Novel mechanisms of Na\textsuperscript{+} retention in obesity: phosphorylation of NKCC2 and regulation of SPAK/OSR1 by AMPK

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Davies M, Fraser SA, Galic S, Choy S, Katerelos M, Gleich K, Kemp BE, Mount PF, Power DA. Novel mechanisms of Na\textsuperscript{+} retention in obesity: phosphorylation of NKCC2 and regulation of SPAK/OSR1 by AMPK. Am J Physiol Renal Physiol 307: F96–F106, 2014. First published May 7, 2014; doi:10.1152/ajprenal.00524.2013.—Enhanced tubular Na\textsuperscript{+} reabsorption of salt is important in the pathogenesis of obesity-related hypertension, but the mechanisms remain poorly defined. To identify changes in the regulation of salt transporters in the kidney, we examined Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter (NKCC2) and Na\textsuperscript{+}-Cl\textsuperscript{-}cotransporter (NCC) expression, mechanisms regulating the activity of these transporters, and cytoskeletal interactions. The STE-20-type kinases STE-20/SPAK and SPS1-related proline-alanine-rich protein kinase (SPAK) are key effectors in the regulation of these transporters; however, their contributions to Na\textsuperscript{+} retention in obesity are poorly understood.

Studies examining the relationship between expression of renal Na\textsuperscript{+} transporters and obesity-related Na\textsuperscript{+} retention have produced inconsistent results (1, 20, 28, 38–40). Overall, increased Na\textsuperscript{+} transporter expression does not appear to adequately explain obesity-related Na\textsuperscript{+} retention. This suggests that increased activity of Na\textsuperscript{+} transporters in obesity occurs by mechanisms other than changes in protein expression. Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter (NKCC2) and Na\textsuperscript{+}-Cl\textsuperscript{-} cotransporter (NCC) are members of the cation Cl\textsuperscript{-} cotransporter family. NKCC2 is found in the loop of Henle, and NCC is found in the distal convoluted tubule. They are responsible for the reabsorption of ~20% and 5–10% of filtered Na\textsuperscript{+}, respectively, under basal conditions (12). In addition to protein expression, mechanisms regulating the activity of these transporters include phosphorylation, membrane trafficking, and cytoskeletal interactions. The STE-20-type kinases STE-20/SPAK and SPS1-related proline-alanine-rich protein kinase (SPAK) and oxidative stress responsive 1 kinase (OSR1) interact with a conserved RXF[V/I] motif present on the intracellular NH2 termini of NKCC2 and NCC (42, 43). These kinases phosphorylate the cotransporters on conserved residues to cause activation. Activating phosphorylation of T101-NKCC2 and T58-NCC (murine sequences, equivalent to T105-NKCC2 and T60-NCC) have been identified as the most important SPAK/OSR1 phosphosites (42, 43). NKCC2 is also activated by phosphorylation at S126 (murine sequence, equivalent to S130 in human NKCC2), which is not a SPAK/OSR1 site (43). This site can be phosphorylated by AMP-activated protein kinase (AMPK) (11), but other kinases may also be involved (43). A recent study (21) has reported enhanced phosphorylation of NCC by SPAK/OSR1, predominant membrane localization of both total and phosphorylated (p)NCC, and an altered SPAK profile in 12-wk-old obese Zucker (ZO) rats. To date, no studies have examined changes in the phosphorylation state of NKCC2 in obesity. AMPK activity has been found to be reduced in the renal cortex of rats with high-fat diet (HFD)-

LARGE OBSERVATIONAL STUDIES have identified obesity as a major risk factor for the development of hypertension (19, 36). Along with animal models of obesity (7, 23, 44), studies of weight loss in humans have supported a causative role of obesity in elevating blood pressure (34). The mechanisms leading to obesity-related hypertension are multifactorial, including activation of sympathetic nervous and renin-angiotensin systems, alterations in adipokine profiles, insulin resistance, and endothelial dysfunction (for a review, see Ref. 8). Volume expansion through renal Na\textsuperscript{+} retention has been identified as a major contributing factor and occurs as a common downstream consequence of dysregulation of the aforementioned pathways. Human and animal studies have demonstrated that obesity leads to increased renal blood flow and an increase in glomerular filtration rate, indicating that renal Na\textsuperscript{+} retention must occur as a result of enhanced tubular reabsorption (5, 16, 41). However, the mechanisms leading to increased tubular Na\textsuperscript{+} reabsorption are poorly understood.

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induced obesity (6), but the effect of obesity on renal SPAK and OSR1 activity is unknown.

The epithelial Na$^+$ channel (ENaC) is found in the collecting duct and is responsible for the reabsorption of ~3% of filtered Na$. Regulation of ENaC activity can occur through hormones such as aldosterone and insulin altering expression levels and membrane trafficking (3). Phosphorylation of ENaC may also be important, but the relevant phosphosites have not yet been clearly identified (29).

Studies examining the effects of obesity on the abundance of renal Na$^+$ transporters have predominantly used lean Zucker and ZO rats (1, 20, 21, 28, 38, 39). ZO rats have a mutation of the leptin receptor and profound hyperinsulinemia. Leptin and insulin affect Na$^+$ excretion (2, 52) and thus potentially affect the analysis of Na$^+$ transporters.

Several studies have found that C57BL/6 mice develop obesity-related hypertension after high fat feeding (35–60% energy from fat) from as early as 8 wk (37, 45, 54) as well as impaired renal salt handling and salt sensitivity (7). C57BL/6 mice fed a HFD develop progressive insulin resistance and membrane localization of NKCC2 and NCC as obesity in C57BL/6 mice to examine the expression, phosphor-

bolic syndrome in humans (24).

Hyperlipidemia, hyperleptinemia, and hypoadiponectinemia in ZO rats (24, 55). Administration of a HFD also produces approximately twofold increase in plasma insulin, which is much more akin to the changes found in human obesity than those seen in ZO rats (24, 55). Administration of a HFD also produces hyperlipidemia, hyperleptinemia, and hypoadiponec tinemia in C57BL/6 mice, thereby mimicking the changes seen with metabolic syndrome in humans (24).

In the present study, we used a model of HFD-induced obesity in C57BL/6 mice to examine the expression, phosphorylation, and membrane localization of NKCC2 and NCC as well as the expression and membrane localization of ENaC. In addition, the effect of obesity on the kinases SPAK/OSR1 and AMPK, which are known to be involved in the regulation of NKCC2 and NCC, was also studied.

MATERIALS AND METHODS

Antibodies. Rabbit antibodies directed against pNKCC1 (T212/217)/pNKCC2 (T96/T101), pNCCK (S126), and pNCN (T58) have previously been described (10). Rabbit antibodies against AMPK-$\alpha$ and pACC1 (S79) have previously been described (33, 50). Mouse antibody against NKCC1/NKCC2 (T4) was obtained from the Developmental Studies Hybridaoma Bank (University of Iowa). Rabbit antibodies against NKCC2, $\alpha$-ENaC, $\beta$-ENaC, and $\gamma$-ENaC were obtained from StressMarq Biosciences. Rabbit antibodies against pSPA K (S383)/pOSR1 (S325), SPAK, OSR1, and NCC were obtained from Merck Millipore (Darmstadt, Germany). Rabbit antibody against pAMPK (T172) was obtained from Cell Signaling Technology (Danvers, MA).

Animals. C57BL/6 mice were maintained under specific pathogen-free conditions with a 12:12-h light-dark cycle, and all experimental procedures used were approved by the Austin Health Animal Ethics Committee. Animals were given free access to food and water. Mouse diets were obtained from Specialty Feeds. Mice were fed a control diet (CD; 5% fat modified AIN93G) or HFD (23% fat modified AIN93G diet (43% energy from fat), with a 15% increase in NaCl) for 14 wk. The NaCl content of the HFD was 15% higher than that of the CD to match NaCl intake between groups (we have previously found that mice eat ~15% less food by weight of the HFD).

Body weights were measured weekly. At 14 wk, blood was collected, and fat pads and kidneys were harvested and snap frozen in liquid nitrogen.

Blood pressures. Systolic blood pressure was measured noninvasively in 14-wk-old mice using lite Life Science (Woodlands, CA) tail-cuff plethysmography equipment and software. Nonanesthetized mice were acclimatized to the technique for 2 days before recordings were used for analysis. Five-consecutive readings were taken for each mouse. Mice used for blood pressure recordings were not used in tissue analysis experiments.

Biochemical analysis of plasma and urine. Blood was collected from the orbital sinus, and plasma was recovered by centrifugation. Serum samples were analyzed by ProSearch Laboratories. Plasma renin concentrations were measured by an enzyme kinetic assay using sheep serum as the angiotensinogen source and subsequent measurement of angiotensin I by radioimmunoassay (31). Insulin, leptin, and resistin levels were measured with luminex using the Mouse Adipokine Magnetic Bead Panel (MADKMAG-71K, Merck Millipore).

Western blot analysis. Kidneys were excised rapidly and snap frozen in liquid nitrogen. Kidneys were sliced in half transversely. Tissue was sliced from the superior pole for the preparation of cortical preparations. The inferior half of the kidney was used for “whole kidney” (cortex and medulla) preparations. The isolated medulla was not examined due to concerns of ischemic activation of AMPK when dissecting free the medulla. Lysates were prepared using a glass on glass Dounce homogenizer in lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40, 0.27 M sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and protease inhibitor cocktail (1 tablet per 10 ml, Roche Diagnostics, Basel, Switzerland)]. Homogenates were centrifuged at 10,000 $g$ for 15 min at 4°C, and the protein concentration in the supernatants was measured using the Bradford method (Bio-Rad protein assay kit, Bio-Rad Laboratories, Hercules, CA). Homogenates were stored at ~8°C until required. Immunoprecipitations for NKCC2 were performed using the T4 antibody and equal protein concentrations of whole kidney lysates from HFD- and CD-fed mice. Anti-mouse IgG-agarose was used to immunoprecipitate immune complexes.

Samples were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) using the Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membrane was blocked in 10% BSA in Tris-buffered saline (TBS) for 1 h and then incubated in primary antibody. The optimal antibody concentration and duration of incubation were determined for each antibody. After being washed in TBS-0.05% Tween 20, the membrane was incubated for 30 min in FITC-conjugated secondary antibody (Dako, Glostrup, Denmark). Antibody complexes were detected with anti-FITC POD (Roche Diagnostics), and immunoreactive proteins were detected by enhanced chemiluminescence with the Western Lightning system (Perkin Elmer). If the membrane was to be probed with another primary antibody, antibody bound to the membrane was stripped by incubation in Reblot stripping solution (Chemicon) for 15 min. Quantification of Western blots was performed by densitometry with analysis using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence microscopy of mouse kidneys. Tissue was perfusion fixed with 4% paraformaldehyde (BDH, Poole, UK), processed, and embedded in paraffin. Four-micrometer sections were blocked with 10% BSA in TBS before incubation with the primary antibody. Alexa fluor 488-conjugated secondary antibody (Invitrogen, Life Technologies Australia, Mulgrave, Victoria, Australia) was applied, and sections were visualized on a Zeiss 510 Meta Scanning Confocal Microscope (Zeiss, Jena, Germany).

Natriuretic experiments. The natriuretic response to furosemide has been used by others as a marker of the in vivo activity of NKCC2 (18, 28, 38, 40). Mice (14 wk old) were administered intraperitoneal injections of 16.5 mg/kg furosemide (Sigma-Aldrich). Mice were placed in metabolic cages, and urine was collected for 3 h. One week before, urine was collected after intraperitoneal injections of an equal volume of vehicle (saline). Na$^+$ and creatinine concentrations were measured using a Roche Hitachi Cobas c-series autoanalyzer. Na$^+$-
to-creatine ratios after furosemide were compared with baseline levels to give a marker of the relative in vivo activity of NKCC2.

**Treatment of cells with A-769662 and low-NaCl solutions.** Cells were grown to 80% confluence and incubated in serum-free DMEM for 16 h before experiments. Cell stimulations were performed with the AMPK activator A-769662 (100 μM) for 60 min. Normal NaCl solution (pH 7.4) contained 100 mM NaCl, 25 mM NaHCO₃, 0.96 mM NaH₂PO₄, 0.24 mM Na₂HPO₄, 5 mM KCl, 1.2 mM MgSO₄, 1.0 mM CaCl₂, and 5.5 mM glucose. For low-NaCl solution (pH 7.4), the NaCl was substituted with 200 mM mannitol; osmolalities were equal (274 mosM). Treatment with NaCl solutions was for 30 min.

**Mouse embryo fibroblast isolation and immortalization.** Mouse embryo fibroblasts (MEFs) were isolated from wild-type (WT) or AMPK-β⁻/⁻ knockout (KO) embryos at days 12–13 post-conception. The head and internal organs were removed, and the remaining embryonic body minced and trypsinized. After being washed in PBS, the cell suspension was passed through a 40-μm cell strainer and plated in 10-cm dishes. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and passaged at 1:2 or 1:4 ratios. In culture, cell growth was changed every 2–3 days. Once cells resumed growth, passaging continued at 1:4 or 1:6. Cultures were considered immortalized when they showed uniform morphology and a rapid proliferation rate, which occurred for both genotypes around passages 22–25.

**AMPK activity.** AMPK-α₁ was immunoprecipitated from 150 μg of lysates overnight at 4°C using anti-AMPK-α₁ antibody bound to protein A-Sepharose beads. After washes in PBS, immunoprecipitates were resuspended in assay buffer containing 50 mM HEPEs, 1 mM DTT, 5% glycerol, and 0.05% Triton X-100 (pH 7.3). Activity was determined by phosphorylation of SAMS peptide in a reaction containing 100 μM SAMS, 200 μM [γ⁻⁵²P]ATP, 100 μM AMP, and 10 mM MgCl₂ at 30°C in a 40-μl volume. Reactions were terminated after 8 min by spotting 25 μl of the reaction mixture onto P81 phosphocellulose paper (Whatman) and washing in 1% phosphoric acid. Radioactivity was measured by liquid scintillation counting (Tri-Carb 2000, Packard Instrument).

**Statistics.** Statistics were performed using Instat (version 3.05, GraphPad Software, San Diego, CA). Data are presented as means ± SD. Multiple group means were compared by ANOVA followed by a post hoc test. Comparison of means from two groups was performed by an unpaired t-test. P values of <0.05 were considered significant.

**RESULTS**

Characteristics of HFD-fed C57BL/6 mice. At 14 wk, the percent weight gains in the two groups were 11% and 32% for CD- and HFD-fed mice, respectively (P < 0.001; Table 1). Abdominal obesity, as measured by abdominal fat pad weight, was significantly greater in HFD-fed mice. Kidney weight was higher in HFD-fed mice, and protein concentrations of kidney lysates produced in equal volumes of lysis buffer were also greater in these mice, indicating that the increased kidney weights were not solely due to fat accumulation. Systolic blood pressures were significantly higher in HFD-fed mice (112 vs. 97 mmHg, n = 10, P < 0.05). HFD-fed mice developed hyperinsulinemia, hyperleptinemia, and hyperresistinemia, consistent with the expected changes of obesity-induced metabolic syndrome. There was a trend toward an increase in renin levels in HFD-fed mice, but this did not achieve statistical significance.

**Expression and phosphorylation of Na⁺ transporters in kidneys from HFD-fed mice.** In whole kidney preparations, no difference in the abundance of NKCC2 was found between groups (Fig. 1A). S126 phosphorylation of NKCC2 immuno-precipitated from whole kidney preparations was increased ~2.5-fold in HFD-fed mice (P < 0.001), whereas T96/T101 phosphorylation was unchanged (Fig. 1B). In the renal cortex, however, HFD-fed mice had a 39% increase in the ratio of pNKCC2 (T96/T101) to total NKCC2 (P < 0.001; Fig. 1C). Cortical NKCC2 abundance was reduced by 17% in HFD-fed mice (P = 0.034). There was a trend toward a small increase in the abundance of pNKCC2 (T96/T101), but this did not meet statistical significance (P = 0.16).

No differences in the abundance or T58 phosphorylation of NCC were found in the renal cortex between groups (Fig. 1D). Reductions of 46% and 38% in the expression of the ENaC β-subunit were observed in whole kidney (Fig. 1E) and cortical (Fig. 1F) preparations from HFD-fed mice (P = 0.03 and P = 0.02, respectively). In contrast, no changes in the abundance of ENaC α- or γ-subunits were found.

**Cellular distribution of NCC, NKCC2, and ENaC in kidneys from obese mice.** Staining of sections for total NKCC2 and pNKCC2 with T4 and pNCC (T92/T101) antibodies, respectively, revealed dominant apical membrane staining with no detectable staining at the basolateral membranes, indicating that NKCC2 is the predominant NKCC isoform detected by these antibodies in the kidney (Fig. 2). There did not appear to be any difference in the membrane localization of total NKCC2 or pNKCC2 (T96/T101) between groups. The distribution of pNKCC2 (S126) could not be assessed. Anti-NCC antibody and anti-pNCC (T58) antibody produced dominant apical staining of cells in the distal convoluted tubule, and no differences were noted between groups. Cytoplasmic staining of β-ENaC was seen in the principal cells of the collecting ducts with no staining of intercalated cells, similar to the cellular distribution previously reported in the mouse kidney (27). The intensity of luminescence was reduced in HFD-fed mouse

| Table 1. Characteristics of C57BL/6 mice fed control and high-fat diets for 14 wk |
|---------------------------------|---------------------------------|------------------|
| **Control Diet** | **High-Fat Diet** | **P Value** |
| Initial weight, g | 25.6 ± 0.7 | 25.1 ± 1.3 | 0.259 |
| Final weight, g | 28.6 ± 1.3 | 33.1 ± 1.6 | <0.0001 |
| Kidney weight, g | 0.24 ± 0.04 | 0.30 ± 0.03 | <0.0001 |
| Protein concentration in the kidney lysate, mg/ml | 10.6 ± 1.0 | 12.5 ± 1.6 | 0.003 |
| Abdominal fat pad weight, g | 0.65 ± 0.15 | 1.09 ± 0.31 | 0.003 |
| Systolic blood pressure, mmHg | 97 ± 13 | 112 ± 15 | <0.05 |
| Insulin, pmol/l | 9,468 ± 8,490 | 32,160 ± 19,392 | 0.005 |
| Leptin, pmol/l | 156.9 ± 93.5 | 459.5 ± 201.3 | 0.0005 |
| Resistin, pmol/l | 629.1 ± 90.7 | 831.1 ± 246.3 | 0.026 |
| Renin, μmol/l·h⁻¹ | 51.4 ± 37.6 | 74.6 ± 32.8 | 0.16 |

Values are shown as means ± SD.
preparations (not formally quantified), but no difference in cellular localization was detected between groups. Similarly, there was no difference detected in ENaC subunit distribution (data not shown).

Natriuretic responses to furosemide.
The finding of increased activating phosphorylation of NKCC2 would be predicted to increase the activity of the transporter. To verify that NKCC2 activity was increased in vivo, natriuretic responses to furosemide were measured in 14-wk-old CD- and HFD-fed mice (Fig. 3). HFD-fed mice had a significantly greater increase in the urinary Na\(^{+}\)/H\(^+\)-to-creatinine ratio from baseline in response to furosemide (7.6- vs. 16.3-fold increases for CD- and HFD-fed mice, respectively, \(P = 0.047\)).

Increased phosphorylation of SPAK/OSR1 in HFD-fed mice.
Activating phosphorylation of NKCC2 at T96/T101 is mediated by the STE-20 kinases SPAK and OSR1 (43). Therefore, the effect of HFD on SPAK and OSR1 activity was studied. A 26% increase of pSPAK (S383)/pOSR1 (S325) (pSPAK/pOSR1) was found in the renal cortex of HFD-fed mice (\(P = 0.04\)). A similar difference was found in whole kidney samples (22% increase, \(P = 0.003\); Fig. 4).

Reduced AMPK phosphorylation in kidneys from HFD-fed mice.
The metabolic stress-sensing enzyme AMPK has been identified as a regulator of several ion channels and cotransporters (11, 17, 25) and is known to phosphorylate NKCC2 at S126 (11). AMPK is activated by phosphorylation on the \(\beta\)-subunit at T172 (48). A 32% reduction in pAMPK (T172) was found in the cortex of HFD-fed mice (\(P = 0.01\); Fig. 4). No difference in pAMPK was found between groups in whole kidney samples.

Activation of AMPK inhibits phosphorylation of SPAK/OSR1 in vitro.
Inhibition of renal cortical AMPK has been identified as a pathway involved in the initiation of renal injury in obesity (6). The HFD led to changes in both SPAK/OSR1 and AMPK activity. Furthermore, both SPAK/OSR1 (43) and AMPK (11) are reported to coimmunoprecipitate with NKCC2. We proposed that inhibition of cortical AMPK may be involved in the observed increase in pSPAK/pOSR1.
A-769662, an allosteric activator of AMPK, was used to examine the effect of AMPK activation on SPAK/OSR1 activity in Madin-Darby canine kidney (MDCK) cells. Activation of AMPK was confirmed with a radiometric assay (Fig. 5A) and by demonstrating enhanced phosphorylation of the AMPK substrate acetyl-CoA carboxylase at S79 (Fig. 5B). Treatment with A-769662 reduced pSPAK/pOSR1 by 46% (P < 0.01; Fig. 5C). Treatment with A-769662 did not affect the expression of SPAK or OSR1 (Fig. 5D), indicating that the reduction in pSPAK/pOSR1 was due to a reduction in phosphorylation.

To determine whether the effects of A-769662 were mediated through AMPK, MEFs were derived from various AMPK-deficient mouse strains. AMPK exists as a heterotrimer composed of a catalytic α-subunit and regulatory β- and γ-subunits. Different isoforms of the subunits exist, which are expressed variably in different cell lines. The AMPK subunit compositions of the MEF cell lines were determined by Western blot analysis. The greatest reduction in the AMPK catalytic (α) subunit was seen in MEFs derived from AMPK-β1 conditional KO mice crossed to total AMPK-β2−/− mice (β1floxed/β2 KO), with no catalytic α-subunit and minimal β1-subunit detectable by Western blot analysis (Fig. 6A). AMPK activity was measured in MEFs under basal conditions and after stimulation with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide [AICAR (1 mM); Fig. 6B]. Consistent with the Western blot data, β1floxed/β2KO MEFs had the greatest reduction in AMPK activity, with minimal activity detectable even after stimulation with AICAR (Fig. 6B). This MEF line was used for subsequent experiments to determine the role of AMPK in the regulation of SPAK/OSR1 and is subsequently referred to as MEF-AMPK−/−.

In MEF-AMPK−/− (WT) cells, the AMPK activator A-769662 produced a 34% reduction in pSPAK/pOSR1 (P < 0.01), whereas it had no effect on pSPAK/pOSR1 in MEF-AMPK−/− cells, consistent with the reduction in pSPAK/pOSR1 being an AMPK-mediated effect (Fig. 6C).

SPAK/OSR1 are phosphorylated by with no lysine kinase (WNK) kinases. This pathway is activated by low extracellular Cl− concentration (32). To further examine the role of AMPK in modulating phosphorylation of SPAK/OSR1, AMPK MEFs were stimulated by low-NaCl solution. The activation of SPAK/OSR1, as measured by pSPAK/pOSR1, was significantly greater in MEF-AMPK−/− cells (4.6-fold increase) compared with WT cells (1.9-fold increase, P < 0.01), con-
DISCUSSION

The present study demonstrates an increase in activating phosphorylation of NKCC2 in mice with HFD-induced obesity, which is associated with evidence of increased activity of NKCC2 in vivo. A 2.5-fold increase in S126 phosphorylation was demonstrated, which did not appear to be mediated by increased AMPK activity. An increase in T96/T101 phosphorylation was also identified in the renal cortex. However, this change was accompanied by a reduction in cortical NKCC2 expression, meaning that the overall abundance of cortical pNKCC2 (T96/T101) was unchanged between groups. The increased rate of phosphorylation at the T96/T101 site is likely a consequence of increased activity of SPAK/OSR1, and in vitro studies have identified a new role for AMPK in modulating SPAK/OSR1 activity and, hence, T96/T101 phosphorylation of NKCC2.

There is strong evidence that changes in the function of NKCC2 affect blood pressure. An inactivating mutation in NKCC2 leads to the hypotensive, salt-wasting, Bartter syndrome (49). NKCC2 activity can be modulated by phosphorylation, and the most important regulatory phosphosites are T101 and S126 (43). Individual mutation of each of these sites leads to a 30–40% reduction of NKCC2 ion transport, and combined mutation of the two sites almost abolishes activity (43). The effects of obesity on the phosphorylation of NKCC2 have not been previously examined, but increased T96/T101 phosphorylation of NKCC2 due to activation of SPAK has recently been reported in the Milan hypertensive rat (4). Changes in S126 phosphorylation have not been previously studied in animal models of hypertension.

The finding of markedly enhanced phosphorylation of NKCC2 at the S126 site in this animal model of obesity is predicted to promote Na+/H+ retention and volume expansion, which are known contributors to the development of obesity-induced hypertension. What effect the increase in phosphorylation of cortical NKCC2 at the T96/T101 sites would have on renal Na+/H+ reabsorption is unclear as this appears to be offset by a reduction in cortical NKCC2 expression, such that the absolute abundance of pNKCC2 (T96/T101) was unchanged between groups. No other changes that would increase Na+/H+ retention were identified in NKCC2, NCC, or ENaC, suggesting that the phosphorylation changes identified in NKCC2 may be of key importance in the pathogenesis of salt retention in established obesity. To verify that the changes found in NKCC2 phosphorylation affected activity in vivo, natriuretic responses to furosemide were measured. Studies of obese rats, both ZO (28) and HFD fed (40), have previously reported an increased natriuretic response to furosemide. In keeping with these studies, the natriuretic response to furosemide was significantly greater in HFD-fed mice in the present study, consistent with increased activity of NKCC2.

AMPK is a heterotrimeric serine-threonine kinase, which plays a key role in cellular energy metabolism. AMPK is known to phosphorylate NKCC2 at S126 (11). PKA has also been reported to phosphorylate this site (15). After HFD, active AMPK was unchanged in whole kidney preparations and reduced in the renal cortex. This suggests that a kinase other
than AMPK is likely to be responsible for the increased S126 phosphorylation of NKCC2 seen after HFD, although regional activation of AMPK in particular cell types cannot be fully excluded by the available data. The increased S126 phosphorylation could be due to increased activity of PKA or another yet-to-be identified kinase.

Although T96/T101 phosphorylation of NKCC2 was increased in the renal cortex, the absolute abundance of pNKCC2 (T96/T101) was unchanged between groups, due to a reduction in the cortical expression of NKCC2. A study (38) in Zucker rats has also demonstrated a reduction in cortical NKCC2 expression in ZO rats compared with lean control rats. The only previous study to examine changes in NKCC2 expression using a HFD model did so in Fischer Norway rats (40). That study reported no change in cortical abundance but increased levels of NKCC2 in the outer stripe of the inner medulla. Of note, rats that received a HFD did not achieve a statistically significant gain in weight compared with control rats (100 ± 10 vs. 97 ± 4g) and organs were removed after 8 wk. Although there was some evidence of insulin resistance, other metabolic parameters, such as aldosterone, were not different between the groups. It may be that a reduction in cortical expression of NKCC2 occurs in obesity later than changes in T96/T101 phosphorylation as a compensatory mechanism, potentially explaining the different findings between this study and ours. Of note, in the Fischer rat study, the HFD-fed rats did have an increased natriuretic response to furosemide, indicating increased activity of NKCC2 in vivo. While this may be explained by the increased medullary expression of NKCC2 they found, the results of the present study predict that the increased response to furosemide may have been due to enhanced activating phosphorylation of NKCC2 in the obese animals.

In the present study, in contrast to the findings in the cortex, expression and T96/T101 phosphorylation of NKCC2 in preparations from the whole kidney were unaltered between groups. This suggests there may be differential regulation of...
NKCC2 expression between the cortex and medulla and implies that NKCC2 abundance is unchanged or elevated in the medulla of HFD-fed mice. This finding is in keeping with the results of the study of Riazi et al. (38) in Fischer rats detailed above as well as findings by the same group in Zucker rats (40). The findings of the present study also suggest that phosphorylation of NKCC2 may be differentially regulated between the cortex and medulla. This has not previously been studied in models of obesity but has recently been reported to occur in response to angiotensin II infusion (35).

Despite an increase in cortical pSPAK/pOSR1, and in contrast to a recent study in ZO rats (21), no differences in the expression, phosphorylation, or cellular distribution of NCC was found in the present study in HFD-fed mice. In their study, Komers et al. (21) proposed the enhanced phosphorylation they observed was due to the hyperinsulinemia of ZO rats. They demonstrated that insulin treatment increased T53 phosphorylation of NCC in vitro in both human embryonic kidney-293 cells transfected with tetracycline-inducible NCC and mouse distal convoluted tubular cells. The ZO rats in their study achieved plasma insulin levels in excess of 40 ng/ml, and this concentration was used to treat the cells in vitro. This concentration is much greater than that observed in mice fed a HFD and levels found in human obesity. Insulin levels, therefore, could explain differences in the findings between the present study and that of Komers et al. (21). It is also possible that phosphorylation changes at T53 are differentially regulated from changes at T58, although both are known to be SPAK/OSR1 sites (42).

Similar to previous studies (1, 28, 39), expression levels of the ENaC subunits did not change in synchrony with one another. Levels of β-ENaC were reduced in the kidneys of HFD-fed mice, whereas expression of the other subunits was unchanged. Confocal microscopy did not identify any differ-
ences in the cellular localization of the subunits. However, as staining was predominantly cytoplasmic, it is hard to draw firm conclusions about any effect on cell surface expression. To our knowledge, no studies have previously explored ENaC expression in a HFD model of obesity. Previous studies in ZO rats have yielded inconsistent results, and it is probably not valid to compare between models given the profound hyperinsulinemia in ZO rats, as insulin is known to affect ENaC activity. The significance of the reduction in β-ENaC found in the present study is unclear.

Phosphorylation of cortical NKCC2 at pT96/T101 is due to either of the STE-20 kinases SPAK or OSR1. SPAK and OSR1 are themselves activated through phosphorylation by WNK kinases, which, in turn, respond to changes in the intracellular Cl\(^{-}\) concentration. The chief activating phosphatases are T243 (SPAK) and T185 (OSR1) (53). SPAK and OSR1 are also phosphorylated by WNK kinases at S383 and S325, respectively (53). Phosphorylation of the latter sites has been used as a marker of activation of SPAK/OSR1 by WNK kinases (51).

In the present study, HFD-fed mice had increased phosphorylation of SPAK/OSR1 on S383/S325 in the cortex and whole kidney compared with CD-fed mice. Interestingly, phosphorylation of cortical NKCC2 at the SPAK/OSR1 site was increased in HFD-fed mice, whereas that of NCC was unchanged, suggesting that SPAK/OSR1 activity is regulated differentially in the thick ascending limb of the loop of Henle (TAL) and distal convoluted tubule. This is consistent with findings of recent studies in SPAK\(^{-/-}\) mice, which have suggested different roles of SPAK isoforms and OSR1 in the different areas of the tubule (13, 26, 30, 47).

In keeping with the results of previous studies (6, 22), there was a reduction in pAMPK in the cortex of HFD-fed mice. A reduction in whole kidney pAMPK could not be demonstrated, suggesting differential regulation of AMPK between the cortex and medulla. Possibly this relates to the reliance of the cortex on fatty acid oxidation, whereas glycolysis is more important in the medulla (14, 47). Inhibition of AMPK activity in the kidney has been proposed as one of the mechanisms leading to the development of obesity-related kidney disease (6). AMPK and SPAK/OSR1 share a similar distribution in the kidney (9, 56), and both kinases are known to independently associate with NKCC2 (11, 43). These observations, along with the finding that HFD led to changes in the activity of both kinases, led us to postulate that reduced renal AMPK activity might contribute to the activation of the SPAK/OSR1/NKCC2 pathway with HFD.

To examine the role of AMPK in the regulation of SPAK/OSR1, MDCK cells were treated with the specific AMPK activator A-769662, causing increased AMPK activity and a reduction in the phosphorylation of SPAK/OSR1. This finding is consistent with AMPK exerting an inhibitory effect on the phosphorylation of SPAK/OSR1. Experiments using MEF-AMPK\(^{-/-}\) cells demonstrated that the effect of A-769662 was mediated via AMPK and was not an off-target effect. Furthermore, there was a significantly greater increase in pSPAK/pOSR1 in response to low-NaCl solution in MEF-AMPK\(^{-/-}\) cells than in MEF-AMPK\(^{+/+}\) cells, consistent with AMPK negatively regulating SPAK/OSR1 phosphorylation. Although acute activation of AMPK was found to inhibit phosphorylation of SPAK/OSR1, interestingly, MEF-AMPK\(^{-/-}\) cells had a lower basal level of pSPAK/pOSR1 than WT cells. The cause of this reduction has not yet been determined, but the finding suggests that constitutive absence or suppression of AMPK activity could regulate SPAK/OSR1 through different mechanisms than those invoked by acute changes in AMPK activity.

A limitation of the present study is a presumption that the cell culture results in MDCK cells and MEFs represent the signaling events that occur in cells of the TAL. Nonetheless, the AMPK/SPAK/OSR1 pathway has been shown to be conserved in both cell types, and SPAK/OSR1 and AMPK are known to be expressed in the TAL, so it is likely that this assumption is valid. Future direct studies of this pathway in cultured TAL cells could be of interest.

In conclusion, this study found that obesity produced by HFD led to increased activating phosphorylation of NKCC2 and increased activity of NKCC2 in vivo. There was a marked increase in phosphorylation at S126. Expression of NKCC2 was reduced in the cortex, but T96/T101 phosphorylation of NKCC2 was increased, which was associated with increased pSPAK/pOSR1. Furthermore, HFD led to reduced cortical AMPK activity, which in vitro studies have suggested is associated with reduced negative regulation of pSPAK/pOSR1. Taken together, these data identify increased activating phosphorylation of NKCC2 and dysregulation of an AMPK/SPAK/OSR1/NKCC2 pathway as contributors to the pathogenesis of obesity-related hypertension.

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

AUTHOR CONTRIBUTIONS

REFERENCES
Increased phosphorylation of NKCC2 in obesity


