Effects of 20-HETE on Na\(^{+}\) transport and Na\(^{+}\)-K\(^{+}\)-ATPase activity in the thick ascending loop of Henle

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Yu M, Lopez B, Santos EA, Falck JR, Roman RJ. Effects of 20-HETE on Na\(^{+}\) transport and Na\(^{+}\)-K\(^{+}\)-ATPase activity in the thick ascending loop of Henle. Am J Physiol Regul Integr Comp Physiol 292: R2400–R2405, 2007. First published February 15, 2007; doi:10.1152/ajpregu.00791.2006.—Previous studies have indicated that 20-hydroxyeicosatetraenoic acid (20-HETE) inhibits Na\(^{+}\) transport in the medullary thick ascending loop of Henle (mTALH), but the mechanisms involved remain uncertain. The present study compared the effects of 20-HETE with those of ouabain and furosemide on intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_i\)), Na\(^{+}\)-K\(^{+}\)-ATPase activity, and \(^{86}\)Rb\(^{+}\) uptake, an index of Na\(^{+}\) transport, in mTALH isolated from rats. Ouabain (2 mM) increased, whereas furosemide (100 μM) decreased, [Na\(^{+}\)]\(_i\) in the mTALH of rats. Ouabain and furosemide inhibited \(^{86}\)Rb\(^{+}\) uptake by 91 and 30%, respectively; 20-HETE (1 μM) had a similar effect as ouabain and increased [Na\(^{+}\)]\(_i\), from 19 ± 1 to 30 ± 1 mM. 20-HETE reduced Na\(^{+}\)-K\(^{+}\)-ATPase activity by 30% and \(^{86}\)Rb\(^{+}\) uptake by 37%, but it had no effect on \(^{86}\)Rb\(^{+}\) uptake or [Na\(^{+}\)]\(_i\) in the mTALH of rats pretreated with ouabain. 20-HETE inhibited \(^{86}\)Rb\(^{+}\) uptake by 12% and increased [Na\(^{+}\)]\(_i\) by 19 mM in mTALH pretreated with furosemide. These findings indicate that 20-HETE secondarily inhibits Na\(^{+}\) transport in the mTALH of the rat, at least, in part by inhibiting the Na\(^{+}\)-K\(^{+}\)-ATPase activity and raising [Na\(^{+}\)]\(_i\).

cytochrome P-4504A; 20-hydroxyeicosatetraenoic acid; sodium-potassium-adenosinetriphosphatase; medullary thick ascending loop of Henle; rat

20-HYDROXYEICOSATETRAENOIC ACID (20-HETE) is the primary metabolite of arachidonic acid (AA) produced by enzymes of the cytochrome P4504A (CYP4A) family in the kidney (31, 36). The results of previous studies indicating that chronic administration of CYP inhibitors promote the development of salt-sensitive hypertension in rats (16, 34) suggest that 20-HETE plays an important role in the regulation of Na\(^{+}\) transport and the long-term regulation of arterial pressure. 20-HETE is avidly produced in the proximal tubule (20, 27), where it inhibits Na\(^{+}\) transport (29) and Na\(^{+}\)-K\(^{+}\)-ATPase activity (9, 26, 28, 33).

20-HETE also regulates Na\(^{+}\) transport in the medullary thick ascending loop of Henle. Escalante et al. (7) and Carroll et al. (6) first reported that 20-HETE was produced in the medullary thick ascending loop of Henle (mTALH) and that it inhibited Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) transport. Subsequent studies have revealed that 20-HETE inhibits reabsorption of Na\(^{+}\) and Cl\(^{-}\) in the mTALH of rats perfused in vivo (40) or in vitro (18) and that it reduces the activity of both the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (1) and the Na\(^{+}\)H\(^{+}\) exchanger (14).

Despite the importance of 20-HETE in the regulation of Na\(^{+}\) transport in the mTALH, its mechanism of action remains uncertain. Na\(^{+}\) uptake via the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter and Na\(^{+}\)/H\(^{+}\) exchanger is dependent on the Na\(^{+}\) gradient across the apical membrane that is maintained by Na\(^{+}\)-K\(^{+}\)-ATPase activity. Since 20-HETE inhibits Na\(^{+}\)-K\(^{+}\)-ATPase activity in the proximal tubule (26, 28) and the same subunit is expressed in the mTALH (10, 23, 38), it is possible that 20-HETE secondarily inhibits Na\(^{+}\) transport in this nephron segment by raising the intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_i\)). However, a previous study concluded (8) that 20-HETE inhibits Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter directly, because it decreased rather than increased the Na\(^{+}\) content of rabbit mTALH cells. However, only the steady-state Na\(^{+}\) content of the cells was measured by flame photometry after extensive washing of the cells with Na\(^{+}\)-free medium, and interpretation of this type of measurement can be difficult, since mTALH volume is regulated by opening of nonelective cation channels and cells lose intracellular Na\(^{+}\) and K\(^{+}\) when exposed to osmotic gradients (15). More recently, Na\(^{+}\)-sensitive fluorescent probes have been developed that allow for the dynamic measurement of changes in [Na\(^{+}\)]\(_i\) in proximal tubules (30, 39) and mTALH (19). Thus the present study reexamined the effect of 20-HETE on [Na\(^{+}\)]\(_i\) in the mTALH of rats by using the Na\(^{+}\)-sensitive dye sodium-binding benzofuran isophthalate acetoxy methyl ester (SBFI AM) to determine whether 20-HETE secondarily influences Na\(^{+}\) by inhibiting Na\(^{+}\)-K\(^{+}\)-ATPase activity or acts directly on the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter or Na\(^{+}\)/H\(^{+}\) exchanger.

MATERIAL AND METHODS

Animals. Experiments were performed on 6-wk-old male Sprague-Dawley (SD) rats weighing ~150 g, purchased from Harlan Laboratories (Indianapolis, IN). They were maintained in the Animal Care Facility at the Medical College of Wisconsin, which is approved by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by the Animal Care and Use Committee of the Medical College of Wisconsin.

Effect of 20-HETE on [Na\(^{+}\)]\(_i\), in mTALH. These experiments compared the effects of the Na\(^{+}\)-K\(^{+}\)-ATPase inhibitor ouabain (2 mM; Sigma, St Louis, MO) and 20-HETE (1 μM) on [Na\(^{+}\)]\(_i\) in mTALH microdissected from the kidney of rats. The rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the left kidney was quickly removed, sliced into 2-mm-thick sections, and stored in a low-Ca\(^{2+}\) Tyrode solution containing (in mM) 145 NaCl, 1 MgCl\(_2\), 6 KCl, 0.05 CaCl\(_2\), 4.2 NaHCO\(_3\), 10 glucose, and 10 HEPES (pH 7.4) with 2 succinate, 5 L-alanine, 2 L-glutamine, and 0.06% BSA. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Na-lactate at 4°C. mTALH were microdissected from the inner strip of outer medulla, placed on a glass coverslip that had been coated with Cell-Tak (BD Biosciences, San Jose, CA) to facilitate tubule adherence, and stored at 4°C until use. The intact mTALH were incubated with 10 μM of the Na\(^+\)-sensitive fluorescence probe SBF1 AM (Invitrogen, Carlsbad, CA) in a low-Ca\(^{2+}\) Tyrode solution containing 0.02% Pluronic F-127 (Invitrogen) for 60 min at room temperature. After loading, the tubules were transferred to a 1-mL perfusion chamber mounted on the stage of an inverted microscope and superfused with physiological HEPES buffer (pH 7.4) bubbled with 100% O\(_2\) containing (in mM) 135 NaCl, 1 MgCl\(_2\), 3 KCl, 1.5 CaCl\(_2\), 5.5 glucose, 2 K\(_2\)HPO\(_4\), 5 l-alanine, and 10 HEPES. 17-Octadecynoic acid (17-ODYA; 10 μM) and indomethacin (4 μM) were included in the bathing solution to block the endogenous formation of 20-HETE and to prevent the metabolism of 20-HETE by cyclooxygenase. The tubules were allowed to equilibrate in the dark for 20 min at 37°C before the baseline level of [Na\(^+\)] was determined.

[Na\(^+\)], measured using an InCyt Im2 imaging system (Intra-cellular Imaging, Cincinnati, OH) mounted on an inverted microscope (Nikon TS-100Fi) equipped with an ultraviolet ×40 fluorescence objective. The cells were alternatively excited at wavelengths of 340 and 380 nm, and the fluorescent images from three to six regions in each tubule were recorded at an emission wavelength of 510 nm. The data obtained for different regions of the same tubule were averaged and expressed as a single value per tubule. Two to three microdissected tubules were studied per rat. At the end of each experiment, the tubules were permeabilized by adding the ionophore amphotericin B (30 μM; Sigma) to the bath, and an in situ calibration curve for [Na\(^+\)], in each region was constructed by sequentially decreasing the Na\(^+\) concentration of the bath from 135 to 67, 34, 17, 8, and 4 mM. [Na\(^+\)], was calculated from the fluorescence intensity ratios obtained by using excitation and emission wavelengths of 340/380 and 510 nm, respectively, and the in situ standard curve generated at the end of each experiment. In each experiment, baseline [Na\(^+\)], was recorded for 2 min during a control period, and then vehicle (ethanol), ouabain (2 mM), or 20-HETE (1 μM) was added to the bath, and [Na\(^+\)], was recorded during a 10-min experimental period. The final concentration of ethanol in the bath was <0.1%. The effects of vehicle or 20-HETE on [Na\(^+\)] were also studied in mTALH tubules pretreated with furosemide (100 μM; Sigma) to block the Na\(^+\)-K\(^+\)-2Cl\(^−\) cotransporter.

**Effect of 20-HETE on Na\(^+\)-K\(^+\)-ATPase activity in mTALH.** These experiments examined the effect of 20-HETE on Na\(^+\)-K\(^+\)-ATPase activity in the mTALH of the rat. Intact mTALH were bulk isolated from the outer medulla of rats by using the rapid sieving method as previously described by Trinh- Tran-Tan et al. (37). Briefly, the kidney was flushed with 10 mL of physiological salt solution (PSS) containing 1 mg/mL collagenase (type II, 190 U/mg; Worthington Biochemical, Freehold, NJ), 300 U/mg hyaluronidase (Sigma), and 10,000 U/mg soybean trypsin inhibitor (Sigma). The inner stripe of the outer medulla was incubated in PSS containing 0.3 mg/mL collagenase, hyaluronidase, and trypsin inhibitor for three 15-min periods at 37°C. The supernatant was poured through a 70-μm nylon sieve and rinsed with 5 mL of PSS. The retained tissue enriched with intact mTALH (>95%) was resuspended in 100 μl of 100 mM potassium phosphate (pH 7.25) containing 30% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The mTALH were homogenized and then centrifuged at 9,000 g for 15 min, and the supernatant was collected. The protein concentration of these samples was measured using the Bradford method (Bio-Rad protein assay; Bio-Rad Laboratories, Hercules, CA) (4) with BSA as a standard.

Na\(^+\)-K\(^+\)-ATPase activity was determined using a K\(^+\)-p-nitrophosphatase (pNPPase) assay that avoids the problems of the large endogenous phosphate pool and competition for ATP utilization from mitochondria (35). Each batch of tubules was divided into three aliquots and incubated with vehicle (ethanol), 20-HETE (1 μM), or ouabain (2 mM) in 1 mL of assay buffer (pH 7.4) containing (in mM) 50 Tris·HCl, 10 MgSO\(_4\), and 90 KCl for 30 min at 37°C in a shaking water bath in an atmosphere containing 100% O\(_2\). The final concentration of ethanol in the reaction was <0.1%. The tubules were then centrifuged for 1 min at 1,200 g, and the pellets were homogenized in 0.8 ml of sucrose buffer (250 mM) containing deoxycholic acid (2.4 mM). The protein concentration of these samples was measured using the Bradford method (4) and the K\(^+\)-pNPPase activity was determined by incubating 10 μg of homogenate protein according to the method described by Murer et al. (24). The K\(^+\)-pNPPase activity in mTALH was determined as the difference between total and ouabain-insensitive K\(^+\)-pNPPase activity.

**Effect of 20-HETE on Na\(^{86}\)Rb\(^+\) uptake in mTALH.** These experiments examined the effects of 20-HETE on Na\(^{86}\)Rb\(^+\) uptake, an index of Na\(^+\) transport, in the mTALH of the rat. Intact mTALH were isolated from the outer medulla of rats as described above. The tubular suspensions were preincubated with vehicle (ethanol), 20-HETE (1 μM), ouabain (2 mM), or furosemide (100 μM) in 200 μl of HEPES buffer (pH 7.4) for 15 min at 37°C in a shaking water bath in an atmosphere containing 100% O\(_2\). The final concentration of ethanol in the reaction was <0.1%. Na\(^{86}\)Rb\(^+\) (0.2 μCi) was added to the tubular suspensions and incubated for 5 min at 37°C in the presence of 100% O\(_2\). Our
preliminary experiments indicated that uptake of $^{86}$Rb$^+$ in isolated mTALH of the rat is linear for up to 2 min. Isotope uptake was terminated by adding 550 μl of an ice-cold stop solution containing (in mM) 150 NaCl, 10 BaCl₂, and 10 HEPES to the tubular suspensions, followed by 550 μl of a mixture of dioctyl phthalate and silicone oil (1.75:1). The tubules were centrifuged at 11,000 g for 30 s to pellet the tubules and remove the incubation medium. The supernatant was discarded, and the bottom of the tube containing the mTALH pellet was cut off into a scintillation vial. The $^{86}$Rb$^+$ activity in the pellet was determined using a gamma counter (TM Analytic, Elk Grove Village, IL). Other aliquots of the same mTALH suspension were homogenized for measurement of the protein concentration using the Bradford method to normalize $^{86}$Rb$^+$ uptake per milligram of protein. Ouabain-insensitive $^{86}$Rb$^+$ uptake was determined in separate suspensions of tubules treated with 2 mM ouabain. Subtraction of $^{86}$Rb$^+$ uptakes in the presence and absence of ouabain provided the estimate of ouabain-sensitive $^{86}$Rb$^+$ uptake, an index of Na$^+$ transport.

**Statistical analysis.** Data are presented as means ± SE. Either a paired t-test or a one-way ANOVA for repeated measures followed by a Holm-Sidak post hoc test was used to determine the significance of differences between mean values in different treatment groups (13). A $P$ value <0.05 was considered to be statistically significant.

![Fig. 2. Effects of ouabain (2 mM) and 20-hydroxyeicosatetraenoic acid (20-HETE; 1 μM) on intracellular Na$^+$ concentration ([Na$^+$]) in mTALH of rats. Representative tracings are shown of [Na$^+$], after treatment of mTALH with ouabain (top) or 20-HETE (middle). Bottom: summary of the effect of vehicle, 20-HETE, and ouabain on [Na$^+$] in mTALH. Numbers in parentheses indicate the total number of mTALH studied. †$P$ < 0.05, significant difference from the corresponding value in mTALH treated with vehicle.](image)

![Fig. 3. Effect of furosemide (50 μM) and 20-HETE (1 μM) on [Na$^+$] in mTALH of rats. Top: representative tracing of effects of 20-HETE on [Na$^+$], after pretreatment of cells with furosemide. Middle: summary of the effect of vehicle and 20-HETE on [Na$^+$] in mTALH. Bottom: summary of the effect of vehicle and 20-HETE on [Na$^+$] in mTALH after blockade of the Na$^+$-K$^+$-2Cl$^-$ cotransporter with furosemide. Numbers in parentheses indicate the total number of mTALH studied. *$P$ < 0.05, significant difference from the corresponding baseline value. †$P$ < 0.05, significant difference from the corresponding value in mTALH treated with vehicle.](image)
RESULTS

Effect of 20-HETE on \([\text{Na}^+]/\text{H}^+\) in mTALH. A representative tracing of an in situ standard curve for \([\text{Na}^+]/\text{H}^+\) in an amphotericin B (30 μM)-permeabilized mTALH is presented in Fig. 1. The F340/F380 fluorescence intensity ratio increased to the maximal value following addition of amphotericin B to the bath. It fell sequentially as the \([\text{Na}^+]/\text{H}^+\) concentration in the bath was reduced from 135 to 4 mM (Fig. 1, top). A representative in situ standard curve of F340/F380 fluorescence intensity ratio as a function of the \([\text{Na}^+]/\text{H}^+\) concentration of the bath is presented in Fig. 1, bottom. The F340/F380 fluorescence intensity ratio increased as the \([\text{Na}^+]/\text{H}^+\) concentration of the bath was increased from 4 to 135 mM.

Representative tracings comparing the effects of ouabain and 20-HETE on \([\text{Na}^+]/\text{H}^+\) in mTALH microdissected from the kidney of rats are presented in Fig. 2. Ouabain (Fig. 2, top) increased \([\text{Na}^+]/\text{H}^+\) from 20 to 40 mM. 20-HETE (Fig. 2, middle) had a similar effect and raised \([\text{Na}^+]/\text{H}^+\) from 16 to 35 mM. The composite results from all experiments are summarized in Fig. 2, bottom. Baseline \([\text{Na}^+]/\text{H}^+\) averaged 19 ± 1 mM. Addition of ouabain to the bath increased \([\text{Na}^+]/\text{H}^+\) by 19 ± 1 mM. 20-HETE had an effect similar to that of ouabain and significantly increased \([\text{Na}^+]/\text{H}^+\) in mTALH of rats by 11 ± 1 mM. It had no effect on \([\text{Na}^+]/\text{H}^+\) in tubules pretreated with ouabain.

The effect of 20-HETE on \([\text{Na}^+]/\text{H}^+\) in mTALH of rats after blockade of the \([\text{Na}^+]/\text{K}^+]/\text{2Cl}^-\) cotransporter with furosemide is presented in Fig. 3. Representative tracings of the effects of furosemide (100 μM) and 20-HETE (1 μM) are presented in Fig. 3, top. Baseline \([\text{Na}^+]/\text{H}^+\) averaged 25 ± 3 mM. Addition of furosemide decreased \([\text{Na}^+]/\text{H}^+\) in the mTALH by 6 mM; however, \([\text{Na}^+]/\text{H}^+\) still increased by 18 mM after the addition of 20-HETE to the bath (Fig. 3, middle). The increase in \([\text{Na}^+]/\text{H}^+\) in furosemide-treated mTALH after addition of 20-HETE was significantly greater than that observed in tubules treated with vehicle alone (19 ± 3 vs. 4 ± 2 mM; Fig. 3, bottom).

Effect of 20-HETE on \([\text{Na}^+]/\text{K}^+]/\text{ATPase activity in mTALH. The results of this study are presented in Fig. 4. 20-HETE significantly reduced the K^+-pNPPase activity in mTALH of rats by 25% compared with that seen in tubules treated with vehicle alone.}

Effect of 20-HETE on \(^{86}\text{Rb}^+\) uptake in mTALH. The results of these experiments are presented in Fig. 5. 20-HETE reduced ouabain-sensitive \(^{86}\text{Rb}^+\) uptake by 37% in the mTALH of rats compared with that seen in tubules treated with vehicle alone (Fig. 5, top). Ouabain (2 mM) reduced \(^{86}\text{Rb}^+\) uptake by 91%. 20-HETE had no additional effect on \(^{86}\text{Rb}^+\) uptake in the mTALH of rats pretreated with ouabain (Fig. 5, middle).
Furosemide (100 μM) reduced $^{86}$Rb$^+$ uptake by 30% (Fig. 5, bottom). 20-HETE further reduced $^{86}$Rb$^+$ uptake by 12% in mTALH pretreated with furosemide (Fig. 5, bottom).

**DISCUSSION**

Previous studies have indicated that 20-HETE plays a critical role in the regulation of Na$^+$ transport in the mTALH (18, 40). In this regard, 20-HETE has been reported to inhibit $^{86}$Rb$^+$ uptake, an index of Na$^+$ transport, in the mTALH of rabbits in vitro (7, 8) and Cl$^-$ transport in the loop of Henle of rats perfused in vivo (40) and in vitro (18). Other studies have indicated that 20-HETE inhibits the activity of Na$^+$/H$^+$ exchanger (14) and the Na$^+$/K$^+$/2Cl$^-$ (1) cotransporter in mTALH isolated from the kidney of rats. However, the mechanism by which 20-HETE regulates Na$^+$ transport in this portion of nephron remains uncertain. In the proximal tubule, 20-HETE inhibits Na$^+$/K$^+$/2Cl$^-$ cotransporter activity by stimulating PKC to phosphorylate the serine-23 residue in the α-subunit of this enzyme (26). The α-subunit of Na$^+$/K$^+$/2Cl$^-$ATPase that is expressed in the mTALH is identical to that expressed in the proximal tubule (10, 23, 38). Therefore, it would be reasonable to assume that 20-HETE also inhibits Na$^+$/K$^+$/2Cl$^-$ATPase activity in the mTALH and secondarily inhibits Na$^+$ transport by raising [Na$^+$], and diminishing the driving force for Na$^+$ entry (5, 11). On the other hand, the activity of both the Na$^+$/H$^+$ exchanger (14) and Na$^+$/K$^+$/2Cl$^-$ cotransporter (1) can be modified by phosphorylation by PKC. Thus it is possible that 20-HETE might have a direct effect to inhibit Na$^+$ entry by either of these pathways. To explore the mechanism by which 20-HETE regulates Na$^+$ transport in the mTALH, the present study compared the effects of 20-HETE with those of ouabain (rabbits vs. rats) or methodological differences. Escalante et al. (8) measured the effect of 20-HETE on the Na$^+$ content in rabbit mTALH cells by flame photometry after extensive washing of the tubules with a Na$^+$-free medium, whereas in the present study [Na$^+$], was measured dynamically, using the Na$^+$-sensitive dye SBFI, in mTALH microdissected from the outer medulla of rats. It is possible that changes in Na$^+$ content of the mTALH may not accurately reflect changes in [Na$^+$]; if the cells shrink or swell during an experiment, since mTALH cell volume is regulated by opening of nonselective cation channels and these cells lose intracellular Na$^+$ and K$^+$ when exposed to osmotic gradients (15). The other major difference is that the present experiments were performed in the presence of 17-ODYA and indomethacin to block the endogenous formation of 20-HETE and to prevent the metabolism of 20-HETE by cyclooxygenase to 20-hydroxy-PGE$_2$. Indeed, we found that a higher concentration of 20-HETE was needed to alter [Na$^+$], if the tubules were not pretreated with 17-ODYA. Moreover, previous studies have indicated that PGE$_2$ can inhibit Na$^+$ transport in the mTALH secondary to activation of PKA that directly phosphorylates and inhibits the activity of the Na$^+$/H$^+$ exchanger (3).

In summary, the results of the present study indicate that 20-HETE secondarily inhibits Na$^+$ transport in the mTALH of rat, at least in part, by inhibiting the Na$^+$/K$^+$/2Cl$^-$ATPase and raising [Na$^+$]). These results are consistent with recent findings that a deficiency in the renal production of 20-HETE contributes to the elevated Na$^+$ transport in the mTALH (18, 40) and to the development of salt-sensitive hypertension (32) in Dahl salt-sensitive rats. They are also consistent with recent findings that knockout of CYP4A10 (25) or CYP4A14 (17) genes contribute to the development of hypertension in mice and with genetic studies linking a T8590C polymorphism in the CYP4A11 gene that reduces the 20-HETE formation with the development of hypertension in three independent human populations (12, 21, 22).

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