2\) and increased tubular Na\(^+\) transport with a subsequent reduction in cortical PO\(_2\).
and medullary PO₂ that is improved by acute NADPH oxidase inhibition.

Perspectives and Significance

Diabetic patients are at increased risk for development of hypertension and already in uncomplicated early diabetes display increased Na⁺ retention (8, 23, 36). One contributing factor might be increased tubular electrolyte transport due to increased oxidative stress resulting in volume expansion and hypertension. Increased tubular transport contributing to Na⁺ retention induced by enhanced O₂⁻⁻ production by the NADPH oxidase is now evident in both proximal tubule and mTAL (41). More research is needed to further clarify the regulation of tubular electrolyte transport and the potential link to increased arterial blood pressure in diabetes.

DISCLOSES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.P. and F.P. conception and design of research; P.P. performed experiments; P.P. and F.P. analyzed data; P.P., P.H., and F.P. interpreted results of experiments; P.P. and F.P. prepared figures; P.P. drafted manuscript; P.P., H., and F.P. edited and revised manuscript; P.P., P.H., and F.P. approved final version of manuscript.

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