Inhibitors of 20-HETE Formation Promote Salt-Sensitive Hypertension in Rats

Kimberly M. Hoagland, Averia K. Flasch, Richard J. Roman

Abstract—This study examined whether chronic blockade of epoxyeicosatrienoic acids (EETs) and/or 20-hydroxyeicosatetraenoic acid (20-HETE) formation promotes development of salt-sensitive hypertension. Changes in blood pressure, renal cytochrome P450 metabolism of arachidonic acid, and 20-HETE excretion in response to a high salt diet were measured in rats chronically treated with 1-aminobenzotriazole (ABT, 50 mg/kg per day) to block EETs and 20-HETE formation or N-hydroxy-N'-(4-butyl-2 methylphenyl) formamidine (HET0016, 10 mg/kg per day) that selectively reduces 20-HETE formation. ABT reduced blood pressure in rats fed a low salt (0.4% NaCl) diet, but blood pressure rose by 20 mm Hg after these rats were switched to a high salt (8% NaCl) diet for 10 days. HET0016 had no effect on blood pressure in rats fed a low salt diet; however, blood pressure rose by 18 mm Hg after the rats were fed a high salt diet. 20-HETE formation in kidney homogenates rose by 30% and epoxygenase activity doubled when rats were fed a high salt diet. Chronic treatment with ABT and HET0016 inhibited the renal formation of 20-HETE by ~90%. Renal epoxygenase activity decreased by 76% in ABT-treated rats and was not significantly altered in rats treated with HET0016. 20-HETE excretion rose from 470 to 570 ng/d when the rats were switched from the low to the high salt diet. 20-HETE excretion fell by 68% and 85% in rats that were chronically treated with ABT and HET0016. These results suggest that chronic blockade of the formation of 20-HETE promotes the development of salt-sensitive hypertension in rats. (Hypertension. 2003;42[part 2]:669-673.)

Key Words: rats, Dahl ■ metabolism ■ arachidonic acids ■ blood pressure ■ hypertension, sodium-dependent

The role of the cytochrome P450 (P450) metabolites of arachidonic acid (AA) in the development and maintenance of hypertension remains uncertain in part because this pathway has both prohypertensive and antihypertensive actions.1 20-Hydroxyeicosatetraenoic acid (20-HETE) inhibits Na+ transport in the proximal tubule and thick ascending limb of the loop of Henle (TALH), and compounds that induce the renal formation of 20-HETE lower blood pressure in Dahl salt-sensitive (DS) rats. Similarly, epoxyeicosatrienoic acids (EETs) inhibit Na+ transport in the proximal tubule and collecting duct, and increasing the renal levels of EETs with inhibitors of soluble epoxide hydrolase (sEH) reduces blood pressure in spontaneously hypertensive rats (SHRs) and angiotensin II–induced hypertensive rats. However, 20-HETE is also a potent vasoconstrictor, and many investigators have reported that blocking the vascular effects of 20-HETE may contribute to its antihypertensive effect in SHR and other experimental models of hypertension.

Part of the problem in defining the role of the P450 metabolites of AA in the control of blood pressure has been the lack of selective inhibitors of the pathway that chronically block the formation of 20-HETE and EETs in vivo. Inhibitors that are commonly used to inhibit the formation of EETs and 20-HETE in vitro, such as 17-octadecynoic acid (17-ODYA), dibromododecyl methylsulfimide (DDMS), and methylsuphonyl-6-(2-propargyloxy-phenyl)-hexanamide (PPOMS), bind to plasma proteins, and in our hands are not effective in reducing the renal formation of EETs and 20-HETE after systemic administration. More recently, 1-aminobenzotriazole (ABT) has been reported to reduce the renal formation of 20-HETE and/or EETs when given acutely to rats, and N-hydroxy-N'-(4-butyl-2 methylphenyl) formamidine (HET0016) has been shown to be the most selective inhibitor of the renal formation of 20-HETE.

Therefore, in the current study, we examined the effects of chronic administration of ABT and HET0016 on the renal metabolism of AA, the urinary excretion of 20-HETE, and blood pressure in Sprague-Dawley (SD) rats fed a low or a high salt diet.

Methods

Animals

Experiments were performed in adult male SD rats weighing between 300 and 350 g purchased from Harlan (Indianapolis, Ind). The rats were housed in stainless steel metabolic cages in a chronic monitoring facility at the Medical College of Wisconsin, approved by the American Association for the Accreditation of Laboratory Animal Care. The rats were fed a purified diet (AIN-76 A) purchased from Dyets, Inc, that contained either 0.4% (cat no. 113755) or 8%...
NaCl (cat no. 100078) by weight. The rats had free access to food and water throughout the study. All protocols were approved by the Animal Welfare Committee at the Medical College of Wisconsin and were in accordance with the National Institute of Health’s “Guide for the Care and Use of Laboratory Animals.”

**Surgical Preparation of Animals for Chronic Study**

The rats were anesthetized with an intramuscular injection of ketamine (40 mg/kg), xylazine (2.5 mg/kg), and acepromazine (0.6 mg/kg). Microcatheters (Braintree Scientific, Inc) catheters were chronically implanted into the left femoral artery and vein for measurement of mean arterial pressure (MAP) and intravenous drug administration. The catheters were tunneled subcutaneously, exteriorized between the scapulae, and protected by a stainless steel spring attached to a dual-channel swivel device anchored above the cage so that blood pressure could be continuously monitored in conscious, unrestrained rats. After surgery, the rats received enrofloxacin (20 mg/kg) to prevent infection. The catheters were flushed daily with 0.3 mL isotonic saline containing heparin (500 U/mL).

**Experimental Protocol**

The rats were maintained on a low salt diet containing 0.4% NaCl for 5 days to recover from surgery. Baseline MAP was then recorded on 3 consecutive control days while the rats remained on the low salt diet. The arterial catheters were connected to transducers interfaced with a computerized data acquisition system, and systolic, diastolic, and mean arterial blood pressure and heart rate were continuously recorded at a frequency of 300 Hz for 5 hours per day between 10:00 AM and 3:00 PM. MAP was averaged over 1-minute intervals, and a single value was determined for each recording session. The rats were divided into 4 treatment groups and received either ABT (50 mg/kg per day IV, n=5) to block the renal formation of EETs and 20-HETE,10,12 HET0016 (10 mg/kg per day IV, n=5) to selectively inhibit the formation of 20-HETE,14,15 or the vehicles for ABT (0.9% NaCl, n=5) and HET0016 (10% lecithin in 0.9% NaCl solution, n=4). After administration of the P450 inhibitors or the vehicles, the rats remained on the low salt diet and MAP was recorded for 2 days. Then, the rats were switched to a high salt (8% NaCl) diet and MAP was recorded for an additional 10 days. Other experiments were performed in rats treated with ABT (n=5) and HET0016 (n=5) that were maintained on the low salt diet throughout the experiment to determine whether the blood pressure responses to these inhibitors were salt-dependent.

Urine samples were collected during the control period and on day 10 of the high salt diet for measurement of 20-HETE excretion. The samples were collected into glass bottles placed on dry ice that contained 5 mg triphenylphosphine to prevent oxidation of 20-HETE. The concentration of 20-HETE in the urine samples was determined through the use of a fluorescent high-performance liquid chromatography (HPLC) assay, as previously described.12

**Renal P450 AA Metabolism Assay**

The effects of chronic treatment of rats with ABT and HET0016 on renal metabolism of AA by renal cortical homogenates were studied. Crude homogenates, instead of renal microsomes, were used in these experiments to avoid dilution of the tissue levels of the drugs, since HET0016 is a reversible competitive inhibitor of the formation of 20-HETE. In these experiments, the chronically treated rats were anesthetized with sodium pentobarbital (50 mg/kg IV), and the kidneys were removed. The kidneys were homogenized in 1 mL of a 10-mmol/L potassium buffer (pH 7.7) containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 7.5 μmol/L protease inhibitor cocktail (Sigma, cat no. P-8340). The homogenates were centrifuged for 15 minutes at 3000g and for 30 minutes at 11 000g. The protein concentrations of the homogenates were determined with the use of the Bradford method with bovine γ-globulin as a standard. P450 enzyme activity was determined by incubating samples (1 mg protein) with [14C]-AA (0.2 μCi, 1.8 μmol/L) in 0.5 mL of a 100-mmol/L potassium phosphate buffer (pH 7.4) containing 10 mmol/L MgCl, 1 mmol/L EDTA, 1 mmol/L NADPH, and an NAPDH-regenerating system (10 mmol/L isocitrate and 0.16 U/mL isocitrate dehydrogenase) at 37°C for 15 minutes. These reactions were run without adding cold AA because it competes with the competitive inhibitor HET0016. Acidic lipids were extracted with ethyl acetate and dried overnight in a stream of N2.

**Statistical Analysis**

Values are presented as mean±SEM. Significance of differences between mean values were determined with the use of an ANOVA followed by the Student-Newman-Keuls post hoc test. A value of P<0.05 for a 2-tailed test was considered statistically significant.

**Results**

**Effects of ABT and HET0016 Treatment on Blood Pressure**

The effects of chronic blockade of the renal formation of EETs and 20-HETE with ABT or 20-HETE alone with HET0016 on MAP are summarized in Figure 1. Since there were no differences in MAP in the rats that received different vehicles for HET0016 and ABT, the data from these two control groups were combined. Blood pressure did not change during the protocol in the vehicle-treated rats. In rats fed a low salt diet, MAP fell by ≈10 mm Hg on the first day of ABT treatment (Figure 1A) and remained significantly below control throughout the study. In contrast, HET0016 had no effect on MAP in rats fed a low salt diet (Figure 1B). MAP gradually increased by ≈20 mm Hg in both the ABT-treated and HET0016-treated rats when the rats were switched to a high salt diet for 10 days. In contrast, MAP did not increase in rats treated with ABT or HET0016 that were maintained on a low salt diet throughout the study.

**Effect of ABT and HET0016 Treatment on Renal Metabolism of AA**

The effects of ABT and HET0016 on the formation of 20-HETE, EETs, and diHETEs in renal homogenates were presented in Figure 2A. ABT and HET0016 reduced the synthesis of 20-HETE by ≈90% in rats fed either a low or a high salt diet. ABT also reduced cortical epoxygenase activity by 50% in the rats maintained on a low salt diet and by 76% in the rats fed high salt diet for 10 days. However, epoxygenase activity was not significantly altered in rats that were treated with HET0016.

**Effect of ABT and HET0016 Treatment on Urinary Excretion of 20-HETE**

The effects of ABT and HET0016 on the excretion of 20-HETE are summarized in Figure 2B. The excretion of 20-HETE averaged 470±21 ng/d when the rats were fed a low salt diet. 20-HETE excretion increased by 20% when rats were fed a high salt diet for 10 days. ABT and HET0016 treatment reduced 20-HETE excretion by ≈70% and ≈90%, respectively.
Discussion

In the current study, ABT reduced blood pressure by \( \approx 10 \text{ mm Hg} \) in SD rats fed a low salt diet. This fall in MAP is consistent with previous findings in the SHR, in which inhibition of the renal formation of 20-HETE with ABT, sodium 10-undecynyl sulfate (10-SUYS), or antisense oligonucleotides have been reported to reduce blood pressure in this strain. ABT has also been reported to lower blood pressure in deoxycortico-sterone acetate (DOCA) salt–hypertensive rats and in rats with angiotensin II–induced hypertension. This may indicate that the reduction in blood pressure after ABT administration is mediated by some action that is independent of blockade of the formation of EETs and/or 20-HETE. In support of this view, other investigators have demonstrated that ABT does inhibit the activity of several other P450 enzymes besides those that produce EETs and 20-HETE.

The current study also compared the effects of chronic blockade of the renal formation of 20-HETE with ABT or HET0016 on blood pressure in SD rats fed a high salt diet. MAP gradually rose by 20 mm Hg over a 10-day period in rats that were treated with ABT or HET0016. In contrast, blood pressure was not significantly altered in the SD rats fed a low salt diet that were treated with ABT and HET0016. This fall in MAP is consistent with previous studies indicating that ABT blocks the formation of both EETs and 20-HETE in the kidney after chronic administration. This would suggest that the difference in the blood pressure response seen in rats treated with HET0016 and ABT may be due to an effect of ABT to inhibit formation of EETs. However, this hypothesis is not consistent with previous reports demonstrating that EETs are potent vasodilators, promote natriuresis, and have antihypertensive properties. This may indicate that the reduction in blood pressure after ABT administration is mediated by some action that is independent of blockade of the formation of EETs and/or 20-HETE. In support of this view, other investigators have demonstrated that ABT does inhibit the activity of several other P450 enzymes besides those that produce EETs and 20-HETE.

Figure 1. Effects of blockade of P450 metabolism of AA on blood pressure in SD rats fed a high salt diet. Blood pressure was recorded from conscious SD rats chronically treated with either ABT (A) to block the formation of EETs and 20-HETE or HET0016 (B) to selectively block the formation of 20-HETE. Mean±SEM values are presented. Numbers in parentheses indicate number of rats studied in each group. *P<0.05 vs baseline blood pressure. †P<0.05 vs corresponding value in vehicle-treated rats.

Figure 2. Effect of ABT and HET0016 on renal metabolism of AA (A) and urinary excretion of 20-HETE (B) in SD rats fed a low and/or high salt diet. Epoxygenase activity indicates EETs+DiHETEs formation. Mean±SEM values are presented. Numbers in parentheses indicate number of rats studied in each group. *P<0.05 vs vehicle-treated rats.
a high salt diet that were treated with vehicle or in rats treated with ABT and HET0016 that were maintained on a low salt diet throughout the study. Both ABT and HET0016 were equally effective in blocking the formation of 20-HETE in the kidney and in reducing the urinary excretion of 20-HETE. Taken together, these findings suggest that chronic blockade of the renal formation of 20-HETE promotes the development of salt-sensitive hypertension in otherwise salt-insensitive SD rats. This finding is consistent with previous studies, indicating that a deficiency in the renal formation of 20-HETE plays a critical role in elevating loop Cl– transport, resetting pressure natriuresis and the development of hypertension in DS rats. Our findings are also consistent with those of Stee et al., who demonstrated that chronic blockade of the renal formation of 20-HETE with an intrarenal infusion of 17-ODYA induced salt-sensitive hypertension in Lewis rats; it also fits with a recent report by Laffer et al. showing that 20-HETE excretion is linked to sodium sensitivity of blood pressure in humans.

The finding that a reduction in the renal formation of 20-HETE is associated with the development of salt-sensitive hypertension in normally salt-resistant SD rats suggests that 20-HETE may contribute to the renal adaptation to elevations in Na+ intake. However, the current results indicate that elevations in salt intake have little effect on the renal production or excretion of 20-HETE in SD rats. These results are consistent with previous reports showing that the renal formation of 20-HETE either did not change or fell when Lewis, Brown-Norway, SD, DR, and DS rats are fed a high salt diet.22,25–28 Together, these findings indicate that 20-HETE in the kidney plays an important role in the chronic regulation of Na+ excretion and blood pressure, but elevations in renal 20-HETE production per se do not contribute to the inhibition of Na+ transport associated with elevations in salt intake.

Renal epoxygenase activity increased in rats fed a high salt diet in the current study. This finding confirms earlier reports by Makita and colleagues and Holla and coworkers, suggesting that epoxygenase activity is influenced by salt intake and that an inability to upregulate epoxygenase activity may contribute to the development of hypertension in DS rats. Our findings also are consistent with recent findings that administration of inhibitors of sEH, to elevate the renal formation of EETs, lowers blood pressure in SHR rats and attenuates angiotensin II–induced hypertension. However, in the current study, there was no difference in the salt sensitivity of blood pressure in SD rats treated with either ABT or HET0016, even though renal epoxygenase activity was inhibited in rats treated with ABT but not in rats treated with HET0016. This finding suggests that it is likely that the fall in renal 20-HETE levels rather than the levels of EETs determines salt sensitivity of blood pressure in rats.

The mechanism responsible for the rise in blood pressure in ABT-treated and HET0016-treated rats fed a high salt diet remains to be determined; however, our results are consistent with the hypothesis that inhibition of renal 20-HETE production increases tubular reabsorption of Na+. In this regard, 20-HETE is normally produced by proximal tubules and inhibits Na+ reabsorption in this nephron segment by inhibiting Na+/K+–ATPase activity. EETs have also been reported to inhibit Na+/K+–ATPase activity, and inhibit Na+ transport in the proximal tubule and rabbit cortical collecting duct, and inhibit vasopressin-stimulated water reabsorption in the collecting duct. 20-HETE also plays a critical role in the regulation of Cl– transport in the TALH and inhibits Na+/K+/2Cl– transport by blocking K+ channels in the apical membrane of TALH cells, thereby limiting the availability of K+ for transport via Na+/K+/2Cl– transporters. This reduces the lumen positive transepithelial potential and reduces passive reabsorption of Na+ in this nephron segment. The findings from the current study, when coupled with these previous observations, suggest that the hypertensive effect observed by lowering renal 20-HETE levels may involve Na+ retention; however, additional work that demonstrates altered renal Na+ handling is necessary to accept this hypothesis.

**Perspectives**

The current study is the first to demonstrate that chronic blockade of the renal formation of 20-HETE increases blood pressure in normally salt-insensitive SD rats fed a high salt diet. The hypertensive effect of 20-HETE inhibitors is salt-dependent, since blood pressure did not increase in rats treated with ABT or HET0016 that were fed a low salt diet. This finding is consistent with the view that the renal formation of 20-HETE influences Na+ excretion and the regulation of blood pressure during dietary salt loading.

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