Low Na intake suppresses expression of CYP2C23 and arachidonic acid-induced inhibition of ENaC

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THE KIDNEY PLAYS AN IMPORTANT role in maintaining the balance between Na intake and Na excretion and thus in the regulation of extracellular volume (31). Although only 10% filtered Na is reabsorbed in the distal nephron, the Na absorption in the distal nephron plays a key role in determining the final renal Na excretion and is also tightly regulated by hormones and dietary Na intake (31). Defective regulation of Na transport in the distal nephron has been shown to cause diseases such as Liddle syndrome and pseudohypoaldosteronism type II (2, 13, 29).
animals were used in each experimental group. The rats were anes-
or on a Na-D for 2 wk until the day of the experiment. Three to five
weighing 200 to 250 g were maintained either on a normal Na (0.5%)
and ENaC in the apical membrane.

Measurement of EET. The isolated CCDs were placed in a tube
containing ice-cold Na Ringer (0.5 ml). Eicosanoids in the tubule and
media were acidified to pH 4.0 with 9% formic acid. We added 2 ng
D$_{11}$ 11,12-EET in the tube as internal standard, the samples were
evacuated to dryness and the lipid residue was subsequently resus-
posed in methanol. After extraction, the CCD tubules were homog-
ized and the protein concentration was measured. The samples
were purified by reverse phase (RP)-HPLC on a C$_{18}$ Bondapak column
(4.6 × 25 mm) using a linear gradient from acetonitrile:water:acetic
acid (62:5.3:5.0:0.05%) to acetonitrile (100%) over 20 min at a flow
rate of 1 ml/min. The fraction containing 11,12-EET was collected on
the basis of the elution profile of standards monitored by ultraviolet
absorbance (205 nm). The fractions were evaporated to dryness and
resuspended in 100 µl of acetonitrile. HPLC fractions containing
11,12-EET were derivatized as described earlier (4). The derivatized
11,12-EET was dried with nitrogen and resuspended in 50 µl of
iso-octane for gas chromatography-mass spectrometry (GC-MS) anal-
yses. A 1-µl aliquot of derivatized CYP-derived AA metabolites,
dissolved in iso-octane, was injected into a GC (Hewlett-Packard
5890) column (DB-1ms; 10.0 m, 0.25-mm inner diameter, 0.25-µm
film thickness, Agilent). We used temperature programs ranging from
150–300°C at rates of 25°C/min, respectively (16). Methane was used as
a reagent gas at a flow resulting in a source pressure of 1.3 Torr and
the MS (Hewlett-Packard 5989A) was operated in electron capture
ionization mode. The endogenous 11,12-EET (ion mz 319) was iden-
tified by comparison of GC retention times with authentic D$_{11}$
11,12-EET (mz 327) standards.

Immunocytochemical staining. Kidneys were perfused with 50 ml
PBS containing heparin (40 U/ml) followed by 200 ml of 4% para-
formaldehyde. After perfusion, the kidneys were fixed with 4% para-
formaldehyde for 12 h and dehydrated. We used Leica900 cryostat
 Leia to cut kidney slices which were dried at 42°C for 1 h.
The slides were washed with 1× PBS for 15 min, and permeabilized
with 0.4% Triton dissolved in 1× PBS buffer containing 1% BSA and
0.1% lysine (pH 7.4) for 15 min. Kidney slices were blocked with 2%
goat serum for 1 h at room temperature and then incubated with antibodies to aquaporin 2 (AQP2; Alomone, Jerusalem, Israel), THP
(ICI, Pharmaceutical, Aurora, OH), and CYP2C23 (a gift from Dr. J.
H. Capdevila, Vanderbilt University) for 12 h at 4°C. Slides were
thoroughly washed with 1× PBS followed by incubation in second
antibody mixtures in 0.4% Triton X-100 dissolved in 1× PBS for 2 h
at room temperature.

In situ microperfusion of distal tubule. Male Sprague-Dawley rats
weighing 200 to 250 g were maintained either on a normal Na (0.5%)
or on a Na-D for 2 wk until the day of the experiment. Three to five
animals were used in each experimental group. The rats were anes-
ethetized by intraperitoneal injection of 100 to 150 mg/kg body wt of
inactin (5-ethyl-5-(1-methylpropyl)-2-thiobarbituric acid) purchased
from Sigma. Animals were kept on a thermostatically controlled
surgical table to maintain body temperature at 37°C.

The technique for microperfusion of distal tubules is similar to that
described previously (8, 33). Briefly, a late distal tubule (from the late
distal convoluted tubule to the initial part of the CCD) was selected
and perfused from the kidney surface at a rate of 12 nl/min. Distal
tube fluid was collected from the second or third segment on the
kidney surface ahead of an oil block. After each collection, the pipette
was withdrawn from the tubule into the oil covering the surface of the
kidney and a small amount of oil was aspirated into the tip of the
collection pipette to prevent evaporation of the sample. One collection
was made in each perfused tubule and three to five collections were
obtained in each kidney. The perfusion solutions were contained 20
µCi/ml of low-sodium [3H]methoxy-inulin for measuring volume
absorption, and 0.1% FD & C green dye for identification of the
perfused loops. The composition of the perfusion solution was as
follows (in mM): 115 NaCl, 25 NaHCO$_3$, 4 KCl, 1 CaCl$_2$, 5 Na-
acetate, 2.5 Na$_2$HPO$_4$, 0.5 NaH$_2$PO$_4$. Solutions was bubbled at
room temperature with 5% CO$_2$-95% O$_2$ before use. The pH was
adjusted to 7.4 with a small amount of NaOH or HCl. The rate of fluid
absorption ($J_w$) was analyzed by the [3H]inulin concentrations in the
perfusate and collected fluid. The concentrations of Na in the perfu-
sion solution and tubular fluid samples were measured by ultramicro-
atomic absorption spectrophotometry (8, 33) and compared with
known standards. Rates of Na$^+$ ($J_{Na}$) absorption were expressed per
millimeter of distal tubular length.

Solution and statistics. The bath solution for patch-clamp exper-
iments contained (in mM) 140 NaCl, 1.8 KCl, 1.8 MgCl$_2$, and
10 HEPES (pH 7.4). The pipette solution was composed of (in mM)
140 NaCl, 1.8 KCl, 1.8 MgCl$_2$, and 5 HEPES (pH 7.4). AA was
obtained from Nu-Check (Elysian, MN) and 11,12-EET was pur-
chased from Biomol. N-methylsulfonyl-6-(propargyloxyphenyl)hex-
amide (MS-PPOH), an inhibitor of CYP epoxygenase (32), was
synthesized in Dr. Falck’s laboratory, Southwestern Medical Center at
Dallas. The data are presented as means ± SE. We used Student’s
t-tests to determine the statistical significance. If the P value was
<0.05, the difference was considered to be significant.

RESULTS

High Na intake has been shown to stimulate CYP2C44 expression
in the kidney and urinary EET excretion in mice and that defective regulation of CYP2C44 in response to high
Na intake resulted in hypertension (20). To further explore the
relationship between dietary Na intake and CYP epoxygenase
activity, we examined the effect of Na restriction on the
expression of CYP2C3, a rat homolog of mouse CYP2C44
(25). We expect that if high Na upregulates CYP epoxygenase,
low Na intake should have an opposite effect on CYP2C3. Figure 1 is a typical immunostaining showing the expression of
CYP2C3 in the renal cortex and outer medulla of rats on
either a normal-Na diet (0.5%); Fig. 1A) or Na-D for 2 wk (Fig.
1B). It is apparent that Na restriction significantly diminished the expression of CYP2C3 in the kidney. We next used AQP2
as a marker of CD and THP as a marker of the thick ascending
limb (TAL) to determine whether CYP2C3 was expressed in
both nephron segments. We confirmed the previous finding that
CYP2C3 is expressed in the CCD (Fig. 2) (34). In
addition, the enzyme was also expressed in the OMCD. Figure 2 is a confocal image demonstrating the expression of
CYP2C3 in AQP2-positive CCD (top) and OMCD (bottom).
Furthermore, confocal images demonstrated that CYP2C3 was
expressed in the cortical and medullary TAL (Fig. 3).
However, as expected, Na restriction suppressed the expression
The finding that Na restriction decreased the expression of CYP2C23 was also supported by Western blotting in which the effect of Na intake on the total protein expression of CYP2C23 was examined. Figure 4A is a Western blot demonstrating that the expression of CYP2C23 was significantly diminished in the kidney from rats on Na-D for 14 days (30 ± 5% of the control value, \( P < 0.01 \)), whereas it was increased by 150 ± 20% (\( n = 4, P < 0.01 \)) in those on a high Na intake (4%) compared with the control value. Since CYP2C23 in rats is mainly responsible for converting AA to 11,12-EET (11), it is conceivable that decreases in CYP2C23 expression should reduce the 11,12-EET level in the CCD. To test this hypothesis, we used GC-MS to measure the content of 11,12-EET in the isolated CCD. Results summarized in Fig. 4B show that the level of 11,12-EET in isolated CCDs was 1.45 ± 0.5 ng/mg protein from control rats and that Na restriction decreased 11,12-EET to 0.64 ± 0.17 ng/mg protein in the CCD (\( n = 4 \) rats). Thus Na restriction decreased both the expression of CYP2C23 and the endogenous level of 11,12-EET in the CCD.

Since 11,12-EET mediates the inhibitory effect of AA on ENaC (34), a decrease in CYP2C23 expression in the CCD is expected to attenuate the inhibitory effect of AA on ENaC. We used the patch-clamp technique to examine the effect of AA on ENaC in the CCD of rats on a Na-D for 2–3 days or for 13–15 days. The reason for selecting rats on Na-D for 2–3 days rather than using animals on a normal-Na diet is that it is technically difficult to identify ENaC in the CCD from rats on a normal-Na diet. Western blot has also confirmed that the expression of CYP2C23 in renal cortex and outer medulla was significantly higher in rats on Na-D for 3 days (75 ± 8% control value, \( n = 4 \)) than those on Na-D for 14 days (31 ± 5%; Fig. 4C). Thus it is still justified to use the rats on 3-day Na-D as control to examine the role of CYP epoxygenase in the regulation of ENaC. Figure 5A is a representative channel recording showing that application of 15 μM AA inhibited ENaC and reduced \( N_P \) from 1.9 to 0.4 in the CCD of the rats on Na-D for 3 days. In contrast, application of the same amount of AA modestly inhibited ENaC and decreased \( N_P \) from 1.6 to 1.2 in the CCD from rats on Na-D for 2 wk (Fig. 5B). Data summarized in Fig. 6 show that the dose response of the AA effect on ENaC in the CCD from rats on a Na-D for 3 and 14 days, respectively. Application of 15 μM AA decreased channel activity by 77 ± 8% (\( n = 6, P < 0.01 \)) in the CCD from rats on a Na-D for 3 days. In contrast, it reduced channel activity only by 35 ± 5% (\( n = 5, P < 0.05 \)) in the CCD from rats on a Na-D for 14 days.
This indicates that the ENaC is less responsive to AA in the CCD from rats on Na-D for 2 wk than that for 3 days. This is not due to a decreased sensitivity of ENaC to 11,12-EET because application of 100 nM 11,12-EET inhibits ENaC in the CCD of rats on Na-D for 14 days (control, 1.4 ± 0.2; EET, 0.2 ± 0.06) to the same extent as in those for 3 days (control, 1.3 ± 0.2; EET, 0.2 ± 0.06; Fig. 7). Thus the sensitivity of ENaC to 11,12-EET was not altered by Na restriction. The role of CYP epoxygenase in the regulation of ENaC activity was further demonstrated in experiments in which effect of MS-PPOH on ENaC was examined in the CCD of rats fed on a normal-Na diet. Figure 8 is a representative recording showing that inhibition of CYP epoxygenase with 10 μM MS-PPOH increases channel activity from 0.01 ± 0.01 to 0.35 ± 0.1 (n = 11, P < 0.01).

After showing that CYP epoxygenase-dependent AA metabolism plays a role in the regulation of ENaC activity, we used a microperfusion technique to examine the effect of inhibiting CYP epoxygenase on Na transport in the distal nephron. It is expected that inhibition of CYP epoxygenase should increase Na absorption in the distal nephron from rats on a normal-Na diet but have a minimal effect in rats on Na-D. A late distal tubule (from the late portion of the distal convoluted tubule to the initial part of the CCD) was selected and perfused from the kidney surface for the study. Data summarized in Fig. 9 show that inhibition of epoxygenase with MS-PPOH (10 μM) significantly increased Na absorption in the distal nephron from 200 ± 28 to 322 ± 27 pmol·min⁻¹·mm⁻¹ (n = 5). The stimulatory effect of MS-PPOH must be related to CYP epoxygenase activity because MS-PPOH had no significant effect on Na absorption in the rats fed on Na-D for 14 days. Figure 8 shows that Na transport before MS-PPOH was 211 ± 31 pmol·min⁻¹·mm⁻¹ in the distal nephron of K-restricted animal. This value was not significantly different from that observed in rats on a normal-Na diet. A similar finding has been reported by other investigators (28) and it is speculated that the Na delivery rate to the distal nephron normally used in microperfusion study was not high enough to demonstrate that low Na intake alters Na absorption. Thus a high perfusion rate may be required to saturate the capacity of Na absorption in the distal nephron. Although the Na absorption rate in the distal nephron between normal and Na-restricted rats was the same under our experimental conditions, inhibition of CYP epoxygenase with MS-PPOH failed to stimulate Na absorption and it was 221 ± 16 pmol·min⁻¹·mm⁻¹ in rats on Na-D for 14 days.

DISCUSSION

The aim of the present study was to explore the role of CYP epoxygenase-dependent AA metabolism in mediating the effect of Na intake on ENaC activity and Na transport in the distal nephron. We selected the Na-restricted rats rather than those on a high-Na diet in the patch-clamp experiments because ENaC activity was suppressed in the CCD from rats on a high-Na diet. Thus it is difficult to study the effect of AA on
Fig. 4. A: Western blot showing the effect of Na intake on the CYP2C23 expression. B: summarizes data showing the effect of low Na intake on 11,12-EET levels in the isolated CCDs. C: Western blot demonstrating the expression of CYP2C23 in the renal cortex and outer medulla in rats on Na-deficient diet (Na-D) for 3 and 14 days. The homogenates from renal cortex and outer medulla (100 μg) were used for the Western blot, and the primary and secondary antibody was diluted in 1:1,000 and 1:2,000, respectively.
ENaC in rats on a high Na intake or even on a normal-Na diet. As an alternative, we carried out the study in Na-restricted rats for 3 or 14 days to prove the principal that Na intake regulates the CYP epoxygenase-dependent AA metabolism which in turn affects ENaC activity and Na transport in the distal nephron. Three lines of evidence suggest that Na intake regulates the activity of CYP epoxygenase in the distal nephron: 1) Western blot shows that high Na increased while low Na decreased CYP2C23 expression; 2) the immunostaining of CYP2C23 was almost absent in the CD from rats on Na-D for 14 days; and 3) the level of 11,12-EET in the CCD of rats on a Na-D diet was significantly lower than those on a control Na diet. This is consistent with previous findings that high Na intake increases CYP epoxygenase-dependent AA metabolites such as 11,12-EET (17). Although we did not examine the expression of CYP epoxygenases other than CYP2C23 which are also expressed in the kidney (25), CYP2C23 is a main CYP epoxygenase responsible for converting AA to 11,12-EET in the kidney (11). This notion is also supported by the finding that a diminished expression of CYP2C44, a mouse homolog to rat CYP2C23, decreases urinary EET excretion in CYP4A10(−/−) mice (20). In addition to CYP epoxygenase, AA can also be metabolized by CYP hydroxylation in the kidney. We and others previously demonstrated that 20-HETE, a CYP hydroxylase-dependent AA metabolite, inhibited Na transport in the TAL by blocking apical K channels and Na-Cl-K cotransporter (5, 10). However, it is unlikely that the CYP-hydroxylase-dependent AA metabolism plays a significant role in mediating the effect of AA on ENaC because inhibition of CYP hydroxylase failed to block the effect of AA on ENaC (34).

Several studies have suggested that CYP epoxygenase regulates renal Na transport. It has been reported that 5,6-EET inhibits Na transport in the rabbit CCD (26). The effect of 5,6-EET on Na transport is possibly mediated by a cyclooxygenase-dependent pathway because the effect of 5,6-EET was abolished by indomethacin. We have previously shown that adenosine inhibits ENaC activity by a CYP epoxygenase-dependent pathway (35). It is possible that stimulation of A1 adenosine receptor increases AA release
which is converted to 11,12-EET and blocks ENaC (34). The view that AA-induced inhibition of ENaC depends on CYP epoxygenase activity is supported by two lines of evidence: 1) The inhibitory effect of AA was attenuated from rats on Na-D for 2 wk which had a low CYP epoxygenase activity and 2) inhibition of epoxygenase increased ENaC activity in the CCD of rats on a normal-Na diet. Because 11,12-EET inhibits ENaC in the CCD with the same potency in rats on Na-D for 14 days as that for 3 days, this indicates that the attenuated AA effect on ENaC was due to a diminished CYP-epoxygenase activity. We speculate that the sensitivity of ENaC to AA in the CCD from rats on control (0.5%) Na diet and on a high Na intake should be much higher than that in rats on Na-D.

The notion that CYP epoxygenase AA metabolism plays a role in the regulation of Na transport in the distal nephron under physiological conditions was further indicated by the microperfusion study in which inhibition of CYP epoxygenase increased Na transport in the distal nephron in rats on a control Na diet. Because distal nephron includes connecting tubule and the initial portion of CCD where ENaC is located, it is assumed that inhibition of CYP epoxygenase should decrease 11,12-EET levels and thus increase Na transport by stimulation of ENaC activity. Since Na restriction downregulates the expression of CYP epoxygenase in the distal nephron, inhibition of CYP epoxygenase had no effect on ENaC in rats on a Na-D. Thus our data provide further evidence to support the role of CYP epoxygenase, especially CYP2C23/44, in the regulation of renal Na transport.

The role of CYP epoxygenase-derived EETs in the regulation of Na transport was best demonstrated in CYP4A10(/−/−) mice in which deletion of the CYP4A10 gene also suppressed the expression of CYP2C44, a major enzyme which converts AA to EETs in mice (20). As a consequence, the mice developed hypertension even if they were fed a normal-Na diet (0.5%). Three lines of evidence indicate that the hypertension in CYP4A10(/−/−) mice was the result of defective regulation of ENaC by CYP2C44-dependent AA metabolism. First, Na intake failed to regulate the expression of CYP2C44 and renal 11,12-EET levels. Second, application of AA failed to inhibit ENaC in the CCD from CYP4A10(/−/−) mice while 11,12-EET was able to inhibit ENaC. Third, treatment of animals with amiloride restored the normal blood pressure in CYP4A10(/−/−) mice. Therefore, CYP2C44/23 is involved in mediating the effect of Na intake on renal Na transport.

The mechanism by which Na intake regulates the expression of CYP2C23 is not clear. Because high Na suppresses while low Na increases the plasma level of aldosterone, it is possible that the renin-angiotensin-aldosterone pathway may play an important role in the regulation of CYP epoxygenase activity. The stimulatory effect of aldosterone on ENaC takes place not only at the level of transcription and translation but also at the posttranslational level by increasing the channel open probability (14) or the ENaC number in the apical membrane (21). However, the mechanism by which aldosterone increases the channel open probability is not completely understood. Aldosterone has been shown to increase SGK activity which increases the surface number and channel open probability of ENaC (18, 19). It has also been demonstrated that aldosterone stimu-
lates methylation of ENaC and increases channel open probability of ENaC (27). The present observation that low Na intake diminished the inhibitory effect of AA on ENaC suggests that decreases in the 11,12-EET level induced by low Na intake are, at least in part, responsible for the aldosterone-mediated increase in channel open probability. This notion is also supported by the finding that channel open probability in the MS-PPOH-treated CCD is significantly higher than that without the CYP epoxygenase inhibitor (34). We speculate that the effect of aldosterone on ENaC activity is partially mediated by regulation of CYP epoxygenase and 11,12-EET levels in the CCD.

The present study has shown that CYP2C23 is expressed not only in the CCD but also in the TAL. However, the role of CYP2C23 in the TAL has not been explored. Because the expression of CYP2C23 in the TAL was also regulated by Na intake, this strongly suggests that CYP epoxygenase should also play a role in the regulation of Na transport in the TAL. Further experiments are needed to explore the role of EET in the regulation of Na transport in the TAL. In summary, the present study suggests that CYP epoxygenase-dependent AA metabolites play a role in mediating the effect of Na intake on Na transport in the distal nephron. High Na intake stimulates the expression of CYP epoxygenase and increases the formation of 11,12 EET which inhibits Na absorption. In contrast, Na restriction downregulates the expression of CYP epoxygenase and decreases the concentration of 11,12-EET. As a consequence, the channel open probability of ENaC increased. We concluded that low Na suppresses the expression of CYP2C23 in the CCD and decreases 11,12-EET levels which are partially responsible for increasing ENaC channel open probability in the CCD from Na-restricted rats.

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