Interaction Between P450 Eicosanoids and Nitric Oxide in the Control of Arterial Tone in Mice


Objective—Epoxyeicosatrienoic acids (EETs) serve as endothelial-derived hyperpolarizing factors (EDHF), but may also affect vascular function by other mechanisms. We identified a novel interaction between EETs and endothelial NO release using soluble epoxide hydrolase (sEH) −/− and +/+ mice.

Methods and Results—EDHF responses to acetylcholine in pressurized isolated mesenteric arteries were neither affected by the sEH inhibitor, N-adamantyl-N′-dodecylurea (ADU), nor by sEH gene deletion. However, the EDHF responses were abolished by catalase and by apamin/charybdotoxin (ChTx), but not by iberiotoxin, nor by the cytochrome P450 inhibitor PPOH. All four EETs (order of potency: 8,9-EET >14,15-EET = 5,6-EET > 11,12-EET) and all 4 dihydroxy derivatives (14,15-DHET= 8,9-DHET=11,12-DHET > 5,6-DHET) produced dose-dependent vasodilation. Endothelial removal or L-NAME blocked 8,9-EET and 14,15-DHET-dependent dilations. The effects of apamin/ChTx were minimal. 8,9-EET and 14,15-DHET induced NO production in endothelial cells. ADU (100 µg/mL in drinking water) lowered blood pressure in angiotensin II–infused hypertension, but not in L-NAME–induced hypertension. Blood pressure and EDHF responses were similar in L-NAME–treated sEH+/+ and −/− mice.

Conclusions—Our data indicate that the EDHF response in mice is caused by hydrogen peroxide, but not by P450 eicosanoids. Moreover, P450 eicosanoids are vasodilatory, largely through their ability to activate endothelial NO synthase (eNOS) and NO release. (Arterioscler Thromb Vasc Biol. 2009;29:54-60.)

Key Words: eicosanoids ■ soluble epoxide hydrolase ■ NO synthase ■ L-NAME ■ EDRF

The endothelium releases nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF).1,2 Epoxyeicosatrienoic acids (EETs) are cytochrome P450 epoxygenase (CYP)-derived metabolites of arachidonic acid (AA) that may be EDHFs.3,4 Other candidates include K+ ions and hydrogen peroxide (H2O2).5–7 Endothelial cell hyperpolarization spreads to adjacent vascular smooth muscle cells (VSMCs) through myo-endothelial gap junctions.8,9 Calcium-activated potassium channels, most probably the SK4 (IKc4) and SK3 (SKc3) expressed on the endothelium, are the end-cellular gateway mediating hyperpolarization, and subsequent EDHF relaxation.2,4,10–13 EETs convincingly cause hyperpolarization.14–16 They can induce vasodilation in certain vascular beds by increasing the open-state probability of calcium-activated potassium (BK) channels.4,15,17 The soluble epoxide hydrolase (sEH) metabolizes EETs to dihydroxy derivatives (DHET), sEH inhibition could enhance EET activity.18 Blood pressure decreased in spontaneously hypertensive rats (SHR) given an sEH inhibitor.19 sEH inhibition also lowered blood pressure in rats given angiotensin II (Ang II).20 Thus, sEH could contribute to Ang II–induced hypertension21 and salt-sensitivity.22 Even deoxy cortisolone acetate (DOCA)-salt hypertension was ameliorated with sEH inhibition.23 Finally, male sEH gene-deleted (−/−) mice had lower blood pressures than sEH +/+ mice.24 EETs could also affect vascular function by other mechanisms. A novel inhibitory interaction between CYPs and H2O2 was recently identified.25 CYP epoxygenases were directly inhibited by H2O2, an interaction that could modulate EET bioavailability. We studied interactions between P450 eicosanoids, EDHF, and NO using sEH −/− and +/+ mice. Our findings may have important implications for the development and use of pharmacological sEH inhibitors.

Methods

Animals and Blood Pressure Measurements

The local council on animal care (American Physiological Society criteria) approved all protocols in FVB/N mice. We performed radiotelemetry, Ang II osmotic minipump infusion (1.44 mg/kg/d), and N(omega)-nitro-L-arginine methyl ester via the drinking water (L-NAME; 5 mg L-NAME per 10 mL tap water) as outlined.

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elsewhere. After a 7- to 10-day recovery, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP), and heart rate (HR) recordings were obtained for 3 days (baseline values) and continued thereafter. Mice received N-adamantyl-N’-dodecylurea (ADU 100 μg/mL) via the drinking water over 7 days. In additional experiments, we studied the effects of L-NAME on blood pressure in male sEH +/- and sEH --/-- mice backcrossed over 6 generations to FVB/N.

sEH Activity
We prepared renal microsomes using 1.5 kidney per male adult FVB/N +/- animal. The sEH activities were determined using the 100 000g supernatants obtained during the preparation of renal microsomes and using [1-14C]-14,15-epoxyeicosatetraenoic acid (EET). [1-14C]-14,15-EET was prepared by chemical oxidation of radiolabeled AA according to Falcik et al. To measure the sEH activity in blood vessels, mesenteric arteries were isolated and cleaned from fatty tissue. Vessels were incubated with [1-14C]-14,15-EET, and the 14,15-DHET produced and the remaining 14,15-EET were extracted with ethylacetate and analyzed by RP-high-performance liquid chromatography (HPLC).

Vessel Experiments
Intact 2nd or 3rd order branches of mesenteric arteries of adult male sEH +/- or --/-- mice were obtained. Thereafter, we carefully removed the connective tissue with scissors. The arteries then were mounted onto two glass cannulas in an arteriograph with continuous superfusion (3 to 5 mL/min) of oxygenated physiological salt solution (PSS) at 37°C.29,30 Dose-response curves for 5,6-EET, superfusion (3 to 5 mL/min) of oxygenated physiological salt solution (PSS) at 37°C.29,30 Dose-response curves for 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET were performed using vessels isolated from male sEH +/- mice preconstricted with U46619 (100 nmol/L). In some experiments, the endothelium was removed by intraluminal application of an air bubble mice. Mice background crossed onto C57BL/6ByJ or FVB/N were used.

Determination of EET and DHET-Levels
EET and DHET levels were determined in red blood cells, plasma samples, and isolated mesenteric arteries from male +/- FVB/N mice (n=5) using liquid chromatography-tandem mass spectrometry.

Statistics
We used analysis of variance (repeated measures where indicated), Duncan multiple range tests, Bonferonni-corrected t tests, and Student t tests as indicated. A probability value <0.05 was accepted as significant. Fiducial limits are given in mean±SEM.

Methodological details are given in the supplemental materials (available online at http://atvb.ahajournals.org).

Results
sEH Activity in Mesenteric Arteries
All four EETs (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET; total 243±56 ng/g, ratio 52:16:11:21) were present in the vessel wall of mesenteric arteries (see supplemental Table I). EETs were also detected in red blood cells (total 542±79 ng/g) and blood plasma (see supplemental Table I). The functional expression of sEH in the mesenteric artery was tested with the sEH inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (ADU). Figure 1A shows the dose-response curve with an IC50 value for ADU of ~10 nmol/L. ADU efficiently inhibited the sEH activity present in the cytosolic fraction of mouse renal homogenates. Using 10 μmol/L 14,15-EET as the substrate, ADU produced significant inhibition already in the low nmol/L range and almost completely abolished the hydrolysis at a concentration of 1 μmol/L. We analyzed the effect of ADU on the sEH activity in vessels derived from the mesenteric artery tree, as shown in Figure 1B. sEH protein expression was detected by Western blotting in arteries from sEH +/- mice, but not from --/-- mice (inset). Vessels isolated from male sEH +/- mice, but not from male sEH --/-- mice, hydrolyzed 14C-labeled 14,15-EET within 30 minutes. DHET production by the wild-type vessels was significantly reduced by preincubation with ADU at concentrations of 1 and 10 μmol/L. Thus, sEH is present and metabolically active in mesenteric and possibly other arteries of mice, and can be effectively blocked by ADU.

EDHF Response Is Most Probably Caused by H2O2, not by P450 Eicosanoids
We next examined whether or not EDHF-dependent vasodilation is affected by inhibiting sEH. Under basal conditions, vasorelaxation by acetylcholine (ACh) was equipotent between sEH +/- and --/-- arteries, as shown in Figure 2A and 2B. Vasoconstriction by 60 mmol/L KCl and vascular reactivity to U46619 expressed as % of KCl showed no differences. Similar results were obtained in isolated mesenteric arteries and isolated perfused mesenteric beds of female sEH +/- and --/-- mice (not shown). Next, we studied EDHF-dependent relaxation in vessels by treatment of the vessels with L-NAME plus indomethacin. ACh-dependent relaxation was significantly reduced in vessels treated with L-NAME alone or L-NAME plus indomethacin, as shown for male vessels in Figure 2B. However, the relaxation was not different between sEH +/- and --/-- mice. Similar results were obtained in female vessels (n=5, not shown). Note that this relaxation was completely abolished by apamin/ChTx, suggesting that activation of both endothelial small and intermediate-conductance Ca2+-activated K+ channels (SK3, SK4) is crucial in EDHF-dependent signaling and relaxation.
in mouse mesenteric arteries.\textsuperscript{7,31} In contrast, iberiotoxin was not effective in inhibiting EDHF-dependent relaxation, indicating that large-conductance \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) (BK) channels in arterial smooth muscle cells play no role in the EDHF response in this vascular preparation. EDHF-dependent dilation was completely inhibited by catalase 1000 U/mL (30 to 45 minutes preincubation; Figure 2A and 2B right bar), but not by 6-(2-propargyloxyphenyl)hexanoic acid (PPOH, 10 \(\mu\text{mol/L}\), 30 minutes), a selective CYP inhibitor, or by 14,15-epoxyeicoso-5(Z)-enoic acid (EEZE) at 10 \(\mu\text{mol/L}\) for 20 minutes, an EET antagonist (Figure 2A, \(n=4\) in each group; Figure 2B, \(n=4\) to 10 in each group). We next measured \(\text{H}_2\text{O}_2\) production in endothelial cells of intact mesenteric arterioles using a laser confocal microscope with CM-H\(_2\)DCFDA, a peroxide-sensitive fluorescence dye. In these experiments, the endothelial monolayer was loaded with CM-H\(_2\)DCFDA, which can be clearly distinguished from underlying smooth muscle cells, as shown in supplemental Figure IIA and IIB. All experiments were performed in the presence of indomethacin 5 \(\mu\text{mol/L}\) and L-NAME 100 \(\mu\text{mol/L}\) (\(n=4\) in each group). A significant increase in the dichlorofluorescein fluorescence was observed in endothelial cells stimulated by acetylcholine (ACh 1 \(\mu\text{mol/L}\)) as compared with controls or tissues treated with catalase (1000 U/mL). Removal of endothelial cells (-E) abolished the dichlorofluorescein signal in response to acetylcholine. Only smooth muscle layer was observed in (Figure IIA, images D and D'). Taken together, these results suggest that \(\text{H}_2\text{O}_2\), but not EETs, significantly contributes to EDHF-mediated dilation in mouse mesenteric arteries, which is in line with previous findings.\textsuperscript{7,32,33}

**Vasodilator Effects of EETs and DHETs**

Vasodilator responses were tested in the U46619-preconstricted (60 mm Hg) arteries. As shown in Figure 3, all 4 EETs (order of potency: 8,9-EET >14,15-EET >5,6-EET >11,12-EET) and all 4 DHETs (14,15-DHET >8,9-DHET >11,12-DHET >5,6-DHET) produced dose-dependent vasodilation. 8,9-EET was the most potent EET, with \(\approx50\%\) dilation at \(\approx3 \mu\text{mol/L}\). 14,15-DHET, 8,9-DHET, and 11,12-DHET were the most potent DHET, with \(\approx50\%\) dilation at \(\approx0.3 \mu\text{mol/L}\).

The 8,9-EET-dependent vasodilations were slightly inhibited by the SK3/SK4 channel blockers apamin/ChTx, as shown in Figure 4 (\(n=6\) in each group). In contrast, dilations by the less potent EETs (ie, 5,6-EET, 11,12-EET, and 14,15-EET) were not affected by apamin/ChTx (not shown). Apamin/ChTx slightly inhibited 14,15-DHET- (Figure 5, \(n=6\) in each group), 8,9-DHET-, and 11,12-DHET-dependent dilations, but had no effect on 5,6-DHET–dependent dilations (supplemental Figure I). Together, these results demonstrate that DHETs are up to \(\approx10\)-fold more potent vasodilators in mouse mesenteric arteries than EETs. EETs/DHETs exhibit their vasodilatory properties without any, or with very little, involvement of SK3/SK4 channels.

**EET/DHET-Dependent Dilation Is Mediated by eNOS Activation**

To study the mechanisms of P450 eicosanoid-induced vasodilation, we used the most potent metabolites, namely 8,9-EET and 14,15-DHET. As shown in Figures 4 and 5, 8,9-EET– and 14,15-DHET–induced vasodilations were dependent on intact endothelium (panels A and C, \(n=6\) in each group). In the presence of SK3/SK4 channel blockers, a prominent residual dilation to 8,9-EETs and 14,15-DHETs...
occurred (panels D). This response indicates the presence of a major additional endothelial vasodilator mechanism. To determine whether or not EETs/DHETs activate eNOS to produce vasodilation, N-(omega)-nitro-L-arginine methyl ester (L-NAME) was coadministered with apamin/ChTx. Figures 4 and 5 panels B and D show that the dilation in response to both 8,9-EET and 14,15-DHET was completely inhibited by L-NAME/Apamin/ChTx; n=6 in each group). Indomethacin (5 μmol/L, 30 minutes preincubation) had no effect. Figures 4 and 5 panels E show that both 8,9-EET and 14,15-DHET are able to induce NO production in primary mouse aortic endothelial cells. These effects were inhibited by L-NAME (100 μmol/L, n=4 experiments in each group). Thus, our results suggest that EETs/DHETs can modulate the bioavailability and/or action of NO to produce vasodilation.

L-NAME–Induced Hypertension Is Resistant to sEH Inhibition

Figure 6A shows the telemetric blood pressure values of mice given L-NAME (panel A, n=6) for 7 days followed by ADU. Depicted are baseline values, the days 5 to 7 under L-NAME treatment, and the days 5 to 7 given L-NAME with ADU. Mice given L-NAME exhibited a prompt increase in mean arterial blood pressure (MAP) from 103 ± 1 mm Hg to 112 ± 2 mm Hg (panel A). The combination of L-NAME with ADU increased blood pressure slightly to 114 ± 2 mm Hg. In addition, L-NAME induced elevated blood pressure in sEH−/− mice that was not different compared to sEH+/− mice (Figure 6B, n=6 each). In contrast, ADU reduced blood pressure in Ang II–induced hypertension. Mice were given Ang II for 7 days followed by 7 days with additional ADU.
treatment. Ang II infusion increased blood pressure (MAP) from 100/110 mm Hg to 132/132 mm Hg. After ADU, MAP in these mice was reduced to 114/110 mm Hg. These values did not reach initial blood pressures; however, the blood pressure reduction was highly significant (supplemental Figure I, n=6).

To determine whether or not endothelial relaxation is modulated by L-NAME, endothelial-dependent dilation of isolated mesenteric arteries of FVB/N +/- and +/- mice was examined. Figure 6C shows the results. L-NAME-treated animals showed reduced ACh-dependent vasodilation. Noteworthy, this dilation was not affected by the presence of L-NAME in the bath chamber. This response was neither affected by ADU nor by genetic sEH deletion (n=6 in each group). Thus, chronic treatment of mice with L-NAME leads to EDHF responses that cannot be modified by sEH inhibition in the vessel wall in L-NAME-induced hypertension.

In an additional experimental series, we studied the effects of catalase on EDHF-dependent vasodilation of L-NAME treated mice. Vasorelaxation by acetylcholine (ACh) in the presence of L-NAME was equipotent between sEH +/- and --/- arteries of L-NAME treated animals, as shown in Figure 6D. Catalase 1000 U/mL inhibited this EDHF response. However, the effects of catalase were not different between sEH +/- and --/- mice. Indomethacin 5 μmol/L had no additional effects (n=4 to 7 in each group).

### Discussion

This study is the first to investigate the vascular interaction between P450 eicosanoids, SEH, and NO in mice. The novel findings are 3-fold. First, both EETs and DHETs are vasodilatory in mesenteric arteries largely through their ability to activate eNOS and NO release. Second, the ACh-induced EDHF response is predominantly caused by H₂O₂, but not by P450 eicosanoids. Thus, the effect is not related to sEH inhibition. Third, in contrast to Ang II–infused hypertension, L-NAME hypertension is not affected by sEH inhibition.

### EDHF in Mouse Mesenteric Arteries

Little is known about the contribution of EETs and DHETs to vascular tone in mice, although these epoxides have been implicated in EDHF-mediated relaxation of certain vascular beds in other species. Thus far, we are not aware of any study showing vasomotor effects of EETs/DHETs in the vasculature of mice. We found that all four EETs and all four DHETs produced dose-dependent vasodilation of mouse mesenteric arteries. The effects were endothelium-dependent, but not or only slightly inhibited by apamin/ChTx. In contrast, apamin/ChTx completely blocked the EDHF response. These results suggest that EETs/DHETs (alone or in combination) do not function as an EDHF in mice, which should be solely dependent on SK4/SK3 channels. Moreover, we observed that EDHF responses in mesenteric arteries were not affected by any measures that influence EET generation or action, including CYP inhibition by PPOH, sEH inhibition by ADU or by gene deletion, or EET antagonism by EEZE. Instead, our data show that the EDHF response is completely inhibited by catalase and accompanied by H₂O₂ production, which is in line with previous findings. Taken together, our results present evidence that P450 eicosanoids do not significantly contribute to EDHF-mediated dilation in mesenteric arteries of mice. Our data strongly support the notion that H₂O₂ is an EDHF in this vessel, which causes hyperpolarization, most probably via activation of endothelial SK3/SK4 channels in endothelial cells, which spreads to adjacent vascular smooth muscle cells (VSMC) through myo-endothelial gap junctions and produces subsequent EDHF relaxation. Specifically, the gap junction components Cx40, Cx43, and Cx37 have been recently implicated in the EDHF signal spread from endothelial to smooth muscle cells in mouse mesenteric arteries.

### Prominent Role of NO in Vasodilation by EETs/DHET

The vasculature can be exposed not only to endogenously produced P450 eicosanoids, but also from nonvascular
Role of sEH in Blood Pressure Regulation

sEH inhibition has been reported to lower blood pressure in several forms of hypertension. Our data confirm previous findings that Ang II–induced hypertension is reduced by pharmacological sEH inhibition.19–22 The novel finding of the present study is that L-NAME hypertension is insensitive to pharmacological or genetic sEH inhibition. These data suggest that the role of sEH in blood pressure regulation depends on the type of secondary hypertension. We propose that sEH inhibition is ineffective in lowering blood pressure in L-NAME hypertension for the following reasons: (1) L-NAME hypertension is primarily attributable to increased vascular tone and diminished NO release in vessels; (2) EETs and DHETs are both potent vasodilators which primarily rely on intact eNOS activity; (3) EDHF responses in L-NAME–treated mice are not modified by sEH inhibition, but remain sensitive to catalase. The mechanism of how sEH inhibition ameliorates Ang II–induced hypertension in mice is largely unclear, but may involve renal and cardiac mechanisms rather than changes in peripheral arterial resistance.21

In summary, our data demonstrate that P450 eicosanoids have an important impact on bioavailability of NO in the vasculature. This interaction between 2 EDRFs plays an important role in the modulation of vasomotor tone in the mesenteric microcirculation and has important implications for blood pressure regulation by sEH inhibition in mice and possibly other species. This pathway may increase in importance during cardiovascular disease, when NO production is reduced.

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Disclosures

None.

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20. Oltman CW, Weintraub NL, VanRollins M, Dellsperger KC. Epoxycyclo-


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Supplemental Material

**Figure I.** (panel A) Effects of ADU on Ang II-induced hypertension in male FVB/N +/+ mice are shown (n=6). (Panel B) Vasodilator responses to 5,6-DHET, 8,9-DHET and 11,12-DHET were tested in U46619-preconstricted pressurized (60 mmHg) mouse mesenteric arterioles. The vasodilator responses of these compounds were tested alone or in the presence of charybdoxin (ChTx, 100 nM) and apamin (APA, 100 nM). (n=4-6 as indicated in each group). *, p< 0.05.

**Figure II.** (Panels A and B) ACh-induced production of H$_2$O$_2$ by the endothelium in intact small mesenteric arteries. Images on the left serve as controls prior to addition of ACh.

**Table I.** Detection of EETs and DHETs in blood samples and isolated mesenteric arteries from wild-type FVB/N mice. The total amounts of all regioisomeric EETs and DHETs are given as mean±SEM (n=5). The relative contribution of the individual regioisomers is expressed in % of total EETs and DHETs, respectively. n/a- non applicable due to the very low amounts of DHETs present in mesenteric arteries.

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<th>Tissue</th>
<th>EETs [ng/ml(g)]</th>
<th>DHETs [ng/ml(g)]</th>
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<th>DHET-regioisomers</th>
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<td></td>
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<td>% of total EETs</td>
<td>% of total DHETs</td>
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<td>21 : 11 : 16 : 52</td>
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**Detailed Methods**

*Animal protocols:* Male FVB/N mice (Charles River, Germany) were allowed free access to standard chow (0.25% sodium, SNIFF Spezialitäten GmbH, Soest, Germany) and drinking water ad libitum. All protocols were approved by the local council on animal care that corresponds to the requirement of the American Physiological Society. For radiotelemetry, mice were anesthetized with isoflurane (CuraMed Pharma GmbH, Karlsruhe, Germany). The pressure-sensing catheter of blood pressure device (TA11PA-C20; Data Sciences Int., St. Paul, USA) was advanced via the right carotid artery into the ascending aorta and the transmitter was placed into a subcutaneous pocket along the flank. The mice were synchronized to a light-dark schedule of 12:12 hours with lights on at 6 AM. All mice were allowed 7 to 10 days recovery from surgery before baseline blood pressure and heart rate values were recorded for 3 days via radio frequency signals and collected using the Dataquest A.R.T. system, version 2.1 (Data Sciences Int.). The data were sampled every 5 min for 10 sec continuously day and night with a sampling rate of 1000 Hz. Systolic, diastolic, mean arterial pressure, and heart rate were recorded using the DATAQUEST software. Heart rate was computed from the pulse intervals of the blood pressure recordings.\(^1\)\(^2\)

After a 7 to 10-day recovery, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP), and heart rate (HR) recordings were obtained for 3 days (baseline values) and continued thereafter while Ang II (n=6, bwt: 24±04 g, 1.44 mg/kg/day) or N(omega)-nitro-L-arginine methyl ester (L-NAME; n=6, bwt 26±04 g, 5 mg L-NAME pro 10 ml tap water) were given. We gave Ang II by osmotic minipumps implanted into the abdominal cavity; L-NAME was given via the drinking water. After 1 week of Ang II or L-NAME treatment, the mice also received N-adamantyl-N’-dodecylurea (ADU 100 µg/ml) via the drinking water over 7 days. We used MAP and HR values of day 5\(^{th}\) to 7\(^{th}\) of Ang II or L-NAME treatment to characterize the effects of these substances. We also used the 5\(^{th}\) to 7\(^{th}\) of additional ADU supplementation to characterize the effect of ADU on Ang II or
L-NAME-induced blood pressure changes. In additional experiments, we studied the effects of L-NAME on blood pressure in sEH +/+ and sEH -/- mice (n=6 each, bwt 27±1 g, and 28±1 g, respectively). These mice were backcrossed over 6 generations to FVB/N. L-NAME was given via the drinking water. Values are given as mean±SEM; n represents the number of animals tested.

**Enzyme determinations:** Renal microsomes were prepared using one and a half kidney per male FVB/N +/+ animal. The renal capsule and the fatty tissues were carefully removed. Kidneys were minced and homogenized in four to five volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 150 mM potassium chloride, 2 mM EDTA, 2 mM DTT, 1 µM FAD and FMN, and 0.25 mM PMSF in a motor-driven Teflon-glass Potter Elvehjem homogenizer. After differential centrifugation (10 min, 1,000 x g; 20 min, 10,000 x g; 90 min, 100,000 x g), the 100,000 x g pellet (microsomes) was suspended and homogenized in 50 mM Tris-HCl buffer, pH 7.7, containing 20 % glycerol, 5 mM EDTA, and 1 mM DTT. Aliquots were snap frozen in liquid nitrogen and stored at -80°C.

The sEH activities were determined using the 100,000 x g supernatants obtained during the preparation of renal microsomes. Reactions were performed at 37 °C for 10 min in 100 µl 100 mM potassium phosphate buffer pH 7.2 containing 10 µM [1-U14C]-14,15-epoxyeicosatetraenoic acid (EET). [1-U14C]-14,15-EET was prepared by chemical oxidation of radiolabeled AA according to Falck et al. The reactions were started by adding the 100,000 x g supernatant (1 µg of protein), stopped and extracted with ethylacetate and analyzed by RP-HPLC. To measure the sEH activity in blood vessels, mesenteric arteries were isolated and cleaned from fatty tissue (see below). The mesenteric tree was separated into four equal parts and distributed into Eppendorf tubes containing 200 µl 100 mM potassium phosphate buffer pH 7.2. The vessels were preincubated for 10 min at 37°C in the presence of ADU (1 or 10 µM) or vehicle (0.5 % DMSO). 10 µl of [1-U14C]-14,15-EET (dissolved in same buffer
immediately before use) were added to give a final concentration of 10 µM and the vessels were incubated for additional 30 min. The 14,15-DHET produced and the remaining 14,15-EET were extracted with ethylacetate and analyzed by RP-HPLC as described.

_Synthesis of N-adamantyl-N’-dodecylurea (ADU):_ ADU was synthesized essentially as described for a set of analogous compounds by Morisseau et al. Briefly, 1.85 g (10 mMol) of dodecylamine (Fluka) were dissolved in 100 ml hexane. To this solution, 1.77 g (10 mMol) of 1-adamantyl-isocyanate (Aldrich) in 20 ml of hexane were added under stirring at room temperature. After 16 h, the solid product was filtered and washed with cold hexane. The product was then recrystallized twice from hexane and dried. This procedure yielded 3.3 g (91.1 % yield) of a white crystalline solid. To make ADU compatible with drinking water, the compound was incorporated into cyclodextrin: 10 mg ADU were added to 20 ml 5 % (2-hydroxypropyl)-ß-cyclodextrin and dissolved by vigorous stirring followed by sonication and dilution to 100 ml. The final solution contained 100 µg ADU per ml and 1 % (2-hydroxypropyl)-ß-cyclodextrin in tap water. For in vitro studies such as inhibition of sEH in vessels or tissue homogenates, ADU was dissolved in DMSO.

_Vessel experiments on intact pressurized arteries:_ Male FVB/N mice (see above), male sEH +/+ or -/- mice (20-25 g, 10-12 weeks, each) were killed under ether. The sEH -/- mice originate from a colony developed by the Boehringer Ingelheim Pharmaceuticals, Inc. However, these mice were further back-crossed over 9 generations onto C57BL/6ByJ. Then heterozygous F9 animals were crossed to obtain a homozygous knockout (-/-) and homozygous wild-type line (+/+). +/+ and -/- sEH mice showed total EET levels of 489±46 ng/g and 558±55 ng/g in red blood cells (n=5 per group). The corresponding serum levels
were $53 \pm 11$ ng/ml and $139 \pm 33$ ng/ml (+/+ vs. -/-; n=4 per group, p<0.05). If not otherwise indicated, those -/- and +/+ mice were used in the present study.

Intact 2nd or 3rd order branches of mesenteric arteries were obtained and quickly transferred to cold (4°C) oxygenated (95% O$_2$/5% CO$_2$) physiological salt solution (PSS) of the following composition (in mM): 119 NaCl, 4.7 KCl; 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$; 2.5 CaCl$_2$, 1.2 MgSO$_4$; and 11 glucose. Thereafter, we carefully removed the connective tissue with scissors. The arteries then were mounted onto two glass cannulas in an arteriograph with continuous superfusion (3 to 5 mL/min) of oxygenated PSS at 37°C. One cannula was connected to a reservoir, which produced the desired intravascular pressure by a pressure control system (PS/200, Living Systems Instrumentation, Burlington, Vermont, USA); the other cannula was connected to a pressure monitor (PS/200, Living Systems Instrumentation, Burlington, Vermont, USA). Leaking vessels were discarded at any stage of the experiment to ensure complete non-flow conditions. After a 20-minute equilibration period, intravascular pressure was increased gradually from 2 mm Hg to 60 mm Hg. After an additional period of 30 minutes and before starting the experiment, the vessel was constricted with KCl 60 mmol/L to assess its contractility. To elucidate a role of endothelium, responses to 10 µM acetylcholine were studied in endothelium-intact vessels preconstricted by U46619 (100 nM) in the absence or presence of L-NAME alone (300 µM, 20 min preincubation) or in combination with indomethacin (5 µM, 20 min preincubation), catalase (1000 U/ml, 20 min preincubation), 6-(2-propargyloxyphenyl)hexanoic acid (PPOH, 10 µmol/L, 30 min), charybdotoxin (ChTx; 100 nM, 20 min preincubation), apamin (100 nM, 20 min incubation), carbenoxolone (100 µM, 30-45 min) and/or iberiotoxin (100 nM, 20 min preincubation). Similar experiments were performed on female sEH +/- and -/- mice. Activity of PPOH was assessed in control experiments on renal arteries of rats, where EDHF responses were partially inhibited by PPOH in distal main, 1st to 2nd order branches of renal arteries.
In another set of experiments, dose-response curves for 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, and 14,15-DHET were performed using vessels isolated from male FVB/N +/- mice preconstricted with U46619 (100 nM) in the absence and presence of charybdotoxin (ChTx) and apamin (100 nM each, 20 min preincubation). In other experiments, dose-response curves for P450 eicosanoids were performed using vessels from male FVB/N +/- mice preconstricted with U46619 (100 nM) in the presence of L-NAME (300 µM, 20 min preincubation) in combination with indomethacin (5 µM, 20 min preincubation) and ChTx and apamin (100 nM each, 20 min preincubation). In some experiments, the endothelium was removed by intraluminal application of an air bubble. Successful removal of the endothelium was assessed by a lack of vasodilation in response to acetylcholine (10 µmol/L).

The vessel diameter was measured using a video-microscopic system (Nikon Diaphot, Duesseldorf, Germany), connected to a computer with appropriate software (TSE, Bad Homburg, Germany) for detection of changes of vessel diameter. All vessels tested were from different animals. All drugs were applied to the bath solution, namely from the extraluminal side of the vessel.

Vessel experiments on isolated mesenteric beds: The mesenteric vascular bed was perfused as previously described. Briefly, wild-type sEH +/- or -/- mice (20-25 g, 10-12 weeks) were killed under ether. The superior mesenteric artery was cannulated at its junction with the abdominal aorta, put in an organ chamber, and perfused using a peristaltic pump (WPI, Germany, or Bioscience Tools, San Diego, CA) at constant flow (1.5-3.5 mL/min, 30 mmHg) with oxygenated (95% O₂ - 5% CO₂) KHS. The composition of KHS (in mM) was 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 1.2 Mg₂SO₄, 11.1 glucose, 2.5 CaCl₂. The bath solution was continuously oxygenated with a gas mixture of 95% O₂ plus 5% CO₂, and kept at 37°C (pH, 7.4). Perfusion pressure was continuously determined by a pressure transducer (PM/4,
Living Systems Instrumentation, Burlington, Vermont, USA) and recorded on a polygraph or using Powerlab data acquisition and software (ADInstruments, Bella Vista, Australia). Since flow was maintained at a constant rate, changes in perfusion pressure were used as an index of changes in the resistance of the arteries, and a rise or a decrease in the perfusion pressure indicated either vasoconstriction or vasodilatation, respectively. After an equilibration period of 60-80 minutes and before starting the experiment, the vascular mesenteric bed was contracted with KCl 60 mM to assess its contractility. Subsequently, KCl was removed and dose-response curves for 8,9-EET and 14,15-DHET were performed using beds isolated from male +/+ mice preconstricted with U46619 (100 nM) in the absence and presence of charybdotoxin and apamin (100 nM each, 20 min preincubation). To elucidate a role of endothelium, responses to 10 µM acetylcholine were studied in mesenteric beds preconstricted by U46619 (100 nM) in the absence or presence of ADU (10 µmol/L, 20 min preincubation).

High external potassium solutions were made by iso-osmotic substitution of NaCl with KCl in the KHS. All drugs were applied intraluminally, i.e. from the internal side of the vessels. Values are given as mean±SEM; n represents the number of vessels or mesenteric beds tested.

Isolation of endothelial cells from mouse aorta: Primary aortic endothelial cells were obtained using a modified protocol Kobayashi et al. Briefly, two male mice (C57BL/6, 8-10 weeks) were killed by cervical dislocation. After the abdominal cavity was opened, the aortas were removed and placed in ice-cold PBS. The composition of PBS was (in mM): NaCl 137, KCl 2.7, NaH₂PO₄ 1.4, Na₂HPO₄ 4.3 (pH 7.4, Gibco). The aorta was dissected out from the aortic arch to the abdominal aortas, and immersed in 20% FBS-DMEM containing 1,000 U/ml of heparin. The fat and connective tissue were removed with fine forceps under a stereoscopic microscope. A 25-guage cannula was inserted into the proximal portion of the aorta. After ligation at the site with a silk thread, the inside of the lumen was washed with serum-free DMEM. The other side was bound and filled with collagenase type II solution (2
mg/ml, dissolved in serum-free DMEM). After incubation for 45 min at 37 °C incubation, endothelial cells were removed from the aorta by flushing with 5 ml of DMEM containing 20% FBS. Endothelial cells were collected by centrifugation at 1,2000 rpm for 10 minutes. Then, the pellet was gently resuspended by pipette with 2 ml of 20% FBS-DMEM and cultured in a 35 mm culture dish. To remove smooth muscle cells, after 2 hr incubation at 37 °C, the medium was removed, the cells were washed with warmed PBS, and medium G (DMEM, 20% FBS, 100 U/ml penicillin-G, 100 µg/ml streptomycin, 2 mM L-glutamine, 1X non-essential amino acids, 1X sodium pyruvate, 25 mM HEPES (pH 7.0 -7.6), 100 µg/ml heparin). The isolated cells were confirmed as endothelial cells by PECAM-1 staining.

Measurement of nitric oxide (NO) by laser confocal fluorescent microscopy: Fluorimetric measurements were performed on primary aortic endothelial cells using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Melville, NY, USA) mounted on an inverted IX81 Olympus microscope, equipped with a 10X objective (NA 0.5). The circular coverslips seeded with the mice aortic endothelial cells were pinned in a specially designed chamber for imaging. Intracellular NO production was monitored using fluorescent NO indicator DAF-FM diacetate. Endothelial cells seeded on glass coverslips were incubated at room temperature for 30 min in normal physiological saline solution (NPSS; 130 mmol/L NaCl, 5.6 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, 11 mmol/L glucose, 10 mmol/L HEPES, pH 7.4 with NaOH) at a concentration (i.e., 1 µM) of DAF-FM diacetate (Invitrogen). NO detection was made by measuring fluorescence intensity excited at 495 nm and emitted at 515 nm with the confocal system. Changes in intracellular NO production were displayed as a ratio of fluorescence relative to the intensity (F1/F0). The data presented were obtained from four independent experiments. During experiments, cells were maintained in NPSS at room temperature.
Measurements of $\text{H}_2\text{O}_2$ production in endothelial cells: The method of hydrogen peroxide detection was modified from Matoba et al.\textsuperscript{13} Small mesenteric arteries of male C57BL/6 mice (8–10 weeks of age) were cut into rings and then opened longitudinally. The vascular strip was loaded with 5 µM 5-(and-6)-chloromethyl-2',7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA; Molecular Probes, Cat. No. C6827) for 30 min at room temperature. Then, the artery segments were placed on a slide glass and observed using a laser confocal microscope. Fluorimetric measurements were performed using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc.,Melville, NY) mounted on an inverted IX81 Olympus microscope, equipped with a 20X water-immersion objective (NA 0.5). Fluorescence intensity excited at 495 nm and emitted at 515 nm was measured for 5 min. Changes in intracellular H$_2$O$_2$ were expressed as F1/F0 ratios where F1 and F0 was the fluorescence intensity at a specific time and at the initiation of image recording. Fluorescence images of the endothelium were obtained before and 3 minutes after the application of ACh, which was achieved by dropping 5 µl HEPES buffer containing 10 µM ACh onto the slide glass using a micropipette. Relative fluorescence intensity was calculated using images obtained under basal conditions without ACh. The inhibitory effect of the pretreatment with indomethacin 5 µM, L-NAME 100 µM, catalase 1250 U/ml on the ACh-induced increase in fluorescence intensities was determined. All measurements were performed in HEPES buffer of the following composition (in mM): Na\textsuperscript{+} 143, K\textsuperscript{+} 5.9, Mg\textsuperscript{2+} 1.2, Ca\textsuperscript{2+} 1.6, H$_2$PO$_4$\textsuperscript{-} 1.2, Cl\textsuperscript{-} 150.9, glucose 11, HEPES 5.

Determination of endogenous EETs and DHETs: Blood samples (~0.5 ml) were obtained from anesthetized mice by cardiac puncture and separated in the presence of EDTA. Red blood cells (RBCs) were pelleted by centrifugation at 1000xg for 10 min. The cell-free supernatant (plasma) and the RBCs (after aspirating the buffy layer) were immediately frozen.
in liquid nitrogen and stored at -80°C. The mesenteric artery tree was excised and the periadventitial fat and connective tissue were removed using scissors. The remaining vascular tissue was briefly dried, weighed and frozen as above. For the subsequent analysis 100-200 µl of plasma, 50-100 mg of the RBC pellet or 10-30 mg of vascular tissue were used. The samples were supplemented with 100 µl of an internal standard mixture containing 100 ng/ml of each 20-HETE-d6 (Cayman Chemical) and 14,15-EET-d8 (Biomol). Alkaline hydrolysis was performed by adding 100 µl 10N NaOH and 500 µl methanol and incubating the samples after vortexing for 30 min at 60°C. The samples were then neutralized with acetic acid and adjusted to pH 6 by adding 2 ml sodium acetate buffer. Undissolved material was removed by centrifugation. Solid-phase extraction of the CYP eicosanoids was performed using a Varian Bond-Elut-Certify II column as described. The extract was dried under a stream of nitrogen gas and reconstituted with 50 µl of acetonitrile. The extracts were analyzed using the triplequadrupole tandem mass spectrometer Agilent 6410 combined with Agilent 1200 HPLC-System. A Zorbax Eclipse Plus-C18 4.6 x 150 mm x 1.8 µm column was used as stationary phase and acetonitrile / aqueous ammonium acetate solution (0.01 mol/L) as mobile phase. Acetonitrile was increased from 30 to 90 % during first 10 min and hold for further 5 min. The triplequadrupole mass spectrometer was equipped with electrospray ionisation source in negative mode. The analytes were detected using multiple reaction monitoring (MRM). Fragmentor voltage, collision energy, and product ion profile was optimized for each substance separately (Tab. 1). The calibration curves (authentic standard substances obtained from Cayman Chemical versus 20-HETE-d6 and 14,15-EET-d8) were linear in the range from 0.5 to 50 ng. Each sample was analyzed twice using 20 µl of the corresponding extracts. The results were calculated using the Agilent Mass Hunter Quantification software in ng per ml or g wet weight of the original sample.

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<th>Compound</th>
<th>Precursor Ion</th>
<th>Product Ion</th>
<th>Fragmentor</th>
<th>Collision energy</th>
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**Chemicals and drugs:** Ang II, U46619, indomethacin, and acetylcholine were obtained from Sigma (Taufkirchen, Germany). All salts and drugs were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany). All drugs were applied by addition to the bath solution.

**Statistics:** We used analysis of variance (repeated measures where indicated), Duncan’s multiple range tests, Bonferonni-corrected T tests and Student’s T tests as indicated. A p value <0.05 (*) was accepted as significant. n.s., not significant. Fiducial limits are given in mean±SEM.


Fig. I online
Fig. II online