Reduction of renal triglyceride accumulation: effects on proximal tubule Na\(^+\)/H\(^+\) exchange and urinary acidification

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Bobulescu IA, Dubree M, Zhang J, McLeroy P, Moe OW. Reduction of renal triglyceride accumulation: effects on proximal tubule Na\(^+\)/H\(^+\) exchange and urinary acidification. Am J Physiol Renal Physiol 297: F1419–F1426, 2009. First published August 19, 2009; doi:10.1152/ajprenal.00177.2009.—One main pathophysiological mechanism underlying the increased risk for uric acid nephrolithiasis in humans with the metabolic syndrome is the excretion of unduly acidic urine, in part because of reduced excretion of the main urinary buffer, ammonium. The Zucker diabetic fatty (ZDF) rat, an established rodent model of the metabolic syndrome, has similar urinary abnormalities, attributed in part to lower expression and activity of the principal mediator of proximal tubule ammonium excretion, brush-border membrane Na\(^+\)/H\(^+\) exchanger 3 (NHE3). These defects are associated with renal tubular steatosis in ZDF rats, but the causal relationship between renal steatosis and defective urinary acidification has not been investigated in vivo. We hypothesized that reduction of renal steatosis would commensurately normalize urinary acidification parameters. We treated ZDF rats with thiazolidinediones to reduce nonadipose tissue steatosis. Four weeks of treatment reduced renal triglyceride accumulation and restored urinary acidification parameters in ZDF rats to levels comparable to their lean littermates; urinary acidification was not affected by treatment in lean rats. To further document the direct effects of fat, we showed that functional abnormalities induced by fat loading in a cell culture model of proximal tubule steatosis and lipotoxicity can be reversed by fat removal but not by thiazolidinediones alone. Together, these findings support the causative role of renal steatosis in the pathogenesis of urinary acidification defects, demonstrate reversibility upon lipid removal, and highlight a potential therapeutic strategy for renal abnormalities in the metabolic syndrome.

Na\(^+\)/H\(^+\) exchanger 3; metabolic syndrome; renal lipotoxicity; ammonium; uric acid nephrolithiasis

HUMANS WITH FEATURES of the metabolic syndrome excrete an overly acidic urine and, as a consequence, have an increased propensity for uric acid nephrolithiasis (9, 16, 27, 36, 42, 45). Low urine pH in these patients has been attributed in part to decreased excretion of the principal urinary buffer, ammonium (NH\(_3\)) (9, 18, 21, 37, 42), but the pathophysiology underlying this defect is unknown.

NH\(_3\) excreted in the urine originates principally from the mitochondrial metabolism of glutamine to glutamate and then to 2-oxoglutarate, releasing ammonia (NH\(_3\)) in the proximal tubule cell (35). Intracellularly produced NH\(_3\) can either diffuse across the brush border and be trapped in the tubular lumen by protonation to NH\(_4^+\) or can be protonated in the cell and cross the lipid bilayer as NH\(_3\) \(\rightarrow\) Na\(^+\)/H\(^+\) exchanger 3 (NHE3) is important for both transport processes by extruding the H\(^+\) necessary for luminal trapping of NH\(_3\), and by functioning as a Na\(^+\)/NH\(_4^+\) exchanger (24, 33–35). Downstream of the proximal tubule, NH\(_3\)/NH\(_4^+\) is in part retrieved in the thick ascending limb of Henle’s loop, concentrated in the renal medulla, and resecreted in the collecting duct, while some is returned to the systemic circulation (35). Decreased NH\(_4^+\) excretion in the urine can result from insufficient production, defective extrusion into the proximal tubule lumen, or downstream transport defects resulting in reduced net renal excretion.

One abnormality associated with organ dysfunction in the metabolic syndrome is the disrupted balance of nonadipose tissue lipid uptake, synthesis, and utilization, leading to intracellular accumulation of free nonesterified fatty acids (FFA), triglycerides, and lipid metabolites, a phenomenon generally referred to as steatosis (43, 48, 54). Cellular lipid overload may result in dysfunction and injury in nonadipose tissues, a process that has been termed lipotoxicity and has been linked to organ dysfunction in the heart, skeletal muscle, liver, and pancreatic \(\beta\)-cell (43, 48, 50, 54). Lipotoxicity may also occur in the kidney, but its effects on renal function are incompletely understood (2, 51, 54). The proximal tubule may be particularly susceptible to lipotoxicity, because of its critical role in retrieving the FFA-bearing albumin normally filtered at the glomerulus (5, 20, 41, 54). Elevated FFA levels in the metabolic syndrome (39, 49) result in a higher FFA-to-albumin molar ratio and may expose the proximal tubule to an increased load of FFA from both the interstitium and the tubule lumen (5, 54).

Zucker diabetic fatty (ZDF) rats, an established model of obesity and metabolic syndrome (10), have a urinary biochemical profile highly similar to that of humans with the metabolic syndrome, including impaired NH\(_4^+\) excretion, low urinary pH, and high titratable acidity (TA) (6). These abnormalities have been attributed in part to reduced proximal tubule brush-border NHE3 expression and activity and are associated with increased circulating FFA and renal proximal tubule steatosis (6). Incubation of proximal tubule-like opossum kidney (OKP) cells (12) with a mixture of FFA carried on albumin resulted in intracellular triglyceride accumulation, impaired NHE3 activity and regulation, and reduced NH\(_4^+\) production, supporting a causal link between proximal tubule lipotoxicity and defective urinary acidification (6).

One important question regarding the functional abnormalities associated with proximal tubule lipotoxicity is whether reducing fat accumulation can restore or preserve normal function. This question not only bears relevance to the pathophysiology of renal lipotoxicity but may also have clinical therapeutic implications. We tested this, using the ZDF rat model as well as a proximal tubule cell culture model. We
examined renal lipid accumulation, urinary biochemistry, and proximal tubular brush-border NHE3 in ZDF rats treated with two members of the thiazolidinedione (TZD) family of peroxisome proliferator-activated receptor-γ (PPARY) agonists. These drugs have been shown to reduce skeletal muscle, heart, and liver steatosis in humans and animal models (4, 22, 38, 47, 52, 57), primarily by redistributing lipids to the adipose tissue (44, 55). The direct effects of intracellular lipid reduction and TZD treatment were also examined in lipid-loaded OKP cells, a cell culture model of proximal tubule lipotoxicity (6).

**EXPERIMENTAL PROCEDURES**

**Materials and supplies.** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except where otherwise noted, and except for the following: cell culture media, 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and human recombinant insulin (Invitrogen, Carlsbad, CA); penicillin-streptomycin (Cambrex, East Rutherford, NJ); EZ-Link sulfo-NHS-Ss-biotin and immunopure immobilized streptavidin (Pierce, Rockford, IL); protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN); horseradish peroxidase-labeled anti-mouse IgG and enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ); polyvinylidene difluoride membranes (Immobilon, Millipore, Billerica, MA).

**Animals.** The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center. Experiments were conducted in accordance with institutional guidelines and with the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). Thirty-two male 8-wk-old ZDF (fa/fa) rats and their lean wild-type (+/+) littermates were a generous gift from Dr. Roger Unger (University of Texas Southwestern Touchstone Center for Diabetes Research). Animals were fed standard rodent diets (Harlan Teklad, Madison, WI) with or without troglitazone (Sankyo, Tokyo, Japan; 400 mg/kg chow) or rosiglitazone (Sankyo, Tokyo, Japan; 4 mg/kg chow) for 4 wk. The effects of troglitazone and rosiglitazone on renal acidification and lipid parameters were indistinguishable, and therefore the results are reported collectively. For urine collections, rats were pair fed standard chow in metabolic cages and 24-h urine samples were collected under mineral oil with added mannitol, 18 HEPES, 5 EGTA, pH 7.5), and lipids were extracted (280 mM mannitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5) with a Potter-Elvehjem Teflon homogenizer, equilibrated for 2 h at 4°C, centrifuged at 48,000 g, and resuspended with a 27-gauge syringe needle at a final concentration of 10 mg/ml. Extravesicular solution [120 mM N-methyl-d-glucamine (NMDG)-gluconate], 20 mM HEPES, pH 7.5] with fresh acridine orange (Invitrogen) added to a final concentration of 6 μM was loaded in a cuvette equipped with a magnetic mini stir bar. Acridine orange fluorescence was monitored in a computer-controlled spectrophotometer (λexcitation = 493 nm, λemission = 530 nm; QM-800/300, Photon Technology International, London, ON, Canada). Fluorescence was rapidly quenched by addition of acid-loaded BBMV (a volume containing 150 μg protein) in the absence of Na+ and under constant stirring. Addition of extravesicular Na+ (Na+–glucanate to a final concentration of 30 mM) activated Na+/H+ exchange and resulted in a proportional increase in fluorescence. Replacement of Na+–glucanate with NMDG-glucanate was used to monitor Na+–independent quenching. Na+/H+ exchange activity was estimated as the initial rate of increase in fluorescence on addition of Na+–glucanate, from which the fluorescence increase with NMDG-glucanate (typically <1/10th of that with Na+–glucanate) was subtracted. Quantitative comparisons were made between BBMV preparations at the same time from rats killed on the same day, and the data were expressed as Na+/H+ transport activity of BBMV.

**NHE3 antigen in BBMV.** Rat kidney cortex BBMV prepared as above were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) containing fresh protease inhibitors and cleared by centrifugation (14,000 g, 30 min), and protein content was determined by the method of Bradford. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (7). Briefly, samples containing the same amount of total protein were heated for 2 min at 95°C in loading buffer, size fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes with a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA). Membranes were blocked in nonfat milk, probed overnight at 4°C with the 3H3 monoclonal anti-NHE3 antibody at 1:1,000 dilution (7), washed (5 × 10 min, 0.05% Tween 20 in PBS), incubated with a horseradish peroxidase-labeled secondary antibody for 1 h, washed as above, and visualized by enhanced chemiluminescence. Equivalent loading was confirmed by stripping and reprobing with a monoclonal anti-β-actin antibody (Sigma) as previously described (6). Quantification of protein abundance was performed with Scion/NIH Image software (Scion, Frederick, MD).

**Cell culture.** OKP cells (12) were maintained in a humidified 95% air–5% CO2 incubator at 37°C, in high-glucose (450 mg/dl) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). FFA were prepared as previously described (6) with a mixture of albumin-bound oleate and palmitate (molar ratio 2:1) carried on albumin. Confluent cells were rendered quiescent by incubation in isolation buffer containing fresh protease inhibitors, and crude membranes were obtained by centrifugation at 48,000 g for 1 h at 2°C (Beckman J2–21M; JA-20 rotor; Beckman Coulter, Fullerton, CA). Pellets were resuspended, homogenized in a Dounce glass homogenizer, and subjected to Mg2+ aggregation by addition of MgCl2 to a final concentration of 15 mM at 4°C for 20 min. Aggregated membranes were removed by centrifugation at 3,000 g for 10 min at 2°C (Beckman Allegra 21R; Beckman Coulter), and the supernatant was subjected to two additional rounds of Mg2+ aggregation as above (pellets discarded), followed by centrifugation at 48,000 g for 30 min at 2°C. The resulting pellet enriched in BBMV was used for Na+/H+ exchange activity experiments and for immunoblotting.

Na+/H+ exchange activity in BBMV. Na+/H+ exchange activity was measured in fresh BBMV with the acridine orange fluorescence quenching method (53) as previously described (6). Briefly, the BBMV pellet obtained as above was rehomogenized in intravesicular solution [280 mM mannitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5] with a Potter-Elvehjem Teflon–glass homogenizer, equilibrated for 2 h at 4°C, centrifuged at 48,000 g, and resuspended with a 27-gauge syringe needle at a final concentration of 10 mg/ml. Extravesicular solution [120 mM N-methyl-d-glucamine (NMDG)-glucanate, 20 mM HEPES, pH 7.5] with fresh acridine orange (Invitrogen) added to a final concentration of 6 μM was loaded in a cuvette equipped with a magnetic mini stir bar. Acridine orange fluorescence was monitored in a computer-controlled spectrophotometer (λexcitation = 493 nm, λemission = 530 nm; QM-800/300, Photon Technology International, London, ON, Canada). Fluorescence was rapidly quenched by addition of acid-loaded BBMV (a volume containing 150 μg protein) in the absence of Na+ and under constant stirring. Addition of extravesicular Na+ (Na+–glucanate to a final concentration of 30 mM) activated Na+/H+ exchange and resulted in a proportional increase in fluorescence. Replacement of Na+–glucanate with NMDG-glucanate was used to monitor Na+–independent quenching. Na+/H+ exchange activity was estimated as the initial rate of increase in fluorescence on addition of Na+–glucanate, from which the fluorescence increase with NMDG-glucanate (typically <1/10th of that with Na+–glucanate) was subtracted. Quantitative comparisons were made between BBMV preparations at the same time from rats killed on the same day, and the data were expressed as Na+/H+ transport activity of BBMV.

**Renal triglyceride, plasma fatty acids, and urine biochemistry.** Kidney cortical tissue was homogenized on ice with a Polytron (Brinkmann Instruments, Westbury, NY) in isolation buffer (in mM: 300 mannitol, 18 HEPES, 5 EGTA, pH 7.5), and lipids were extracted by the method of Folch et al. (19). Tissue triglycerides were measured according to the method of Danno et al. (15) with a triglyceride determination kit (Sigma, St. Louis, MO). For plasma and urine biochemistry, a Cobas Mira Plus Chemistry Autoanalyzer (Roche Diagnostics, Basel, Switzerland) was used to measure urinary NH3 (glutamate dehydrogenase assay), creatinine (kinetic alkaline picrate method), citrate (citrate lyase method), and plasma nonesterified fatty acids (enzymatic kit, Wako Chemicals, Richmond, VA). TA was measured by titrating the urine samples collected under oil to pH 7.4 with 0.1 N NaOH and an automated burette (Radiometer, Copenhagen, Denmark).

**Preparation of BBMV.** Renal cortical BBMV were prepared by the Mg2+ aggregation method as previously described (6). Briefly, fresh kidney cortical samples were homogenized as above in ice-cold
serum-free low-glucose (100 mg/dl) DMEM-containing vehicle (albumin) for a total of 72 h, as follows: to study lipotoxicity, FFA were added to the cells during the last 12 h of serum deprivation; to study lipotoxicity reversal, cells were serum deprived for 24 h and then FFA were added for 12 h, followed by 36 h of incubation in FFA-free medium before experiments. Before assays, cells were incubated with or without insulin (10⁻⁵ M) for 2 h. For rosiglitazone treatment, confluent OKP cells were incubated with 0.75 mM FFA carried on albumin or albumin alone for 24 h in serum-free medium supplemented with 25 mM N₂-H₂-oxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, pH 7.4), with addition of either vehicle (dimethyl sulfoxide) or excipient-free rosiglitazone (Cayman Chemical, Ann Arbor, MI) at the indicated concentrations.

Apical membrane NHE3 antigen in OKP cells. Confluent OKP cells were rinsed with ice-cold isotonic wash buffer (in mM: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, 2 KH₂PO₄, 1 MgCl₂, 0.1 CaCl₂, pH 7.4) and incubated with 3 ml of biotinylation buffer (1.5 mg/ml sulfo-NHS-SS-Biotin, 2 mM CaCl₂, 150 mM NaCl, 10 mM triethanolamine, pH 7.4) for 90 min with horizontal motion at 4°C. After biotin labeling, cells were washed twice with 6 ml of quenching buffer (in mM: 1 MgCl₂, 0.1 CaCl₂, 100 glycine in PBS, pH 7.4, for 20 min at 4°C) and lysed in RIPA buffer with protease inhibitors as previously described (7). Lysates were cleared by centrifugation (14,000 × g, 4°C, 30 min; Denville 260D, Denville Scientific, Metuchen, NJ), diluted to 2.5 mg/ml protein (as measured by the method of Bradford) with RIPA buffer, and equilibrated overnight at 4°C with streptavidin-agarose beads on an end-over-end rotator. Beads were washed three times with solution A (in mM: 50 Tris-HCl, pH 7.4, 100 NaCl, 5 EDTA), two times with solution B (in mM: 50 Tris-HCl, pH 7.4, 500 NaCl), and once with solution C (50 mM Tris-HCl, pH 7.4), each time with recovery by centrifugation at 1,000 g for 1 min. Beads were then heated to 95°C in 2.5× loading buffer, and the supernatant was subjected to SDS-PAGE and immunoblotting as above.

Apical Na⁺/H⁺ exchange activity in OKP cells. Intracellular pH (pHi) was monitored with a computer-controlled spectrofluorometer (λ excitation: 500 and 450 nm, λ emission: 530 nm) in cells grown on glass coverslips and loaded with the intracellularly trapped pH-sensitive dye BCECF as described previously (7, 32). Calibration of the 500- to 450-nm fluorescence ratio to pHi was performed with the K⁺/nigerin method. Apical Na⁺/H⁺ exchange activity was estimated from the initial rate of the Na⁺-,K⁺-dependent pHi increase after nigericin-induced acid load, in the absence of CO₂/HCO₃⁻. Intracellular buffer capacity (β) measured with the NH₄Cl pulse method was not significantly different between the different incubation conditions (not shown).

Statistics. Statistical analysis was performed with Student’s t-test and ANOVA as specified in Figs. 1–5. Results are presented graphically as means and SD. For animal experiments, n = 8 per condition unless otherwise noted.

RESULTS

Effect of TZD on plasma and renal lipid abnormalities in ZDF rats. Compared with their lean littermates, ZDF rats have higher levels of FFA in the plasma and accumulate excess triglycerides in the renal cortex (6). Treatment of ZDF rats with PPARγ agonists has been shown to reduce plasma FFA (13, 52) and lipid accumulation in the heart, skeletal muscle, and liver (22, 52), but the effect on kidney triglyceride accumulation has not been studied. ZDF rats treated with TZD for 4 wk gained more weight than untreated ZDF rats, but their average decapsulated kidney weight was not different (Table 1). Treatment with TZD significantly decreased plasma FFA levels and renal cortical triglyceride content in ZDF rats, while having no detectable effect in their lean littersmates (Fig. 1).

Effect of TZD on urinary acidification parameters in ZDF rats. We showed previously (6) that high circulating FFA and renal triglyceride accumulation in ZDF rats are associated with urinary acidification defects, primarily due to inappropriate NH₄⁺ excretion. The causal relationship between lipid abnormalities and defective NH₄⁺ production and/or transport has been tested in vitro in cell culture but not in vivo (6). We examined causality in vivo by reducing plasma FFA and the development of renal steatosis in ZDF rats with TZD treatment. The amelioration of lipid abnormalities in TZD-treated ZDF rats was accompanied by changes in urinary acidification parameters including urinary pH, NH₄⁺, and TA to values close to those in lean rats, while TZD treatment in lean rats, in the absence of lipid abnormalities, had no effect on urinary acidification (Fig. 2, A–C). Of note, urinary citrate was lower in ZDF rats compared with their lean littersmates, and this difference was also reversed by TZD treatment (Fig. 2D). These differences could not be attributed to acid-base disturbances or differential acid or alkali intestinal absorption, since acid-base status and urinary sulfate and potassium excretion were not different between the groups (Table 1).

Table 1. Plasma and urinary parameters in ZDF rats with or without TZD treatment

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Lean + TZD</th>
<th>ZDF</th>
<th>ZDF + TZD</th>
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<tbody>
<tr>
<td>Body and kidney weights</td>
<td></td>
<td></td>
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<tr>
<td>Body wt, g</td>
<td>288±15</td>
<td>294±19</td>
<td>395±13*</td>
<td>456±28†‡</td>
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<tr>
<td>Kidney wt, g</td>
<td>1.19±0.07</td>
<td>1.21±0.09</td>
<td>1.51±0.15*</td>
<td>1.56±0.19†</td>
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<td>Kidney/body wt, %</td>
<td>0.414±0.038</td>
<td>0.413±0.047</td>
<td>0.382±0.046*</td>
<td>0.342±0.054*</td>
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<tr>
<td>Blood chemistry</td>
<td></td>
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<tr>
<td>pH</td>
<td>7.38±0.01</td>
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<td>7.38±0.02</td>
<td>7.37±0.03</td>
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<tr>
<td>Na⁺, meq/l</td>
<td>141.5±2.12</td>
<td>142.5±2.35</td>
<td>139.63±2.43</td>
<td>140.5±3.87</td>
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<tr>
<td>K⁺, meq/l</td>
<td>4.68±0.62</td>
<td>4.73±0.48</td>
<td>5.58±1.49</td>
<td>5.48±1.45</td>
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<tr>
<td>Cl⁻, meq/l</td>
<td>105.17±1.47</td>
<td>104.33±1.97</td>
<td>102±3.27</td>
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<tr>
<td>HCO₃⁻, meq/l</td>
<td>24.50±2.43</td>
<td>24.33±2.66</td>
<td>25.38±1.65</td>
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<tr>
<td>Anion gap, meq/l</td>
<td>16.52±1.52</td>
<td>18.57±3.40</td>
<td>17.83±2.67</td>
<td>18.48±3.35</td>
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<td>Urinary markers of acid-base intake</td>
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<tr>
<td>SO₄²⁻/creatinine, μmol/mg</td>
<td>29.43±5.31</td>
<td>29.04±4.3</td>
<td>29.55±4.87</td>
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<tr>
<td>SO₄²⁻, μmol/24 h</td>
<td>206.37±37.44</td>
<td>212.6±31.45</td>
<td>233.75±38.56</td>
<td>220.4±35.32</td>
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<td>K⁺/creatinine, meq/mg</td>
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<tr>
<td>K⁺, meq/24 h</td>
<td>1.36±0.32</td>
<td>1.48±0.51</td>
<td>1.65±0.46</td>
<td>1.53±0.38</td>
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</table>

Values are means ± SD. ZDF, Zucker diabetic fatty; TZD, thiazolidinedione. *P < 0.01 vs. untreated lean rats; †P < 0.01 vs. TZD-treated lean rats; ‡P < 0.01 vs. untreated ZDF rats (n = 15 or 16).

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NHE3 antigen and activity in ZDF rats treated with TZD. The Na\(^+\)/H\(^+\) exchanger NHE3 is critical for NH\(_4\)\(^+\) transport in the proximal tubule (24, 33–35), and reduced expression and activity of NHE3 at the proximal tubule brush-border membrane may account at least in part for the decreased urinary NH\(_4\)\(^+\) and pH in ZDF rats (6). We next tested whether the amelioration of lipid abnormalities and urinary acidification defects in ZDF rats treated with TZD is accompanied by a corresponding increase in proximal tubule NHE3 expression and function. Rosiglitazone treatment was previously shown to have no effect on renal cortical NHE3 protein in ZDF rats (40), but this finding does not distinguish between brush-border NHE3, which participates in luminal transport, and intracellular NHE3. In addition, NHE3 activity can change without commensurate changes in protein abundance (8, 31). To determine the effect of TZD on NHE3 function at the lumen of the proximal tubule, we measured NHE3 antigen and Na\(^+\)/H\(^+\) exchange activity in isolated proximal tubule BBMV. TZD treatment was accompanied by a significant increase in brush-border NHE3 protein abundance and activity in ZDF rats, while having no detectable effect in lean rats (Fig. 3).

Reversibility of lipotoxic effects in OKP cells incubated with fatty acids. ZDF rats have a constellation of metabolic abnormalities, and TZD treatment may improve renal acidification and NHE3 function by acting on mechanisms independent of renal lipid content. We used FFA-incubated OKP cells to investigate the effect of intracellular lipid reduction in the absence of other potential confounding factors. OKP cells avidly take up FFA carried on albumin from the culture medium and store them predominantly as triglycerides in intracellular lipid droplets (46). OKP cells also appear to be rather susceptible to lipotoxicity. While a high dose of FFA (1.5 mM) causes irreversible cellular damage by promoting apoptosis, a low dose of FFA (0.75 mM), with no significant effect on apoptosis or baseline NHE3 activity, is sufficient to abolish the insulin stimulation of NHE3 function (6). We examined the reversibility of this phenomenon in lipid-loaded OKP cells maintained in a nutrient-poor medium to promote lipid catabolism, mitochondrial beta-oxidation of intracellular FFA, and hence recovery from lipotoxicity. Compared with cells studied immediately after lipid loading (Fig. 4A), intracellular lipid droplets were markedly reduced in cells allowed to recover for 36 h (Fig. 4B) and the regulation of NHE3 by insulin was partly restored (Fig. 4C). Cells maintained in nutrient-poor medium continued to reduce their intracellular lipid stores and remained viable in culture for up to 6 days, but NHE3 activity and insulin response gradually decreased with prolonged serum and nutrient deprivation in both vehicle- and FFA-incubated cells (not shown). These cell culture artifacts

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**Fig. 1.** Thiazolidinedione (TZD) treatment reduces plasma and renal lipid abnormalities in Zucker diabetic fatty (ZDF) rats. Eight-week-old ZDF rats and lean littermates were treated with TZD for 4 wk. Plasma free fatty acids (FFA, **A**) and renal cortical triglyceride content (**B**) in ZDF and lean rats treated with TZD or control rats (Con) are shown. \(^*\)P < 0.05 by 1-way ANOVA.

**Fig. 2.** Improvement of urinary acidification defects with TZD treatment in ZDF rats. Eight-week-old ZDF rats and lean littermates were treated with TZD for 4 wk. Urinary pH (**A**), urinary NH\(_4\)\(^+\) excretion (**B**), urine titratable acidity (TA; **C**), and urine citrate excretion (**D**) in ZDF and lean rats treated with TZD or control rats are shown. \(^*\)P < 0.05 by 1-way ANOVA.
seen with prolonged serum starvation make these later time points unsuitable for the study of NHE3 regulation.

Effect of rosiglitazone on NHE3 activity in OKP cells. Our findings in lean control rats do not support a direct effect of TZD on urinary acidification in the absence of renal steatosis (Fig. 2). However, the presence of intracellular lipid deposits may alter the transcriptional and metabolic makeup of the proximal tubule, and it is theoretically possible that TZD may have a direct effect on urinary acidification in the altered environment of the steatotic kidney. This effect, if present, would not be related to the reduction of steatosis but rather to TZD, which would drastically alter the interpretation of our data. To examine this possibility, we incubated control and lipid-loaded OKP cells with different concentrations of rosiglitazone for 24 h. Rosiglitazone treatment had no effect on intracellular lipid content in OKP cells, as estimated by Oil Red O staining (not shown). Incubation of OKP cells with either 1/1000M or 10/1000M rosiglitazone had no effect on apical Na+/H+ activity (Fig. 5). Incubation with a suprapharmacological dose of the drug (100/1000M), two orders of magnitude higher than the maximum concentration of total rosiglitazone reached in human plasma after an 8-mg oral dose (14), led to a significant increase in apical Na+/H+ activity, but this increase was not different in control and lipid-loaded cells (Fig. 5).

DISCUSSION

One renal manifestation of the metabolic syndrome in both humans and ZDF rats is an excessively acidic urine, attributed in part to impaired urinary NH4+ excretion (6, 9, 16, 27, 28). Decreased NHE3 expression and activity in the proximal tubule brush border contribute to this defect in ZDF rats, although other factors such as decreased glutamine metabolism due to mitochondrial substrate competition by excess intracellular FFA could also play a role (6). Impaired luminal acid extrusion via NHE3 could also explain the reduced excretion of urinary citrate in ZDF rats, since intracellular acidification in the proximal tubule upregulates both citrate reabsorption and citrate metabolism (1, 29). Hypocitraturia in these animals cannot be attributed to differences in acid or alkali intestinal absorption, given that urinary sulfate and potassium were not different in ZDF and lean rats. These functional abnormalities are accompanied by renal steatosis with predominant tubular localization in ZDF rats and can be reproduced in part in a cell culture model of proximal tubule lipotoxicity, OKP cells incubated with FFA (6). Renal steatosis and lipotoxicity may thus be an important factor in the pathophysiology of renal acidification defects associated with the metabolic syndrome.

Fig. 3. Effect of TZD treatment on brush-border membrane Na+/H+ exchanger 3 (NHE3) antigen and Na+/H+ exchange function in ZDF and lean rats. Eight-week-old ZDF rats and lean littersmates were treated with TZD for 4 wk. A: representative immunoblot. B: summary of immunoblot data. C: summary of Na+/H+ exchange data. *P < 0.05 by 1-way ANOVA.

Fig. 4. Recovery of insulin-stimulated NHE3 activity in opossum kidney (OKP) cells after intracellular lipid reduction. OKP cells were incubated with 0.75 mM nonesterified fatty acids (FFA) or vehicle (albumin) for 12 h, with or without incubation with FFA-free medium for the next 36 h. A and B: Oil Red O staining of lipids after 12 h of incubation with FFA (A) and after incubation of lipid-loaded cells with FFA-free medium for 36 h (recovery; B). C: insulin regulation of apical Na+/H+ exchange activity in cells incubated with vehicle (albumin), FFA, and FFA followed by recovery. Baseline Na+/H+ exchange activity (filled bars) was not significantly different between groups. *P < 0.05 by unpaired Student’s t-test comparing insulin to control in each group (n = 12/group).

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We have shown that TZD treatment in ZDF rats reduces renal steatosis. The antisteatotic action of TZD has been described in humans and animal models of the metabolic syndrome in the liver, heart, and skeletal muscle (4, 22, 38, 47, 52, 57), but to our knowledge the effect of TZD on renal triglyceride content has not been previously examined. Reduction of renal fat with TZD in ZDF rats was accompanied by a shift in urinary NH4 excretion with TZD in ZDF rats was accompanied by a shift in urinary NH4 excretion and reduced Na+/H+ exchange, having as one consequence improved urinary acidification and, in humans, decreased risk for uric acid stone formation.

Renal steatosis and the antisteatotic action of TZD in the kidney are likely to have multiple effects beyond urinary acidification. Several lines of evidence in both humans and animal models have associated TZD treatment with surrogate markers of renal protection in type 2 diabetes and the metabolic syndrome (3, 23, 30, 56), but whether this effect is at least in
part due to decreased steatosis in the kidney requires further exploration.

Importantly, whether humans accumulate fat in the kidney and whether renal steatosis causes damage in a similar fashion are currently unknown. A comprehensive approach to renal steatosis is required to establish its importance in human pathophysiology, including experiments aimed to characterize its correlation with systemic disease and its effects on various aspects of renal function, including but not limited to urinary acidification.

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