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Soluble epoxide hydrolase inhibitor, AUDA, prevents early salt-sensitive hypertension

Jing Li[1], Mairead A. Carroll[1], Praveen N. Chander[2], John R. Falck[3], Bhavani Sangras[3], Charles T. Stier [1]

[1]Department of Pharmacology, New York Medical College, Valhalla, New York 10595, [2]Department of Pathology, New York Medical College, Valhalla, New York 10595, [3]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390

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1. ABSTRACT

In stroke-prone spontaneously hypertensive rats (SHRSP) end-organ damage is markedly accelerated by high-salt (HS) intake. Since epoxyeicosatrienoic acids (EETs) possess vasodepressor and natriuretic activities, we examined whether a soluble epoxide hydrolase (sEH) inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), to inhibit the metabolism of EETs, would protect against pathologic changes in SHRSP. Seven-week-old male SHRSP were treated as follows: normal salt (NS), NS + AUDA, HS and HS + AUDA. Systolic blood pressure (SBP) (205 +/- 4 v 187 +/- 7 mmHg) and proteinuria (3.7 +/- 0.2 v 2.6 +/- 0.2 mg/6 h), but not plasma EETs (11.0 +/- 0.9 v 9.7 +/- 1.1 ng/ml), were significantly increased at 9 weeks of age in HS v NS SHRSP. HS was associated with fibrinoid degeneration and hypertrophy of arterioles in the kidney and perivascular fibrosis and contraction band necrosis in the heart. AUDA ameliorated these early salt-dependent changes in saline-drinking SHRSP and increased plasma levels of EETs but did not affect water and electrolyte excretion. sEH inhibition may provide a therapeutic strategy for treating salt-sensitive hypertension and its sequelae.

2. INTRODUCTION

The mechanisms underlying salt sensitivity of blood pressure elevation and tissue injury remain to be defined. Studies have shown that sodium intake may be linked to target organ damage including cardiac and renal disease in a manner that is independent of blood pressure (1). The stroke-prone spontaneously hypertensive rat (SHRSP) is a well-established model of hypertension in which end-organ damage is salt-dependent. SHRSP develop severe hypertension, malignant nephrosclerosis, cardiac injury and stroke (2-4). Of particular note is that the treatment of SHRSP with excess dietary salt dramatically increases the onset stroke, myocardial infarction and kidney damage. Consistent with a role for the renin-angiotensin system (RAS) was the finding that saline-drinking SHRSP display a paradoxical increase in plasma renin activity with time despite continued salt-loading (5-7).

In previous studies, we have shown that angiotensin-converting enzyme (ACE) inhibitors (7-9), angiotensin II (Ang II) subtype-1 (AT_1) receptor blockers (5, 10) and the mineralocorticoid receptor antagonists

spironolactone (11) and eplerenone (12) markedly reduce stroke, proteinuria and vascular injury in the absence of blood pressure lowering in saline-drinking SHRSP. Consistent with a central pathophysiological role of aldosterone in the saline-drinking SHRSP and the ability of aldosterone to stimulate the epithelial sodium channel (ENaC), we found that amiloride and other agents that inhibit ENaC function offer major protective effects against the development of proteinuria, renal microvascular damage and stroke (2, 4, 13). These data indicate that ENaC inhibition subserves an important vascular protective effect in saline-drinking SHRSP. Recent studies have described epoxyeicosatrienoic acids (EETs) as having a direct inhibitory effect on ENaC activity (14). Since EETs may serve as endogenous ENaC inhibitors, we reasoned that interventions which increase the levels of EETs may have a major impact on the pathologic changes that occur in saline-drinking SHRSP.

EETs are metabolites of arachidonic acid, the formation of which may be highly sensitive to high-salt intake (15, 16). EETs are potent vasodilators involved in the action of endothelium-derived hyperpolarizing factor that can lower blood pressure and increase renal sodium excretion (17). The hydrolysis of EETs to their corresponding *vic*-dihydroxyeicosatrienoic acids (DHETs) products is generally regarded as a mechanism by which the biologic actions of the EETs can be largely eliminated (18, 19). This conversion is catalyzed by the enzyme soluble epoxide hydrolase (sEH) (20) which can be selectively inhibited *in vivo* and *in vitro*. In kidneys of SHR compared with WKY, increased EET hydrolysis consistent with elevated expression of sEH has been reported (21). Potent and stable urea-based inhibitors of sEH have been used for long-term *in vivo* studies (15, 22). One of these agents, 2-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA) (23), exhibits increased water solubility with maintenance of potency (24) making it amenable to oral administration. Placement of AUDA in the drinking water of SHRSP (25 mg/L) starting at 6 weeks of age reduced the size of cerebral infarcts (25). This cerebrovascular protective effect of sEH inhibition occurred independently of changes in arterial blood pressure. In Ang II-induced hypertensive rats, renal cortical sEH protein expression was found to be significantly elevated compared with normotensive controls and chronic administration of the sEH inhibitor N-cyclohexyl-N-dodecyl urea lowered arterial blood pressure (19, 26, 27). On the basis of the beneficial cardiovascular properties of EETs, we hypothesize that sEH inhibition would provide protection against pathophysiological changes in saline-drinking SHRSP.

3. MATERIALS AND METHODS

3.1. Experimental animals

Experiments were performed using 21 male SHRSP (generation F-85) from our colony at New York Medical College. These animals were bred from National Institutes of Health stock, which was derived from the SHRSP/A3N substrain as described previously (4). Animals were weaned at four weeks of age and allowed water to drink *ad libitum* and fed standard rodent diet (Purina Lab Chow 5001; Ralston-Purina, St. Louis, MO). Animals were housed in a room maintained at an ambient temperature of 23 +/- 2°C with a 12-h light (06:00 to 18:00 h) and 12-h dark (18:00 to 06:00 h) cycle. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23) and the Institutional Animal Care and Use Committee at New York Medical College approved all procedures.

3.2. Experimental procedures

Throughout the study, all animals were fed Purina Lab Chow which contains approximately 0.38% sodium and 1.23% potassium by weight (8). At approximately 7 weeks of age, the animals were divided into the following treatment groups: 1) SHRSP given water to drink and no drug treatment (normal salt (NS) intake; (n = 4), 2) SHRSP given water to which AUDA was added at 25 mg/L (NS + AUDA; n=4), 3) SHRSP given 1% NaCl to drink and no drug treatment (high salt (HS) intake; n = 7), and 4) SHRSP given 1% NaCl to drink to which 12.5 mg/L of AUDA was added (HS + AUDA; n = 6). β-cyclodextrin (500 mg, Sigma Chemical Co., St. Louis, MO) and 70 % ethanol (0.75 ml) was used to facilitate the dissolution of AUDA and was added to each liter of drinking fluid prepared for each group. The concentration of AUDA in the 1% NaCl drinking solution was decreased by half as the fluid intake in these animals was twice that of the NS-treated SHRSP. AUDA was synthesized in the laboratory of Dr. John R. Falck. Animals were housed in metabolic cages for the last 3 days of the study. A 6-h urine collection was obtained on the day before the study ended for measurement of urinary protein excretion and on the final day systolic blood pressure (SBP) and heart rate were determined using tail-cuff plethysmography (CODA 2 non invasive blood pressure apparatus, Kent Scientific Corporation, Torrington, CT). Rats were then anesthetized (65 mg/kg of sodium pentobarbital i.p.) and blood was drawn through a midline incision from the abdominal aorta into a 10-mL syringe that has been rinsed with 3,000 Units/ml of sodium heparin (4). Approximately 7 ml of blood was placed into a purple-top EDTA tube for the preparation of plasma for the measurement of EETs and DHETs (16, 28). Kidneys and heart from all animals were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. The tissues were cut at 2 to 4 μm and stained with hematoxylin and eosin (H and E) and periodic acid-Schiff reagent (PAS) for examination by light microscopy. The prevalence of renal damage was quantified by counting the number of vascular profiles exhibiting either hypertrophy or fibrinoid degeneration. Vascular hypertrophy was defined as a variable degree of mural thickening. It was often uneven when evaluated in cross-sectioned or longitudinally cut blood vessels. The latter frequently displayed alternating areas of constriction and dilation with a sausage-shaped appearance. Fibrinoid degeneration of vessels was defined as the absence of myocytic nuclei in conjunction with hypereosinophilia in an area of the vessel wall and/or accumulation of brightly, PAS positive globular material in the vessel wall. The data are expressed as the total number of vessels affected per field of 200 glomeruli. Urinary

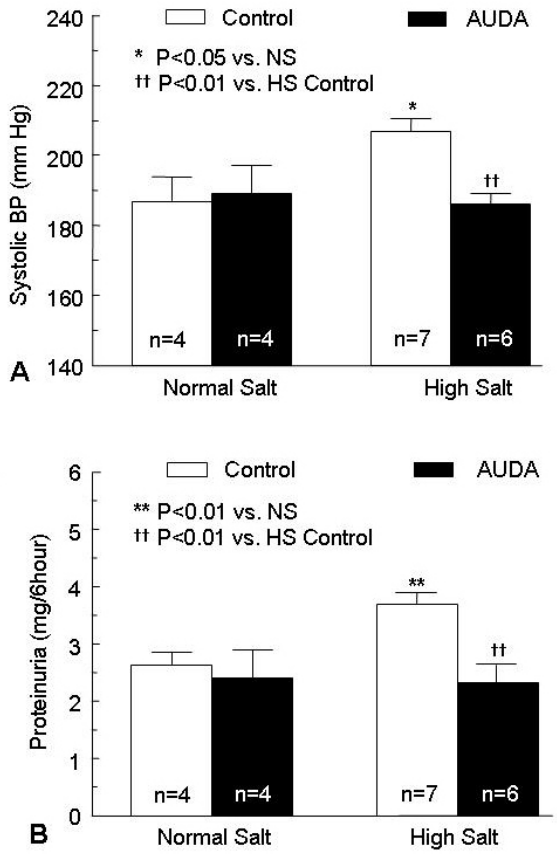

Figure 1. Systolic blood pressure (SBP) (A) and urinary protein excretion (B) of stroke-prone spontaneously hypertensive rats at nine weeks of age. Animals were untreated or given AUDA starting at 7 weeks of age. At 7 weeks and 3 days of age, some animals in each treatment group either continued to receive water or were given 1% NaCl to drink. All animals were fed Purina Rodent Chow throughout the study. sEH inhibition prevented the salt-sensitive component of hypertension and increases in urinary protein excretion in SHRSP. Values are means +/- SE.

protein excretion was measured by the sulfosalicyclic acid turbidity method as previously described (8).

3.3. Quantitation of plasma EETs and DHETs by GC-MS

500 µl frozen plasma from rats in each of the groups that received NS and HS were thawed on ice and prepared for analysis. 1 ng each of deuterated (D_8) 8, 9-EET, 11, 12-EET and 14, 15-EET (Biomol, Plymouth Meeting, PA), and 1ng each of D_8 8, 9-DHET, 11, 12-DHET and 14, 15-DHET (synthesized by us), were added as internal standards before extraction. Samples were diluted with 500 µl purified water and total plasma lipid was extracted twice with 2 ml chloroform/methanol (2:1) and evaporated to dryness. 1 ml KOH (1M) was added to samples to hydrolyze esterified EETs and DHETs. After sitting at room temperature for 90 min, samples were acidified to pH 4.0 with 1M HCl and total plasma EETs and DHETs were extracted twice with 2 volumes of ethyl acetate and evaporated to dryness and reconstituted in 50 µl methanol for HPLC purification.

The samples were purified by RP-HPLC on a C_{18} µ Bondapak column (4.6 x 24 mm) by using a linear gradient from acetonitrile:water:acetic acid (62.5:37.5:0.05%) to acetonitrile (100%) for 20 min at a flow rate of 1 ml/min. Fractions containing EETs and DHETs were collected on the basis of the elution profile of standards monitored by ultraviolet absorbance (205 nm) and were derivatized to pentafluorobenzyl esters, and DHETs were further derivatized to trimethylsilyl ethers. Samples were dried with nitrogen and resuspended in 50 µl of isooctane until gas chromatography-mass spectrometry (GC-MS) analyses. 1 µl aliquots were injected into a GC column (DB-1; 15.0 m; 0.25 mm, i.d.: 0.25 µm film thickness, Supelco) using a temperature program ranging from 180°C to 300°C at a rate of 25°C/min for DHETs and a temperature program ranging from 150°C to 300°C at a rate of 30°C/min for EETs. The MS was operated in electron capture chemical ionization mode and metabolites identified by selective ion monitoring and by comparison of GC retention times with standards: Sample EETs were identified (ion *m/z* 319) and quantified by calculating the ratio of abundance of D_8 EET (m/z 327) and DHETs by the ratio of abundance of ion *m/z* 481 to *m/z* 489. The highly labile 5, 6-EET was not measured (16, 29).

3.4. Statistical analysis

Significant effects with respect to treatment and time were determined by a two-way analysis of variance. Data with only one grouping variable were analyzed by one-way analysis of variance, followed by post-hoc analysis using the method of Bonferroni. Data were analyzed using the BMDP software package (BMDP Statistical Software, Los Angeles, CA). Correlation analysis was performed using the Pearson correlation coefficient. Differences between means were considered statistically significant at $P < 0.05$. Data are expressed as means +/- SE.

4. RESULTS

4.1. Blood pressure and heart rate

Figure 1A shows the results for pre-terminal measurement of SBP by tail-cuff plethysmography. SHRSP maintained on 1% NaCl displayed a significantly higher level of SBP than animals in either the NS or the NS + AUDA groups. The SBP of HS-treated SHRSP in the present study was the same as that reported previously (4). However, SBP was significantly lower in SHRSP on HS + AUDA compared with SHRSP on HS alone ($P<0.01$). In contrast, AUDA had no effect on SBP in SHRSP that were maintained on NS intake. Treatment of SHRSP on HS intake resulted in a level of SBP that was indistinguishable from that in the NS and NS + AUDA groups. Heart rate averaged from 392 to 427 beats per minute and did not differ among the groups (Table 1).

4.2. Urinary protein excretion

Figure 1B shows the results for urinary protein excretion which was measured on the day before the study

Table 1. Heart rate and urinary excretion data

		NS	NS + AUDA	HS	HS + AUDA
Heart rate	beats/min	425+/-2	427+/-20	392+/-11	417+/-11
Urine flow	ml/day	10.8+/-0.4	11.1+/-1.3	25.0+/-1.0 [2]	27.7+/-3.7 [2]
Urine Na concentration	mEq/L	149+/-12	167+/-8	322+/-10 [2]	332+/-19 [2]
Urine Na excretion	mEq/day	1.62+/-0.16	1.83+/-0.19	8.03+/-0.41 [2]	8.91+/-0.85 [2]
Urine K concentration	mEq/L	255+/-14	272+/-11	150+/-6 [1]	153+/-16 [1]
Urine K excretion	mEq/day	2.90+/-0.23	3.34+/-0.10	3.71+/-0.12	4.00+/-0.29
Urine Na/K		0.58+/-0.02	0.61+/-0.02	2.18+/-0.11 [2]	2.22+/-0.11 [2]
Number of rats		4	4	7	6

[1] $P < 0.05$, [2] $P < 0.01$ vs NS; NS = normal salt, HS = high salt, AUDA = 2-(3- adamantan-1-yl-ureido)-dodecanoic acid; Values are mean +/- SEM

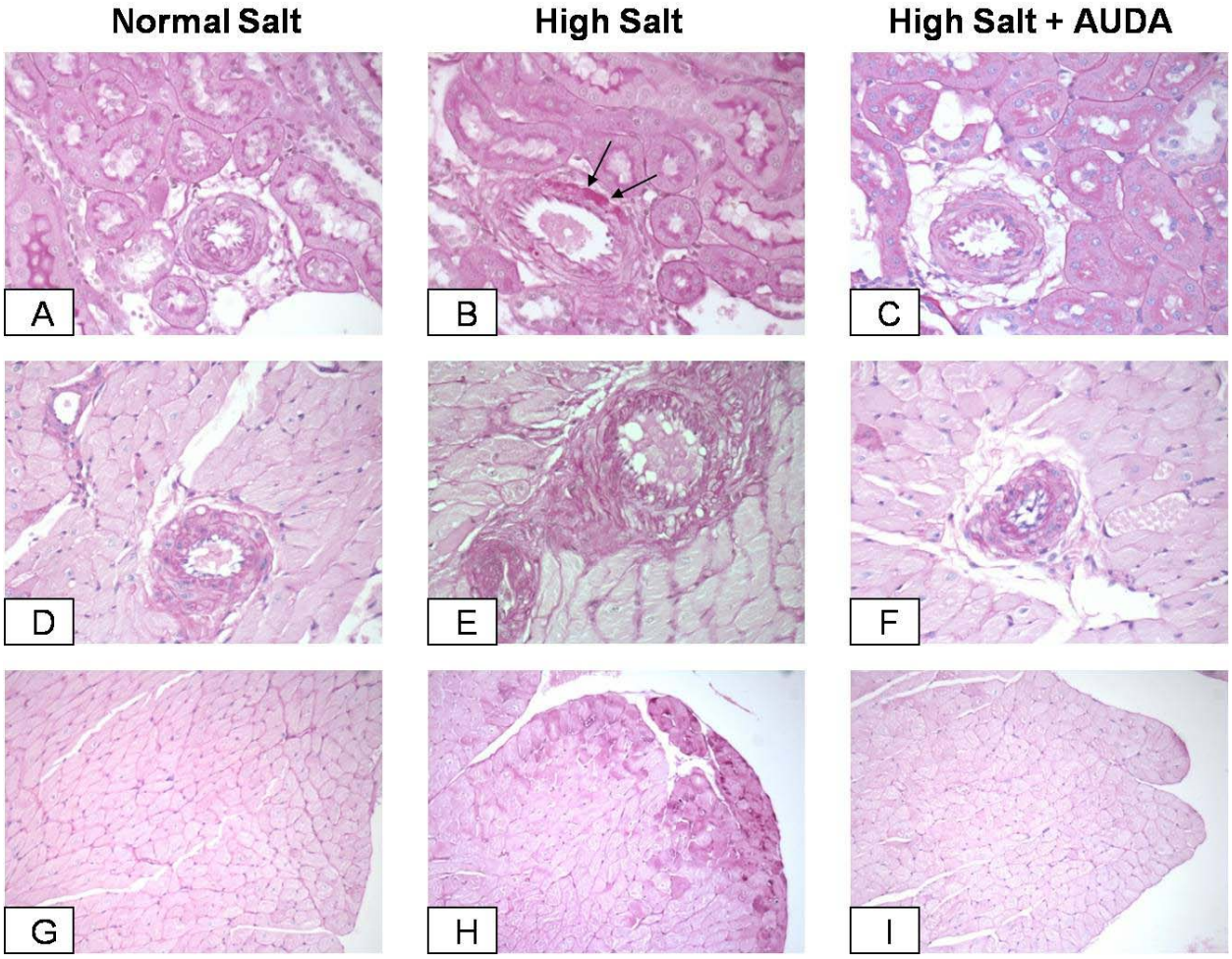

Figure 2. Representative PAS-stained photomicrographs (X100) of sections of kidney (A-C) and heart (D-I). In the kidney, medial hypertrophy and focal fibrinoid degeneration (arrows) in occasional arteries in the HS group was seen (B). The NS (A) and HS + AUDA (C) groups were generally devoid of similar changes. Medium-sized coronary arteries often showed prominent perivascular fibrosis and focal mural thickening in HS (E) compared with NS (D). Vascular pathology was largely abrogated in similar vessels in the AUDA + HS group (F). Hypereosinophilia in the subendocardial myocardium with mild focal contraction band necrosis in HS (H) compared to generally normal appearance in NS (G) was seen. These lesions were markedly abrogated by AUDA treatment (I).

ended. SHRSP on NS intake excreted low levels of protein into the urine and this was unaffected by treatment with AUDA. In contrast, SHRSP maintained HS exhibited a mild but highly significant increase in urinary protein excretion which did not occur in those animals that were treated with AUDA. Thus, AUDA prevented the

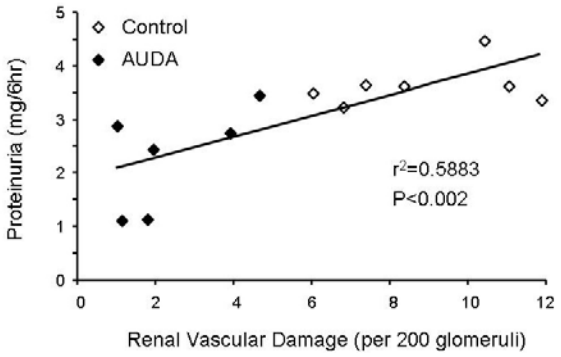

Figure 3. Diagram depicting the correlation between preterminal urinary protein excretion and the histologic evaluation of renal vascular damage expressed as the number of vascular profiles affected per field of 200 glomeruli in saline-drinking SHRSP that were chronically treated with either AUDA or vehicle (control). The Pearson correlation analysis revealed an r value of 0.7255 and a P value of < 0.002.

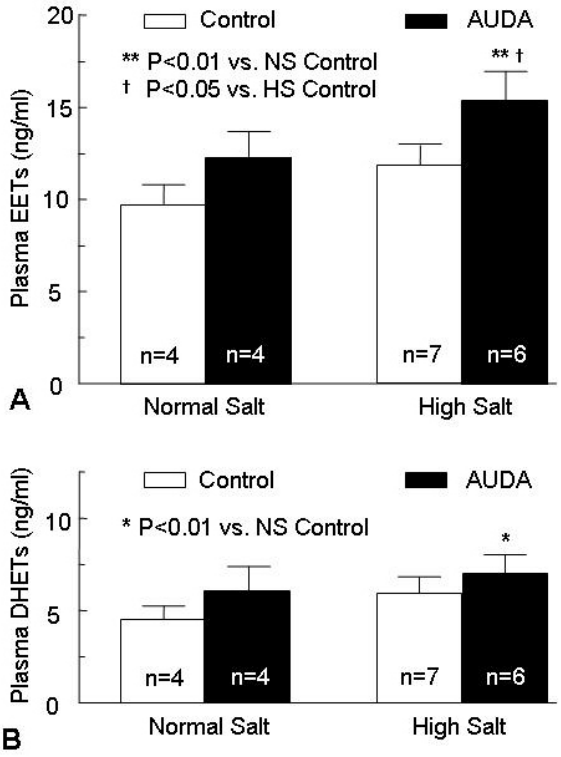

Figure 4. Plasma concentrations of EETs (A) and DHETs (B). AUDA had no effect in SHRSP that were maintained on NS intake. Plasma levels of EETs were significantly increased in animals on HS+AUDA compared with NS or HS controls, whereas, plasma levels of DHETs were significantly increased in animals treated with HS+AUDA compared with NS controls. Values are means +/- SE.

early increase in urinary protein excretion that was caused by HS intake in young SHRSP.

4.3. Cardiac and renal pathology

SHRSP on HS intake compared to NS intake showed mild histological evidence of damage in the heart and kidney. The changes in the heart were primarily related to focal perivascular fibrosis and in many cases subendocardial hypereosinophilia with focal contraction band necrosis. In the kidneys, there was evidence of fibrinoid degeneration of vessels in the medium-sized arteries. In addition, there were occasional areas of ischemic tubules and rarely lymphocytic infiltration. Many renal vessels showed moderate hypertrophy and vacuolation. These changes in the heart and kidneys were abrogated in HS-treated SHRSP that were treated with AUDA as is illustrated in Figure 2. The total renal vascular damage assessed as the number of vascular profiles exhibiting hypertrophy and fibrinoid degeneration was significantly less in SHRSP on HS + AUDA (2.38+/-0.62 per 200 glomeruli) compared with SHRSP on HS alone (8.82+/-0.86 per 200 glomeruli) (P<0.0001). Correlation analysis revealed a strong positive association between urinary protein excretion and renal vascular damage (P<0.002, Figure 3). The values for both parameters were highest for SHRSP on HS alone and lowest for the HS + AUDA SHRSP, with no overlap between the groups.

4.4. Plasma levels of EETs and DHETs

Figure 4 shows the results for the plasma concentration of EETs (A) and DHETs (B) in the four groups of animals that were studied. Although plasma EETs tended to be greater in the NS + AUDA and HS groups compared with the NS-treated SHRSP, these differences did not achieve statistical significance. However, in SHRSP maintained on HS + AUDA plasma levels of EETs were significantly elevated compared with either the NS or HS groups of SHRSP. Plasma levels of DHETs showed a similar profile as for those of EETs but were approximately 2-fold lower. Plasma levels of DHETs were significantly higher in SHRSP treated with HS + AUDA compared with the NS control group.

4.5. Urine volume and electrolyte excretion

Table 1 shows the results for the terminal collection of urine and illustrates no difference between the untreated and AUDA-treated SHRSP regardless of salt status. Urinary sodium concentration was significantly greater and urinary potassium concentration was significantly lower, respectively, in HS-treated SHRSP compared with SHRSP on NS intake. Urinary sodium excretion was significantly greater in SHRSP with HS intake. Although urinary potassium tended to be greater in HS-treated SHRSP, there was no overall difference among the groups. Treatment with AUDA did not affect the concentration or excretion of sodium or potassium regardless of the salt status of the animals. Although terminal body weight of SHRSP given HS was greater than those given NS, the initial body weight of SHRSP in the NS groups, measured at 6.5 weeks of age, was also slightly lower. Thus, the change in body weight over the course of the study did not differ between the NS and HS groups and this was also unaffected by treatment with AUDA.

5. DISCUSSION

In the present study, SBP was significantly lower in saline-drinking SHRSP that were treated with AUDA and did not differ from the NS control and NS + AUDA groups. Thus, AUDA eliminated the salt-sensitive component of BP elevation in SHRSP but did not affect BP in non-salt loaded SHRSP. In our previous studies with ACE inhibitors, AT_1 receptor antagonists and mineralocorticoid receptor antagonists in SHRSP maintained on 1% NaCl, plus Stroke-Prone Rodent Diet, we observed an initial period (1-2 weeks) of mild BP lowering which was not sustained during the remainder of the study despite continued HS intake and pharmacological treatment. Whether the BP lowering observed at 10-days in the present study would be maintained for longer periods of time remains to be determined. Of note also is that the animals in the present study were placed on a 1% NaCl drinking solution but did not receive Stroke-Prone Rodent Diet which has reduced potassium content. Thus, maintenance of saline-drinking SHRSP on Purina Lab Chow which has higher potassium content than Stroke-Prone Rodent Diet (1.23% vs. 0.71% by weight, respectively) (8) may have enabled a blood pressure lowering effect to be more readily demonstrable. Future studies will be needed to evaluate the chronic, long-term effects of AUDA on blood pressure and end-organ damage in these animals. The effect of AUDA on blood pressure has been examined in several different studies (22, 25, 30-33). AUDA at 130 μg/ml in the drinking water did not affect BP in normotensive mice but reduced BP in mice infused with 1 mg/kg per day of Ang II (22). Targeted disruption of the sEH gene has been shown to effect resting baseline BP in mice (34, 35). The BP phenotype in these animals is presumed to be due to an increase in the vasoactive arachidonic acid metabolites. Male knockout sEH mice have lower blood pressure than wild-type mice (35). Treatment with AUDA had no effect on SBP in SHRSP maintained on NS intake which is consistent with previous reports in which no effect on blood pressure was observed in SHRSP that were treated with AUDA, 25 mg/liter in the drinking water, for up to 6 weeks (25).

In the present study, we were able to detect early changes in urinary protein excretion that were associated with placing SHRSP on a HS intake. Although this change was mild, it was highly significant and was prevented by treatment with AUDA. We have previously reported that there is a strong correlation between urinary protein excretion and glomerular injury in saline-drinking SHRSP (36). Typically SHRSP placed on HS intake exhibit a low level of urinary protein excretion for several weeks which then increases progressively over time, ultimately developing renal lesions of fibrinoid necrosis. Thus, the effects observed in the present study would be commensurate with the prevention of subtle early changes which may subsequently progress to severe damage. Consistent with a mild level of urinary protein excretion observed in SHRSP on HS intake alone in this study, histological analysis revealed mild focal and scattered renal lesions. Importantly, enumeration of renal vascular damage revealed a significant reduction by treatment with AUDA that was positively correlated with reduced urinary protein excretion.

Treatment of saline-drinking SHRSP with AUDA elevated the levels of EETs in plasma. This increase is reflective of EETs in both the free and acylated forms as a base hydrolysis was performed to liberate EETs. Likewise an increase in DHETs was observed with AUDA in HS-treated SHRSP. The plasma level of EETs and DHETs we observed were similar to those reported by Dorrance *et al.* (25). However, unlike the latter study where an increase in the ratio of EETs to DHETs was found after six weeks of AUDA treatment, we did not observe such a change after 10 days of AUDA administration. Therefore, a change in the ratio of EETs in relationship to DHETs, which is usually taken as an index of sEH activity, may not relate to the results of our studies. In addition to metabolism by sEH and β oxidation, reacylation of EETs is another important route for the terminal disposition of EETs. sEH inhibition may prolong the biological half-life of EETs, to support a beneficial pharmacodynamic action, and this may result in increased reacylation and tissue levels of EETs as other pathways become more important in the final disposition of EETs. Our results would support the notion that AUDA increases the tissue levels of EETs. Problems with contamination precluded an analysis of the urine samples from this study for EETs and DHETs.

EETs have been described to possess diuretic and natriuretic activity at many levels within the kidney (17). They can inhibit Na/K ATPase, decrease fluid reabsorption in the proximal tubule, antagonize the effects of vasopressin and most recently they have been reported to block the action of aldosterone to stimulate sodium reabsorption in the collecting duct (14). Therefore, it would be anticipated that treatment with AUDA might be associated with increases in urinary salt and water excretion. However, we were unable to discern such effects. The concentration and excretion of sodium and potassium in the urine was unaffected by treatment with AUDA. Likewise, there was no effect on the urinary sodium-to-potassium ratio which is thought to be a sensitive index of the modulation of the renal tubular action of aldosterone. Previous studies reported that the effect of thiazide diuretics on water and electrolyte excretion is transient and not sustained. Thus, we cannot rule out the possibility that subtle changes in electrolyte excretion may be responsible, in part, for some of the effects of AUDA in our study. Further, the absence of a clear diuretic and natriuretic effect of AUDA in HS-treated SHRSP may be the consequence of the lower blood pressure in these animals. Thus, AUDA may have shifted the renal pressure-natriuresis curve such that water and electrolyte excretion could be sustained at a much lower arterial pressure. Interestingly, EETs also have anti-inflammatory activity (37) and this may have also contributed to the protection of saline-drinking SHRSP against the development of pathology observed with AUDA treatment.

In conclusion, the sEH inhibitor AUDA, prevented the early salt-dependent rise is arterial pressure in saline-drinking SHRSP. This effect was associated with a

reduced level of urinary protein excretion and elevated levels of EETs in plasma reflective of both the free and acylated forms. The effect of AUDA was not associated with evidence of sustained increases in water and electrolyte excretion. Although the possibility that AUDA has alternate actions cannot be excluded, the results suggest that inhibition of soluble epoxide hydrolase may provide a therapeutic strategy for treating salt-sensitive hypertension and its sequelae.

6. ACKNOWLEDGMENTS

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Abbreviations: ACE: angiotensin-converting enzyme, Ang II: angiotensin II, AT1: angiotensin II subtype-1, AUDA: 2-(3-adamantan-1-yl-ureido)dodecanoic acid, CYP: cytochrome P450, DHETs: dihydroxyeicosatrienoic acids, EETs: epoxyeicosatrienoic acids, GC-MS: gas chromatography-mass spectrometry, HS: high salt, NS: normal salt, RAS: renin-angiotensin system, SBP: systolic blood pressure, sEH: soluble epoxide hydrolase, SHRSP: stroke-prone spontaneously hypertensive rats

Key Words: Epoxyeicosatrienoic Acid, Salt-Sensitive Hypertension, Soluble Epoxide Hydrolase Inhibition, Stroke-Prone Spontaneously Hypertensive Rats

Send correspondence to: Charles T. Stier, Jr., Ph.D., Department of Pharmacology, Basic Science Building, New York Medical College, Valhalla, New York 10595, Tel: 914-594-4138, Fax: 914-594-4273, E-mail: charles_stier@nymc.edu

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