Modest dietary $K^+$ restriction provokes insulin resistance of cellular $K^+$ uptake and phosphorylation of renal outer medulla $K^+$ channel without fall in plasma $K^+$ concentration

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Chen, Pei, John P. Guzman, Patrick K. K. Leong, Li E. Yang, Anjana Perianayagam, Elisa Babilonia, Jennifer S. Ho, Jang H. Youn, Wen Hui Wang, and Alicia A. McDonough. Modest dietary $K^+$ restriction provokes insulin resistance of cellular $K^+$ uptake and phosphorylation of renal outer medulla $K^+$ channel without fall in plasma $K^+$ concentration. Am J Physiol Cell Physiol 290: C1355–C1363, 2006. First published December 14, 2005; doi:10.1152/ajpcell.00501.2005.—Extracellular $K^+$ is tightly regulated to maintain normal membrane excitability. Extracellular fluid (ECF) $K^+$ homeostasis is maintained by the coordinated regulation of kidney and muscle (16). Kidneys match $K^+$ output to $K^+$ input by adjusting active $K^+$ secretion and absorption. Extrarenal tissues are primarily responsible for acute ECF [$K^+$] regulation (4). This is evident in that 50% of an acute $K^+$ load is not excreted in the urine within the first 6 h. Skeletal muscle, which contains the biggest intracellular fluid (ICF) $K^+$ stores, rapidly buffers changes in ECF $K^+$. After meals, insulin drives $K^+$ from ECF to ICF via activation of Na$^+$$-K^+$$-ATPase$; during and after exercise, Na$^+$$-K^+$$-ATPase$ actively returns $K^+$ that was released to the ECF back to the ICF, stimulated at least in part by catecholamines.

When $K^+$ output exceeds input, as occurs in dietary $K^+$ restriction, the kidneys avidly retain $K^+$ by shifting from $K^+$ secretion to $K^+$ absorption in the cortical collecting duct (CCD) and renal output falls to near zero. The molecular mechanisms of this renal $K^+$ conservation are becoming evident (15). Decreased $K^+$ intake and the associated reduction in plasma [$K^+$] stimulates renal expression of a protein tyrosine kinase (PTK), specifically cSrc, which phosphorylates the inward rectifier $K^+$ channel 1.1 (Kir1.1; ROMK1 in kidney) (28), which is responsible for $K^+$ secretion in the CCD. The phosphorylation of ROMK enhances ROMK internalization, reduces the $K^+$ channel number on the cell surface in the CCD (14), and thus reduces $K^+$ secretion to the point at which net reabsorption of $K^+$ occurs.

Because $K^+$ loss via excretion and sweat persists during dietary $K^+$ restriction, plasma [$K^+$] falls. As a homeostatic response, muscle Na$^+$$-K^+$$-ATPase$ $\alpha_2$-isoforms levels decrease, reducing the capacity to pump $K^+$ into the muscle. As a result, $K^+$ is redistributed from muscle ICF stores to the ECF, buffering the fall in plasma [$K^+$] (16, 17). In addition, we have demonstrated that after just 2 days of $K^+$ restriction, when there is only a slight decrease in plasma [$K^+$] and before a
significant decrease in muscle Na\(^+\) pump abundance, there is a decrease in insulin-stimulated cellular K\(^+\) uptake (6).

Recently, Choi et al. (5) examined whether rats fed a high-fat diet (66% for 15 days) developed insulin resistance to cellular K\(^+\) uptake along with the well-characterized insulin resistance to cellular glucose uptake observed during high-fat feeding. Insulin sensitivity was assessed using simultaneous euglycemic, euclidean clamps in conscious rats. Although there was no significant fall in plasma [K\(^+\)], insulin-stimulated cellular K\(^+\) uptake was significantly depressed in the high-fat diet group, evidence for insulin resistance to cellular K\(^+\) uptake. However, urinary K\(^+\) excretion was much lower in the rats fed a high-fat diet, strong evidence for reduced K\(^+\) intake. Indeed, dietary K\(^+\) intake was reduced due to lower K\(^+\) content in typical high-fat diets and consumption of less mass of the high-calorie, high-fat chow. Insulin-stimulated cellular K\(^+\) uptake was normalized in the high-fat diet group by dietary K\(^+\) supplementation, demonstrating that a high-fat diet does not directly provoke insulin resistance of cellular K\(^+\) uptake.

The purpose of the current study was to determine the relationship between dietary K\(^+\) restriction and activation of cellular mechanisms to maintain plasma [K\(^+\)] levels. The results demonstrate that when dietary K\(^+\) intake was reduced to 33% of control level for 2 wk, there was no change in either plasma [K\(^+\)] level or aldosterone concentration or in the expression levels of muscle Na\(^+\)-K\(^+\)-ATPase or renal H\(^+\)-K\(^+\)-ATPase or ROMK. However, there is a significant decrease in insulin-stimulated cellular K\(^+\) uptake and renal K\(^+\) conservation, the latter of which can be accounted for by an increase in expression of the PTK cSrc and increased phosphorylation of ROMK.

METHODS

**Animals and diets.** All animal experiments were approved by the University of Southern California Keck School of Medicine IACUC and conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (10–12 wk of age, 200–225 g) were maintained at a constant temperature (21°C) under a 12:12-h artificial light-dark cycle with unlimited access to rat chow and water. Animals were fed a 1.0% K\(^+\) diet or a 0.33% K\(^+\) diet for 14–16 days. In the study of ROMK regulation, a third set of rats was fed a 0 K\(^+\) diet for 4 days. All Chow types were obtained from Harlan Teklad (Madison, WI).

**Metabolic cage.** At 7 and 15 days after treatment, rats were placed in metabolic cages during a 17-h period for urine collection. Collection bottles contained 5 ml of mineral oil and were wrapped in sufficient gel ice to remain cool overnight. Urine volume was determined by weighing bottles before and after 17-h collection. Na\(^+\) concentration ([Na\(^+\)]) and [K\(^+\)] were measured with a FLM 3 flame photometer (Radiometer, Copenhagen, Denmark) using Li\(^+\) as an internal standard. Overnight Na\(^+\) and K\(^+\) excretion were calculated as urinary [Na\(^-\)] and [K\(^-\)] multiplied by urine volume, divided by time in the metabolic cage, with the results expressed in micromoles per hour.

**Insulin-sensitive cellular K\(^+\) uptake and K\(^+\) excretion.** As described in detail previously (6), each rat was placed in an individual cage with a wired floor and the distal one-third of its tail drawn through a hole placed low on the side of the cage and secured with a rubber stopper and tape to protect tail blood vessel catheters during experiments. Animals were acclimated to their tails being tethered for 3 days while they were free to move about and allowed unrestricted access to food and water. Under local lidocaine hydrochloride anesthesia with the rats snugly wrapped in towels, an infusion catheter was inserted into the tail vein the day before the experiment and an arterial catheter was inserted into the tail artery for blood sampling 6 h before the experiment (∼0700). The animals were returned to their cages with their tails secured as previously described and were free to move about. The patency of the arterial catheter was maintained by slow (0.016 ml/min) infusion of heparinized (10 U/ml) saline.

To measure insulin-stimulated decrease in plasma [K\(^+\)], rats were fasted for 6 h (0700–1300), and then baseline [K\(^+\)] and glucose concentration measurements were performed in triplicate at 1300. Insulin (Lilly, Indianapolis, IN) diluted in saline was infused through a tail vein catheter at 5 mU·kg\(^-1\)·min\(^-1\) for 1.5 h and then at 50 mU·kg\(^-1\)·min\(^-1\) for another 1.5 h. At 10-min intervals during the infusions, 60-µl blood samples were collected for immediate measurement of plasma [glucose] and [K\(^+\)]. Plasma [glucose] was clamped at the baseline value by infusion of 20% dextrose via a Y adaptor through the tail vein catheter at variable rates determined empirically.

To determine K\(^+\) excretion during insulin infusion, urine was collected for 3 h from the bottom of the cage with a fine-mesh placed below the wire floor to prevent fecal contamination. This urine was combined with that from the urinary bladder collected at the end of the experiment while the rats were under pentobarbital sodium anesthesia. Because the urinary bladder was not emptied at the beginning of the experiment, it is possible that the urine collected during clamping included some that was in the bladder before insulin infusion had begun. Although this circumstance would lead to overestimation of urinary K\(^+\) excretion during clamping, the error appears to have been small because the amount of K\(^+\) in the bladder was typically ~15% of the total amount collected during the 3-h clamp. Urinary [K\(^+\)] and [Na\(^+\)] were measured using flame photometry.

To quantify the rate of insulin-stimulated cellular K\(^+\) and glucose uptake, the same protocol described in the previous paragraph was used with both plasma [glucose] and plasma [K\(^+\)] levels being clamped at baseline values by infusion of 20% dextrose and 150 mM KCl via a Y adaptor through the tail vein catheter at variable rates determined empirically. Also, insulin was infused at only one rate (5 mU·kg\(^-1\)·min\(^-1\)) for the entire 3-h clamp period. At the end of the clamp, the animals were anesthetized with pentobarbital sodium and whole gastrocnemius and renal outer medullae were dissected out, frozen in liquid N\(_2\), and stored at −70°C for later analysis of muscle [K\(^+\)], [Na\(^+\)], and Na\(^-\)-K\(^+\)-ATPase activity as well as renal transporter abundance.

**Assays in blood and skeletal muscle.** Blood samples collected every 10 min during experiments were centrifuged at 13,400 rpm for 15 s. Ten microliters of plasma were collected for [K\(^+\)] and [Na\(^+\)] measurement using flame photometry. Ten microliters of plasma were taken for [glucose] measurement using a glucose oxidase method in a Glucose Analyzer II (Beckman Coulter, Fullerton, CA). Aldosterone concentration was measured in 200-µl samples of plasma collected using a Coat-A-Count kit (Diagnostic Products, Los Angeles, CA) while the rats were under pentobarbital sodium anesthesia. The results were read by interpolation on a plot of the percentage of aldosterone bound against aldosterone concentration.

For measurement of muscle [Na\(^+\)] and [K\(^+\)], whole gastrocnemius muscle samples were thawed, blotted, trimmed of connective tissue, weighed, and homogenized in 0.3 M TCA (1:50 dilution; w/vol) for 5 min with a Tissuemizer homogenizer (Teledyne Tekmar, Mason, OH) and then centrifuged at 2,500 rpm for 20 min to remove cell debris. [K\(^+\)] and [Na\(^+\)] in muscle TCA extracts were measured using flame photometry and expressed as micromoles per gram wet weight.

Na\(^+\)-K\(^+\)-ATPase activity was measured in triplicate in total membrane preparations of whole gastrocnemius muscle (3,000 g supernatant) as the rate of P\(_i\) generated during a 15-min period in the absence minus the presence of 2 mM ouabain (sufficient to inhibit both α\(_i\)- and α\(_j\)-isoforms of Na\(^-\)-K\(^+\)-ATPase) and expressed as micromoles P\(_i\) liberated per milligram of protein per hour as described in detail previously (6).
**Immunoblot analysis of K⁺ transporters.** The renal outer medulla and gastrocnemius samples were dissected, and a low-speed supernatant (S₁₀₀) was prepared for immunoblot analysis as previously described in detail for kidney (29) and muscle (24). To assess the total pool size of transporters, S₁₀₀ protein concentration was determined by performing BCA in triplicate (Pierce Biotechnology, Rockford, IL), and then a constant amount of protein (alongside one-half of the amount to verify linearity) was assessed using immunoblot analysis. ATPase samples were denatured for 30 min at 37°C, and samples for ROMK were denatured at 60°C for 15 min in SDS-PAGE sample buffer, resolved on 7.5% SDS-PAGE gels, and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). For proteins in the renal outer medulla, the amount of protein assayed and the antibodies used for detection were as follows: 60 µg of detection of ROMK with anti-ROMK (APC-001, 1:100 dilution; Alomone Labs, Jerusalem, Israel), 16 µg for the detection of the colonic isoform of H⁺-K⁺-ATPase with HKA109 (1:200 dilution; Alomone Labs, Jerusalem, Israel), 80 µg for the detection of aquaporin-2 (AQP2) with anti-AQP2 (1:1,000 dilution; M. Knepers, Cornell University), and 80 µg for the detection of a 2-isoform with McHered (1:2,000 dilution; M. Kashgarian, Yale University, New Haven, CT). These blots were incubated with sections of Na⁺-K⁺-ATPase and aldosterone, and 80 µg for the detection of the gastric isoform of H⁺-K⁺-ATPase with HKA111 (1:500 dilution; provided by R. Silver, Cornell University), and 80 µg for the detection of aquaporin-2 (AQP2) with anti-AQP2 (1:1,000 dilution; M. Knepper, National Institutes of Health, Bethesda, MD). Twenty-four micrograms of whole gastrocnemius protein were assayed for the detection of Na⁺-K⁺-ATPase α₂-isoform with McHered (1:2,000 dilution; produced by our laboratory) and the detection of the Na⁺-K⁺-ATPase α₁-isoform with MAb 464.6 (1:200 dilution; M. Kashgarian, Yale University, New Haven, CT). These blots were incubated with secondary antibodies, either Alexa Fluor 680-labeled goat-anti-rabbit or goat-anti-mouse (Molecular Probes, Eugene, OR), detected using an Odyssey infrared imaging system (LI-COR, Lincoln, NE) and quantitated using the accompanying LI-COR software.

**ROMK regulation.** To determine the effect of 15-day 1% vs. 0.33% K⁺ diet on PTK cSrc levels and ROMK phosphorylation, mixed renal cortex and outer medulla from four sets of rats were collected on the same day, snap-frozen in liquid N₂, and stored at −80°C until assay. The assays of cSrc abundance and ROMK phosphorylation were conducted as described in detail previously (14, 28). In brief, tissue was suspended in RIPA solution [50 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 1 mM sodium molybdate, 1 mM para-nitrophenylphosphate, and 1 mM EDTA] containing protease and phosphatase inhibitors (1:8 dilution; wt/vol). The samples were left on ice for 15 min, homogenized with mortar and pestle, incubated with DNase (5 µg/ml) at 4°C for 60 min, and centrifuged at 3,000 rpm for 10 min at 4°C to remove debris. Protein concentrations were measured twice (BSA protein assay; Pierce Biotechnology) to ensure accuracy. For assay of cSrc abundance, 80 µg of extract protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes, blocked, and incubated with anti-cSrc (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). To assess constant protein loading, the same blots were reprobed with anti-α-actin (1:10,000 dilution; Sigma, St. Louis, MO).

ROMK phosphorylation was assayed after immunoprecipitation as described previously (14), starting with tissue extracts prepared as described above. Antibodies to ROMK (Alomone Labs) were added to the renal samples at a ratio of 4 µg antibody per 1 mg protein with 25 µl of protein A/G plus agarose (Santa Cruz Biotechnology) for an additional 2 h at 4°C. Samples were pelleted, washed twice with 1 ml of PBS containing 10 µl/mg saturated PMSF and 10 µl/mg protease inhibitor cocktail, mixed with 25 µl of 2× SDS sample buffer, and boiled for 5 min, and then the supernatant was resolved by SDS-PAGE, transferred onto nitrocellulose membranes, blocked, rinsed, and probed with either anti-ROMK (1:250 dilution) or anti-tyrosine phosphorylation (4G10, 1:1,000 dilution; Upstate Biotechnology, Lake Placid, NY). For cSrc ROMK and tyrosine phosphorylation detection, antibody-antigen complexes were detected using ECL (Amerham Pharmacia Biotech, Piscataway, NJ), and the intensity of the corresponding band was determined using densitometry. To ensure constant ROMK levels, the same Western blots were probed with 4G10 and anti-ROMK antibody.

**Statistical analysis.** Data are expressed as means ± SE except where SD is indicated. The significance of the differences in mean values between groups was evaluated using an unpaired two-tailed t-test. Differences were considered significant at *P* < 0.05.

**RESULTS**

**Effect of 0.33% K⁺ diet on Na⁺ and K⁺ in plasma, muscle, and urine.** Rats fed a 0.33% K⁺ diet or 1.0% K⁺ diet were compared after 15 days. As summarized in Table 1, body weight, plasma [K⁺], plasma [Na⁺], muscle K⁺ and Na⁺ stores, as well as plasma aldosterone concentration, were unchanged. The plasma [K⁺] values were measured in seven sets of animals (see Fig. 3). Another six sets of rats used in the study (see Fig. 2) had baseline plasma [K⁺] of 4.0 ± 0.02 (both 0.33% K⁺ and 1.0% K⁺ diet groups). In addition, plasma and muscle [Na⁺] and [K⁺] remained unchanged even after 30 days of being fed the 0.33% K⁺ diet compared with the 1.0% K⁺ diet (*n* = 4 pairs of rats; data not shown). These findings indicate that total body K⁺ stores were maintained while rats were fed a diet of 0.33% K⁺. Despite the fact that plasma [K⁺] was unaltered, urinary K⁺, collected overnight in metabolic cages, fell 80%: from 117 µmol/l in rats fed the 1.0% K⁺ diet to 24 µmol/l in rats fed the 0.33% K⁺ diet, evidence of significant K⁺ conservation in the absence of an "error signal," i.e., a change in plasma [K⁺] from normal levels (Fig. 1). The fact that the fall in urinary [K⁺] (80%) was greater than the decrease in K⁺ intake (66%) probably reflects the fact that there are routes of persistent K⁺ loss, such as in the feces. Overnight urinary volume and urinary Na⁺ (Fig. 1) were not different between the two groups; that is, there was no evidence of diuresis previously associated with K⁺-free diets and hypokalemia (1, 21).

**Effect of 0.33% K⁺ diet on insulin-sensitive plasma K⁺ clearance.** Muscle actively participates in plasma K⁺ regulation by acutely clearing K⁺ into the ICF after a meal, driven by insulin stimulation, and by chronically donating K⁺ from muscle ICF to ECF during K⁺ deprivation. In addition, muscle becomes resistant to insulin stimulation of cellular K⁺ uptake during K⁺ deprivation (6). Therefore, we tested the hypothesis that insulin-stimulated cellular K⁺ uptake would be decreased after 15 days on the 0.33% K⁺ diet, a response that could help to preserve plasma [K⁺] in the face of decreased dietary K⁺.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1.0% K⁺ Diet</th>
<th>0.33% K⁺ Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt. g ± SD</td>
<td>1.32 ± 0.5</td>
<td>3.18 ± 0.5</td>
</tr>
<tr>
<td>Plasma [K⁺], mM ± SE</td>
<td>7.40 ± 0.03</td>
<td>4.00 ± 0.05</td>
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<tr>
<td>Plasma [Na⁺], mM ± SE</td>
<td>7.12 ± 1.25</td>
<td>125.7 ± 1.9</td>
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<tr>
<td>Plasma aldosterone, pg/ml ± SE</td>
<td>5–6</td>
<td>44 ± 3.5</td>
</tr>
<tr>
<td>Whole gastrocnemius K⁺, µmol/g wet wt ± SE</td>
<td>5–6</td>
<td>103.0 ± 1.2</td>
</tr>
<tr>
<td>Whole gastrocnemius Na⁺, µmol/g wet wt ± SE</td>
<td>5–6</td>
<td>20.5 ± 1.5</td>
</tr>
<tr>
<td>Whole gastrocnemius Na⁺-K⁺-ATPase activity, µmol P/mg protein 4-h ± SE</td>
<td>5–6</td>
<td>2.67 ± 0.18</td>
</tr>
</tbody>
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Values are means ± SE except where SD is indicated.
We also hypothesized that insulin-stimulated glucose uptake would be unchanged. Specifically, conscious rats fed either the 1.0% or the 0.33% K⁺ diet for 15 days were infused with 5 mU of insulin·kg⁻¹·min⁻¹ for 90 min and 50 mU of insulin·kg⁻¹·min⁻¹ from 90 to 180 min. Plasma glucose levels were clamped at baseline with glucose infusion. As shown in Fig. 2, the insulin-stimulated fall in plasma [K⁺] in the 0.33% K⁺ diet group (to 3.65 ± 0.06 mM between 70 and 90 min and to 3.47 ± 0.07 mM between 150 and 180 min) was significantly blunted compared with the fall in the 1.0% K⁺ diet group (to 3.56 ± 0.03 mM between 70 and 90 min and to 3.19 ± 0.06 mM between 150 and 180 min). As shown in Fig. 2B, the amount of K⁺ excreted in the urine during the 180-min insulin infusion (measured in mmol/kg), determined by pooling urine collected from the bottom of the cage with urine from the bladder, was not significantly depressed in the 0.33% K⁺ diet group compared with the 1.0% K⁺ diet group. For comparison, when expressed, in the units used in Fig. 1, the urinary K⁺ was 124 μmol/h in the 1.0% K⁺ diet group and 112 μmol/h in the 0.33% K⁺ diet group. This lack of a significant difference in the two groups is likely due to 1) fluid infusion to accomplish glucose concentration clamping during insulin infusion and 2) significant insulin-provoked fall in plasma [K⁺] in the two groups. Thus, there is no evidence that the depressed insulin-stimulated K⁺ clearance is due to increased fractional K⁺ reabsorption; rather, the blunted decrease in plasma K⁺ likely represents insulin resistance of cellular K⁺ uptake analogous to the results of our previous study in 0 K⁺-fed rats (6).

The next series aimed to quantify the decrease in insulin-sensitive cellular K⁺ uptake and to test the hypothesis that there is no change in insulin-sensitive glucose uptake after 0.33% K⁺ dietary intake. Rats were infused with insulin, during which time both plasma [K⁺] and plasma [glucose] levels were clamped at baseline to quantitate the magnitude of K⁺ and glucose clearance in response to insulin; that is, the amount of glucose and K⁺ infused to maintain plasma levels at baseline was assumed to be equivalent to the amount cleared into the intracellular compartment, because there was no change in urinary excretion. The results in Fig. 3, A and C, demonstrate that plasma [K⁺] and plasma [glucose] levels were maintained close to baseline levels, with little variability during the clamp. The amount of K⁺ infusion (Kinf) necessary to maintain these baseline concentrations during insulin infusion was significantly less in the low-K⁺ diet group. Specifically, the average Kinf during the last 60 min was 36.2 ± 11.8 μmol·kg⁻¹·min⁻¹ in the 0.33% K⁺ diet group and 68.2 ± 10.4 μmol·kg⁻¹·min⁻¹ in the 1.0% K⁺ diet group (P < 0.01); that is, a 45% fall in insulin-stimulated cellular K⁺ uptake in the 0.33% K⁺ diet group. In contrast, the amount of glucose infusion (Ginf, μmol·kg⁻¹·min⁻¹) needed to maintain baseline plasma glucose levels was the same in the 0.33% vs. 1.0% K⁺ diet groups, verifying that insulin-stimulated cellular uptake of glucose was unaltered by this modest dietary K⁺ reduction.

**Effect of altered K⁺ diet on renal ROMK regulation.** To determine the molecular mechanisms affecting renal K⁺ conservation in the absence of a change in plasma [K⁺], we focused on the regulation of renal ROMK (K1r1.1), which is responsible for K⁺ secretion in the CCD. During K⁺ deprivation and accompanying hypokalemia, expression of the PTKs cSrc and cYes increase and phosphorylate ROMK, which provokes ROMK internalization, a response that reduces K⁺ excretion (14, 15, 28). We tested the hypothesis that both renal PTK cSrc and cSrc phosphorylation increase in rats fed a 0.33% K⁺ diet for 20 days compared with rats fed a 1.0% K⁺ diet, despite the lack of a change in plasma [K⁺]. A third group of rats was fed a 0 K⁺ diet for 4 days as a positive control for the expected increase in cSrc expression and ROMK1 phosphorylation. Plasma [K⁺] in mM (mean ± SD, n = 4) in the three groups at the time of euthanasia were 4.15 ± 0.61 (1% K⁺ diet), 4.15 ± 0.78 (0.33% K⁺ diet), and 2.85 ± 0.2 (0 K⁺ diet). Incidentally, this set of rats was studied in metabolic cages (Fig. 1). An immunoblot assay of a constant amount (80 μg) of mixed renal cortex and outer medulla supported our hypothesis (Fig. 4). Compared with the 1.0% K⁺ diet group, cSrc expression was increased 50 ± 20% in the 0.33% K⁺ diet group and 110 ± 20% in the 0 K⁺ diet group. Likewise, immunoblot analysis of tyrosine phosphorylation in immunoprecipitated ROMK indicates that compared with rats fed the 1.0% K⁺ diet, ROMK phosphorylation was increased by 50 ± 20% in the 0.33% K⁺ diet group and 150 ± 20% in the 0 K⁺ diet group. These results demonstrate that the 4-day
0 K⁺ diet, as expected, increased PTK cScr expression and ROMK phosphorylation as plasma [K⁺] falls to 2.8 mM and that cScr expression and ROMK phosphorylation also increased after the 15-day 0.33% K⁺ diet despite the absence of a change in plasma [K⁺].

Effect of 0.33% K⁺ diet on K⁺ transporter abundance. In response to K⁺ deprivation, the abundance and activity of the muscle Na⁺⁺-K⁺-ATPase α₂-isofrom, but not the α₁-isofrom, decreases, facilitating the transfer of K⁺ from muscle stores to ECF (16), and the abundance of renal H⁺⁻K⁺-ATPase increases, perhaps facilitating active K⁺ reabsorption (25). Likewise, the abundance of ROMK in the kidney decreases, facilitating a decrease in K⁺ secretion (19). The fall in ROMK abundance is suggested in ROMK immunoprecipitation in the 0 K⁺ diet group, as shown in Fig. 4B. We tested the hypothesis that these changes in K⁺ transporter abundance would also be evident in rats fed the 0.33% K⁺ diet. Homogenates from kidney medulla and from whole gastrocnemius from six pairs of rats fed the 1.0% vs. 0.33% K⁺ diet for 15 days were prepared, and a constant amount was analyzed by immunoblot with transporter-specific antibodies. Figure 5 shows three samples from each group and demonstrates that there was no detectable difference in the expression of ROMK, the colonic isoform of H⁺⁻K⁺-ATPase, the gastric isoform of H⁺⁻K⁺-ATPase, or muscle Na⁺⁺-K⁺-ATPase α₂-isofrom expression in rats fed the 0.33% vs. the 1.0% K⁺ diet for 15 days, disproving our hypothesis. We also determined that as previously reported for K⁺ deprivation, there was no change in muscle Na⁺⁺-K⁺-ATPase α₁-isofrom expression (data not shown). Muscle Na⁺⁺-K⁺-ATPase activity, measured under Vmax conditions, was unchanged in the 0.33% K⁺ diet group (Table 1). In addition, renal medullary AQP2 abundance, which decreases during hypokalemia and contributes to the concentration defect and diuresis (1, 21), was not significantly decreased by 15-day 0.33% K⁺ diet (0.79 ± 0.22) compared with same time on 1% K⁺ diet (1.0 ± 0.25) (means ± SD, mean density in 1% K⁺ diet group defined as 1.0, n = 6 pairs, P > 0.05, blots not shown). This finding correlates with the lack of change in urine output after rats were fed the 0.33% K⁺ diet (Fig. 1). These results suggest that renal K⁺ conservation is mediated primarily by down-regulation of the transport activity of a constant pool of ROMK, but we cannot rule out the activation of existing H⁺⁻K⁺-ATPases. The results also indicate that the decrease in insulin-stimulated cellular K⁺ uptake is not secondary to a decrease in muscle Na⁺⁺-K⁺-ATPase α₂-isofrom pool size or Vmax activity.
nominally K from our groups, start with feeding rodents diets that are observed in the present study after 15 days on the 0.33% K concentration. These changes, including a decrease in insulin-responsiveness, would act to buffer a fall in plasma [K+] during dietary restriction. The results demonstrate that homoeostatic mechanisms are activated before a significant fall in extracellular K. In other words, a fall in plasma [K+] is not the error signal that activates the [K+] conserving homeostatic responses. Some studies of K deprivation, including those from our groups, start with feeding rodents high potassium diets that are nominally K free, which causes plasma [K+] to fall by as early as 2 days (6, 14, 26). In these K deprivation studies, a wide array of changes in transporter protein levels occur, including decreases in muscle Na+/K+-ATPase α2-subunit levels (27), decreases in renal ROMK (19) and AQP2 (1, 21), and increases in renal colonic H+-K+-ATPase (18, 25), as well as a fall in muscle stores of K (26). These changes in K+ and water transporter levels and muscle K+ pool size were not observed in the present study after 15 days on the 0.33% K+ diet, providing evidence that these changes may be provoked by a fall in plasma [K+]. Even when rats were maintained on the 0.33% K+ diet for 30 days (results not shown), no changes in plasma [K+] or abundance of muscle Na+/K+-ATPase α2-isoform, renal ROMK, H+-K+-ATPase, or AQP2 were observed, demonstrating that within 15 days of starting the 0.33% K+ diet, the rats were at steady state. In contrast, we observed a marked change in expression of the PTK cSrc that can phosphorylate ROMK, leading to its inactivation via retraction from the cell surface (14, 15). The retraction of ROMK would increase net K+ reabsorption in the CCDs mediated by existing pools of H+-K+-ATPase. These findings suggest two categories of responses to the low-K+ diet: a first line of defense activated by an undefined signal (perhaps sensing of K+ ingestion), leading to a decrease in insulin-stimulated cellular K+ uptake and renal K+ conservation, and a second line of defense activated when plasma [K+] falls in which muscle Na+ pump α2-isoform expression decreases to facilitate a transfer of K+ from ICF to ECF and in which ROMK and H+-K+-ATPase abundance changes occur, consistent with renal K+ conservation.

Can the findings reported in this study of rodents subjected to one-third normal K+ intake be applied to humans? To our
Compared with rats fed the 1.0% K\(^+\) and with anti-ROMK to verify equivalent levels of ROMK in samples, immunoblot analysis with 4G10 used as the probe, an antibody that reacts with tyrosine-phosphorylated ROMK level in mixed renal cortex and outer medulla. Which plasma [K\(^+\)] was maintained within a normal range. The results of this study in rats suggest that the hallmark of such a K\(^+\)-poor diet would be pronounced renal K\(^+\) conservation, even if plasma [K\(^+\)] were maintained within the normal range. Humans who consumed a diet containing only 20 mM K\(^+\)/day (one-sixth of the IOM-recommended amount) for 9 days did not maintain normal serum [K\(^+\)]; rather, serum [K\(^+\)] fell from 4.1 to 3.5 mmol/l. In addition, there was evidence of elevated blood pressure, renal Na\(^+\) and K\(^+\) retention, and increased free water clearance (10). Whether some or all of these changes would be evident in individuals with normal serum [K\(^+\)] remains to be determined.

After a meal, insulin stimulates cellular uptake of K\(^+\) as well as glucose (7). This point is critically important because it is possible to consume as much K\(^+\) in one meal as is found in the entire ECF (16), and insulin-stimulated clearance of K\(^+\) into skeletal muscle prevents life-threatening cardiac complications of acute hyperkalemia. Of the many homeostatic systems of the body, the K\(^+\) and glucose homeostatic systems are distinctive in that they share acute regulation by insulin. This feature suggests the potential for interactions or cross talk between the two systems. However, the findings of this study and our previous studies indicate that insulin’s actions on glucose and K\(^+\) cellular uptake are independently regulated. The current study demonstrates that insulin-sensitive K_{inf} (Fig. 3) is suppressed by ∼45% during the 0.33% K\(^+\) diet and that insulin-sensitive G_{inf} is unchanged. In our previous study (6), we measured K_{inf} and G_{inf} in response to the 0 K\(^+\) diet and observed dramatic insulin resistance to cellular K\(^+\) uptake: K_{inf} fell by 80% at 2 days and by 94% after 10 days on the 0 K\(^+\) diet (along with a fall in muscle Na\(^+\)-K\(^+\)-ATPase a2-isoform levels at 10 days). In contrast, rats that were fed a high-fat diet (supplemented with K\(^+\) to normalize K\(^+\) intake to that in the control group) exhibited the expected insulin resistance to cellular glucose uptake (20% decrease in G_{inf}) without a change in K_{inf} (5). Altogether, these findings demonstrate that insulin’s actions on glucose and K\(^+\) uptake are independently regulated by dietary fat and K\(^+\) content. A correlation between K\(^+\) intake and insulin-sensitive K\(^+\) clearance is physiologi-

Fig. 4. Effect of 0%, 0.33%, and 1.0% K\(^+\) diets on renal ROMK regulation. A: representative immunoblots of mixed renal cortex and outer medulla show the expression of cSrc in 80 μg of homogenate protein. The blot was reprobed with β-actin to verify equivalent loading. Compared with 1.0% K\(^+\) diet, cSrc expression was increased by 50 ± 20% in rats fed the 0.33% K\(^+\) diet and by 110 ± 20% in rats fed the 0 K\(^+\) diet (n = 5 sets). B: immunoblot analysis of tyrosine-phosphorylated ROMK level in mixed renal cortex and outer medullary samples after immunoprecipitation of ROMK channels, followed by immunoblot analysis with 4G10 used as the probe, an antibody that reacts with PTK and with anti-ROMK to verify equivalent levels of ROMK in samples. Compared with rats fed the 1.0% K\(^+\) diet, ROMK phosphorylation was increased by 50 ± 20% in rats fed the 0.33% K\(^+\) diet and by 150 ± 20% in rats fed the 0 K\(^+\) diet (n = 5 sets).

knowledge, there have been no studies conducted to date of humans who consumed diets with K\(^+\) restricted to approximately one-third of a normal diet, analogous to this study, in which plasma [K\(^+\)] was maintained within a normal range. The recent Institute of Medicine (IOM) report on dietary reference intakes for water, K\(^+\), Na\(^+\), Cl\(^-\), and sulfate (http://www.nap.edu/catalog/10925.html) recommended “adequate intake” of K\(^+\) set at 4.7 g (120 mmol/day) for all adults to decrease blood pressure, reduce the risk of kidney stones, and possibly decrease bone loss. However, the IOM study also reported that at present, dietary K\(^+\) intake is considerably lower than this recommendation in the United States, where the median intake has been measured at 2.8–3.3 g (72–84 mmol)/day for men and 2.2–2.4 g (56–61 mmol)/day for women. Because the current median K\(^+\) consumption is only from ∼50% (women) to ∼60% (men) of that recommended by the IOM, we conclude that in the United States, the consumption of modestly reduced K\(^+\) diets maintains plasma [K\(^+\)] within a normal range. The results of this study in rats suggest that the hallmark of such a K\(^+\)-poor diet would be pronounced renal K\(^+\) conservation, even if plasma [K\(^+\)] were maintained within the normal range. Humans who consumed a diet containing only 20 mM K\(^+\)/day (one-sixth of the IOM-recommended amount) for 9 days did not maintain normal serum [K\(^+\)]; rather, serum [K\(^+\)] fell from 4.1 to 3.5 mmol/l. In addition, there was evidence of elevated blood pressure, renal Na\(^+\) and K\(^+\) retention, and increased free water clearance (10). Whether some or all of these changes would be evident in individuals with normal serum [K\(^+\)] remains to be determined.

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Fig. 5. Lack of effect of 0.33% K\(^+\) diet on K\(^+\) transporter abundance. Homogenates were prepared as described in METHODS from renal medulla and whole gastrocnemius from 6 pairs of rats fed the 1.0% vs. the 0.33% K\(^+\) diet for 15 days. A constant amount of homogenate protein was resolved by SDS-PAGE: 60 μg for ROMK, 16 μg for the colonic isoform of H^+-K^+-ATPase, 40 μg for the gastric isoform of H^+-K^+-ATPase, and 24 μg of whole gastrocnemius protein for the Na^+-K^+-ATPase α2-isoform. Blots were probed with specific antibodies as described in METHODS. No significant differences were detected; 3 representative samples from each group are shown.
cally advantageous, because theoretically it would blunt acute hypokalemia after a K⁺-poor, carbohydrate-rich meal. The molecular mechanisms responsible for the differential sensitivity of glucose transport vs. K⁺ transport to insulin remain to be determined.

During hypokalemia, the kidneys are capable of remarkable K⁺ conservation, excreting only 1% of the filtered load. Hypokalemia brought on by a 0% K⁺ diet is also accompanied by urinary concentration defect and changes in the expression of many renal transporter proteins (8) as discussed above. In the present study, when rats were fed 0.33% K⁺ diets, pronounced K⁺ conservation was observed, despite a lack of change in plasma [K⁺] and no accompanying diuresis. We postulate that filtered K⁺ load is constant and that there is a specific change in renal K⁺ handling with no accompanying change in Na⁺ handling along the nephron, because urinary volume and urinary [Na⁺] are unaltered; thus the urinary concentration defect and changes in transporter protein levels appear to be secondary to the fall in plasma [K⁺]. The one renal protein that did increase in response to the 0.33% K⁺ diet was the PTK cSrc. Previous studies conducted at the Wang laboratory (28) established that the abundance of PTKs, including cSrc and cYes, increase as early as 1 day after initiation of the 0 K⁺ diet in rats and that there is a strong correlation between the duration of the 0 K⁺ diet, the increase in cSrc expression, and the stimulatory effect of PTK inhibitors on apical small-conductance K⁺ channel activity measured in isolated tubules, with the latter representing a measure of channels inactivated by phosphorylation. Subsequent studies conducted by the Wang group established that the activation of PTK causes phosphorylation of ROMK and retraction of ROMK out from the apical membrane and into internal vesicles (14, 15). The present study establishes that cSrc expression and ROMK tyrosine phosphorylation increase, even though plasma [K⁺] does not fall.

The maintenance of chronic K⁺ balance traditionally has been understood on the basis of the concept of negative feedback control (12, 13); during K⁺ restriction, decreased plasma [K⁺] decreases renal K⁺ excretion directly and indirectly (by decreasing aldosterone secretion), which normalizes extracellular [K⁺] level (11). Rabinowitz (22, 23) challenged this view and proposed that K⁺ excretion can be increased without or before increases in extracellular [K⁺] via a mechanism controlled by sensing of K⁺ intake (i.e., sensing of local increases in [K⁺] in splanchic areas during K⁺ intake), representing a feedforward control concept. The results of the present study indicate that there is a set of changes that can occur as a function of decreased K⁺ intake without a change in plasma [K⁺] and another set that occurs when plasma [K⁺] falls. A crucial component of feedforward control is sensing of K⁺ intake. Morita et al. (20) recently provided evidence for K⁺ sensors in the hepatoporal region, specifically that hepatic afferent nerve activity increased in response to intraportal K⁺ injection in a dose-dependent manner and that intraportal KCl infusion elicited immediate kaliuresis with no significant change in plasma [K⁺]. Further studies are warranted to confirm and characterize K⁺-sensing mechanisms in the hepatoporal region or in other splanchic regions. The theoretical advantage of feedforward control is that it could promote rapid control of output function in anticipation of a change, in this case preventing a fall in plasma [K⁺], which may be critical to prevent fluctuations that would compromise cardiac function. The combination of both feedforward and feedback control mechanisms may provide both the speed and stability to potassium homeostasis.

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