Role of 20-HETE in Elevating Chloride Transport in the Thick Ascending Limb of Dahl SS/Jr Rats

Osamu Ito, Richard J. Roman

Abstract—This study examined the role of endogenous 20 hydroxyeicosatetraenoic acid (20-HETE) in elevating Cl⁻ transport in the medullary thick ascending loop of Henle (MTAL) of 9-week-old male Dahl salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) rats perfused in vitro. Basal transepithelial voltage (Vₑ; 14.9±0.9 versus 10.1±0.5 mV) and net lumen-to-bath Cl⁻ flux (Jₑ) (155±6 versus 127±5 pEq · min⁻¹ · mm⁻¹) were significantly greater in MTAL isolated from SS/Jr rats (n=16) than in those obtained from SR/Jr rats (n=16). Blockade of the synthesis of 20-HETE with 17-octadecynoic acid (17-ODYA; 10 μmol/L) increased Vₑ from 9.9±0.8 to 13.1±1.0 mV and Jₑ from 127±7 to 152±8 pEq · min⁻¹ · mm⁻¹ in the MTAL of SR/Jr rats (n=8), but it had no significant effect on Vₑ or Jₑ in the MTAL of SS/Jr rats (n=8). Exogenous 20-HETE (1 μmol/L) decreased Vₑ from 14.8±0.6 to 10.5±0.6 mV and Jₑ from 155±10 to 116±6 pEq · min⁻¹ · mm⁻¹ in MTAL of SS/Jr rats (n=8), but it had no effect on Vₑ or Jₑ in the MTAL of SR/Jr rats (n=8). The expression of P4504A2 protein in the MTAL of SS/Jr rats was approximately half of that seen in the MTAL of SR/Jr rats. These results indicate that endogenously formed 20-HETE regulates transepithelial voltage and Cl⁻ transport in the MTAL and that a diminished production of 20-HETE contributes to an elevation in Cl⁻ transport in the MTAL of SS/Jr rats. (Hypertension. 1999;33(part II):419-423.)

Key Words: kidney ■ sodium ■ potassium ■ ion transport ■ cytochrome P450 ■ hypertension, salt-sensitive ■ blood pressure

Renal transplantation studies have indicated that some form of renal dysfunction underlies the development of hypertension in Dahl salt-sensitive (SS/Jr) rats; however, the factors responsible for altering kidney function are still unknown.1,2 We and others have reported that the pressure-natriuresis relationship is reset toward higher pressures before the development of hypertension in Dahl salt-sensitive/John Rapp substrain (SS/Jr) rats.2-6 Previous micropuncture studies have indicated that Cl⁻ reabsorption is elevated in the loop of Henle, somewhere between the accessible portions of the late proximal and early distal tubule in Dahl SS/Jr rats.5,7-9 However, the exact nephron segment involved could not be identified because this portion of the nephron spans 4 distinct segments, including the Pars recta, thin ascending and descending limbs, and the thick ascending loop of Henle. Moreover, it remains to be determined whether the alterations in sodium and chloride reabsorption seen in Dahl SS/Jr rats are related to intrinsic differences in the regulation of ion transport in a specific cell type in the kidney or are secondary to strain differences in renal hemodynamics, physical factors, the levels of circulating hormones and paracrine factors, or all of these that modify sodium transport in vivo.

Recent observations have suggested that changes in the renal metabolism of arachidonic acid by enzymes of the P4504A family may contribute to the elevation in loop Cl⁻ transport and the development of hypertension in Dahl SS/Jr rats. In this regard, the formation of 20 hydroxyeicosatetraenoic acid (20-HETE) is reduced, and the levels of P4504A protein are lower in the renal outer medulla of SS/Jr rats than in normotensive strains of rats.10,11 Previous studies have also indicated that 20-HETE is the primary metabolite of arachidonic acid that is produced in the thick ascending limb of the loop of Henle.12,13 20-HETE is a potent inhibitor of Na⁺-K⁺-2Cl⁻ transport in these cells.13,14 Thus, it is possible that a deficiency in the formation of this substance could contribute to the elevation in loop Cl⁻ transport seen in SS/Jr rats. Our recent finding that in vivo perfusion of the loop of Henle of SS/Jr rats with exogenous 20-HETE normalizes loop Cl⁻ transport supports this view.9 This hypothesis is also consistent with previous observations that (1) a genetic marker within P4504A2 gene cosegregates with blood pressure in a F2 cross of SS/Jr and Lewis rats,11 (2) induction of the renal production of 20-HETE attenuates the development of hypertension in SS/Jr rats,15 and (3) chronic inhibition of 20-HETE formation in the outer medulla induces salt-sensitive hypertension in Lewis rats.16

The purpose of the present study was to determine whether Cl⁻ transport and transepithelial potential are elevated in the medullary thick ascending limb of Henle (MTAL) of SS/Jr rats perfused in vitro and to compare the effects of blockade...
of 20-HETE formation with 17-octadecynoic acid (17-OYA) and exogenous 20-HETE on Cl⁻ transport in the MTAL of SS/Jr and SR/Jr rats. In addition, the expression of P4504A protein was compared in the MTAL of Dahl SS/Jr rats and of a salt-resistant/John Rapp strain (SR/Jr) of rats.

Methods
Experiments were performed on 9-week-old male SS/Jr, SR/Jr, Lewis, and Sprague-Dawley rats. The SR/Jr, Lewis, and Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN), and the SS/Jr rats were obtained from a colony maintained at the Medical College of Wisconsin by brother-sister mating since 1991. The rats were housed in an Animal Care Facility that is approved by the American Association for Accreditation of Laboratory Animal Care. The rats were maintained on a low salt diet (0.4% NaCl) throughout the study. All protocols received prior approval by the Animal Welfare Committee of the Medical College of Wisconsin.

In Vitro Microperfusion of the MTAL
The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). MTALs were dissected from the outer medulla with fine forceps and perfused in vitro at a rate of 20 nL/min, according to the technique of Burg et al. Bath temperature was maintained at 37°C, and the perfusion and the bath solutions contained (in mmol/L) 135 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 2 KH₂PO₄, 5.5 glucose, 5 L-alanine, and 10 HEPES (pH 7.4) and were equilibrated with 100% oxygen.

Transepithelial voltage (Vₜₑ) was measured between Ag-AgCl wires inserted into 0.9% NaCl–4% agar bridges that were attached to the perfusion pipette and the bath. Net lumen-to-bath Cl⁻ flux (Iₒ) was also measured in some experiments. In these experiments, the perfusion rate was lowered to 5 nL/min, and the Cl⁻ concentration of the perfusate and collected fluid was measured with a microtitrator (model F-25; WPI).

Control Vₜₑ and Iₒ were measured after a 60-minute equilibration period. Then, 17-OYA (10 μmol/L), an inhibitor of the formation of 20-HETE, or 20-HETE (1 μmol/L) was added to the bath, and 20 minutes later Vₜₑ and Iₒ were redetermined.

Bulk Isolation of MTAL
MTALs were isolated with the method of Trinh-Trang-Tan et al. The kidney was flushed with 10 mL physiological salt solution containing 1 mg/mL collagenase (type II, 190 U/mg, Worthington Biochemical), hyaluronidase (300 U/mg, Sigma), and soybean trypsin inhibitor (10 000 U/mg, Sigma). The inner stripe of the outer medulla was incubated in physiological salt solution containing 0.3 mg/mL collagenase, hyaluronidase, and trypsin inhibitor for 3 15-minute periods at 37°C. The supernatant was poured off, and the retained tissue enriched with MTALs (model F-25; WPI).

Control Vₜₑ and Iₒ were measured after a 60-minute equilibration period. Then, 17-OYA (10 μmol/L), an inhibitor of the formation of 20-HETE, or 20-HETE (1 μmol/L) was added to the bath, and 20 minutes later Vₜₑ and Iₒ were redetermined.

Immunoblot Analysis
Proteins were separated by electrophoresis on a 10%–20% gel, 8.5% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in buffer containing 10% nonfat dry milk. The membrane was then incubated for 2 hours with a 1:4000 dilution of a P4504A polyclonal antibody (Duich Chemicals) followed by a 1-hour incubation with a 1:2000 dilution of a secondary antibody (Santa Cruz Biotechnology). Immunoblots were developed using an enhanced chemiluminescence kit (ECL, Amersham). The relative intensities of the bands in the 50- to 52-kDa range were quantified with a densitometer (Personal Densitometer SI; Molecular Dynamics).

Results
Basal Cl⁻ Transport in the MTAL of Dahl SS/Jr and Normotensive Rats
A comparison of Vₜₑ and Jₒ in MTAL of SS/Jr and SR/Jr rats is presented in Figure 1. Basal Vₜₑ in the MTAL of Dahl SS/Jr rats (n = 16) was significantly greater than that seen in SR/Jr rats (n = 16) or in normotensive Sprague-Dawley (10.2 ± 1.2 mV, n = 8) or Lewis rats (6.6 ± 0.9 mV, n = 8). Baseline Jₒ in the MTAL of SS/Jr rats was also significantly greater than that seen in SR/Jr rats (Figure 1).

Effect of 17-OYA on Cl⁻ Transport in the MTAL
A comparison of the effects of blockade of 20-HETE formation with 17-OYA on Vₜₑ and Jₒ in the MTAL of SS/Jr and SR/Jr rats is presented in Figure 2. The addition of 17-OYA (10 μmol/L) to the bath had no effect on Vₜₑ and Jₒ in the MTAL of SS/Jr rats. In contrast, Vₜₑ and Jₒ increased significantly in the MTAL of SR/Jr rats after the addition of 17-OYA. A similar effect of 17-OYA on Vₜₑ was also observed in the MTAL of normotensive Sprague-Dawley rats (from 10.2 ± 1.2 to 13.8 ± 1.3 mV, n = 8, P < 0.001) and Lewis rats (from 6.6 ± 0.9 to 8.6 ± 0.9 mV, n = 8, P < 0.005). To determine whether this effect was specific to blockade of the metabolism of arachidonic acid by P-450, we examined the effects of indomethacin on Vₜₑ in the MTAL of SR/Jr rats. The addition of indomethacin (10 μmol/L) to the bath had no significant effect on Vₜₑ (10.3 ± 0.7 versus 10.3 ± 0.6 mV, n = 8) in these experiments.

Effect of 20-HETE on Cl⁻ Transport in the MTAL
A comparison of the effects of exogenous 20-HETE (1 μmol/L) on Vₜₑ and Jₒ in the MTAL of SS/Jr and SR/Jr rats.
is presented in Figure 3. The addition of 20-HETE (1 \( \mu \)mol/L) to the bath significantly reduced \( V_t \) and \( J_{\text{Cl}} \) in the MTAL of SS/Jr rats, but it had no significant effect in the MTAL of SR/Jr rats.

Expression of P4504A Proteins in the MTAL of Dahl Rats

A comparison of the levels of P4504A enzymes responsible for the formation of 20-HETE in the MTAL of SS/Jr and SR/Jr rats is presented in Figure 4. One immunoreactive band was detected in homogenates prepared from the MTAL of SS/Jr and SR/Jr rats. In comparison with rat liver standards, this isoform migrated like the P4504A2 isoform. The level of P4504A protein in the MTAL of SR/Jr rats was twice as high as that seen in the MTAL of SS/Jr rats. In other experiments, we confirmed that the levels of P4504A protein were also higher in MTAL isolated from Sprague-Dawley and Lewis rats than in MTAL of Dahl SS/Jr rats (data not shown).

Discussion

The present study examined whether Cl\(^{-}\) transport is elevated in the MTAL of Dahl SS/Jr perfused in vitro and the hypothesis that a deficiency in the production of 20-HETE in the MTAL contributes to alterations in the renal handling of sodium and chloride previously reported in this strain of rats in vivo.\(^2\)\(^{-}\)\(^6\) The results indicate that Cl\(^{-}\) reabsorption is elevated in the MTAL of SS/Jr rats perfused in vitro as compared with that seen in the MTAL of SR/Jr rats. \( V_{\text{w}} \) is also 30% higher in the MTAL of SS/Jr rats as compared with the levels seen in normotensive SR/Jr, Sprague-Dawley, and Lewis strains of rats. The significance of these findings is that they are the first to identify the MTAL as the renal cell type in which tubular reabsorption of sodium and chloride is elevated in SS/Jr rats. Indeed, all previous work has indicated that sodium reabsorption is actually reduced in all other nephron segments in Dahl SS/Jr rats, perhaps as an adaptation to chronic sodium retention.\(^7\)

Another important aspect that we found is that there is a phenotypic difference in the regulation of Cl\(^{-}\) transport in the MTAL of Dahl SS/Jr rats that can still be demonstrated in vitro and that is therefore independent of strain differences in renal hemodynamics, the levels of circulating hormones and paracrine factors, or both that influence sodium reabsorption in vivo. Thus, measurement of \( V_{\text{w}} \) and \( J_{\text{Cl}} \) in the MTAL may serve as valuable intermediate phenotypes to identify chro-
mosomal regions and candidate genes that influence salt-sensitivity in this model. Moreover, the present findings suggest that all of the factors that regulate Cl⁻ transport in the MTAL can now be considered to be viable candidate genes for the development of hypertension. These include subunits of Na⁺,K⁺-ATPase, the Na⁺,K⁺,2Cl⁻ cotransporter, and the 70- and 30-pS apical K⁺ channels that regulate Vᵢ in the MTAL. Indeed, a molecular variant of the α subunit of Na,K-ATPase has been linked to alterations in blood pressure and red cell transport in Dahl SS/Jr rats.2² Mutations in the Na⁺,K⁺,2Cl⁻ cotransporter have also been shown to alter blood pressure in humans.2³ It should also be noted that the activity of this transporter is regulated by hormones and paracrine factors that act via the cAMP, cGMP, protein kinase C, and cytochrome P-450/arachidonic acid pathways. Thus, components of these signal transduction pathways can also be considered as potential candidate genes for salt-sensitivity in Dahl SS/Jr rats.

Previous studies have indicated that 20-HETE is the primary metabolite of arachidonic acid produced in thick ascending limb cells of rabbits and that 20-HETE inhibits rubidium uptake, an indirect index of Na⁺ transport in the MTAL cells of rabbits and that 20-HETE inhibits rubidium uptake, an indirect index of Na⁺ transport in the MTAL cells of rabbits. Thus, components of these signal transduction pathways can also be considered as potential candidate genes for salt-sensitivity in Dahl SS/Jr rats.

Previous studies have indicated that 20-HETE is the primary metabolite of arachidonic acid produced in thick ascending limb cells of rabbits and that 20-HETE inhibits rubidium uptake, an indirect index of Na⁺ transport in the MTAL.24 Blockade of this channel should reduce Vᵢ and perhaps below the threshold concentration needed to inhibit Cl⁻ transport. Moreover, exogenous 20-HETE reduced Cl⁻ transport in the MTAL of SS/Jr rats, but it had no effect on Cl⁻ transport in Dahl SR/Jr rats in which the endogenous levels of 20-HETE were below the threshold concentration needed to inhibit Cl⁻ transport. These findings provide direct evidence that there is a deficiency in the formation of 20-HETE contributes to the elevation in Cl⁻ transport in the MTAL of Dahl SS/Jr rats.

In summary, the results of the present study indicate that there is a phenotypic elevation in Cl⁻ transport in the MTAL of Dahl SS/Jr rats and provides direct evidence that there is a deficiency in the formation of 20-HETE contributes to the elevation in Cl⁻ transport in the MTAL of Dahl SS/Jr rats. Overall, these findings provide a strong rationale for further evaluation of the P450A4 gene as a candidate for mediating salt-sensitivity in Dahl SS/Jr rats.

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**References**


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