Effect of dietary K intake on apical small-conductance K channel in CCD: role of protein tyrosine kinase

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Wei, Yuan, Peter Bloom, Daohong Lin, Ruimin Gu, and Wen Hui Wang. Effect of dietary K intake on apical small-conductance K channel in CCD: role of protein tyrosine kinase. *Am J Physiol Renal Physiol* 281: F206–F212, 2001.—We have used Western blot to examine the expression of cSrc protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP)-1D in the renal cortex, and the patch-clamp technique to determine the role of PTK in mediating the effect of dietary K intake on the small-conductance K (SK) channel in the cortical collecting duct (CCD). When rats were on a K-deficient (KD) diet for 1, 3, 5, and 7 days, the expression of cSrc increased by 40, 90, 140, and 135%, respectively. In contrast, the expression of cSrc in the renal cortex from rats on a high-K (HK) diet for 1, 2, and 3 days decreased by 40, 60, and 75%, respectively. However, the protein level of PTP-1D was not significantly changed by dietary K intake. The addition of 1 μM herbimycin A increased NP0, a product of channel number (N) and open probability (P0) in the CCD from rats on a normal diet or on a KD diet. The increase in NP0 was 0.30 (normal), 0.45 (1-day KD), 0.65 (3-day KD), 1.55 (5-day KD), and 1.85 (7-day KD), respectively. Treatment of the CCD with herbimycin A from rats on a KD diet increased NP0 per patch from the control value (0.7) to 1.4 (1-day KD), 1.6 (3-day KD), 2.6 (5-day KD), and 3.5 (7-day KD), respectively. In contrast, HK intake for as short as 1 day abolished the effect of herbimycin A. Furthermore, the expression of ROMK channels in the renal cortex was the same between rats on a KD diet or on a HK diet. Moreover, treatment with herbimycin A did not further increase NP0 in the CCDs from rats on a HK diet. We conclude that dietary K intake plays a key role in regulating the activity of the SK channels and that PTK is involved in mediating the effect of the K intake on channel activity in the CCD.

THE CORTICAL COLLECTING DUCT (CCD) IS RESPONSIBLE FOR K secretion and Na reabsorption. For K secretion, K enters the cell through basolateral Na-K-ATPase and then diffuses into the lumen via the apical K channels (6). Although two types of K channels, Ca2+-activated K channel and the small-conductance K (SK) channel, are identified in the apical membrane of principal cells of the CCD, it is generally agreed that the SK channel is mainly responsible for K exit across the apical membrane (3–4, 7, 10, 18). It is well established that hormones such as aldosterone and vasopressin play an important role in the regulation of K secretion (6). In addition, dietary K intake plays a key role in modulating renal K secretion: an increase in K intake stimulates, whereas a low-K intake inhibits, K secretion (6). The effect of dietary K intake on K secretion is, at least in part, achieved by modifying the number of the SK channels in the CCD. We and others (13, 18) have shown that the number of SK channels increased by three to four times in the CCD from rats on a high-K (HK) diet than from those on a normal diet. The effect of HK intake on channel activity is not mediated by aldosterone because infusion of aldosterone failed to increase the number of the SK channels (13). In previous studies, we have demonstrated that inhibition of protein tyrosine kinase (PTK) increased the number of the SK channels in the CCD obtained from rats on a K-deficient (KD) diet (21). Moreover, blocking protein tyrosine phosphatase (PTP) reversibly decreased the number of SK channels in the CCD from animals on a HK diet (22). Also, we have demonstrated that the protein level and activity of cSrc are significantly higher in the renal kidneys from rats on a KD diet than from those on a HK diet. We have proposed that an increase in tyrosine phosphorylation of SK channels reduces, whereas an increase in tyrosine dephosphorylation augments, the number of SK channels. In the present study, we have extended our investigation by examining changes in protein levels of cSrc and PTP-1D in the renal cortex from rats on a HK diet or on a KD diet. We have also investigated the effect of inhibiting PTK on channel activity to determine the relationship between cSrc expression and channel activity in the CCD.

METHODS

Preparation of CCDs. Pathogen-free Sprague-Dawley rats of either sex (5 wk) were used in the experiments and were purchased from Taconic Farms, (Germantown, NY). The animals were put on a HK diet (wt/wt, 10%) or on a KD diet (Harlan Teklad, Madison, WI) for different days before use. The weight of the rats used for experiments was 100 g. The rats were killed by cervical dislocation, and the kidneys were removed immediately. Several thin slices of the kidney (<1...
mm) were cut and placed in an ice-cold Ringer solution until dissection. The dissection was carried out at room temperature, and two watch-make forceps were used to isolate the single CCD. To immobilize the tubules, we placed them on a 5 × 5-mm cover-glass coated with Cell-Tak (Becton Dickinson, Bedford, MA) and then transferred them to a chamber (1,000 µl) mounted on an inverted Nikon microscope. The CCDs were superfused with HEPES-buffered NaCl solution, and the temperature of the chamber was maintained at 37 ± 1°C by circulating warm water around the chamber. The CCD was cut open with a sharpened micropipette to expose the apical membrane.

**Patch-clamp technique.** An Axon model 200A patch-clamp amplifier was used to record channel current. The current was low-pass filtered at 1 KHz by an eight-pole Bessel filter (model 902LPF; Frequency Devices, Haverhill, MA) and digitized by an Axon interface (Digitada 1200). Data were acquired by an IBM-compatible Pentium computer (Gateway 2000) at a rate of 4 KHz and analyzed using the pClamp software system 6.04 (Axon Instruments, Burlingame, CA). Channel activity was defined as $NP_o$, a product of channel number ($N$) and open probability ($P_o$) that was calculated from data samples of 60 s duration in the steady state as follows:

$$NP_o = \Sigma (1t_1 + 2t_2 + \ldots it_i)$$  \hspace{1cm} (1)

where $t_i$ is the fractional open time spent at each of the observed current levels. The slope conductance of the channel was calculated by measurement of the K current at several observed current levels.

**Tissue preparation for Western blot.** Five to six rats were used for each set of experiments to examine the effect of dietary K intake on cSrc, cYes, and PTP levels in the renal cortex. The rats were from the same clone and were the same age. Five days after receiving them, we started to feed the rats with a different K diet according to the protocol. The plasma Na and K concentrations were measured with flame photometry (Corning 480) in the rats on a normal chow, on a KD diet for 7 days, or on a HK diet for 3 days (Table 1). The rats were killed on the same day, and the renal cortex was cut and homogenized. The tissue was suspended in RIP solution (1:8 ratio, wt/vol) containing (in mM) 10 NaCl, 1 NaF, 1% NP-40 (tergitol), 50 Tris·HCl, 1% Triton (×100), 0.1% SDS, 1.5 NaVO₄, 1 sodium malybdate, 1 paranitrophenyl-phenyl phosphate, and 1 EDTA. For every 125-mg tissue sample, we added a 25-µl cocktail inhibitor of proteases (Sigma, St Louis, MO). The samples were left on ice for 15 min and were followed by homogenization. The homogenized tissue sample was incubated in the presence of DNAse (1 µl) at 4°C for 60 min and followed by centrifuging at 1,790 rpm for 10 min at 4°C. We have taken the supernatant for measuring protein concentrations. We measured protein concentrations of the whole cell extract twice. Only if the difference between two assays was less than 5% did we consider that the measurement was accurate. Moreover, we stained the gel with Co-massie blue to confirm that the amount of proteins was loaded equally. Also, we performed the same Western blot three times for each sample and normalized the results compared with the control value obtained from rats on a normal diet. If the difference among three measurements was less than 10%, we considered the results reliable. Protein samples extracted from the kidney cortex were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline (TBS), rinsed, and washed with 1% milk in Tween-TBS. The PTP-1D, PTP-1B, and PTP-1C antibodies were purchased from Transduction Laboratories (Lexington, KY) and were diluted at 1:1,000. The cSrc and cYes antibodies were obtained from Santa Cruz (Santa Cruz, CA) and were diluted at 1:1,000 and 1:500, respectively. The antibody of cSrc recognizes the epitope corresponding to amino acids 509–533 in the COOH-terminus of cSrc. In addition, the cSrc antibody has a cross-reaction with cYesp62 and Fynp58, two members of the cSrc family. The cYes antibody reacts with the epitope corresponding to amino acids 3–18 in the NH₂-terminus of cYes and has no cross-reaction with other Src PTK. The immunogen of the PTP-1D antibody is located between amino acids 1 and 177 in the NH₂-terminus and has no cross-reaction with other PTPs. The ROMK antibody was obtained from Alomone Laboratories (Jerusalem, Israel) and has been previously characterized by Mennitt et al. (11). The protein concentration used for the immunoblot was 50 µg. The proteins were detected and quantitatively analyzed by fluorescence phosphorimaging.

**Experimental solutions and statistics.** The pipette solution contained (in mM) 140 KCl, 1.8 MgCl₂, and 10 HEPES (pH = 7.4). The bath solution was composed of (in mM) 140 NaCl, 5 KCl, 1.8 CaCl₂, 1.8 MgCl₂, 5 glucose, and 10 HEPES (pH = 7.4). Herbismycin A was purchased from Biomol (Plymouth Meeting, PA) and dissolved in the DMSO solution. The final concentration of DMSO was less than 0.1% and had no effect on channel activity.

Data are shown as means ± SE, and paired or unpaired Student's $t$-test was used to determine the significance between the two groups. Statistical significance was taken as $P < 0.05$.

**RESULTS**

We have previously demonstrated that dietary K intake can affect the expression of cSrc (21, 22). In the present study, we have extended the investigation to examine the time course of the cSrc expression in the kidneys from rats that were maintained on a KD diet from 0 (control), 1, 3, 5, and 7 days. Figure 1A is a typical Western blot showing the expression of cSrc in the renal cortex from animals on a KD diet for 0, 1, 3, and 5 days. It is apparent that the expression of cSrc increased progressively by prolonged KD adaptation. The expression of cSrc was 140 ± 15% (1 day KD, $n = 6$ rats), 190 ± 46% (3 day KD, $n = 6$ rats), and 240 ± 60% (5 day KD, $n = 6$ rats) of the control value, respectively. The cSrc expression in the renal cortex in rats on a KD diet for 7 days was not different from that for 5 days (235 ± 33%, $n = 5$ rats) (data not shown). We also investigated the expression of cYes, a member of the cSrc family, in the renal cortex from rats on a KD diet. From inspection of Fig. 1B, it is clear that the protein level of cYes progressively increased when the

| Table 1. Plasma Na⁺ and K⁺ concentrations |
|----------------|----------------|
| PNa, meq/l | PK, meq/l | n |
| Normal | 147.3 ± 1.0 | 4.00 ± 0.1 | 4 |
| KD, 7 days | 146.7 ± 1.2 | 2.90 ± 0.1* | 4 |
| HK, 3 days | 148.0 ± 1.5 | 4.70 ± 0.2* | 4 |

Values are means ± SE. PNa and PK, plasma Na and K concentrations, respectively; n, no. of rats; KD and HK, K-deficient diet and high-K diet, respectively. *Significantly different, $P < 0.05$. 

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Animals were on a KD diet (1-day, 135 ± 13%; 3-day, 170 ± 20%; and 5-day KD, 190 ± 20% of the control value, n = 4 rats). However, the expression of PTP-1D was not affected by dietary K intake (Fig. 1C, 1 day, 95 ± 13%, 3 days, 115 ± 22%, and 5 days, 110 ± 20% of the control value, n = 6 rats). Moreover, we did not detect the expression of PTP-1B and PTP-1C in the renal cortex (data not shown).

After finding that dietary K intake affected the expression of cSrc, we examined the effect of herbimycin A on the SK channels to determine whether its effect was correlated with the cSrc level. We confirmed the previous finding that inhibiting PTK increased the number of SK channels in the CCD from rats on a KD diet. Figure 2 is a recording illustrating the effect of 1 μM herbimycin A on the SK channel in a CCD from a rat on a KD diet.
rat on a KD diet for 5 days. Inhibiting PTK opened a K channel in a cell-attached patch within 10–15 min. The measurement of channel conductance (35 pS) and analysis of kinetics revealed that the K channel was a typical SK channel. However, the effect of herbimycin A on channel activity depends on the duration of K depletion because the addition of herbimycin A increased the channel activity only in one out of nine cell-attached patches from rats on a KD diet for 1 day (Table 2). A prolonged time for KD adaptation progressively increased the responsiveness of the SK channels to the agent was observed in 3 out of 10 patches (3-day KD), 4 out of 8 patches (5-day KD), and 16 out of 22 patches (7-day KD), respectively (Fig. 4). Figure 3 shows that inhibiting a PTK-induced increase in NP$_0$/patch was 0.3 ± 0.3 (normal, n = 9), 0.45 ± 0.3 (1-day KD, n = 10), 1.55 ± 0.5 (5-day KD, n = 8), and 1.85 ± 0.3 (7-day KD, n = 22), respectively. The data represent a mean increase in NP$_0$, including the experiments in which the response to herbimycin A was negative (zero increase).

Our findings and those of others (15) confirmed that NP$_0$ was not significantly different between rats on a normal diet or on a KD diet and were 0.75 ± 0.2 (normal, n = 33 patches), 0.7 ± 0.2 (1-day KD, n = 46 patches), 0.65 ± 0.15 (3-day KD, n = 50 patches), 0.62 ± 0.15 (5-day KD, n = 31 patches), and 0.6 ± 0.15 (7-day KD, n = 43 patches), respectively. However, treatment of the CCD with herbimycin A (1 μM) for 20 min increased the NP$_0$/patch to 1.3 ± 0.2 (normal, n = 47 patches), 1.4 ± 0.3 (1-day KD, n = 71 patches), 1.60 ± 0.2 (3-day KD, n = 78 patches), and 2.6 ± 0.3 (5-day KD, n = 49 patches), and 3.5 ± 0.4 (7-day KD, n = 67 patches), respectively (Fig. 4).

In previous experiments, we observed that inhibiting PTK with herbimycin A cannot increase the channel activity in the CCD from rats on an HK diet for 10 days (21). Furthermore, we have demonstrated that the cSrc expression decreased in the renal cortex from rats on an HK diet (21). We have now expanded our study to examine the time course of cSrc expression in the renal cortex from rats on a HK diet. Figure 5 is a Western blot demonstrating the protein level of cSrc and PTP-1D in the renal cortex from rats on a HK diet. Figure 5 is a Western blot demonstrating the protein level of cSrc and PTP-1D in the renal cortex from rats on a HK diet.
consistent with previous observations that the PTP-1D expression was not affected by an HK intake.

We confirmed that NP increased by 4-fold, from \(0.75 \pm 0.2\) to \(3.4 \pm 0.4\) in the CCD from rats on an HK diet for 24 h (15). This increase was not the result of changing the protein level of the SK channels. Figure 6 is a Western blot demonstrating that the ROMK channel protein in the renal cortex and renal medulla in rats on a KD diet was even slightly higher (120 \(\pm 18\%\)) than those on a HK diet, although the difference is not significant \((n = 4\) rats). It is generally believed that the ROMK channel is the key component of the native SK channels in the CCD and in the thick ascending limb (14, 20). We have also investigated the effect of herbimycin A on the activity of the SK channels in the CCD from rats on a HK diet. The addition of herbimycin A (1 \(\mu M\)) failed to increase the number of the SK channels in the CCD from rats on a HK diet (Table 2). Moreover, treatment of the tubule with herbimycin A did not increase the channel activity. Figure 7 summarizes the results of experiments in which NP was measured after the tubules were treated with herbimycin A (1 \(\mu M\)) for 20 min. It is apparent that the effect of herbimycin A on channel activity was completely absent in the CCD from rats on an HK diet. The NP from the CCD treated with herbimycin A was \(3.5 \pm 0.4\) (1-day HK, \(n = 33\) patches), \(3.6 \pm 0.3\) (2-day HK, \(n = 21\) patches), and \(3.7 \pm 0.3\) (3-day HK, \(n = 27\) patches), which are not significantly different from the corresponding values from untreated CCDs.

**DISCUSSION**

The main finding of the present study is that the protein levels of cSrc increased progressively when the rats were maintained on a KD diet whereas they decreased when the rats were on a HK diet. Moreover, we have demonstrated that the effect of herbimycin A on channel activity was augmented progressively in proportion to the increase in cSrc levels. We confirmed Palmer’s observation that the basal level of NP in the CCD from rats on a normal diet for 7 days was not significantly different from that in rats on a normal diet (13). However, the effect of herbimycin A on the channel activity in the CCD from rats on a normal chow was significantly smaller than that observed in the rats on a KD diet for 7 days. Moreover, treatment of the tubule with herbimycin A did not increase channel NP in the CCD from rats on a normal chow to the extent that was observed in the rats on a KD diet. In contrast, inhibiting PTK increased NP in the CCDs from rats on a KD diet for 7 days to the same level as those observed in rats on a HK diet. This suggests that the longer the animals are on a KD diet, the larger the PTK-sensitive pool of SK channels.

We speculate that there could be three pools of SK channels in the CCD under physiological conditions. The first group of SK channels is open and actively involved in K exit across the apical membrane. The second group of SK channels is phosphorylated by PTK and internalized or subjected to endocytosis. The third group of SK channels is not phosphorylated by PTK; however, it remains inactive. When rats are on the KD diet, the expression levels and activity of cSrc increase. Therefore, the tyrosine phosphorylation of SK channels by PTK is enhanced, and the population of the second group is expanded. Accordingly, inhibiting PTK with herbimycin A increases the number of SK channels in the CCD from rats on a KD diet. In contrast, when the animals are on an HK diet, the number of the
active SK channels (pool 1) must increase. Moreover, because the PTK level falls, the population of SK channels that are phosphorylated by PTK is diminished. In this case, inhibition of PTK cannot stimulate the channel activity. In addition, it is possible that the number of the silent SK channels that are not phosphorylated by PTK may also decrease. Also, it is conceivable that only a small fraction of SK channels are phosphorylated by PTK in the CCD from rats on a normal diet. This view is supported by the observation that inhibiting PTK can only modestly increase the channel activity.

Also, we found that the expression of ROMK channels in the renal cortex and medulla from animals on a HK diet was not significantly different from that in those on a KD diet. Because ROMK channels are located in the CCD and cortical thick ascending limb (1), this suggests that the expression of ROMK channels in the CCD may not be affected by dietary K intake. Interestingly, it was recently reported that the ROMK protein level in the membrane fraction decreased in the renal cortex and medulla in kidneys from rats on a KD diet (11). Because we used the homogenized tissue to detect the expression of ROMK channels in the renal cortex and medulla, the difference in the ROMK channel expression between the membrane fraction and the whole homogenized tissue strongly suggests that a significant fraction of ROMK channels are located in intracellular compartments. Our previous observation that inhibiting PTK increased $N_p$ in the CCD from rats on a KD diet for 10 days to the same extent as that observed in rats on a HK diet indicated that a low-K intake-induced decrease in channel activity is mediated by a PTK-dependent endocytosis (21). This notion was further supported by experiments in which inhibiting PTP reduced the number of the SK channels in the CCD from rats on a HK diet, and the effect could be blocked by 20% sucrose that blocks the endocytosis (22). Recently, we further demonstrated that stimulation of PTK increases the endocytosis of ROMK1 in oocytes expressing cSrc and ROMK1 (12). Therefore, our data strongly suggest that PTK plays a key role in mediating the effect of dietary K intake on the K secretion.

It has been shown that the channel activity increased significantly when rats were on a HK diet for as few as 6 h (15). The stimulatory effect of HK intake on the SK channels is not the result of increasing transcription of the gene encoding ROMK channels (5) or increasing the expression of ROMK channels (11). Also, the effect of HK intake cannot be mimicked by aldosterone infusion (13). Although we did not measure the level of cSrc in the renal cortex 6 h after the rats were on an HK diet, it is unlikely that a decrease in PTK expression and dephosphorylation of the SK channels by PTP could account for the HK-induced increase in the channel activity. This conclusion is based on the observation that inhibition of PTK in the CCD from rats on a normal diet did not mimic the effect of an HK diet on channel activity. Therefore, it is possible that the rapid increase in channel activity observed in the CCD from rats on a HK diet for 6 h may have resulted from opening the previously silent SK channels that were not phosphorylated by PTK and that were located in the cell membrane. This hypothesis that the ROMK channel expression in the membrane fraction is not significantly different between the animals on a normal diet or an HK diet is supported by Mennitt et al. (11). However, the signal transduction pathway activating the silent SK channels in response to an increase in K intake is still unclear. Because cAMP-dependent protein kinase [protein kinase A (PKA)] has been shown to play an important role in stimulating SK channels acutely (2, 19), it is possible that PKA may be involved in mediating the effect of an HK intake on channel activity in the CCD. In addition to PKA, we have previously demonstrated that protein kinase C (PKC) and calmodulin-dependent kinase II are also involved in regulating the SK channels (9, 19). Therefore, it is conceivable that an HK diet may stimulate channel activity by downregulating PKC and calmodulin-dependent kinase activity. It is likely that PTK is responsible for modulating the SK channels chronically in response to changing K intake whereas PKA/PKC is involved in regulating channel activity acutely. Further experiments are needed to prove this hypothesis.

In the present study, we confirmed the previous finding that PTP-1D is the major isoform of PTP. In addition, the expression of PTP-1D is not significantly regulated by K intake. Because the tyrosine phosphorylation and dephosphorylation are controlled by an interaction between PTP and PTK (8, 16), changes in PTK expression and activity should be mainly responsible for altering the tyrosine phosphorylation process: an increase in PTK levels should favor tyrosine phosphorylation whereas a decrease in PTK should facilitate tyrosine dephosphorylation. However, changes in PTP activity can still have an important role in regulating the tyrosine phosphorylation/dephosphorylation process. For instance, we have previously demonstrated that inhibiting PTP decreased the $N_p$ of the SK channels in the CCD from rats on an HK diet and that the effect of phenylarsine oxide was abolished by inhibiting PTK (22). This suggests that PTK in the CCD from rats on an HK diet is still active. Therefore, inhibiting PTP can still enhance the PTK-dependent tyrosine phosphorylation in the CCD from rats on an HK diet. Thus it is still possible that the activity of PTP-1D is different in the CCD from rats on a HK diet or on a KD diet. Also, as only cSrc and cYes have been closely examined in this study, it is conceivable that other isoforms of PTK are involved in mediating the effect of dietary K intake on channel activity. We have chosen cSrc and cYes in the present study because both PTKs are the most abundant in the Src family and are ubiquitously distributed in different tissues such as the kidney (17). It is well established that cSrc has been demonstrated to play an important role in regulating a variety of cell functions including modulating channel activity (17). The mechanism by which a low-K intake increases the cSrc PTK level is not clear.
K depletion has an effect on cell proliferation and possibly could change the population of collecting duct cells in the kidney. Although we could not completely exclude the possibility that increasing cSrc PTK levels are partially the result of growing collecting duct cells, it is most likely that increasing cSrc levels are the result of stimulating cSrc expression in the previously existing cells. This conclusion is based on our observation (Wang W, unpublished observations) that an increase in protein expression in the renal cortex from rats on a KD diet can only be observed in <5% of protein bands detected by Comassie blue staining gel compared with control samples. This suggests that increasing cSrc PTK expression in the renal cortex from rats on a KD diet did not result from a nonspecific stimulation of protein synthesis.

We conclude that the interaction between PTK and PTP plays a key role in mediating the effect of dietary K intake on the SK channels in the CCD. K depletion increases the expression of PTK and stimulates tyrosine phosphorylation. In contrast, HK intake decreases the PTK levels and augments tyrosine dephosphorylation.

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