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Effect of renal lipid accumulation on proximal tubule Na^+/H^+ exchange and ammonium secretion

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Abstract

Patients with metabolic syndrome have increased risk of uric acid nephrolithiasis due to lower urinary pH and impaired ammonium excretion. The pathophysiology underlying these urinary changes is unknown. We used two animal models and a cell culture model to study whether the alteration in renal acidification is associated with renal fat infiltration (steatosis). Compared with pair-fed lean control rats, Zucker diabetic fatty rats have higher renal triglyceride content, decreased urinary ammonium and pH, and lower levels of brush border membrane Na^+/H^+ exchanger-3 (NHE3), a major mediator of ammonium excretion. High-fat feeding in Sprague-Dawley rats results in transient lowering of urinary ammonium and pH, with all parameters returning to normal when the animals resumed eating normal chow. This is consistent with an absence of diet-induced renal steatosis in these animals. To examine the direct effect of fat accumulation, we incubated opossum kidney (OKP) cells with a mixture of long-chain fatty acids and found accumulation of intracellular lipids with concomitant dose-dependent decrease in NHE3 activity, surface biotin-accessible NHE3 protein, and ammonium secretion. A lower dose of fatty acids that leads to intracellular lipid accumulation but does not change baseline NHE3 is sufficient to abolish the stimulation of NHE3 by insulin and to partially block the stimulation of NHE3 by glucocorticoid hormones; acid regulation of NHE3 in lipid-loaded OKP cells is not affected. These findings suggest that renal steatosis decreases ammonium secretion in the proximal tubule, in part by reducing NHE3 activity and by impairing the regulation of NHE3 by specific agonists.

Keywords

Na^+/H^+ exchanger-3; free fatty acids; lipotoxicity; Zucker diabetic fatty rat; metabolic syndrome

Type 2 diabetes mellitus and the metabolic syndrome have been associated with a higher prevalence of uric acid nephrolithiasis in humans (20,21,48). The principal abnormality underlying the increased risk for uric acid stones in these patients is low urinary pH, which is attributed in part to decreased excretion of the principal high pH urinary open buffer ammonium (NH_4^+) (13,53). Low urinary pH and impaired NH_4^+ excretion have been closely

linked to features of the metabolic syndrome in both stone formers and non-stone formers (1,37–39,48).

The bulk of NH_4^+ excreted by the kidney is produced in the proximal tubule cell through the mitochondrial metabolism of glutamine and glutamate (47). NH_4^+ in the final urine is derived from the end proximal tubular luminal NH_4^+ minus the amount that is returned to the systemic circulation on transit through the nephron (32,47). NH_4^+ transport across the luminal membrane of the proximal tubular cell can occur directly as NH_4^+ or as nonionic diffusion of NH_3 coupled with luminal trapping by secreted H^+ . The Na^+/H^+ exchanger NHE3 is critical for the transport of NH_4^+ in the proximal tubule both by functioning as a $\text{Na}^+/\text{NH}_4^+$ exchanger and by providing the luminal H^+ required for trapping of the diffused NH_3 in the lumen (9,11,30,45–47). Additional support for the role of NHE3 in NH_4^+ excretion is the fact that when the acid-induced increase in NHE3 activity was blocked by experimental adrenal insufficiency, the acid-induced increase in NH_4^+ excretion was much reduced (29).

In obesity, diabetes, and the metabolic syndrome, the balance among lipid uptake, synthesis, and utilization in nonadipose tissues may become disrupted, leading to intracellular lipid accumulation, cellular dysfunction, and injury (35,54,60,66). This process has been termed lipotoxicity and has been described in multiple tissues including the heart, liver, endocrine pancreas, and skeletal muscle (54,60,62,66). Lipotoxic cellular dysfunction and injury result from accumulation of nonesterified (free) fatty acids (FFA) and their toxic metabolites such as fatty acyl-CoA, diacylglycerol, and ceramide (54,60,66). Lipotoxicity is marked by intracellular accumulation of triglycerides (steatosis). Although triglycerides are not considered toxic per se, they are a reservoir of FFA and a source of toxic metabolites, and hence a measurable indicator of tissue lipotoxicity (33,54,66).

Renal lipotoxicity and its role in the pathogenesis of renal disease are not fully understood (4,64,66). In obesity and the metabolic syndrome, renal lipotoxicity can result from excess delivery of circulating FFA and triglycerides to the kidney (64). The proximal tubule may be particularly vulnerable to lipid accumulation due to its role in the reabsorption of FFA-bearing albumin (25,52).

We hypothesize that steatosis and lipotoxicity in the kidney may impair renal ammoniogenesis and transport, or may interfere with the ability of agonists to stimulate these processes. To test this hypothesis, we examined renal fat accumulation, urinary biochemical abnormalities and the proximal tubular NHE3 in Zucker diabetic fatty (ZDF) rats, an established animal model of obesity and obesity-initiated metabolic syndrome (17). ZDF rats have been previously shown to accumulate excess lipids in other tissues including skeletal muscle (55), heart (69), liver (55), and pancreatic islets (35). We also examined urinary acidification and renal fat in an animal model of diet-induced weight gain, Sprague-Dawley rats fed a high-fat diet. The direct effect of fat accumulation on NHE3 activity, NHE3 regulation by agonists, and NH_4^+ secretion was further studied in opossum kidney (OKP) cells, a cell culture model of the proximal tubule (18).

EXPERIMENTAL PROCEDURES

Materials and supplies

All chemicals were obtained from Sigma (St. Louis, MO), except where otherwise noted, and except for the following: cell culture media and BCECF- acetoxymethyl ester (AM; Invitrogen, Carlsbad, CA); penicillin and streptomycin (Cambrex, East Rutherford, NJ); EZ-Link sulfo-NHS-SS-biotin and immunopure immobilized streptavidin (Pierce, Rockford,

IL); horseradish peroxidase-labeled anti-mouse IgG and enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ); and 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. All animal experiments were performed in accordance with institutional guidelines and with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, 1996). Sixteen Zucker ZDF (*fa/fa*) rats and lean wild-type (+/+) 12-wk-old male rats were a gift from Dr. Roger Unger (Touchstone Center for Diabetes Research, UT Southwestern Medical Center). ZDF rats were fed standard chow (Harlan Teklad, Madison, WI) ad libitum and had free access to water. Sixteen 4-wk-old male Sprague-Dawley rats purchased from Charles River Laboratories (Wilmington, MA) were randomly assigned to a high-fat hypercaloric diet (5.1 Kcal/g, containing 18.4% Kcal from protein, 21.3% Kcal from carbohydrate, and 60.3% Kcal from fat) or the corresponding low-fat control diet (3.7 Kcal/g, containing 20.1% Kcal from protein, 69.8% Kcal from carbohydrate, and 10.2% Kcal fat from fat, Harlan Teklad). After 12 wk on the experimental diets, the rats were switched to standard rodent chow for 1 wk before death to exclude the transient confounding effects that dietary differences may have on urinary acidification parameters. To examine the transient effects of high-fat feeding on kidney lipid content and blood chemistry, an additional eight Sprague-Dawley rats were studied after 1 wk of feeding with the experimental diets, without a switch to normal rodent chow before death. For urine collections, rats were pair-fed, then transferred to metabolic cages, and 24-h urine samples were collected under mineral oil with added thymol crystals. Animals were killed under anesthesia with ketamine-xylazine-acepromazine (100, 10, and 1 mg/kg ip). Blood was collected by cardiac puncture. Kidneys were dissected on ice, and cortical samples were frozen in liquid nitrogen. Transport experiments were always performed with fresh tissue immediately after death.

Urinary biochemical analysis

Urinary ammonium (glutamate dehydrogenase assay, Diagnostic Chemicals, Oxford, CT), creatinine (kinetic alkaline picrate method), and plasma nonesterified fatty acids (Wako Chemicals USA, Richmond, VA) were measured using a Cobas Mira Plus Chemistry Autoanalyzer (Roche Diagnostics, Basel, Switzerland). Titratable acidity was determined by titrating the urine samples collected under oil to pH 7.4 with 0.1 N sodium hydroxide using a PHM82 pH meter, TTT80 titrator, and ABU80 autoburette (all from Radiometer, Copenhagen, Denmark).

Tissue triglyceride measurement and lipid staining

Kidney cortical tissue was dissected, frozen in liquid nitrogen, homogenized on ice with a Polytron (Brinkmann Instruments, Westbury, NY) in isolation buffer (300 mM mannitol, 18 mM HEPES, 5 mM EGTA, pH 7.5), and lipids were extracted according to the method of Folch et al. (23). Total triglycerides were measured using a triglyceride determination kit (Sigma, St. Louis, MO) according to the method of Danno et al. (19). For lipid staining, 4- μ m frozen kidney sections were fixed with 4% paraformaldehyde, rinsed with distilled water, briefly rinsed with 60% isopropanol, and stained with oil red O for 60 min. The sections were then subjected to standard hematoxylin staining, mounted on glass slides, and visualized with a Zeiss LSM510 microscope.

Brush border membrane vesicle preparation

Renal cortical brush border membrane vesicles (BBMV) were prepared by the Mg^{2+} aggregation method (12) with modifications as described previously (44). Briefly, fresh dissected kidney cortex was homogenized with a Polytron as above in ice-cold isolation buffer containing fresh protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml pepstatin A, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin). Crude membranes were pelleted at 48,000 g for 60 min at 2°C (Beckman J2-21M, JA-20 rotor; Beckman Coulter, Fullerton, CA), resuspended, homogenized with a Dounce all-glass tissue homogenizer, and then exposed to three consecutive Mg^{2+} aggregation steps (15 mM $MgCl_2$ final concentration at 4°C for 20 min each time). After each step, the aggregated membranes were removed by centrifugation at 3,000 g for 10 min, at 2°C (Beckman Allegra 21R; Beckman Coulter). BBMV were recovered from the final supernatant by centrifugation at 48,000 g for 30 min, at 2°C. BBMV were loaded with the appropriate intravesicular solution by homogenization with a Potter-Elvehjem teflon-glass homogenizer, equilibrated for 2 h at 4°C, repelleted as above, and resuspended using a 27-gauge syringe needle to a final protein concentration of 10 mg/ml.

NHE3 antigen in the kidney

Immunoblotting was performed as described previously (10). Briefly, rat kidney cortex crude membranes or 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 (1 complete mini EDTA-free protease inhibitor cocktail tablet/10 ml, Roche Applied Science, Indianapolis, IN), cleared by centrifugation (14,000 g, 4°C, 30 min), and protein content was determined by the method of Bradford. Identical amounts of protein were heated for 2 min at 95°C in loading buffer, size-fractionated by SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes. The primary antibodies used were 3H3 monoclonal mouse anti-opossum NHE3, 1:1,000 dilution (10) or no. 1568 polyclonal rabbit anti-rat antiserum raised against the epitope DSFLQADGP EEQLQ, 1:500 dilution (2). Membranes were blocked in nonfat milk, probed overnight at 4°C with the appropriate primary antibody, washed (5 × 10 min in 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. Membranes were also probed for β -actin to confirm equivalent loading (not shown). Briefly, air-dried membranes were reactivated in methanol and stripped (30 min. at 50°C in 0.125 M Tris·HCl, pH 6.7 with 4% SDS and 0.016% β -mercaptoethanol), blocked as above, probed with a monoclonal anti- β -actin antibody (Sigma) overnight at 4°C, and visualized using a horseradish peroxidase-labeled secondary antibody and enhanced chemiluminescence as above. Protein abundances were quantified by densitometry using Scion/NIH Image software (Scion, Frederick, MD).

Na^+/H^+ exchange activity in BBMV

Na^+/H^+ exchange activity was measured in fresh BBMV using the acridine orange fluorescence quenching method (65) with modifications as described previously (44). BBMV were loaded with an intravesicular solution (280 mM mannitol, 5 mM MES, pH 5.5) as described above. The extravesicular solution [120 mM *N*-methyl-D-glucamine (NMDG)-gluconate, 20 mM HEPES, pH 7.5] with 6 μ M acridine orange (Invitrogen) was loaded in a cuvette containing a micromagnetic stir-bar. Acridine orange fluorescence was followed in a computer-controlled spectrofluorometer ($\lambda_{excitation} = 493$ nm, $\lambda_{emission} = 530$ nm; QM-8/2003, Photon Technology International, London, Ontario). Addition of the acid-loaded BBMV (a volume containing 150 μ g total protein) under constant magnetic stirring caused rapid fluorescence quenching. Na^+/H^+ exchange activity was then assayed as the initial rate of increase in fluorescence in response to addition of Na^+ to the extravesicular

space (1 M Na⁺-gluconate added to a final concentration of 30 mM). Replacement of Na⁺-gluconate with NMDG-gluconate provided the Na⁺-independent quenching. Comparisons were always made between BBMV preparations from rats killed on the same day.

Cell culture, incubation with fatty acids, and apoptosis

OKP cells (18) cultured at 37°C in a humidified 95% air-5% CO₂ atmosphere were maintained in high-glucose (450 mg/dl) DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100/g/ml). Confluent cells were rendered quiescent by incubation in serum-free medium for 48 h. For lipotoxicity studies, OKP cells were incubated with a mixture of oleate/palmitate (2:1 molar ratio) or vehicle (albumin) for 24 h before the experiments. This model of cellular lipotoxicity has been described previously (41). The fatty acid mixture was prepared as a 12.7 mM oleate and 6.35 mM palmitate stock solution complexed with 1.8 mM fatty acid-free bovine serum albumin and was added to the culture media at a final concentration of either 1.5 or 0.75 mM total fatty acids. For insulin treatment, insulin was added at a final concentration of 10⁻⁶ M in the culture media. For glucocorticoid treatment, dexamethasone 10⁻⁶ M (10⁻³ M stock solution in ethanol) or vehicle was added to the cell culture media for 3 h before the assays. For acid treatment, OKP cells were incubated for 24 h in serum-free medium acidified to pH 7.0 with HCl. Apoptosis was first assessed by DNA laddering using an Apoptotic DNA-Ladder kit (Roche Applied Science). For quantification of apoptosis, cells were labeled using a terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) dual-color fluorescence assay kit (Apo-BrdU Apoptosis Detection, eBioscience, San Diego, CA) according to the manufacturer's instructions and analyzed on a multiparameter flow cytometer (Becton Dickinson, San Jose, CA).

Cell surface NHE3 antigen in OKP cells

Apical membrane proteins were biotin labeled and affinity purified with streptavidin-agarose as described (10). Briefly, 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 labeling, cells were washed twice with 6 ml of quenching buffer (1 mM MgCl₂, 0.1 mM CaCl₂, 100 mM glycine in PBS, pH 7.4, for 20 min, 4°C) and lysed in RIPA buffer with protease inhibitors as above. Lysates were cleared by centrifugation, and the supernatants were diluted to 2.5 mg/ml of protein with RIPA buffer. Cell lysates of equivalent amount of protein were equilibrated overnight with streptavidin-agarose beads at 4°C. Beads were washed sequentially with solutions A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA), B (50 mM Tris-HCl, pH 7.4, 500 mM NaCl), and C (50 mM Tris-HCl, pH 7.4). Biotinylated proteins were released from beads by heating to 95°C with 2.5× loading buffer and then subjected to immunoblotting as above.

Na⁺/h⁺ exchange activity in OKP cells

NHE3 activity in OKP cells was measured fluorimetrically using the intracellularly trapped pH-sensitive dye BCECF as described previously (43). Briefly, cells grown on glass coverslips were loaded with 10 μM BCECF-AM (30 min at 37°C) and intracellular pH (pH_i) was estimated from the ratio of fluorescence in a computer-controlled spectrofluorometer (λ_{excitation} = 500 and 450 nm, λ_{emission} = 530 nm). The 500/450-nm fluorescence ratio was calibrated to pH_i using K⁺/nigericin. Na⁺/H⁺ exchange activity was assayed as the initial rate of the Na⁺-dependent pH_i increase after an acid load using nigericin in the absence of CO₂/HCO₃⁻, and results are reported as dpH_i/dt. Intracellular buffer capacity (β) was measured by pulsing cells with 20 mM NH₄Cl, according to the formula β = [NH₄Cl]/ΔpH_i.

Intracellular buffer capacity for control and treated cells was not significantly different (not shown). Comparisons were always made between cells of the same passage studied on the same day.

NH₄⁺ production in OKP cells

Confluent OKP cells monolayers were incubated with a mixture of oleate/palmitate (2:1 molar ratio) or vehicle (albumin) for 24 h, followed by serum-free medium for 24 h. Cells were then incubated with pregassed, prewarmed Krebs-Ringer bicarbonate buffer [(in mM) 115 NaCl, 24 NaHCO₃, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 10 HEPES, pH 7.4] with 1 mM glutamine for 30 min. At the end of each experiment, the medium was removed for ammonium assay, and the cells were lysed as above for protein measurement.

Insulin receptor and serum- and glucocorticoid-inducible kinase 1 phosphorylation

Confluent OKP cells were treated with vehicle or insulin for 30 min and lysed with RIPA buffer containing phosphatase and protease inhibitors. Immunoblotting was performed as above. The antibodies used were rabbit anti-human insulin receptor β /IGF1R (phospho Y1158, phospho Y1162, and phospho Y1163; Abcam, Cambridge, MA), mouse anti-human insulin receptor β (BD Biosciences, Franklin Lakes, NJ), rabbit anti-human insulin receptor and serum- and glucocorticoid-inducible kinase 1 (SGK1; Genex Bioscience, Hayward, CA), and rabbit anti-human SGK1 (phospho S78; Cell Signaling, Beverly, MA).

SGK1 kinase activity

Triple-Flag-tagged SGK1 in pCMV 7.1 and purified GST-Nedd4-2 were generous gifts from Drs. Melanie Cobb and Bing-E Xu (UT Southwestern Medical Center). OKP cells were transiently transfected with the triple-Flag-tagged SGK1 construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Confluent cells were serum deprived for 48 h, treated with vehicle or insulin for 30 min, lysed as above, and an in vitro SGK1 kinase assay was performed as described (68). Briefly, Flag-SGK1 was immunoprecipitated using a monoclonal anti-Flag antibody (Sigma) and protein A-Sepharose (Amersham Biosciences, Piscataway, NJ). Reactions containing 10 mM HEPES (pH 7.5), 50 μ M ATP (10–50 cpm/fmol, where cpm is counts/min), 10 mM MgCl₂, 1 mM DTT, 1 mM benzamidine, and 1 μ g of the SGK1-specific substrate GST-Nedd4-2 were incubated for 1 h at 30°C. Assays were terminated by addition of electrophoresis sample buffer and resolved by electrophoresis on 10% polyacrylamide gels. Gels were dried on filter paper and exposed to film overnight. GST-Nedd4-2 phosphorylation by immunoprecipitated SGK1 was quantitated by densitometry as above.

Statistics

All results are reported as means $\pm$ SD. Statistical analysis was performed using ANOVA and Student's *t*-test.

RESULTS

Lipid and urinary abnormalities in ZDF rats

We studied ZDF rats as an established animal model of metabolic syndrome and multiple organ lipotoxicity (35,55,69). The average body weight for ZDF rats before death was 378 ± 11 g, compared with 256 ± 14 g for the lean control animals ($P < 0.001$). Average decapsulated kidney weight was 1.65 ± 0.1 g in ZDF rats and 1.11 ± 0.08 g in control rats ($P < 0.001$); of note, average kidney weight-to-body weight ratio was similar for both groups. Plasma FFA were significantly elevated in ZDF rats (Fig. 1A). ZDF rats accumulate fat in the renal cortex, as shown quantitatively by enzymatic triglyceride measurement (Fig. 1B)

and qualitatively by oil red O staining (Fig. 1C). Accumulation of intrarenal lipid in ZDF rats was detected mostly in renal tubular cells. There was no detectable lipid staining in the kidney sections of lean control animals (not shown). Compared with their lean littermates, ZDF rats have lower urinary pH (Fig. 2A), lower excretion of NH_4^+ (Fig. 2B), and higher titratable acidity (TA) (Fig. 2C).

NHE3 antigen and activity in ZDF rats

The Na^+/H^+ exchanger NHE3 is critical for the transport of NH_4^+ in the proximal tubule (30,45–47). We examined whether renal fat accumulation and urinary abnormalities in ZDF rats are accompanied by a decrease in NHE3 function. Previous studies have reported a decrease in total renal cortical NHE3 protein in ZDF rats (7,51). However, total cortical NHE3 is a measure of both brush border and intracellular NHE3 protein, and NHE3 activity can change independently of changes in protein abundance (42). To accurately assess proximal tubular NHE3 function, we isolated kidney BBMVs from ZDF and lean rats and measured brush border NHE3 protein and Na^+/H^+ exchange activity. The amount of NHE3 antigen in brush border membrane was decreased in ZDF rats (Fig. 3A). Correspondingly, Na^+/H^+ exchange activity measured in renal cortical BBMVs was significantly reduced in ZDF rats compared with their lean littermates (Fig. 3B).

Dietary effects in Sprague-Dawley rats

We used Sprague-Dawley rats fed a high-fat diet to study whether diet-induced weight gain results in renal fat accumulation and urinary acidification defects. After 12 wk on the experimental diets, the average body weight of rats fed a high-fat diet was 415 ± 15 g compared with 384 ± 28 g for the control animals fed a low-fat diet ($P < 0.05$). Rats fed a high-fat diet had lower urinary pH and NH_4^+ excretion compared with control animals, but these differences did not persist when both groups were switched back to a pair-fed standard rodent diet for 1 wk (Fig. 4, A and B). In a separate group of animals studied after 1 wk of feeding with the high- vs. low-fat diets, without a switch to normal rodent chow before death, significantly higher serum FFA levels were noted in the high fat-fed rats (Fig. 4C, left). This difference did not persist in rats switched to a standard rodent diet for 1 wk (Fig. 4C, right). There was no significant difference between high fat- and low fat-fed rats in basic blood chemistry (including fasting serum glucose and electrolytes), kidney weight, renal cortical triglyceride content, and NHE3 protein, both when animals were studied during feeding with the experimental diets and when they were switched to the same standard diet for 1 wk (not shown).

NHE3 antigen, NHE3 activity, and NH_4^+ secretion in OKP cells incubated with fatty acids

We used an in vitro renal cell culture system to examine the causal relationship between fat accumulation and decreased NHE3 protein abundance and activity. OKP proximal tubular cells were incubated directly with a mixture of FFA as described in *Experimental Procedures*. It has previously been shown that OKP cells readily take up FFA and accumulate them intracellularly, primarily as triglycerides (59). Figure 5A shows intracellular lipid accumulation in OKP cells incubated with 0.75 or 1.5 mM FFA. Incubation of OKP cells with 1.5 mM FFA, but not with 0.75 mM FFA, results in a significant decrease in apical membrane NHE3 protein (Fig. 5B), apical Na^+/H^+ exchange activity (Fig. 5C), and NH_4^+ secretion (Fig. 5D). These abnormalities are accompanied by increased apoptosis in cells incubated with the higher dose of FFA, as evidenced by increased DNA laddering (not shown) and by flow cytometric quantitation of apoptotic OKP cells labeled using a dual-color fluorescent TUNEL assay (Fig. 5E).

Regulation of NHE3 in OKP cells incubated with fatty acids

Incubation of OKP cells with a low dose of FFA (0.75 mM) leads to fat accumulation but does not affect NHE3 protein expression or activity. We tested whether at this state NHE3 activity is regulated by agonists previously shown to stimulate NHE3 in OKP cells: Insulin (24,31), glucocorticoids (6,10), and acid (3). Since the acute and chronic regulation of NHE3 by insulin have been shown to involve distinct signaling pathways (24), we examined insulin action on NHE3 after 2 (acute) and 24 h (chronic). As shown in Fig. 6, both the acute and chronic effects of insulin on NHE3 activity are completely abolished by 0.75 mM FFA, and the effect of glucocorticoid (dexamethasone) is blunted by incubation with FFA. The stimulatory effect of acid, which occurs through distinct mechanisms (36,43), is not affected by incubation with FFA. Consistent with the intact response to acid was the finding that acute gastric gavage with NH_4Cl (4 mmol/kg body wt) triggered comparable escalation of renal NH_4^+ excretion and induction of phosphoenolpyruvate carboxykinase transcript in renal cortex in ZDF and lean control rats (not shown).

Insulin signaling in OKP cells incubated with fatty acids

Incubation of OKP cells with FFA completely abolishes the stimulatory effect of insulin on NHE3. We sought to identify whether this defect is due to disruption of the known insulin signaling cascade in OKP cells (24). We examined the most proximal (insulin receptor phosphorylation) and most distal (SGK1 phosphorylation and activation) known signaling events in cells incubated with 0.75 mM FFA or vehicle. As shown in Fig. 7A, both insulin receptor expression and phosphorylation in response to insulin were not affected by incubation with FFA. SGK1 baseline expression and the phosphorylation and activation of SGK1 in response to insulin were also not different between lipid-loaded and control cells (Fig. 7B).

DISCUSSION

We have shown that ZDF rats, an established animal model of obesity and obesity-related metabolic syndrome, have significant accumulation of triglycerides in the kidney. Accumulation of fat in nonadipose tissues of ZDF rats has previously been shown in skeletal muscle (55), heart (69), liver (55), and pancreatic islets (35), but little has been known about fat accumulation in the kidney. Renal steatosis in the ZDF rat is unlikely to be an isolated defect due to leptin receptor deficiency, since fat accumulation has also been demonstrated in the kidneys of wild-type mice with diet-induced obesity (27,28,58). However, species and strain differences in the susceptibility of the kidney to fat accumulation may exist, and we have shown that high fat feeding in Sprague-Dawley rats for 12 wk results in modest weight gain but no renal steatosis. The significance of these findings for human pathophysiology requires further studies, and whether humans with obesity and metabolic syndrome accumulate fat in the kidney remains to be established.

Renal steatosis in ZDF rats is associated with persistently low urinary pH, low urinary NH_4^+ , and high titratable acidity. These features closely resemble the urinary biochemical profile of patients with the metabolic syndrome, with or without uric acid nephrolithiasis (13,14,37,48,49). In contrast, Sprague-Dawley rats fed a high-fat diet are resistant to renal fat accumulation, and the effects of high-fat feeding on urinary acidification do not persist beyond the period of high-fat feeding. This finding suggests that aciduria in this model may be dietary in origin rather than an alteration in the acidification function. One potential cause may be elevated plasma FFA in Sprague-Dawley rats during high-fat feeding, which may transiently impair renal ammoniagenesis by competing with glutamine as an oxidizable substrate in the proximal tubule (5,63).

The pathophysiology of impaired NH_4^+ excretion in the ZDF rat is likely multifactorial. Although substrate competition by FFA may also play a role in this model, decreased urinary excretion of NH_4^+ could be attributed in part to reduced brush border NHE3 protein and activity, which impairs both direct NH_4^+ transport and ionic trapping of NH_3 in the proximal tubule (30,45–47). The predominant tubular localization of renal fat in the ZDF rat is compatible with the hypothesis that renal fat accumulation and tubular lipotoxicity result in reduced NHE3 surface protein and function. We tested this hypothesis in a renal cell culture model.

Exposure of cultured OKP cells to FFA carried on albumin leads to a dose-dependent decrease in apical membrane NHE3 protein and activity, at least in part due to lipotoxicity and lipoapoptosis. In both humans and animals, FFA are key mediators of lipotoxicity (22,56,60,66). Previous studies have shown that circulating FFA are increased in both ZDF rats (35) and obese diabetic humans (50,61) and that the human kidney is an important site of FFA disposal (40). Since most of the plasma FFA are carried on albumin, the proximal tubule cell may be the renal cell type most exposed to FFA overload, owing to its critical role in albumin reabsorption (8,25,52,59,66). Our findings in OKP cells substantiate the causative role of fat accumulation in decreasing renal NHE3 protein and function.

Furthermore, exposure of OKP cells to low levels of FFA, which has no effect on baseline NHE3 expression and activity, abolishes the regulation of NHE3 by insulin. In isolated tubules, kidney slices, and cultured proximal tubule cells, insulin enhances both NH_4^+ secretion (16,34) and the transport activity of NHE3 (24,31). Insulin resistance induced by FFA or FFA metabolites has been previously described in insulin-responsive tissues both in vitro and in vivo, including in human skeletal muscle (15,54,56,57). Our findings in OKP cells suggest that excess FFA delivery may also lead to insulin resistance in the renal proximal tubule, manifested as reduced insulin-stimulated NHE3 activity. Incubation of renal cells with FFA also reduces the ability of glucocorticoid hormones to enhance NHE3 activity. Glucocorticoids stimulate NH_4^+ excretion (26,67), have a permissive effect on insulin stimulation of NHE3 (31), and are important modulators of renal acidification (10). A diminished glucocorticoid effect on NHE3 may also play a role in impaired NH_4^+ excretion in vivo. These effects are agonist specific, as acid stimulation of NHE3 is intact in FFA-incubated cells.

Decreased in vitro production of NH_4^+ by OKP cells incubated with FFA correlates with decreased abundance and activity of NHE3 and is compatible with our in vivo experiments in ZDF rats. Similar to ZDF rats, both NH_4^+ synthesis and transport may be affected in OKP cells, as FFA may act by impairing NHE3 function as well as by competing with glutamine as an oxidizable substrate (5,63). Importantly, while reduced NH_4^+ secretion via NHE3 is likely due to fat accumulation and lipotoxicity (as shown by our experiments in ZDF rats and OKP cells), reduced NH_4^+ synthesis due to substrate competition is a distinct phenomenon which may occur in the absence of lipotoxicity (as shown by our experiments in Sprague-Dawley rats).

Whether these findings in animals and cell culture mirror human pathophysiology requires further investigation. Impaired NH_4^+ excretion in patients with obesity and type 2 diabetes is likely multifactorial and is also not sufficient to explain the entire spectrum of urinary abnormalities found in these patients (13). The link between the metabolic syndrome, low urinary pH, and uric acid nephrolithiasis may ultimately be contingent on a complex pathophysiology, with impaired NH_4^+ excretion being just one element of multiple defects.

We propose that renal lipotoxicity is an integral part of this complex pathophysiology, by causing impaired Na^+/H^+ exchange and NH_4^+ secretion in the proximal tubule.

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Fig. 1.

Plasma and renal lipid abnormalities in the Zucker diabetic fatty (ZDF) rat model. Plasma free fatty acids (FFA; *A*) and renal cortical triglyceride content (*B*) in ZDF rats compared with lean control littermates are shown. * $P < 0.001$ ($n = 8/\text{group}$). *C*: representative oil red O lipid staining of ZDF rat kidney cortex (magnification $\times 40$).

Fig. 2.

Urinary acidification defects in ZDF rats. Urinary pH (A), urinary NH₄⁺ excretion (B), and urine titratable acidity (TA; C) in ZDF rats compared with lean control littermates are shown. * $P < 0.05$ ($n = 8/\text{group}$).

Fig. 3. Reduced proximal tubule brush border Na^+/H^+ exchanger (NHE3) antigen and function in ZDF rats. *A*: representative immunoblots from 6 ZDF and 6 lean rats and summary of data. *B*: Na^+/H^+ exchange activity in brush border membrane vesicles of ZDF rats vs. lean littermates. * $P < 0.001$ ($n = 6/\text{group}$).

Fig. 4.

Dietary effects in Sprague-Dawley rats fed a high-fat diet. Urinary pH (A) and urinary NH_4^+ excretion (B) measured after 12 wk on the research diets (low fat and high fat) and after 1 additional week on standard rodent chow are shown. * $P < 0.01$ ($n = 8/\text{group}$). C: plasma FFA in rats studied during high- and low-fat feeding ($n = 4/\text{group}$) and in high fat- and low fat-fed rats switched to standard rodent diet for 1 wk ($n = 8/\text{group}$). * $P < 0.05$.

Fig. 5.

Fat accumulation results in reduced NHE3 activity, NHE3 function, and NH_4^+ production in opossum kidney (OKP) cells. *A*: oil red O staining of lipids. *B*: representative immunoblot and summary of data for surface NHE3 protein. *C*: NHE3 activity. *D*: NH_4^+ production; $*P < 0.001$ ($n = 12/\text{group}$). *E*: apoptosis measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and flow cytometry. $*P < 0.01$ ($n = 3/\text{group}$).

Fig. 6. Defective NHE3 regulation in OKP cells incubated with FFA. Effect of insulin (acute and chronic; Ins), dexamethasone (Dex), or acid on NHE3 activity in cells incubated with vehicle or 0.75 mM FFA is shown. * $P < 0.05$ ($n = 9$ /group).

Fig. 7. Insulin signaling in OKP cells incubated with FFA. *A*: representative immunoblots of phospho-insulin receptor- β and insulin receptor- β in cells incubated with vehicle or 0.75 mM FFA and treated with insulin or control (Con). *B*: representative immunoblots of phospho-serum- and glucocorticoid-inducible kinase 1 (SGK1) and SGK1 and representative autoradiography of in vitro phosphorylation of SGK1 substrate.