Soluble Epoxide Hydrolase Is a Main Effector of Angiotensin II–Induced Hypertension

Oliver Jung, Ralf P. Brandes, In-Hae Kim, Frank Schweda, Ronald Schmidt, Bruce D. Hammock,
Rudi Busse, Ingrid Fleming

Abstract—The soluble epoxide hydrolase (sEH) metabolizes vasodilatory epoxyeicosatrienoic acids (EETs) to their di-hydroxy derivatives. We hypothesized that the metabolism of EETs by the sEH contributes to angiotensin II–induced hypertension and tested the effects of a water-soluble sEH inhibitor, 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA) on blood pressure. AUDA (130 μg/mL in drinking water) did not affect blood pressure in normotensive animals but markedly lowered it in mice with angiotensin II–induced hypertension (1 mg/kg per day). The effect of AUDA was accompanied by an increase in urinary salt and water excretion. Intravenous application of AUDA (8 mg/kg) acutely lowered blood pressure and heart rate in animals with angiotensin II–induced hypertension but failed to affect blood pressure in animals with phenylephrine-induced hypertension (29 mg/kg per day). AUDA (0.1 μmol/L) selectively lowered vascular resistance in an isolated perfused kidney preparation from angiotensin II–pretreated mice but not from control mice. In the perfused hind limb and in isolated carotid arteries from angiotensin II–treated mice, AUDA was without effect. The ω-hydroxylase inhibitor *N*-methylsulfonyl-12,12-dibromododec-11-enamide, which attenuates formation of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid, decreased tone in carotid arteries from angiotensin II–treated but not from control mice. These data demonstrate that the decrease in blood pressure observed after sEH inhibition in angiotensin II–induced hypertension can be attributed to an initial reduction in heart rate followed by pressure diuresis resulting from increased perfusion of the kidney. Direct vasodilatation of resistance arteries in skeletal muscles does not appear to contribute to the antihypertensive effects of sEH inhibition in mice. (***Hypertension***. 2005;45[part 2]:759-765.)

Key Words: angiotensin ■ lipids

Epoxyeicosatrienoic acids (EETs) are important signaling molecules derived from arachidonic acid by the action of cytochrome P450 (CYP) epoxygenases.[1] Endothelium-derived EETs are potent vasodilators involved in the action of the endothelium-derived hyperpolarizing factor (EDHF),[2] lower blood pressure, and increase renal sodium excretion.[1,3] Consequently, it may be possible to attenuate or prevent the development of hypertension by maintaining high intravascular EET concentrations. The arachidonic acid epoxides are metabolized to their di-hydroxyl derivatives (DHETS) by the soluble epoxide hydrolase (sEH),[4] and this hydrophilic modification facilitates their diffusion out of the cells and renal clearance.[5,6] The activity of the sEH is therefore thought to be a major determinant of EET bioavailability.[4]

The expression of the sEH is high in a number of organs, including the kidney and the liver,[7] and several publications have suggested that the sEH plays a role in regulation of blood pressure. For example, male sEH$^{-/-}$ mice have a lower blood pressure than their control litter mates,[8] and inhibition of sEH using *N*,*N*′-dicyclohexylurea lowers blood pressure in spontaneously hypertensive rats.[4] Another sEH inhibitor, *N*-cyclohexyl-*N*-dodecyl urea, is reported to lower blood pressure in rats made hypertensive by the infusion of angiotensin II.[9] Although the above-mentioned reports were convincing, little is known about the true physiological effects of sEH inhibition.

In the present study, we characterized the effects of the novel sEH inhibitor 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA)[10] on blood pressure in mice with angiotensin II–induced hypertension. Furthermore, we set out to elucidate the mechanism underlying the antihypertensive effects of sEH inhibition by determining renal function and by using isolated preparations of the mouse hind limb (skeletal muscle region) and kidney.

Methods

Animal Experiments

Male C57/b6 male mice (7 weeks) were purchased from Charles River Breeding Laboratories. Experiments conformed to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85 to 23) and were approved by the local government (II25.3 to 19c20/15-F61/16). Osmotic mini-pumps (Alzet) delivering [5val]-angiotensin II (1 mg/kg per day), phenylephrine (29 mg/kg per day), or vehicle (PBS) were implanted under isofuren anesthesia as described.[11]

Blood pressure was recorded in conscious animals using an automated tail-cuff system (Visitech). In another series of experiments, blood pressure was recorded under general anesthesia (isofuren inhalation) via a catheter placed in the right carotid artery connected to a Stathem transducer system as described previously.[12]

AUDA was synthesized as reported previously[10] and was given either with drinking water (1 mg/mL in 1% [2-hydroxypropyl]-β-cyclodextin) for the experiments in which blood pressure was measured over a period of 12 days or as an intravenous infusion (8 mg/kg dissolved in 10% [2-hydroxypropyl]-β-cyclodextrin; volume 160 μL) to determine the acute effects of the substance on blood pressure. In preliminary experiments, these administration protocols resulted in plasma and tissue levels of AUDA of $\approx$50 nmol/L, which is in the range of the IC_{50} of the compound for the sEH.

In some animals, urine samples were collected over 24 hours, 7 days after implantation of mini-pumps and 24 hours after initiation of AUDA treatment in the drinking water.

Isolated Perfused Kidney

Mice were anesthetized with 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid (100 mg/kg IP; Byk Gulden) and ketamine-HCl (80 mg/kg; Curamed). Kidney perfusion was performed in a thermoregulated chamber as described previously.[13] In brief, the aorta and the renal vein were cannulated, and the venous effluent was drained outside the chamber. Kidneys of mice pretreated with angiotensin II were perfused with a modified Krebs–Henseleit solution at a constant pressure of 120 mm Hg and kidneys of untreated mice at 90 mm Hg. The modified Krebs–Henseleit solution contained (in mmol/L) all physiological amino acids in concentrations between 0.2 and 2.0: 8.7 glucose, 0.3 pyruvate, 2.0 L-lactate, 1.0 α-ketoglutarate, 1.0 L-malate, and 6.0 urea, as well as ampicillin (3 mg/100 mL), flucloxacillin (3 mg/100 mL), 6% (wt/vol) bovine serum albumin, and 1 mU/100 mL vasopressin 8-lysine, as well as freshly washed human red blood cells (10% hematocrit). The perfusate was continuously dialyzed against perfusion medium lacking erythrocytes and albumin and gassed with a mixture of 94% O_2 and 6% CO_2. Angiotensin II and AUDA were infused into the arterial limb of the perfusion circuit, and changes in the flow rate were recorded. Angiotensin II infusion was used to preconstrict the resistance vessels of the kidney. Probably as a consequence of downregulation of the angiotensin II type-1 (AT_1) receptor,[14] the constrictor response to angiotensin II was attenuated in kidneys from angiotensin II–treated animals.

Isolated Saline Perfused Hind Limb Preparation

Animals were euthanized by decapitation 7 days after implantation of the mini-pump. A catheter was inserted into the abdominal aorta and advanced to one iliac artery. The hind limb was perfused with constant pressure (100 mm Hg) with the aid of a roller pump and a pressure control valve using Krebs–Henseleit solution as described previously.[15] AUDA (1 μmol/L), angiotensin II (Ang II; 5 nmol/L), and bradykinin (100 μL bolus in 5% glucose, 10^{-9}–10^{-5} mol/L) were administered via a side port, and changes in flow rate were determined using a magnetic flow probe (Föhr Medical Instruments).

Organ Chamber Experiments

Organ chamber experiments were performed as described,[16] in rings of carotid artery from mice pretreated for 7 days with either angiotensin II or vehicle. Arterial rings were preconstricted with phenylephrine to 80% of the contraction elicited by KCl (80 mmol/L) and the relaxation to increasing concentrations of AUDA and to the ω-hydroxylase inhibitor *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) were determined.

Urine Analysis

Urine samples were diluted 1:10 and analyzed using an automated serum analyzer (EML Radiometer) according to the manufacturer's instructions.

Immunohistochemistry

Samples of mouse carotid artery and the kidney were embedded in Tissue-Tek and frozen in isopentan/liquid nitrogen. Cryostat sections (5 μm) were air-dried, fixed in phosphate-buffered formaldehyde solution (5%), permeabilzed using Triton X-100 (0.05%), and blocked with BSA solution (5% in PBS). The sEH was detected using a polyclonal rabbit antibody (1:800 dilution; kindly provided by C. Morriseau, UC Davis, California) and an appropriate secondary antibody coupled to Alexa-488 (Molecular Probes). Nuclei were counterstained using tropro-3 iodine (1:1000). Images were acquired by laser scanning microscopy (LSM 510 meta; Carl Zeiss). Samples from $sEH^{-/-}$ mice served as negative control (Frank J. Gonzalez, National Institutes of Health, Bethesda, Md).

Statistics

All values are the mean$\pm$SEM. Statistical analysis was performed using ANOVA for repeated measurements followed by Fisher least significant difference test or paired or unpaired *t* test, where appropriate.

Results

AUDA Lowers Blood Pressure in Angiotensin II–Hypertensive Mice

AUDA, in contrast to the sEH inhibitors investigated previously, can be prepared in aqueous solution using [2-hydroxypropyl]-β-cyclodextrin and administered with
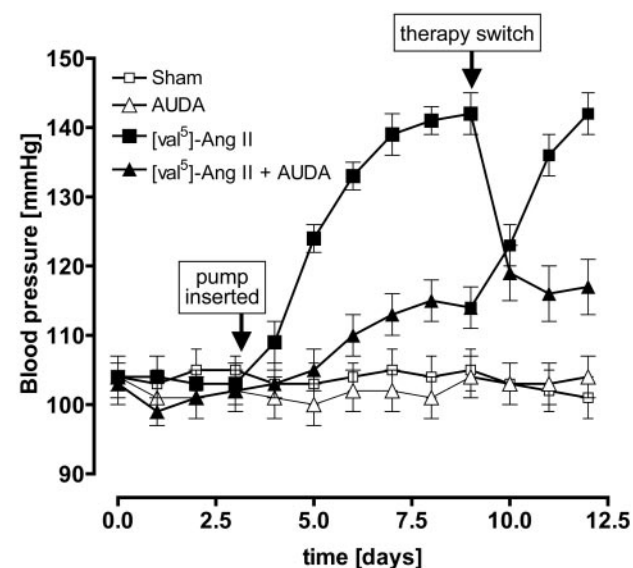

Figure 1. Effect of AUDA per os on blood pressure. Blood pressure was measured daily in conscious mice using the tail-cuff technique, and subgroups were treated with AUDA (130 μg/mL). On day 3 of the experiment, mice were implanted with osmotic mini-pumps delivering either [val5]-angiotensin II (1 mg/kg per day [val5]-Ang II) or vehicle. From day 9 onward (therapy switch), those mice previously receiving vehicle were started on AUDA treatment, whereas AUDA was withdrawn from the previously treated groups (n=8 each group).
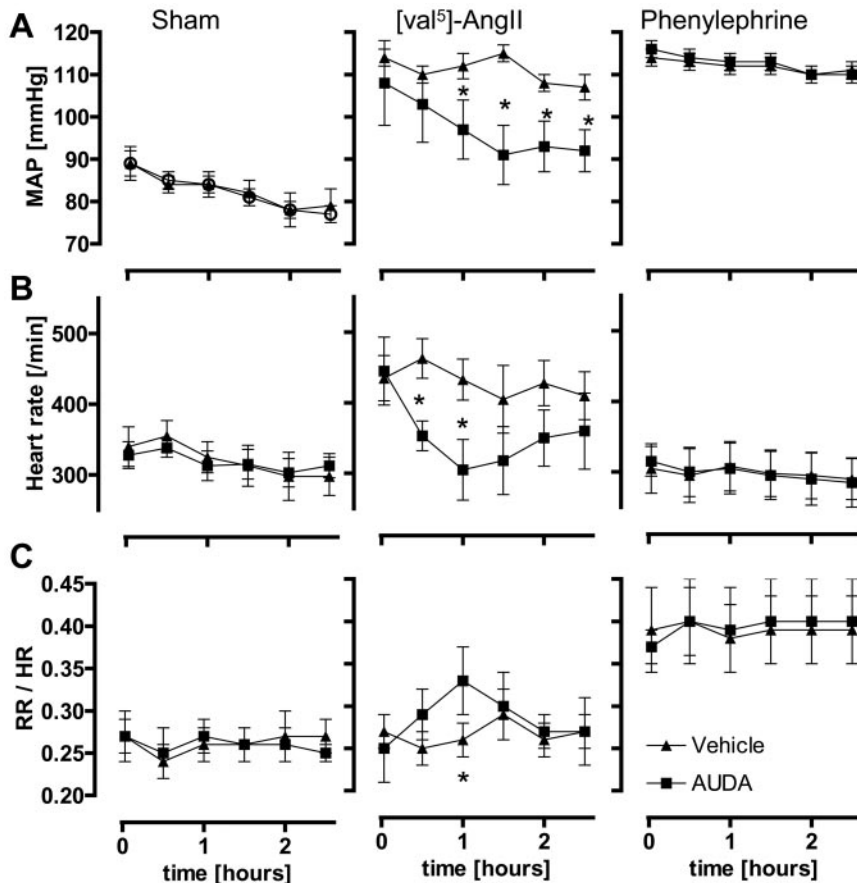
Figure 2. Effect of intravenous AUDA on blood pressure and heart rate. Mice were implanted with osmotic mini-pumps delivering [val[5]]-angiotensin II (1 mg/kg per day), vehicle, or phenylephrine (29 mg/kg per day). Seven days after pump insertion, mice were anesthetized and a catheter was placed in the carotid artery for measurements. AUDA (8 mg/kg) or vehicle was applied by intravenous bolus injection. The graphs show the time course of the effects of AUDA or vehicle given at 0 hours on mean arterial pressure **(A)**, heart rate **(B)**, and the blood pressure to heart rate ration (C; n=6 each group; *$P<0.05$ for vehicle vs AUDA treatment). [val[5]]-AngII indicates [val[5]]-angiotensin II.

drinking water. Treatment of animals with AUDA before the implantation of angiotensin II–containing mini-pumps largely attenuated the hypertensive effect of the peptide. After cessation of AUDA treatment, the blood pressure increased and reached the level of the untreated animals within 4 days. When AUDA treatment was started after induction of angiotensin II–induced hypertension, the blood pressure dropped almost to the level observed in the sham-operated animals. AUDA had no effect on the blood pressure of sham-operated animals (Figure 1).

To better characterize the effects of AUDA on blood pressure, a single bolus of the inhibitor (8 mg/kg) was administered via a catheter placed in the left jugular vein, and blood pressure in the carotid artery was measured. The acute administration of AUDA had no effect in sham-treated animals or animals made hypertensive by the implantation of osmotic mini-pumps containing phenylephrine. In contrast, the inhibitor elicited a significant decrease in the blood pressure of angiotensin II–treated animals (Figure 2). The effect on the diastolic pressure was more pronounced than that on the systolic pressure (data not shown). AUDA significantly lowered the heart rate in angiotensin II–pre-treated animals to the level observed in control animals but did not affect heart rate in either control or phenylephrine-treated mice. The pressure/heart rate ratio, taken as a rough estimate for peripheral resistance (assuming constant stroke volume), was unaffected by AUDA in all 3 groups.

AUDA Selectively Increases the Perfusion of the Kidney in Angiotensin II–Hypertensive Mice

To determine the role of the sEH for the control of the peripheral resistance, isolated organ preparations were used. The effect of AUDA was tested in kidneys and hind limbs from sham- and angiotensin II–treated mice in the presence of angiotensin II. In kidneys isolated from angiotensin II–treated mice, infusion of AUDA significantly lowered vascular resistance, whereas the inhibitor had no effect in control animals (Figure 3). AUDA had no effect on the resistance or the bradykinin-induced vasodilatation of isolated perfused hind limbs from control or angiotensin II–treated mice (Figure 4A).

Inhibition of 20-HETE Formation but Not EET Degradation Relaxes Conduit Vessels From Angiotensin II–Hypertensive Mice

Next, the role of the ω-hydroxylase was compared with that of the sEH on the constrictor tone of conduit vessels. Neither the ω-hydroxylase inhibitor, DDMS, nor AUDA affected the tone of carotid arteries from sham-operated mice. However, DDMS induced a pronounced, concentration-dependent decrease in tone in vessels from angiotensin II–treated animals, whereas AUDA had no effect (Figure 4B and 4C). To exclude the lack of sEH expression in the carotid artery as the reason for this observation, sEH expression was studied. Immuno-histochemistry revealed that the sEH was expressed predominantly in the endothelium (Figure 4D).
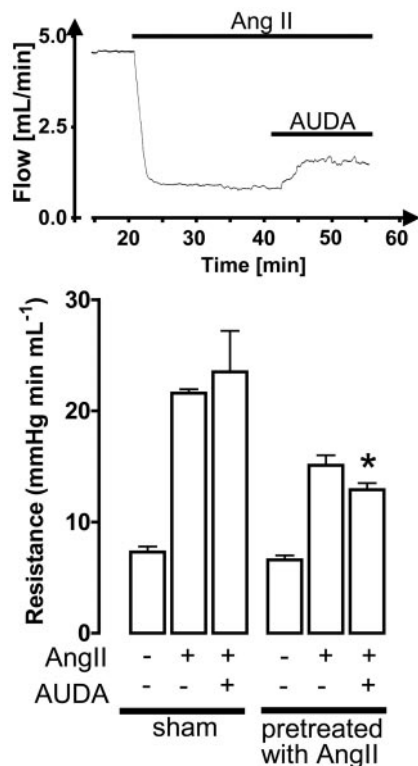
Figure 3. Effect of AUDA on the resistance of the isolated per-fused mouse kidney. Top, Original tracing showing changes in flow in a kidney from an angiotensin II (Ang II)–pretreated mouse. Bottom, Statistical analysis showing the effects of AUDA (100 nmol/L) on the resistance in the isolated kidney preparation after preconstriction with angiotensin II (1 nmol/L). Kidneys were obtained from mice pretreated with angiotensin II (1 mg/kg per day via osmotic mini-pump for 7 days) or vehicle (sham; n=4; *$P<0.05$ before vs during AUDA infusion).

AUDA Increases Urinary Salt and Water Excretion in Angiotensin II–Hypertensive Mice

To determine the effects of AUDA on renal function, urine was collected from mice 6 days after insertion of the osmotic mini-pumps delivering either vehicle or angiotensin II. Urinary flow rate and osmolarity, as well as sodium and potassium excretion, were not different between mice receiving vehicle or angiotensin II. However, AUDA selectively increased urinary flow rate and the excretion of sodium and potassium in mice treated with angiotensin II without having an effect on control animals. In contrast, AUDA did not affect urine osmolarity (Figure 5A). Calculation of the osmotic gap revealed that AUDA also enhanced the excretion of nonionic osmolytes (mainly urea) in mice treated with angiotensin II but not in control animals (data not shown). Immunohisto-chemistry revealed high expression of the sEH in distal tubuli but low levels in the glomeruli (Figure 5B).

Discussion

In the present study, we observed that inhibition of the sEH in mice attenuates the hypertensive effect of angiotensin II but not phenylephrine. The effects of sEH inhibition could be attributed to a reduction in heart rate and an increase in the excretion of salt and water. Vasodilatation, a possible conse-quence of sEH inhibition and mechanism underlying the
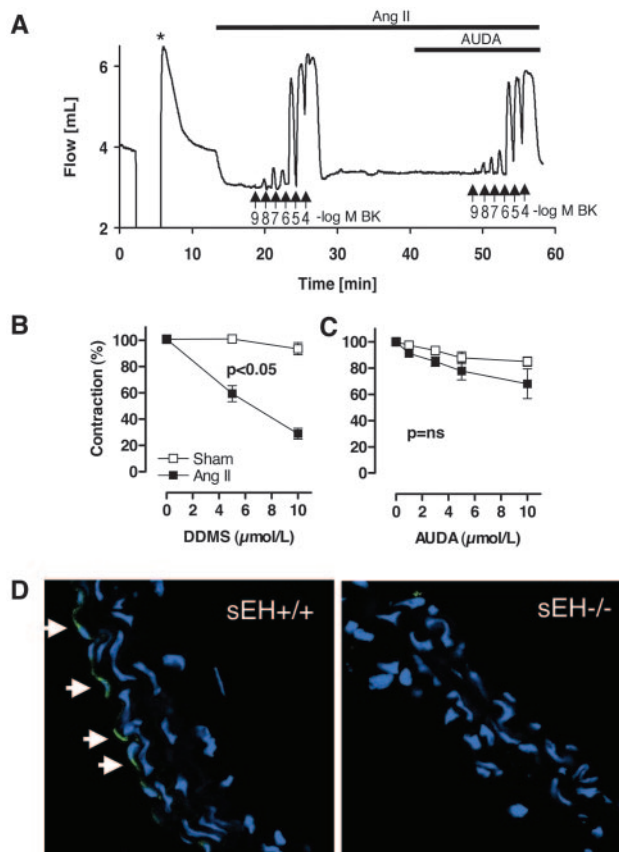

Figure 4. Effect of AUDA on the nonrenal vasculature. A, Origi-nal tracing from the isolated perfused hind limb of a mouse pre-treated with [val⁵]-angiotensin II for 7 days. *Reactive vasodilata-tion after 2 minutes of no flow. Arrows indicate application of bradykinin (BK; 100 μL bolus of the concentration indicated). Vasodilator responses were recorded as increases in flow at a constant perfusion pressure (100 mm Hg). Angiotensin II (5 nmol/L) and AUDA (1 μmol/L) were present in the perfusion medium as indicated by the black bars. B and C, Effect of DDMS (B) and AUDA (C) on the contraction induced by phenyl-ephrine in isolated carotid artery segments from mice pretreated with angiotensin II (1.0 mg/kg per day) or vehicle (sham; n=6 each group; *$P<0.05$ vs sham). D, Expression of the sEH in the carotid artery of wild-type mice (sEH$^{+/+}$) and sEH$^{-/-}$ mice as determined by immunohistochemistry and confocal microscopy. Nuclei are depicted in blue and sEH in green. Arrows point to endothelial cells.

decrease in blood pressure, was restricted to the renal circu-lation despite the fact that the sEH is expressed in the endothelial cells of conduit vessels.

Little is known about the contribution of EETs to vascular tone in mice, although these epoxides have been implicated in the EDHF-mediated relaxation of certain vascular beds in other species.[1] Indeed, it has been demonstrated previously that the bradykinin-induced, EDHF-mediated relaxation of porcine coronary arteries[2] and of the isolated perfused mouse heart[17] is dependent on the activity of CYP enzymes. How-ever, in the present study, AUDA had no effect on the bradykinin-induced vasodilatation of the isolated perfused hind limb. This observation does not exclude an effect of sEH inhibition on EDHF-mediated relaxations because the exper-iments were performed in the absence of cyclooxygenase and NO synthase inhibitors. In such situations, the EDHF re-
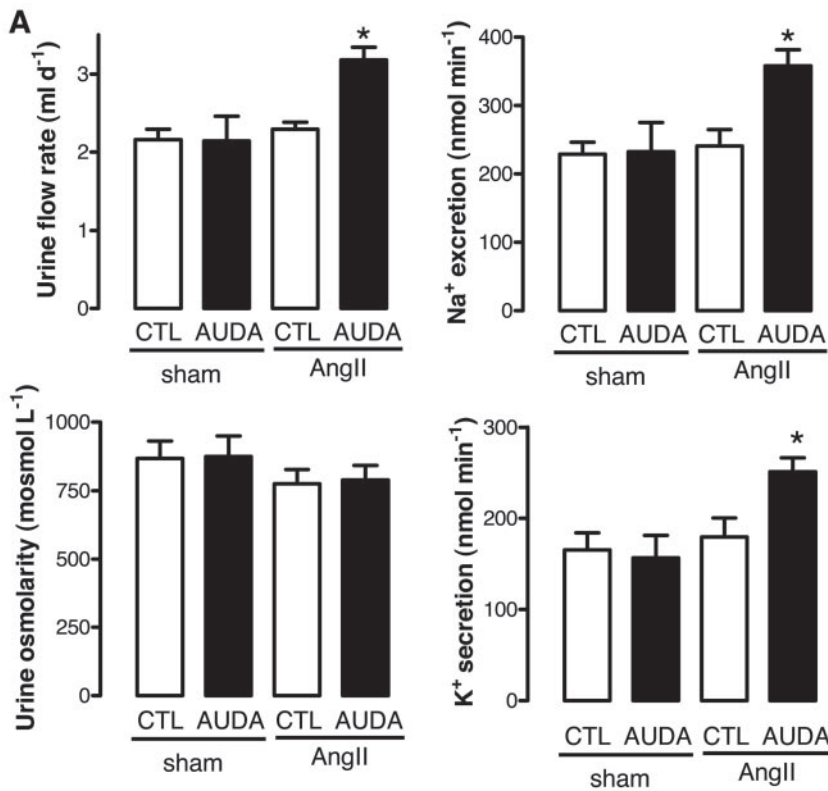
Figure 5. Effect of AUDA on renal function. A, Mice were implanted with osmotic mini-pumps delivering either vehicle (sham) or [val[5]]-angiotensin II (Ang II). Six days after pump implantation, oral administration of AUDA (130 μg/mL) or vehicle (CTL) with the drinking water began. One day later, animals were placed in metabolic cages and urine was collected over 24 hours (n=6 each group; *$P<0.05$ CTL vs AUDA). B, Expression of the sEH in the kidney of wild-type mice (sEH^{+/+}) and sEH^{−/−} mice as determined by immunohistochemistry and confocal microscopy. Nuclei are depicted in blue and sEH in green. Endothelial NO synthase is stained in red. Arrows point to glomeruli.
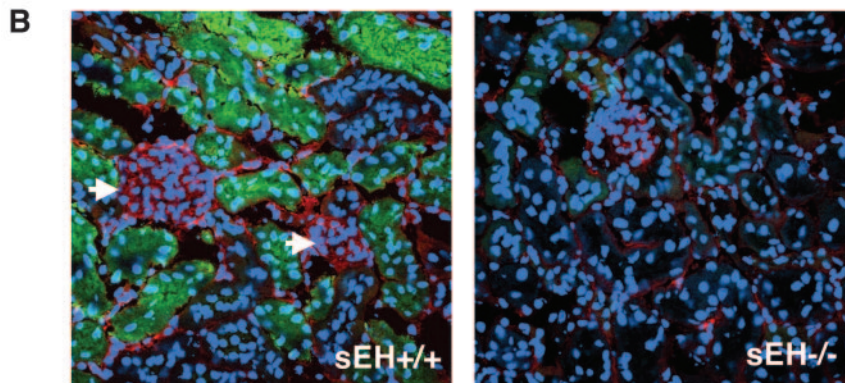
sponse is usually masked by NO and prostacyclin, which are also reported to suppress the formation and action of EDHF.[18,19] Nevertheless, our observation that AUDA lowered vascular resistance in the isolated perfused kidney but had no effect on the baseline resistance of the isolated perfused hind limb indicates that epoxides, such as EETs, which accumulate after inhibition of sEH,[20] are potentially more important for control of vascular resistance in the kidney than in skeletal muscle. Moreover, the rapid hypotensive effect of AUDA observed in vivo can be attributed to a reduction in heart rate, whereas systemic vascular resistance, albeit only indirectly estimated from the blood pressure to heart rate ratio, was completely unchanged. Given that the sEH is expressed in the endothelium of the carotid artery as well as in the kidney, it seems logical to conclude that either the kidney is particularly sensitive to a lipid compound metabolized by the sEH or that such a compound is not formed in the murine hind limb vasculature or in the carotid artery.

AUDA exerted effects only in animals pretreated with angiotensin II without affecting responses in mice made hypertensive by phenylephrine infusion. Thus, it appears that the depressor effects of the sEH inhibitor are restricted to conditions in which the angiotensin system is activated rather than to hypertension, per se. There is at least circumstantial evidence supporting a link between the renin-angiotensin system and the sEH because angiotensin II treatment increases expression of the enzyme,[4] and the renal metabolism of EETs to DHETs is increased in spontaneously hypertensive rats,[21] in which the angiotensin system is activated. On the other hand, the sEH is expressed in control animals and it is questionable whether the upregulation of the sEH and a subsequent increase in the metabolism of EETs can account for the observations of the present study. The situation is further complicated by large variations reported in the expression of the sEH in different species. For example, in human kidney, sEH was detected predominantly in vascular

endothelial cells,[7] whereas we found little or no sEH in the endothelium of the glomeruli but an abundance of the enzyme in distal segments of the murine tubular system. In the rat kidney, the sEH is expressed in the renal cortex and, to a lesser extent, in endothelial cells, as assessed by Western blot analysis.[22]

The effects of sEH inhibitors have been attributed to the accumulation of EETs;[9] indeed, inhibition of the sEH attenuates the metabolism of EETs to DHETs in isolated vessels.[23] However, another eicosanoid (ie, 20-HETE) mediates a large part of the direct vasoconstrictor effects of angiotensin II and inhibits large-conductance K^+_{Ca} channels on smooth muscle cells, which leads to calcium influx[1] and increases the calcium sensitivity of vascular smooth muscle cells.[24] To exclude that the actions of AUDA were a consequence of an unspecific inhibition of the ω-hydroxylase, the effect of AUDA on vascular tone was compared with that of DDMS, an inhibitor of the ω-hydroxylase.[25] Only the latter compound affected the tension of the isolated carotid artery from angiotensin II–treated mice, which underscores the specificity of AUDA but also demonstrates that the sEH has no role in the direct control of carotid artery tone in mice.

AUDA increased the urinary excretion of sodium and potassium to a similar extent in the present study. EETs are reported to affect renal function at several sites; in the proximal tubule and collecting duct, Na^+ transport is inhibited by EETs,[26] and these epoxides have also been reported to inhibit the renal Na^+/K^+-ATPase.[27] Our findings indicate that alterations in the sodium handling in the distal tubulus and the collecting duct cannot account for the effects of AUDA because an action at this site would be expected to decrease potassium excretion. AUDA also increased the excretion of non-ionic osmolytes, as calculated from the ionic gap. Because the absorption of these compounds is dependent on the function of the loop of Henle, it is tempting to speculate that the observed increase in renal perfusion leads to a situation similar to that of pressure diuresis, promoting a excretion of all osmolytes and water through a perfusion-induced reduction of osmolarity in the medulla.

An unexpected result of the present study was that the acute blood pressure–lowering effect of AUDA was a consequence of a negative chronotropic effect of the inhibitor in angiotensin II–treated mice. Certainly, given that EETs affect the open probability of L-type Ca^{2+} channels[28] and cardiac Na^+ channels[29] as well as Ca^{2+}-dependent K^+ channels and ATP-sensitive K^+ channels on ventricular myocytes, direct effects of EETs on the sinoatrial node are conceivable. However, we failed to observe any dromotropic or chronotropic effects of 11,12-EET on the isolated perfused mouse heart (R.P. Brandes, unpublished observations; 2004). On the basis of the results presented, it is not possible to determine whether the effect of AUDA on heart rate was a consequence of a direct effect on the rhythmogenic cells or of central effect on sympathetic outflow. Such alterations in sympathetic outflow may also contribute to the pressure diuretic effect of AUDA. Central effects of sEH inhibition should be considered seriously because a substantial portion of the response to angiotensin II is mediated via central AT1 receptors in the stria vascularis.[30] However, although EETs are known to be generated by astrocytes in response to glutamate release from neighboring neurons[31] and the upregulation of CYP 2C11 elicits neuroprotective effects during transient experimental ischemic attack,[32] little is known about the role and expression of the sEH in the brain.

Perspectives

In the present study, we demonstrated that inhibition of the sEH partially inhibits the hypertensive effect of angiotensin II in mice by increasing the renal excretion of salt and water and by a negative chronotropic effect in the heart. Given that sEH inhibitors also lower the blood pressure in spontaneously hypertensive rats, the sEH can be considered a novel therapeutic target for treatment of hypertension. Indeed, sEH inhibitors prevent the renal damage associated with experimentally induced hypertension.[22] However, because there appear to be marked differences in localization of the sEH within the kidney of different species,[7,22] the consequences of sEH inhibition may differ markedly between the mouse (in which little CYP appears to be expressed in the vascular endothelium) and other species, such as the pig[2] and man,[33] in which CYP-dependent vasodilator responses have been demonstrated. Finally, because the beneficial effects of sEH inhibitors are currently attributed to an increase in the availability of EETs, it will also be necessary to determine the effects of sEH inhibitors in vascular beds that constrict in response to EETs, as observed in some pulmonary vessel.[34]

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