MicroRNA 802 Stimulates ROMK Channels by Suppressing Caveolin-1

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ABSTRACT

Dietary potassium stimulates the surface expression of ROMK channels in the aldosterone-sensitive distal nephron, but the mechanism by which this occurs is incompletely understood. Here, a high-potassium diet increased the transcription of microRNA (miR) 802 in the cortical collecting duct in mice. In addition, high-potassium intake decreased the expression of caveolin-1, whose 3' untranslated region contains the seed sequence of miR-802. In vitro, expression of miR-802 suppressed the expression of caveolin-1, and conversely, downregulation of endogenous miR-802 increased the expression of caveolin-1. Sucrose-gradient centrifugation suggested that caveolin-1 closely associated with ROMK channels, and immunoprecipitation showed that caveolin-1 interacted with the N terminus of ROMK. Expression of caveolin-1 varied inversely with the expression of ROMK1 in the plasma membrane, and caveolin-1 inhibited ROMK1 channel activity. Removal of the clathrin-dependent endocytosis motif from ROMK1 failed to abolish the effect of caveolin-1 on ROMK1 channel activity. Last, expression of miR-802 increased ROMK1 channel activity, an effect blocked by coexpression of caveolin-1. Taken together, miR-802 mediates the stimulatory effect of a high-potassium diet on ROMK channel activity by suppressing caveolin-1 expression, which leads to increased surface expression of ROMK channels in the distal nephron.

sis,14 and cell signaling,15,16 and have been identified in the kidney. Several miRNAs have been shown to play a role in regulation of salt-sensitive hypertension.17,18 However, the role of miRNAs in regulating the effect of HK intake on ROMK channels and K secretion has not been explored. We carried out an miRNA microarray assay in the mouse kidney to examine the effect of HK intake on miRNA profiles and observed that HK intake alters the expression of several miRNAs including miR-802 in the kidney (data not shown), suggesting the possibility that miR-802 regulates ROMK channel activity during high K intake. The aim of this study is to explore the potential target of miR-802 in the collecting duct and to determine the role of miR-802 in regulating ROMK channel activity.

RESULTS

HK Intake Increases miR-802

We first carried out Northern blot experiments to examine whether HK intake stimulated the expression of miR-802 in the mouse kidney using a32P-labeled probe containing oligonucleotides (5’ AAGGATGAATC TTT GTTACTGA) complementary to mmu-miR-802. Figure 1 is a representative Northern blot from four such experiments showing that HK intake increased the expression of the mature form of miR-802 by 120 ± 40% in the mouse kidney. In contrast, HK intake did not affect the expression of U6. The observation that HK intake increased miR-802 expression was also confirmed with real-time PCR experiments showing that HK intake significantly increased miR-802 expression in the mouse kidney in comparison with those on a normal potassium (NK) or low potassium (LK) diet (data not shown). Because HK intake stimulates renal K secretion in the CCD, we examined miR-802 expression in the isolated mouse CCD. We harvested RNA from the isolated CCD of mice on a HK or NK diet for 7 days and carried out qRT-PCR using a pre-miR-802 primer or GAPDH primer to amplify pre-miR-802 and GAPDH mRNA. Their products were subjected to electrophoresis with an agarose gel, and Figure 1B is an agarose gel showing the expression of pre-miR-802 (97 bp) in the CCD. Moreover, HK intake increased the expression of pre-miR-802 in the CCD. Figure 1C summarizes the results from four such experiments showing that HK intake significantly increased pre-miR-802 expression by 140 ± 50% in the CCD in comparison with those on a NK diet.

![Figure 1](image-url)

**Figure 1.** HK intake increases miR-802 transcription. (A) Northern blot shows the effect of HK intake on the mature form of miR-802 and U6 in the mouse kidney. The sequence of oligo probe (mmu-miR-802) used for the Northern blot was 5’ AAGGATGAATC TTT GTTACTGA and the probe was labeled with [γ-32P]. The RNA isolated from the kidney was hybridized with the probe, and the miR-802 was detected by x-ray film. (B) An agarose gel shows the expression of mouse pre-miR-802 (97 bp, NT_039625.7/Mm16_39665_37: C28075137-28075041) amplified from the real-time PCR reaction (30 cycles) in the CCD. GAPDH mRNA isolated from the CCD was used as control. (C) A bar graph summarizes results of real-time PCR experiments showing the relative expression of pre-miR-802 in the isolated CCD of mice on a HK or NK diet. (D) Putative anneal site of mature mouse miR-802 to the mouse or rat caveolin-1 to 3’UTR. The bold fonts indicate the seed sequence.
miR-802 Regulates the Expression of Caveolin-1

After showing that HK intake stimulates miR-802 expression in the mouse kidney and in the CCD, we searched potential target proteins for miR-802 through databases such as Targetscan, Miranda, and miRDB. We noticed that caveolin-1 to 3′ UTR contains a putative binding site of miR-802 at 1771 to 1777 (mouse) or 1773 to 1779 (rat) (Figure 1D). To test whether miR-802 regulates caveolin-1 expression through interacting with its 3′ UTR, we cloned a 2-kb region of the 3′ UTR of the human caveolin-1 gene surrounding the predicted hsa-miR-802 anneal site downstream of a constitutively active luciferase cassette. HEK cells were transfected with the construct containing the caveolin-1 3′ UTR (Cav1–3′UTR) or a mutant of Cav1–3′ UTR (Cav1–3′UTR MUT) in which the seed sequence for hsa-mir-802 was deleted. Co-transfection of pre-miR-802 significantly decreased activity of the luciferase reporter construct containing Cav1–3′UTR (Figure 2A) but not Cav1–3′UTP mutant lacking two putative seed sequences (2606 to 2612 and 2625 to 2630), suggesting that miR-802 is able to modulate the 3′ UTR of caveolin-1.

We next examined whether inhibiting endogenous miR-802 expression would alter the translation of endogenous caveolin-1 in HEK293 cells. We used a method called “microRNA-sponge,”19 in which eight-repeated oligonucleotides complementary to hsa-miR-802 (miR-802-sponger) were incorporated into CXCR4 sponge plasmid. Presumably, miR-802 sponger could “absorb” the mature form of miR-802 in HEK cells, thereby modulating the effect of the endogenous miR-802 on its target genes. Figure 2B is a Western blot showing that transfection of HEK293 cells with the miR-802 sponge significantly increased the expression of endogenous caveolin-1 by 70 ± 10% (n = 5). We also examined whether increasing the expression of miR-802 had an opposite effect and decreased the expression of endogenous caveolin-1 in HEK cells transfected with the hsa-mir-802. Figure 2C is a Western blot showing that the expression of miR-802 decreased the expression of endogenous caveolin-1 by 50 ± 10% (n = 5). Similar results were observed in M-1 cells transfected with a mouse miR-802 mimic. Figure 2D is a Western blot showing that transfection of M-1 cells with commercial available miR-802 mimic for 24 hours decreased the expression of endogenous caveolin-1 by 40 ± 7% (n = 5), whereas application of control oligonucleotides (control) had no significant effect on caveolin-1 expression in comparison to Mock group.

HK Decreases the Expression of Caveolin-1
If miR-802 regulates caveolin-1 expression, we suspect that HK intake should decrease the expression of caveolin-1 because HK intake increases miR-802 expression. This idea was tested by examining the effect of HK intake on caveolin-1 expression.
in the kidney. From inspection of Figure 3A, it is apparent that HK intake decreased the expression of caveolin-1 in both rat and mouse kidneys. Figure 3A also shows that LK intake or K depletion (KD) slightly increased the expression of caveolin-1. The effect of HK intake on caveolin-1 expression was specific because dietary K intake failed to alter the expression of caveolin-2 in the mouse kidney (Figure 3B) and rat kidney (data not shown).

Caveolin-1 Is Associated with ROMK1
Because ROMK channel activity is regulated by dietary K intake, we speculate whether caveolin-1 is involved in mediating the effect of dietary K intake on ROMK channels. Thus, we examined whether caveolin-1 and ROMK were located in the same micro-domain. We used a detergent-free purification method to extract ROMK and caveolin-1 from the mouse kidney. The extracts were analyzed in parallel by centrifugation in a 5 to 45% continuous sucrose gradient. After centrifugation, we collected fractions (200-μl sample) from the bottom to the top, and a total of 60 fractions were harvested. The result of a typical experiment is shown in Figure 4A, showing that ROMK channels were located in caveolin-1–rich fractions from 48 to 54. Caveolin-1 has been shown to bind with endothelial nitric oxide synthase (eNOS), Src kinase (c-Src), and vascular endothelial growth factor (VEGF) receptor, and the putative caveolin-1 binding site has a conserved sequence, such as ΦXΦXXXΦ or ΦXXXXΦXXΦ, where X stands for any amino acid and Φ stands for aromatic amino acid residues (Trp, Phe, Tyr). Through analyzing the ROMK1 sequence, it showed that ROMK1 has three putative caveolin-1 binding motifs in the N terminus (amino acid sequence 62 to 69; 77 to 84; 93 to 100). Thus, we examined whether ROMK1 was associated with caveolin-1 using immunoprecipitation in HEK cells transfected with flag-tagged ROMK1. Figure 4B is a Western blot showing that ROMK1 was co-immunoprecipitated with endogenous caveolin-1. Furthermore, we transfected HEK293 cells with flag-tagged ROMK1 N terminus (ROMK1’s amino acids sequence between 1 and 83) or C terminus (between 181 and 391) and immunoprecipitated cell lysates with flag antibody to determine whether caveolin-1 was co-immunoprecipitated with the ROMK1 N terminus or C terminus. Figure 4C is a Western blot showing that caveolin-1 is immunoprecipitated with the ROMK1 N terminus but not C terminus.

Caveolin-1 Decreases the Surface Expression of ROMK1
Because caveolin-1 has been shown to regulate protein exocytosis and endocytosis, we next examined whether caveolin-1 also regulated ROMK1 channel expression in the plasma membrane using a biotin-labeling technique in M-1 cells transfected with GFP-ROMK1. Figure 5A is a Western blot showing that transfection of M-1 cells with caveolin-1 for 48 hours decreased the surface expression of ROMK1. In contrast, downregulation of endogenous caveolin-1 with siRNA increased the surface expression of ROMK1. Figure 5B is a Western blot showing that treatment of the cells with two kinds of caveolin-1 siRNA for 48 hours increased ROMK channel expression in the plasma membrane of M-1 cells. Data summarized in Figure 5C show that expression of caveolin-1 decreased (by 45 ± 10%), whereas downregulation of caveolin-1 increased (by 55 ± 25%, n = 5) the surface expression of ROMK1 in comparison to the corresponding control.

Caveolin-1 Inhibits ROMK1 Activity
The notion that caveolin-1 may be involved in regulating ROMK channels activity was further tested with the patch-clamp techniques in HEK293 cells transfected with caveolin-1 and GFP-ROMK1. We carried out the perforated whole cell recording to measure the Ba^{2+}-sensitive K currents at 3-hour intervals in the presence or absence of caveolin-1 in GFP-positive cells detected with fluorescence microscope 24 hours after transfection. The reason for using HEK293 cells rather than M-1 cells is that HEK293 cells have low endogenous K currents (<50 pA) in comparison to those of M-1 cells (close to 1000 pA). Expression of caveolin-1 did not significantly decrease K currents in HEK cells transfected with GFP-ROMK1 until 48 hours (data not shown). However, the numbers of ROMK-positive transfected HEK cells decreased sharply 48 hours after transfection. Also, it is difficult to form a high resistance seal in those cells. Therefore, we conducted the patch-clamp experiments 24 hours after transfection in HEK cells treated with brefeldin A (BFA), an agent that blocks the protein export from ER to Golgi apparatus, to disturb the dynamical balance between delivery and endocytosis. Figure 6A is a set of whole cell
channel recordings showing K currents in HEK293 cells transfected with GFP-ROMK1 or GFP-ROMK1 + caveolin-1 in the presence of BFA. It is apparent that the co-expression of caveolin-1 caused a larger decrease in K currents in comparison with those without co-transfection of caveolin-1. Figure 6B summarizes the experimental results showing that expression of caveolin-1 did not significantly affect the basal level of ROMK1 channel activity (no caveolin-1, 1080 ± 20 pA; plus caveolin, 1070 ± 30 pA). However, K currents decreased progressively in the presence of or in the absence of caveolin-1 in cells treated with BFA. Moreover, the co-expression of caveolin-1 caused a larger inhibition of ROMK1 channels in comparison with those without caveolin-1, suggesting that caveolin-1 may either enhance internalization or diminish BFA-insensitive export of ROMK1 channels. Data summarized in Figure 6B show that K currents decreased after a 6-hour BFA treatment from 1070 ± 30 to 250 ± 17 pA in caveolin-1-expressing cells, whereas it decreased from 1080 to 550 ± 25 pA in the absence of caveolin-1. In contrast, ROMK currents slightly decreased for 6 hours in the absence of BFA (K currents, 1010 ± 40 pA in cells treated with methanol—a solvent for BFA).

**Inhibition of Caveolin-1 Prevents ROMK1 Channel Activity Declining**

We also carried out whole cell recording in HEK cells transfected with GFP-ROMK1 to examine the effect of downregulation of caveolin-1 on K currents. Figure 7A is a whole cell recording showing that downregulating endogenous caveolin-1 with siRNA not only increased K currents, presumably through downregulation of endogenous caveolin-1 that inhibited ROMK channels, but also prevented the K current declining in the presence of BFA. In addition, the expression of dominant negative caveolin-1, CAVS80E, in which serine 80 was mutated, also prevented the K current decline in HEK cells transfected with GFP-ROMK1 + CAVS80E (Figure 7B). Figure 7C summarizes the results showing that K currents in HEK cells transfected with ROMK1 + CAVS80E were only modestly smaller (832 ± 20 pA) after 6-hour treatment of BFA than those in the absence of BFA (1070 ± 27 pA). In contrast, the expression of caveolin-1 decreased K currents from 1090 ± 35 to 257 ± 20 pA in HEK cells treated with BFA.

**Deletion of ROMK1’s Clathrin-Endocytosis Motif Fails to Abolish the Effect of Caveolin-1**

ROMK1 channels have been shown to have a tyrosine-based clathrin-dependent endocytosis motif on the C terminus. To determine whether caveolin-1–dependent inhibition of ROMK channels required the tyrosine-based clathrin-dependent endocytosis motif, we examined the effect of caveolin-1 on K currents in HEK293 cells transfected with GFP-ROMK1Δ373-378 (R1Δ373-378), in which the clathrin-dependent endocytosis motif was deleted. Figure 8A is a typical whole cell recording showing that K currents in HEK cells transfected with R1Δ373-378 were not significantly changed within 6 hours in the presence of BFA. However, co-expression of caveolin-1 progressively decreased K currents in the presence of BFA (Figure 8B), although the effect was diminished. Data summarized in Figure 8C show that R1Δ373-378 expression not only increased K current from 1090 ± 35 to 1620 ± 52 pA but also prevented a K current decrease (1503 ± 50 pA, 6 hours after BFA). However, the expression of caveolin-1 decreased K currents to 1010 ± 25 pA in the cells transfected with R1Δ373-378 and treated with BFA for 6 hours. This suggests
that caveolin-1–induced inhibition of ROMK1 channels was at least in part the result of either stimulating clathrin-independent endocytosis or suppressing BFA-insensitive delivery.

**MiR-802 Stimulates ROMK1**

After showing that caveolin-1 regulated ROMK activity, we examined the effect of miR-802 on ROMK1 channels. We measured K currents with the perforated whole cell recording in HEK cells transfected with GFP-ROMK1 + vector, GFP-ROMK1 + pre-miR-802, or ROMK1 + pre-miR-802 + caveolin-1 for 24 hours. Results summarized in Figure 9A show that the expression of pre-miR-802 not only increased K currents under control conditions from 1100 ± 40 to 1460 ± 50 pA (without BFA) but also prevented the ROMK channel activity declining in the presence of BFA for 6 hours (1350 ± 40 pA; n = 5). Figure 9B is an agarose gel showing that expression of pre-miR-802 (1 μg) increases pre-miR-802 by 160 ± 40% (n = 4). Moreover, the effect of miR-802 on ROMK channel was dose-dependent because a decrease in pre-miR-802 vector concentration to 0.5 μg per dish modestly increased K currents to 1290 ± 50 pA (n = 3; data not shown), which was significantly lower than those with 1 μg (1460 pA). The effect of miR-802 on K currents was the result of decreasing caveolin-1, because co-expression of caveolin-1 (without 3’ UTR) abolished the effect of pre-miR-802 and decreased K current from 1100 ± 40 to 300 ± 25 pA (n = 5) in the presence of BFA for 6 hours. Figure 9C is a Western blot showing that miR-802 increased the surface expression of ROMK1 channels by 35 ± 5% (n = 3) in M-1 cells. Thus, the expression of miR-802 stimulated ROMK1 channel activity and surface expression.

**DISCUSSION**

miRNAs are highly conserved endogenous small RNA molecules and play an important role in regulating target protein translation. The precursor of miRNA is pre-miRNA, which is further trimmed by RNase III Drosha into pre-miRNA containing one single hairpin structure. Pre-miRNA is exported into the cytoplasm and further processed by dicer into a mature miRNA containing 22 to 24 nucleotides. One strand of the mature miRNA is loaded into the miRNA-induced silencing complex protein and interacts with 3’ UTR of the target mRNA through imperfect base pairing, thereby inhibiting the expression level of the target protein. A large body of evidence has shown that miRNAs play a role not only in regulating renal function under physiologic conditions but may also be involved in the development of pathologic conditions. For example, miR-15a is involved in regulating the expression of the cell cycle regulator cell division cycle25A, and the decrease in miR-15a expression was associated with accelerating cell proliferation and increasing cyst growth. Alteration in miR-192 expression has been reported to be associated with renal fibrosis through modulating TGF-β/Smad signaling.

The main finding of this study is that HK intake stimulates the expression of miR-802 in the kidney including the CCD.
Although an miRNA microarray assay shows that HK intake changed the expression of miRNAs other than miR-802, HK intake caused the largest increase in miR-802 among all miRNAs whose expression was altered by HK intake. The proteins regulated by miR-802 includes methyl-CpG-binding protein, which is involved in either activation or downregulation of its downstream signaling molecules.30 Although more than hundreds of proteins are suggested to be a potential target for miR-802, we focused on caveolin-1 in the initial study because the expression level of caveolin-1 is regulated by HK diet. Two lines of evidence supported the notion that caveolin-1 is regulated by miR-802: (1) expression of miR-802 decreased the luciferase reporter gene activity in cells transfected with caveolin and (2) expression of miR-802 decreased the expression of caveolin-1 in both HEK and M-1 cells. The role of caveolin-1 in regulating ROMK1 channel activity was strongly suggested by the observation that expression of caveolin-1 decreased the surface expression of ROMK channels in the plasma membrane and enhanced K current declining in cells transfected with ROMK1. However, HK intake has been shown to increase ROMK channel activity by >100%.31,32 In contrast, increasing miR-802 to the level similar to those observed by real-time PCR experiments with the CCD only augments K currents by about 40%. Therefore, it is conceivable that HK induced stimulation of ROMK channel activity through a mechanism other than miR-802–caveolin-1 interaction and that miR-802 is only partially responsible for the effect of HK intake on ROMK channels.

The role of caveolin-1 in mediating the effect of miR-802 on ROMK channels is strongly suggested by experiments in which expression of exogenous caveolin-1 abolished the effect of miR-802 on ROMK channels. Our experiments showed that caveolin-1 is closely associated with ROMK1. First, immuno-precipitation of ROMK1 pulled down the caveolin-1, suggesting that ROMK channels are directly or indirectly associated with caveolin-1. Second, ROMK1 was detected in the caveolin-1–rich fraction detected using gradient centrifugation. The mechanism by which caveolin-1 inhibits ROMK1 channel activity is not completely understood. There are three mechanisms by which caveolin-1 may regulate the surface expression of ROMK1: (1) caveolin-1 may stimulate clathrin-dependent or -independent endocytosis of ROMK channels; (2) caveolin-1 could be involved in a BFA-insensitive ROMK1 export to the plasma membrane; and (3) caveolin-1 could provide a platform for interacting with signaling molecules that regulate ROMK channel trafficking.

Relevant to the first possibility is the report that caveolin-1 regulates the internalization of ATP-sensitive K channels.33 Because caveolin-1 induced K current declining was diminished in the cells transfected with R1Δ373-378 in which clathrin-dependent endocytosis motif was deleted it is possible that caveolin-1 may stimulate clathrin-dependent endocytosis of

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**Figure 6.** Expression of caveolin-1 inhibits ROMK channels. (A) Whole cell recordings show that expression of caveolin-1 (CAV) enhances the decrease in K currents in HEK293 cells transfected with ROMK1 and treated with BFA. The experiments were performed 24 hours after transfection with the perforated whole cell recording at the voltage from −100 to 60 mV with a 20-mV increment. (B) A bar graph summarizes the above data from four such experiments at −100 mV. *Significant difference between 0- (no BFA) and 6-hour treatment of BFA; #Significantly different from the rest of the group.
ROMK. One potential mechanism is that caveolin-1 may affect the function of signaling molecules that regulate clathrin-dependent endocytosis. In addition to stimulating clathrin-dependent endocytosis, caveolin-1 is an important component of caveolae, which is responsible for mediating clathrin-independent endocytosis. Although caveolin-1 is highly expressed in the principal cell of the collecting duct, immunochemical staining failed to detect caveolin-1 expression in the apical membrane. Thus, the lack of caveolae in the apical membrane does not favor the possibility that the effect of caveolin-1 on ROMK1 surface expression was the result of stimulating caveolin-dependent and clathrin-independent endocytosis, although this could also not completely be excluded. Moreover, the finding that caveolin-1 still enhanced the K current declining in cells transfected with R1Δ373-378 strongly suggests that the effect of caveolin-1 on ROMK channels is at least partially caused by stimulating clathrin-independent endocytosis or inhibiting BFA-independent export.

In addition to caveolae, caveolin-1 is also expressed in Golgi apparatus and vesicle structure and is an integral component of trans-Golgi network–derived transport vesicles. Several studies have shown that caveolin-1 modulates the intracellular trafficking between organelles: (1) caveolin-1 interacts with angiotensin II type I receptor as a molecular chaperone and is involved in regulating the angiotensin II type I receptor trafficking through the exocytic pathway; (2) caveolin-1 regulates both delivery and endocytosis of the glutamine transporter, excitatory amino acid carrier; and (3) caveolin-1 regulates epithelial Na channel (ENaC) activity by either modulating apical directed export or acting as a platform for securing regulatory molecules in close proximity to ENaC. Although BFA was used to block the BFA-sensitive export pathway, caveolin-1 may modulate ROMK channel delivery process through a BFA-insensitive protein export mechanism.

Relevant to the third possibility is the report that caveolin-1 interacts with signaling molecules and locks them in caveolae, thereby modulating a variety of signal transduction pathways. For instance, caveolin-1 serves as a scaffolding protein to sequester Ras oncogene and heme oxygenase-1 in caveolae. Therefore, caveolin-1 may indirectly regulate ROMK channel trafficking through interacting with signaling molecules that
regulate either endocytosis of ROMK1 or BFA-independent export of ROMK channels. Further experiments are required to explore the mechanism by which caveolin-1 regulates ROMK channel activity. However, it is safe to conclude that caveolin-1 is a target protein of miR-802 in the CCD and is involved in regulating the surface expression. We hypothesized that an HK intake–induced increase in miR-802 expression may be partially responsible for increasing the ROMK1 channel activity in the apical membrane by modulating renal caveolin-1 levels. We conclude that miR-802 plays an important role in stimulating ROMK channel surface expression by down-regulating caveolin-1 expression.

CONCISE METHODS

Animal Preparation
Male Sprague-Dawley rats (5 to 6 weeks; <90 g) or C57BL/6 mice were used in the experiments, and they were fed with an NK diet (1% K), an LK diet (0.1% K), or an HK diet (2.5%). The method for dissecting the CCD has been previously described. The rats and mice were purchased from Taconic Farms (Germantown, NY) and Jackson Laboratory.

Northern Blot
The total RNA and miRNA from the kidney were harvested with an miRNeasy mini kit (Qiagen) for the preparation of Northern blot. Briefly, tissue (50 mg) of the mouse cortex and outer medulla was lysed with 700 μl QIAzol lysis reagent, and the tissue was homogenized with QIAshredder (Qiagen). The tissue lysate was mixed with 140 μl chloroform and centrifuged for 15 minutes at 12,000 rpm. The sample was diluted by adding 100% ethanol (150% of the lysate volume), vortexed, and loaded into the mini-spin column. After centrifugation, the column was washed, and the total RNA sample was eluted in 30 μl RNase-free water. The quality of the RNA was determined by measuring the ratio of A260/A280. After confirming the quality of the RNA, we carried out electrophoresis. For the prerun, the denatured 15% PAGE gel was conducted at 400 V (40 mA) for 1 hour in 0.5 TBE buffer in Protean III tank (Bio-Rad). The RNA sample was denatured by adding the equal volume of sample buffer containing 10 mM EDTA (pH 8.0), 0.05% xylene cyanol, and bromophenol blue in deionized formamide and incubated for 20 minutes at 65°C.

Figure 8. Co-expression of caveolin-1 also inhibits R1Δ373-378. Typical whole cell K currents in HEK293 cells transfected with ROMK1 mutant, R1Δ373-378, in which the tyrosine based endocytosis motif 373-378 was deleted in the absence of caveolin (A) or in the presence of caveolin-1 (B). The currents were measured from −100 to 60 mV, and the K currents measured at −100 mV are summarized in C.
The expression of pre-miR-802 (miR-802) increases ROMK channel activities. (A) The effect of miR-802 on K currents in HEK293 cells transfected with GFP-ROMK1 (1 μg), GFP-ROMK1 + pre-miR-802 (1 μg), and caveolin-1 (1 μg) + GFP-ROMK1 + pre-miR-802. The K currents were measured with the perforated whole cell recording at a voltage of -100 mV 24 hours after transfection. The construct, pSilencerCMV expressing endogenous pre-miR-802, was used to increase miR-802 expression. (B) An agarose gel shows that transfection of pre-miR-802 vector increases pre-miR-802 expression detected with RT-PCR in HEK 293 cells transfected with either pSilencerCMV-pre-miR-802 (1 μg) or pSilencerCMV-vector (control). The approximate 300 bp contained human pre-miR-802 sequence (93 bp, NT011512.10/HS21_11669: 1087–1098, 2011). (C) MiR-802 increases ROMK expression in the plasma membrane. M-1 cells were transfected with ROMK1 with or without pre-miR-802 for 48 hours. The biotin labeled ROMK1 was immunoprecipitated with neutravidin beads and detected with ROMK antibody. The surface expression of ROMK1 was normalized by calculating the ratio between the surface and the total expression of ROMK1.

The denatured RNA sample was subjected to electrophoresis at 200 V in 1× TBE buffer until bromophenol blue reached the edge. After rinsing, the RNA was wet-transferred into positive-charged nylon membrane and cross-linked with UV Stratalinker 1800 (Stratagene). The mmu-miR-802 probe was prepared by labeling 5′ 32P to DNA oligo, 5′AAGGATGAATCTTTGTTACTGA (bold font, the seed sequence). Briefly, the membrane was prehybridized at 35°C for 30 minutes in 10 ml hybrid buffer (50% deionized formamide, 5× Denhardt’s solution, 0.5% SDS, 2 mg/100 ml boiled salmon sperm DNA, 250 mM NaCl, 50 mM Na-phosphate, 10 mM EDTA, pH7.4). The membrane was incubated with 10 pmol 32P-labeled oligo probe overnight at 37°C and was followed by washing the membrane with 2× SSC containing 0.1% SDS three times. The miR-802 band was detected by placing the membrane on an x-ray film that is in a cassette with the intensify screen.

Real-Time PCR to Detect miR-802 Expression in the CCD
The RNA of the isolated CCD was isolated with RNAqueous-Micro kit (Ambion). The cDNA was generated with Affinity Script RT enzyme from Stratagene (La Jolla, CA). Briefly, 1 μl random primer and 100 ng RNA or single tubule lysate was annealed at 95°C by adding 1 μl DTT, dNTP, and enzyme. The mixture was incubated for 1 hour at 65°C. The primers for the pre-miR-802 were designed using BLAST software. Before conducting the single tube PCR, we validated the RT-PCR with the miRNA purified from the kidney as a template. The miR-802 primers (2.5 μl, 12.5 nM) were mixed with 2 μl cDNA (200 ng) and 12.5 μl 2× SYBR Green master. MxPro3000 (Stratagene) was used to carry out the experiments, and we used 2−ΔΔCt to analyze the comparative expression level of miR-802. GAPDH was used as a control to show the specificity of the effect of a HK intake on miR-802.

Cell Culture and Transfection
M-1 and HEK293 cells were used in this study, and the culture methods were described previously. For each transfection experiment, 2 μg of plasmid DNA per dish was mixed with OPTI-MEM 1 culture medium (Life Technologies) containing 6 μl of TransIT-293 (Mirus, Madison, WI). For biochemical experiments, cells were harvested 48 hours after transfection, whereas they were used 24 hours after transfection for the patch-clamp experiments. The methods for cell lysis and sample preparation have been previously described.

Luciferase Assay
A 2-kb region of the 3′ UTR of the caveolin-1 gene surrounding the predicted hsa-miR-802 anneal site was inserted into the XbaI site of the pGL3 vector (Promega) (downstream of a constitutively active luciferase cassette to form caveolin-1 to 3′ UTR construct). HEK cells were transfected with the caveolin-1 to the 3′ UTR construct or a mutant of the caveolin-1 to 3′ UTR in which the seed sequence for miR-802 was deleted. The cells were also co-transfected with synthetic pre-miR-802 or a negative control pre-miRNA inserted into pSilencer-CMV vector and pRL-SV40 (as transfection control). The ratio among PRL-SV40, pSilencer-CMV-miR-802 and pGL-caveolin-1 was 1:4:4 compared with 3′UTR. The luciferase activity was measured by the Dual-Glo luciferase assay system with Glo-max 20/20 luminometer (Promega).

Immunoprecipitation and Western Blot
Renal cortex and outer medulla were dissected, and the tissue was homogenized. Protein lysate (250 μg) was preclared by incubating with 20 μl protein G beads for 1 hour at 4°C, followed by adding either IgG or the corresponding antibody. The sample was incubated overnight at 4°C, sequentially mixed with 25 μl protein G agarose, and rotated for an additional hour. The proteins were resolved by SDS gel and transferred to a PVDF membrane, and the membrane was blocked with infrared imaging block buffer (Rockland) and exposed to the corresponding primary antibodies.
Biotinylation
The HEK or M-1 cells were transfected with GFP-ROMK1 + vector, caveolin-1 + GFP-ROMK1, or GFP-ROMK1 + siRNA for 48 hours. The cells were gently washed and incubated with 2 mg/ml EZ-Link Sulfo-NHS-SS-Biotin in 1× PBS for 1 hour at 4°C. The biotin labeling was ended with 100 mM glycine in 1× PBS to absorb the unbound biotin. The cells were washed twice with 1× PBS and lysed with RIPA buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) in the presence of a protease inhibitor cocktail and phosphatase inhibitor I and II (Sigma). After centrifugation, the protein sample (100 μg in 20 μl) was mixed with 20 μl immobilized NeutrAvidin Gel in 500 μl RIPA buffer and placed on a rocker overnight at 4°C. The beads fraction was harvested, mixed with 2× sample buffer, boiled for 5 minutes, and resolved by electrophoresis. The biotin-labeled ROMK1 was detected with ROMK antibody or GFP antibody. The surface expression of ROMK1 among different groups was normalized by calculating the ratio between the surface and the total expression of ROMK1.

Electrophysiology Experiments
We followed the protocol described previously to measure the Ba²⁺-sensitive K current in HEK cells transfected with GFP-ROMK1 or other constructs. The cell membrane capacitance was measured and compensated, and K currents were presented as picoamperes/25 pF.

Detergent-Free Purification of Caveolin-1 and ROMK-Rich Fractions
The method described by Song et al. was used for the co-purification of caveolin-1 and ROMK in the mouse kidney. Briefly, the renal tissue dissected from the cortex and outer medulla was added to sodium carbonate–containing buffer (pH = 11.0), homogenized with a Polytron tissue grinder, and sonicated to disrupt the cellular membrane. The tissue lysates were centrifuged for 10 minutes at 12,000 rpm, and the supernatant was harvested. We mixed 5 and 45% sucrose in a 1:9 ratio, added sucrose–containing buffer (pH 6.5) into a tube (final volume is 12 ml) per a peristaltic pump for a 5 to 45% continuous sucrose gradient. The tube was allowed to sit in the cold room overnight, followed by adding 200 μg protein on the top of the tube. The tube was sequentially subjected to centrifugation (Optima LE-80K ultracentrifuge; Beckman Instruments, Palo Alto, CA) at 38,000 rpm and 4°C for 20 hours in a SW40Ti rotor (Beckman). To collect the fractions, a fine tube was inserted into the bottom of the centrifuge tube, and a 200-μl sample was collected through a Biologic Biofrac Fraction Collector (Bio-Rad) from the bottom to the top; a total of 60 fractions were harvested.

Experimental Materials and Statistics
Caveolin-1, caveolin-2, and nonimmune rabbit IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA), and ROMK antibody was obtained from Alomone (Jerusalem, Israel). Flag and GFP antibodies were purchased from Sigma (St. Louis, MO) and Clontech Labs (Mountain View, CA), respectively. We purchased miR-802 mimic and corresponding control oligo from Qiagen (Foster City, CA). To increase the miR-802 expression, a 327-bp nucleotide that was amplified by RT-PCR from genomic sequence of miR-802 was inserted into the pSilencer 4.1-CMV vector (Ambion, Austin, TX). The forward and reversed sequences of primers were 5’-TTACG-GATCCCCATATTTCTCCTTGC and 5’-GAACAGTTG-CAGCCTTTGTGTCAT, respectively. For the negative control experiment, we use the pSilencer 4.1-CMV negative control vector provide by the manufacturers. The dominant negative caveolin-1 construct (Cav-1S80E) is a gift from Dr. Michael Robinson, University of Pennsylvania. GFP-ROMK1 or flag-tagged ROMK1 was sub-cloned into pcDNA3 (Invitrogen, San Diego, CA) as described previously. The data are presented as mean ± SEM. We used a paired t test or one-way ANOVA to determine the statistical significance.

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DISCLOSURES
None.

REFERENCES