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Substituted Adamantyl-Urea Inhibitors of the Soluble Epoxide Hydrolase Dilate Mesenteric Resistance Vessels

Jeffrey J. Olearczyk, Mary B. Field, In-Hae Kim, Christophe Morisseau, Bruce D. Hammock, and John D. Imig

Vascular Biology Center (J.J.O, M.B.F, J.D.I.) and Department of Physiology (J.D.I.), Medical College of Georgia, Augusta, Georgia; and Department of Entomology and Cancer Research Center (I.-H.K., C.M., B.D.H.), University of California, Davis, California

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ABSTRACT

The epoxyeicosatrienoic acids (EETs) have been identified as endothelium-derived hyperpolarizing factors. Metabolism of the EETs to the dihydroxyeicosatrienoic acids is catalyzed by soluble epoxide hydrolase (sEH). Administration of urea-based sEH inhibitors provides protection from hypertension-induced renal injury at least in part by lowering blood pressure. Here, we investigated the hypothesis that a mechanism by which sEH inhibitors elicit their cardiovascular protective effects is via their action on the vasculature. Mesenteric resistance arteries were isolated from Sprague-Dawley rats, pressurized, and constricted with the thromboxane A2 agonist U46619 (9,11-dideoxy-11,9-epoxymethano-prostaglandin F2α). Mesenteric arteries were then incubated with increasing concentrations of the sEH inhibitor 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA). AUDA resulted in a concentration-dependent relaxation of mesenteric arteries, with 10 μM resulting in a 48 $\pm$ 7% relaxation. Chain-shortened analogs of AUDA had an attenuated vasodilatory response. Interestingly, at 10 μM, the sEH inhibitors 1-cyclohexyl-3-dodecylurea, 12-(3-cyclohexyl-ureido)dodecanoic acid, and 950 [adamantan-1-yl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl}urea] were significantly less active, resulting in a 25 $\pm$ 8%, 10 $\pm$ 9%, and -8 $\pm$ 3% relaxation, respectively. Treatment of mesenteric arteries with tetraethylammonium, iberiotoxin, ouabain, or glibenclamide did not alter AUDA-induced relaxation. The AUDA-induced relaxation was completely inhibited when constricted with KCl. In separate experiments, denuding mesenteric resistance vessels did not alter AUDA-induced relaxation. Taken together, these data demonstrate that adamantyl-urea inhibitors have unique dilator actions on vascular smooth muscle compared with other sEH inhibitors and that these dilator actions depend on the adamantyl group and carbon chain length.

The soluble epoxide hydrolase (sEH) enzyme is found in a variety of mammalian tissues, with the highest activity measured in the liver, kidney, intestine, and vascular tissue (Wang et al., 1982; Yu et al., 2004; Newman et al., 2005). Within these tissues, sEH metabolizes endogenous and/or exogenous epoxide-containing compounds to their corresponding diols and/or glycols by catalyzing the addition of water to the epoxide moiety (Morisseau and Hammock, 2005; Newman et al., 2005). An endogenously produced, biologically active group of epoxides that serve as a substrate for sEH are the epoxyeicosatrienoic acids (EETs). EETs are cytochrome P450 epoxygenase metabolites of arachidonic acid that have been identified as important regulatory molecules in the cardiovascular and renal circulations. EETs have been identified as potential endothelial-derived hyperpolarizing factors and have been reported to have specific anti-inflammatory properties (Campbell et al., 1996; Fisslthaler et al., 1999; Node et al., 1999; Falck et al., 2003). The EETs are metabolized by the sEH into their corresponding dihydroxyeicosatrienoic acids, thereby resulting in partial or com-

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ABBREVIATIONS: sEH, soluble epoxide hydrolase; EET, epoxyeicosatrienoic acid; AUDA, 12-(3-adamantan-1-yl-ureido)dodecanoic acid; CUDA, 12-(3-cyclohexyl-ureido)dodecanoic acid; AUOA, 8-(3-adamantyl-ureido)-octanoic acid; AUHA, 6-(3-adamantyl-ureido)-hexanoic acid; AUBA, 4-(3-adamantan-1-yl-ureido)butanoic acid; AADU, 1-adamantyl-3-(12-aminododecyl)urea; 950, adamantan-1-yl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl}urea; DMSO, dimethyl sulfoxide; CDU, 1-cyclohexyl-3-dodecylurea; IbTX, iberiotoxin; MS-PPOH, *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; PBS-T, phosphate-buffered saline containing 0.1% Tween 20; TEA, tetraethylammonium; DMF, dimethyl formamide; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; LC, liquid chromatography; MS, mass spectrometry; TOF, time-of-flight; U46619, 9,11-dideoxy-11,9-epoxymethano-prostaglandin F2α.

plete loss of activity (Moghaddam et al., 1997; Zeldin, 2001; Spector et al., 2004).

As a result of the renal and cardiovascular protective effects afforded by the EETs, inhibiting their metabolism has identified the sEH enzyme as a potential therapeutic target in diseases like hypertension and inflammation. A number of sEH inhibitors have been developed, and their administration in animal models of disease has proved to have beneficial effects. In the spontaneously hypertensive rat, administration of the sEH inhibitor *N*,*N*′-dicyclohexylurea lowered blood pressure (Yu et al., 2000). Another sEH inhibitor, 1-cyclohexyl-3-dodecylurea, also lowered blood pressure in an animal model of angiotensin-dependent hypertension (Imig et al., 2002; Zhao et al., 2004). The sEH inhibitor 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA) lowered blood pressure and decreased renal damage associated with angiotensin-dependent, salt-sensitive hypertension (Imig et al., 2005). In addition to lowering blood pressure, it was demonstrated that sEH inhibition also inhibits the inflammatory response. Administration of 12-(3-adamantane-1-yl-ureido)-dodecanoic acid *n*-butyl ester to spontaneously hypertensive rats exposed to tobacco smoke significantly attenuated the tobacco smoke-induced infiltration of proinflammatory cells into the lung (Smith et al., 2005). The same sEH inhibitor was also reported to decrease lipopolysaccharide-induced tissue injury and mortality (Schmelzer et al., 2005).

Many of the renal and cardiovascular protective effects elicited with sEH inhibition are attributed to the measured decrease in blood pressure. To better understand the mechanism by which sEH inhibition decreases blood pressure, we turned our attention to the vasculature. In the present study, we investigated the hypothesis that a mechanism by which sEH inhibitors elicit their cardiovascular protective effects is via their direct action on the vasculature.

Materials and Methods

Isolated Mesenteric Resistance Vessel Preparation. All animal studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. In addition, all experimental procedures were approved by the Medical College of Georgia Animal Care and Use Committee. Mesenteric artery segments were obtained from male Sprague-Dawley rats (250–310 g) and mounted between two cannulae in a pressure myograph system (Danish Myo Technology model 111P). The interior and exterior of the vessel were oxygenated in (95% O_2/5% CO_2) Krebs physiological salt solution (119.0 mM NaCl, 25.0 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11.0 mM glucose; Sigma-Aldrich) at pH 7.4 and 37°C. Under no flow conditions, over a span of 18 min, the pressure within the vessel was increased at 10 mm Hg increments from 20 to 65 mm Hg. The vessel was then equilibrated at 65 mm Hg for 30 min and remained at that pressure for the duration of the experiment. Lumen diameter measurements were acquired and logged using the MyoView 1.2P user interface (DMT, Aarhus, Denmark). The control lumen diameter was calculated as the mean diameter during the last 15 min of the 30-min equilibration. Vessels were constricted with U46619 (1–3 μM), and the diameter of the constricted vessel was calculated as the mean during the last 2 of 15 min after the addition of U46619. sEH inhibitors AUDA, 12-(3-cyclohexyl-ureido)dodecanoic acid (CUDA), 8-(3-adamantyl-ureido)-octanoic acid (AUOA), 6-(3-adamantyl-ureido)-hexanoic acid (AUHA), 4-(3-adamantan-1-yl-ureido)butanoic acid (AUBA), 1-adamantyl-3-(12-aminododecyl)urea (AADU), and 950 were dissolved in dimethyl sulfoxide (DMSO) and sonicated to make 10^{-1} M stock solutions to be added to the vessel chamber. 1-Cyclohexyl-3-dodecylurea (CDU) was dissolved in 75% ethanol to make a 10^{-2} M stock. Doses of each sEH inhibitor (0.1, 1.0, 10, and 100 μM) were added to the vessel chamber (on the exterior of the vessel) every 5 min, and responses were calculated as the mean lumen diameter during the last 2 min of each dosage. Acetylcholine (10 μM, Sigma-Aldrich) was given at the end of the experimental protocol to ensure endothelial integrity of the vessel, and the final lumen diameter was calculated as the mean diameter over the last 2 min of the acetylcholine response. To ensure that the vehicle into which the drugs were dissolved did not alter vessel reactivity, experiments were conducted using either DMSO or ethanol in the absence of drug. DMSO did not change vascular reactivity at any of the concentrations used in these experiments. The addition of ethanol to the vessel chamber also did not result in a significant change in vascular reactivity at any of the concentrations used in these experiments.

In inhibitor experiments, tetraethylammonium (1 mM; Sigma-Aldrich), ouabain (100 μM; Sigma-Aldrich), and glibenclamide (1 μM; Sigma-Aldrich) were used postconstriction and for 10 to 20 min prior to dosing with AUDA. Iberiotoxin (IbTX; Tocris Bioscience, Ellisville, MO) was reconstituted with deionized water, and a 100 nM dose was given 15 min before constriction with U46619. *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH) was dissolved in 50% ethanol, and a 1 μM dose was given 30 min before constriction with U46619. Potassium chloride (40 mM; Sigma-Aldrich) was used to constrict vessels 20 min prior to addition of AUDA. Endothelium-denuded vessels were prepared by inserting a human hair about which the vessel was rotated using a rolling motion atop a raised, crowned surface in a dish of Krebs physiological salt solution. To ensure that the vessel was no longer capable of an endothelium-related dilation, acetylcholine (100 μM for 5 min) was given. If an acetylcholine-induced relaxation was not observed, the vessel was rinsed and allowed to equilibrate for 10 min before constriction with U46619 and treatment with AUDA. At the end of the experimental protocol, sodium nitroprusside (100 μM) was added to the chamber to demonstrate that the endothelium-denuded vessel was capable of relaxation.

Western Blot Analysis. Mesenteric artery segments were obtained from five male Sprague-Dawley rats (250–310g) of the same size that would be used in the isolated mesenteric resistance vessel preparation. The vessels were homogenized in cell lysis buffer [0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 0.1% NP-40, 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin, and 14 μM E-64], and the homogenate centrifuged at 10,000*g* for 10 min at 4°C to sediment any unhomogenized material. The supernatant was removed, and the protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, IL). Protein was solubilized in Laemmli sample buffer and resolved by electrophoresis using a 10% stacking Tris-glycine acrylamide gel. Protein was electrophoretically transferred to polyvinylidene difluoride membrane and blocked with 3% bovine serum albumin and 10% dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T). After blocking, the membranes were washed several times with PBS-T and incubated with primary antibody for the sEH enzyme (1:2000; a gift from Dr. Bruce D. Hammock, University of California, Davis, CA). Membranes were washed with PBS-T and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (1:25,000; Pierce Biotechnology, Inc.). Protein was visualized using ECL Western Blotting substrate (Pierce Biotechnology, Inc.).

sEH Inhibitors. The synthesis and characterization of AUDA, CUDA, CDU, and AUBA are described elsewhere (Morisseau et al., 2002; Kim et al., 2004).

950. To a solution of adamantyl isocyanate (0.20 g, 1.13 mmol) in DMF (15 ml) was added a solution of 5-amino-1-pentanol (0.17 g,

1.69 mmol) in DMF (15 ml) at 0°C. After stirring for 12 h, an aqueous solution of 1 N HCl (40 ml) was added into the reaction at 0°C, and the mixture was stirred for 30 min. The solid product crystalized was filtered and washed with water (40 ml) and ethyl acetate (20 ml). The resulting solid was dried in the vacuum oven to give 1-adamantan-1-yl-3-(5-hydroxypentyl)urea as a white solid (I; 0.75 g, 100%). To a solution of diethylene glycol monoethyl ether (1 Eq) in tetrahydrofuran (30 ml) was added portion-wise triphenylphosphine (1.1 Eq) and carbon tetrabromide (1.1 Eq) at 0°C. After stirring for 12 h at room temperature, hexane (60 ml) was added to the reaction mixture. This crude mixture was filtered to get rid of triphenylphospine oxide, and the organic solvent dissolving the product was washed with water (60 ml), dried over $MgSO_4$, and concentrated. The residue was purified using silica gel column chromatography (hexane only and hexane:ethyl acetate = 3:1) to give the corresponding bromide II (87%) as an oil. This bromide (1.5 Eq) was added portion-wise to a suspension of 95% sodium hydride (3 Eq) and I (1 Eq) in DMF at room temperature. After stirring for 12 h, water was poured into the reaction mixture, and the product was extracted with ether. The organic solution was dried over $MgSO_4$ and concentrated. The residue was purified using silica gel column chromatography (hexane: ethyl acetate = 3:1) to afford 950 (52%) as a solid. ^{1}H NMR δ ($CDCl_3$) 1.22 (3*H*, t, *J* = 6.9 Hz), 1.37–1.43 (2*H*, m), 1.46–1.53 (2*H*, m), 1.56–1.61 (2*H*, m), 1.64–1.69 (6*H*, m), 1.95–1.99 (6*H*, m), 2.05–2.08 (3*H*, m), 3.11 (2*H*, q, *J* = 6.9 Hz), 3.46 (2*H*, t, *J* = 6.9 Hz), 3.48–3.67 (10*H*, m), 4.21 (1*H*, s), 4.26 (1*H*, s), LC-MS (ESI) *m/z* calculated for $C_{22}H_{40}N_2O_4$ $[M + H]^+$ 397.30, found $[M + H]^+$ 397.31, mp 75°C, Anal. ($C_{22}H_{40}N_2O_4$) C, H, N.

AUOA. To a suspension of 8-aminooctanoic acid (0.48 g, 3.0 mmol) in DMF (20 ml) was added 1-adamantyl-isocyanate (0.48 g, 2.7 mmol) at room temperature. The reaction mixture was stirred and heated at 100°C for 3 h. After cooling down to room temperature, 1 N HCl aqueous solution (25 ml) was added to the reaction and the mixture was stirred for 30 min. The solid crystalline product was filtered and washed with water (20 ml) and ethyl acetate (20 ml). The resulting solid was recrystallized from methanol. The obtained crystal was dried in a vacuum oven to give 0.90 g (98%) of 8-(3-adamantyl-ureido)-octanoic acid as a white solid: mp 140°C; ^{1}H NMR ($CDCl_3$/TMS) 1.37–1.40 (6*H*, m), 1.55–1.58 (2*H*, m), 1.70–1.77 (9*H*, m), 1.98–2.05 (5*H*, m), 2.10–2.16 (3*H*, m), 2.40 (2*H*, t, *J* = 7.4 Hz), 3.13 (2*H*, t, *J* = 6.5 Hz), 4.28 (1*H*, s), 4.47 (1*H*, s) ppm; LC-MS (TOF) *m/z* calculated for $C_{19}H_{33}N_2O_3$ $[M + H]^+$ 337.2491, found $[M + H]^+$ 337.2508 Da.

AUHA. To a suspension of 6-aminohexanoic acid (0.98 g, 7.5 mmol) in DMF (15 ml) was added 1-adamantyl-isocyanate (0.89 g, 5.0 mmol) at room temperature. The reaction mixture was stirred and heated at 100°C for 3 h. After cooling down to room temperature, 1 N HCl aqueous solution (25 ml) was added to the reaction, and the mixture was stirred for 30 min. The solid crystalline product was filtered and washed with water (20 ml) and ethyl acetate (20 ml). The resulting solid was dried in a vacuum oven to give 1.05 g (68%) of 6-(3-adamantyl-ureido)-hexanoic acid as a white solid: mp 159°C; ^{1}H NMR ($CDCl_3$/TMS): 1.60–1.67 (13*H*, m), 1.90–1.96 (5*H*, m), 2.02–2.07 (3*H*, m), 2.36 (2*H*, t, J = 7.2 Hz), 3.09 (2*H*, t, *J* = 6.9 Hz), 4.15 (1*H*, s), 4.33 (1*H*, s) ppm; LC-MS (TOF) *m/z* calculated for $C_{17}H_{29}N_2O_3$ $[M + H]^+$ 309.2178, found $[M + H]^+$ 309.2164 Da.

AADU. To a solution of 1,12-diaminododecane (1.0 g, 5.0 mmol) in DMF (20 ml), adamantyl-isocyanate (0.21 g, 1.1 mmol) at room temperature was added. The reaction mixture was stirred and heated at 100°C for 3 h. After cooling down to room temperature, 150 ml of water was added to the reaction, and the mixture was stirred for 30 min. The yellowish solid was filtered and washed with water (20 ml). The targeted product was purified by chromatography on a silica column equilibrated with ethyl acetate. Product was eluted with a 50:50 mixture of ethyl acetate:ammonia-saturated methanol. The solvent was evaporated, and the obtained crystal was dried in a vacuum oven to give 0.20 g (49%) of 1-adamantyl-3-(12-aminododecyl) urea as a white solid: mp 80°C; ^{1}H NMR ($CDCl_3$/TMS) 1.22–1.33 (19*H*, m), 1.42–1.49 (4*H*, m), 1.55–1.62 (12*H*, m), 2.06 (2*H*, s), 2.68 (2*H*, t, *J* = 7.2 Hz), 3.13 (2*H*, q, *J* = 6.6 Hz), 4.02 (1*H*, s), 4.10 (1*H*, s) ppm; LC-MS (TOF) *m/z* calculated for $C_{23}H_{44}N_3O$ $[M + H]^+$ 337.1440, found $[M + H]^+$ 378.1221 Da.

Statistical Analysis. All data are presented as mean ± S.E.M. In experiments using AUDA, statistical significance between concentrations was determined using an analysis of variance followed by a least significance difference test to identify individual differences. The statistical significance between experimental groups was determined using a two-way analysis of variance and Bonferroni post tests to compare replicate means. $P \leq 0.05$ was considered statistically significant.

Results

The sEH has been identified as a pharmacological target for diseases such as hypertension and inflammation. Carbamate- and urea-substituted sEH inhibitors have been demonstrated to decrease blood pressure in animal models of hypertension. To determine whether or not sEH inhibitors have effects on vascular reactivity, isolated, pressurized, and preconstricted mesenteric vessels from Sprague-Dawley rats were treated with increasing concentrations of AUDA, and the vascular diameter was measured. In experiments using sEH inhibitors or analogs of AUDA, the vessel starting diameter measured 233.6 ± 4.9 μm (n = 51) and measured 79.0 ± 5.3 μm (n = 51) after treatment with U46619. Starting vessel diameters and vessel diameters after treatment with U46619 were not different between the experimental groups. Treatment with AUDA resulted in a concentration-dependent relaxation in mesenteric resistance vessels (Fig. 1). These data obtained using AUDA are repeated on subsequent figures for ease of comparison. To determine whether these vasoactive effects were unique to AUDA, identical experiments were conducted using two additional sEH inhibitors, (CDU), Table 1) and 950 (Table 1). Increasing concentrations of CDU also resulted in dilation of mesenteric resistance arteries (Fig. 1). Interestingly, at a dose of 10 μM, CDU had a statistically lower percent dilation compared with

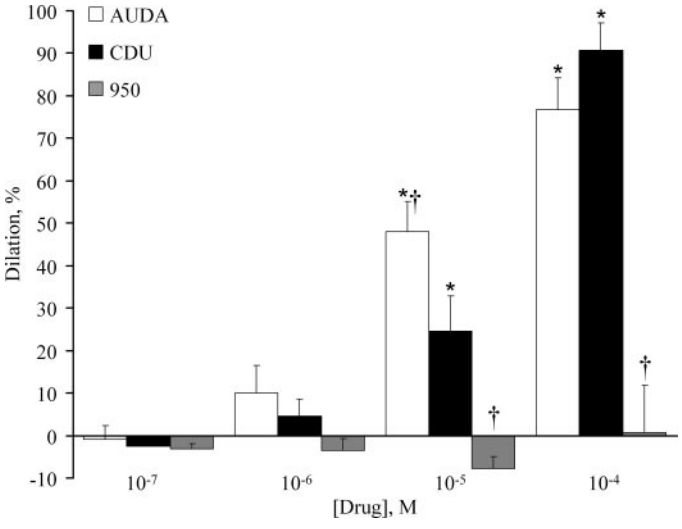
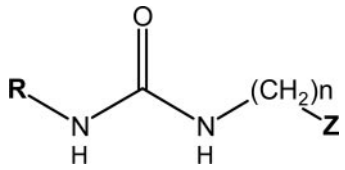
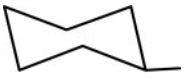


Fig. 1. Vascular reactivity of sEH inhibitors. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized, preconstricted with U46619, and treated with increasing concentrations of the sEH inhibitors AUDA (n = 10), CDU (n = 7), and 950 (n = 6). *, $P < 0.05$, different from previous dose of the same drug; †, $P < 0.05$, different from other drugs at the same dose.

TABLE 1
Structures and IC_{50} of inhibitors of the soluble epoxide hydrolase enzyme

R				
	n	*Z*	Identifier	Human sEH IC_{50}
				nM
	11	COOH	AUDA	3.2
	7	COOH	AUOA	12.9
	5	COOH	AUHA	170.7
	3	COOH	AUBA	684.2
	12	NH_2	AADU	2.6
	5	$(O(CH_2)_2)_2OC_2H_5$	950	14.1
	11	COOH	CUDA	9.5
	11	CH_3	CDU	7.0

AUDA; 25 ± 8% versus 48 ± 7% for CDU and AUDA, respectively. At the highest dose, CDU resulted in a dilation that was not statistically different from that measured with AUDA; 91 ± 7% versus 77 ± 7% dilation for CDU and AUDA, respectively. Treatment of mesenteric resistance vessels with the sEH inhibitor 950 did not induce a measurable change in vessel diameter at any of the concentrations tested.

The sEH enzyme is expressed in a variety of tissues including the kidney, liver, and blood vessels (Enayetallah et al., 2006). To determine whether or not the sEH enzyme is expressed in mesenteric resistance vessels from male Sprague-Dawley rats, vessels were obtained, and the expression of the sEH enzyme was measured by Western blot. The sEH enzyme was identified in the mesenteric resistance vessels from Sprague-Dawley rats (Fig. 2). However, compared with the kidney cortex from Sprague-Dawley rats, sEH protein expression in mesenteric resistance vessels was less per microgram of total protein.

The unique structure of adamantyl-urea inhibitors of the sEH enzyme imparts specific vasoactive properties that are not observed with other sEH inhibitors that could contribute to the reported blood pressure-lowering effects and renal and cardiovascular protective properties. Adamantyl-urea inhibitors are 1,3-disubstituted ureas with an adamantyl group on one side of the urea pharmacophore and a saturated 11-carbon aliphatic chain on the other (Table 1). To investigate the structural elements of adamantyl-urea inhibitors that are responsible for the observed vasoactive actions, structural analogs of AUDA were prepared. These analogs, the structures of which are provided in Table 1, were designed to identify the contributions of the adamantyl group, the carboxylic acid functional group, and the length of the aliphatic carbon chain on vascular reactivity. Changing the adamantyl group of AUDA to a cyclohexyl group, which is the structure of the sEH inhibitor CUDA, significantly attenuated the vasodilatory response (Fig. 3). CUDA resulted in a 10 ± 5% relaxation of mesenteric resistance vessels at the 10 μM dose and a 52 ± 13% relaxation at the 100 μM dose. Replacing the carboxylic acid at the end of the aliphatic chain to a primary amine, AADU, resulted in concentration-dependent dilations of mesenteric resistance vessels that were not different from that of AUDA (Fig. 3). At 10 μM, AADU induced a 55 ± 14% increase in vessel diameter, whereas AUDA induced a 48 ± 7% increase in vessel diameter. Finally, shortening the aliphatic side chain from 12 carbons to eight carbons, AUOA, or

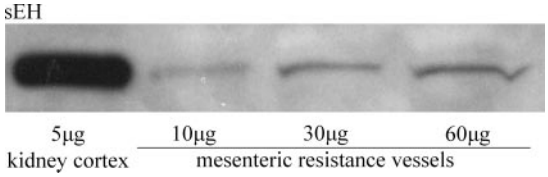


Fig. 2. Expression of the sEH enzyme in rat mesenteric resistance vessels. Mesenteric resistance vessels of the size used in the isolated mesenteric resistance vessel preparation or kidney cortex were obtained from male Sprague-Dawley rats. The vessels were homogenized and increasing concentrations of total protein loaded onto a gel and resolved by electrophoresis. Protein was transferred to polyvinylidene difluoride membrane and the sEH enzyme identified using an anti-sEH primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. sEH was visualized using ECL Western blotting substrate.

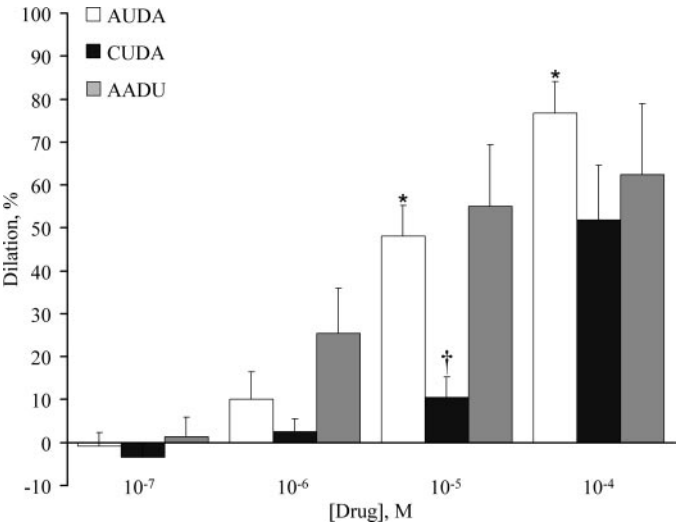


Fig. 3. The effects of the adamantyl group and the carboxylic acid moiety on the vascular reactivity of adamantyl-urea inhibitors of sEH. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized, preconstricted with U46619 and treated with increasing concentrations of CUDA ($n = 5$) or AADU ($n = 6$). *, $P < 0.05$, different from previous dose of the same drug; †, $P < 0.05$, different from other drugs at the same dose.

to four carbons, AUBA, significantly attenuated the vasodilatory response (Fig. 4). AUOA resulted in a $-2 \pm 4\%$ relaxation, and AUBA resulted in a $3 \pm 11\%$ relaxation of mesenteric resistance vessels at the 10 μM dose. Interestingly, shortening the aliphatic side chain from 12 carbons to six carbons, AUHA, altered the tendency for the chain-shortened analogs to decrease activity (Fig. 4). At 10 μM, AUHA induced a $22 \pm 9\%$ relaxation of mesenteric resistance vessels. After treatment with the highest dose of AUDA or its analogs, acetylcholine (10 μM) was added to the isolated mesenteric resistance vessel preparation to ensure endothelial integrity. Acetylcholine increased vessel diameter to 242.5 ± 5.0 μm, a $106 \pm 3\%$ dilation.

Inhibition of the sEH enzyme results in the accumulation of the EETs that are then able to elicit their vasoactive effects. If the mechanism by which adamantyl-urea inhibitors induce vasoreactivity is via increasing EETs levels, then inhibiting EET synthesis would be expected to attenuate the effects of AUDA. To investigate this hypothesis, mesenteric resistance vessels were pretreated with the epoxygenase inhibitor MS-PPOH. Pretreatment of mesenteric resistance vessels with MS-PPOH did not alter the average starting vessel diameter, 224.9 ± 9.1 μm before MS-PPOH treatment and 225.2 ± 9.5 μm after treatment ($n = 4$). After the addition of U46619, the MS-PPOH-pretreated vessels had an average diameter of 150 ± 1.1 μm. MS-PPOH did not statistically alter AUDA-induced vascular reactivity (Fig. 5). In the presence of MS-PPOH, AUDA induced a $43.7 \pm 12.0\%$ relaxation at the 10 μM dose. These data suggest that the mechanism by which AUDA induces its vascular reactivity could be independent of its actions on the sEH enzyme.

Epoxyeicosatrienoic acids are reported to activate calcium-activated potassium channels located in the endothelium and smooth muscle cells, resulting in a decrease in the membrane potential. To determine whether or not the adamantyl-urea inhibitor-induced vasodilation was the result of these inhibitors to activate potassium channels, mesenteric resistance vessels were pretreated with either tetraethylammonium (TEA), a nonspecific potassium channel blocker, or IbTX, a specific large-conductance calcium-activated potassium channel blocker. In experiments using TEA, the average vessel diameter measured 242.7 ± 17.4 μm and measured 74.7 ± 16.3 μm after treatment with U46619 ($n = 5$). Addition of TEA resulted in a stable $22 \pm 8\%$ increase in vessel diameter. Treatment with IbTX did not alter the average starting vessel diameter, 205.2 ± 5.9 μm before IbTX treatment and 200.1 ± 5.2 μm after treatment ($n = 6$). The average diameter of IbTX-pretreated vessels after the addition of U46619 was 75.7 ± 11.8 μm. Neither TEA nor IbTX pretreatment inhibited the observed AUDA-induced relaxation (Fig. 6). In the presence of TEA or IbTX, AUDA induced a $29 \pm 23\%$ or a $34 \pm 9\%$ dilation of mesenteric resistance vessels at the 10 μM dose. The AUDA-induced dilation, how-

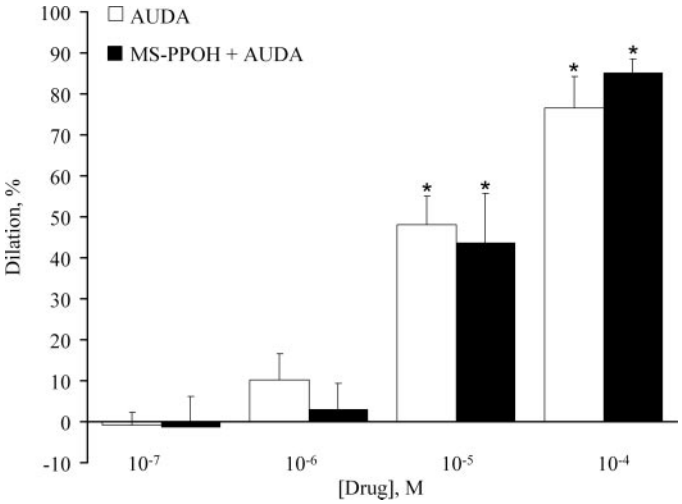


Fig. 5. The effect of epoxygenase inhibition on the vascular reactivity of AUDA. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized and pretreated with MS-PPOH (1 μM, 30 min). The vessels were constricted with U46619 and treated with increasing concentrations of AUDA ($n = 4$). *, $P < 0.05$, different from previous dose of the same drug.

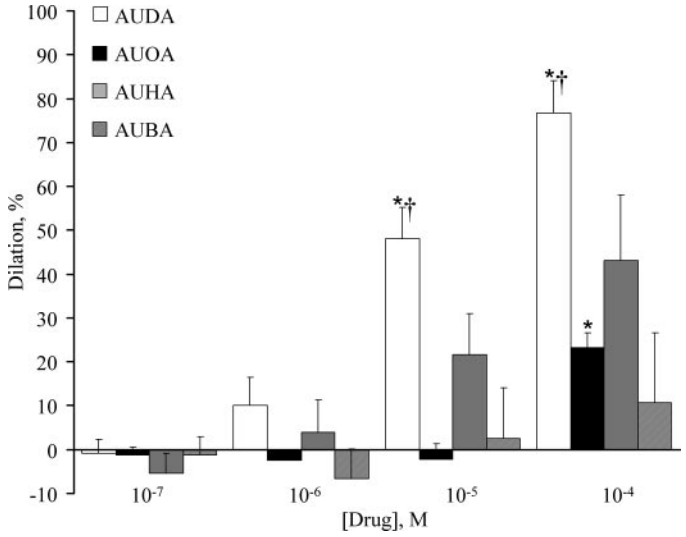


Fig. 4. The effects of chain-shortened adamantyl-urea inhibitors on vascular reactivity. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized, preconstricted with U46619, and treated with increasing concentrations of AUOA ($n = 5$), AUHA ($n = 6$), and AUBA ($n = 5$) from Table 1. *, $P < 0.05$, different from previous dose of the same drug; †, different from AUOA and AUBA.

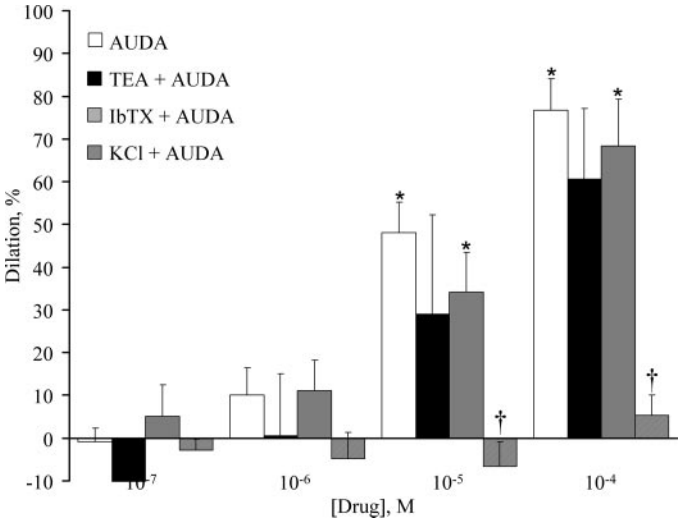


Fig. 6. The effects of potassium channel inhibitors on the vascular reactivity of AUDA. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized, preconstricted with U46619, and pretreated with TEA ($n = 5$), IbTX ($n = 6$), or KCl ($n = 6$). *, $P < 0.05$, different from previous dose of the same drug; †, different from other drugs at the same dose.

ever, was completely blocked when mesenteric vessels were pretreated with potassium chloride (KCl). In these experiments, the starting vessel diameter measured 220.8 ± 14.7 μm and decreased to 131.4 ± 14.2 μm after KCl treatment (n = 6). At a dose of 10 μM, AUDA did not significantly change the vessel diameter, which measured 117.5 ± 17.4 μm (Fig. 6).

Inhibition of AUDA-induced increases in vessel diameter by KCl suggests that adamantyl-urea inhibitors could be influencing potassium flux or altering the activity of some other membrane channel. The epoxyeicosatrienoic acids have been reported to activate the Na^+/K^+-ATPase as well as ATP-sensitive K^+ (K_{ATP}) channels (Pratt et al., 2001; Yang et al., 2005; Ye et al., 2005, 2006). To investigate the possible roles of these channels on AUDA-induced vascular reactivity, mesenteric resistance vessels were treated with ouabain, an inhibitor of the Na^+/K^+-ATPase, or glibenclamide, an inhibitor of the K_{ATP} channel. In experiments using ouabain, the starting vessel diameter measured 216.4 ± 9.7 μm and measured 51.8 ± 2.7 μm after treatment with U46619 (n = 5). Treatment with ouabain did not significantly alter the vessel diameter, 79.2 ± 20.8 μm. AUDA-induced relaxation was not affected by ouabain treatment (Fig. 7). At the 10 μM dose, AUDA induced a 42 ± 12% increase in vessel diameter (Fig. 7). In experiments using glibenclamide, the starting vessel diameter measured 194.2 ± 15.8 μm and measured 102.3 ± 14.0 μm after treatment with U46619 (n = 4). Treatment with glibenclamide resulted in a 28 ± 4% increase in vessel diameter. Glibenclamide treatment did tend to inhibit increases in vessel diameter induced by AUDA; however, these decreases were not statistically significant (Fig. 7).

To determine whether adamantyl-urea inhibitors induce vasodilation indirectly via their actions on the endothelium or directly via their actions on the vascular smooth muscle, experiments were conducted using mesenteric resistance vessels where the endothelium was disrupted. The average vessel diameter measured 168.4 ± 12.9 μm and measured 85.0 ± 8.6 μm after treatment with U46619. Treatment of the preconstricted, denuded vessel with acetylcholine did not statistically alter the vessel diameter, which averaged 93.9 ± 14.3 μm. Disrupting the endothelium did not affect the AUDA-induced relaxation (Fig. 8). At the 10 μM dose, AUDA elicited 48.1 ± 7.0% dilation in nondenuded vessels and 39.5 ± 15.4% dilation in denuded vessels. The addition of sodium nitroprusside (10 μM) to the denuded mesenteric resistance vessel at the end of the experiment increased the vessel diameter to 163.6 ± 10.4 μm, a 158 ± 29% dilation.

Discussion

The sEH enzyme metabolizes epoxide-containing compounds by catalyzing the addition of water to the epoxide moiety (Morisseau and Hammock, 2005; Newman et al., 2005). EETs, cytochrome P450 epoxygenase metabolites of arachidonic acid, are endogenously produced epoxides that serve as substrates for the sEH enzyme. The metabolism of EETs by sEH into the corresponding dihydroxyeicosatrienoic acids results in partial or complete loss of activity (Moghaddam et al., 1997; Zeldin, 2001; Spector et al., 2004). The EETs have been identified as important regulatory molecules in the cardiovascular and renal circulations. As a result, the sEH enzyme has been identified as a therapeutic target for diseases like hypertension and inflammation. Inhibitors of the sEH enzyme have been successfully employed in animal models of hypertension where sEH inhibition was reported to decrease blood pressure and decrease hypertension-induced renal damage. These renal and cardiovascular protective effects are attributed, at least in part, to the blood pressure-lowering effects of the sEH inhibitor. Within the present study, we addressed the hypothesis that a mechanism by which sEH inhibitors elicit their cardiovascular protective effects is via their direct actions on the vasculature.

We demonstrated, using isolated mesenteric resistance vessels, that treatment with adamantyl-urea inhibitors of the sEH enzyme resulted in a concentration-dependent relaxation of the preconstricted vessel. The dilator responses observed in mesenteric resistance vessels occur at concentra-

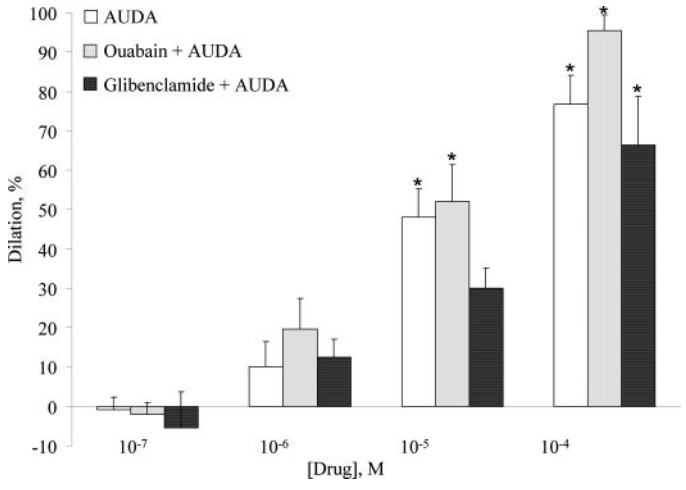


Fig. 7. The effects of inhibition of the Na^+/K^+-ATPase or the K_{ATP} channel on the vascular reactivity of AUDA. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The vessels were pressurized, preconstricted with U46619, and treated with either ouabain (n = 4) or glibenclamide (n = 4). ∗, $P < 0.05$, different from previous dose of the same drug.

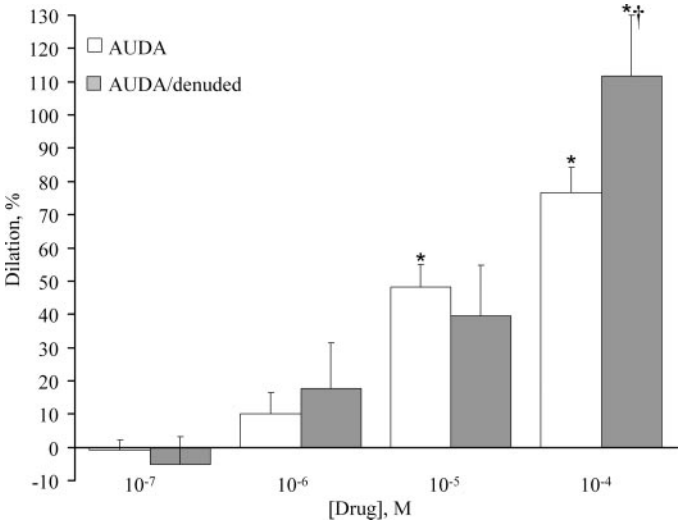


Fig. 8. The effects of endothelial cell disruption on the vascular activity of AUDA. Mesenteric resistance vessels were isolated from Sprague-Dawley rats. The endothelium was disrupted using a human hair and then pressurized, constricted with U46619, and treated with increasing concentrations of AUDA (n = 5). ∗, $P < 0.05$, different from previous dose of the same drug; †, different from other drugs at the same dose.

tions that are above the reported IC_{50} for the sEH enzyme. This response was also demonstrated with the sEH inhibitor CDU but was not observed with the more sterically hindered sEH inhibitor 950. These data suggest that the vasoactive properties measured with adamantyl-urea inhibitors are unique to their structure and are independent of their ability to inhibit the sEH enzyme itself. Adamantyl-urea inhibitors are 1,3-disubstituted ureas with an adamantyl group on one side of the urea pharmacophore and an 11-carbon aliphatic chain on the other (Table 1). To investigate what structural elements of adamantyl-urea inhibitors are responsible for these unique vasoreactive responses, analogs of AUDA were prepared (Table 1). Each of the analogs of AUDA had IC_{50}s for the human sEH enzyme in the nanomolar range.

The sEH inhibitor CUDA exhibited significantly decreased vasoreactivity in mesenteric vessels compared with that measured with AUDA. The structural difference between CUDA and AUDA is that CUDA has a cyclohexyl group on one side of the urea pharmacophore, whereas AUDA has an adamantyl group. These data would suggest that the adamantyl group is an important structural feature that is unique to adamantyl-urea inhibitors and is responsible for the observed vasoactive properties. Interestingly, the adamantyl functional group was determined to be an important structural feature for the inhibition of the sEH enzyme itself (Morisseau et al., 2002). CUDA shares some structural similarity to CDU in that both sEH inhibitors have a cyclohexyl group occupying one side of the urea pharmacophore. This could explain why CDU exhibits a similar reactivity as CUDA in this preparation. However, in addition to having a cyclohexyl group on one side of the urea pharmacophore, the structure of CDU also differs from AUDA in that it has an aliphatic chain terminating with a methyl group instead of a carboxylic acid. This could explain why at the highest concentration, CDU demonstrated similar reactivity to AUDA.

When the negatively charged carboxylic acid at the end of the aliphatic carbon chain of AUDA was changed to a positively charged amine group (Fig. 3, AADU), the vascular reactivity of AUDA was not altered. These data suggest that the terminal functional group at the end of the aliphatic carbon chain is not an important structural feature for the vascular reactivity observed with adamantyl-urea inhibitors. Interestingly, altering this functional group also had little effect on the IC_{50} for the human sEH enzyme. This finding would also suggest that the inability of CDU to induce similar vasodilations was mainly the result of the cyclohexyl functional group, not the methyl group at the end of the aliphatic carbon chain. Changing the length of the aliphatic carbon chain, however, did alter the observed vasoreactivity of adamantyl-urea inhibitors. AUOA, which shortened the length of the aliphatic carbon chain from 12 to eight carbons, significantly attenuated the vasoactive properties of AUDA. The same result was observed with AUBA, which shortened the length of the aliphatic carbon chain from 12 to four carbons. AUHA, which has an aliphatic carbon chain length of six carbons, demonstrated a slightly attenuated vasoreactivity compared with AUDA. The human sEH enzyme IC_{50}s for AUOA, AUHA, and AUBA did not directly correspond to the observed vasoreactivity. Of the chain-shortened analogs, AUOA has the lowest IC_{50} for the human sEH but demonstrated very little vasoreactivity compared with AUDA. AUBA had the highest IC_{50} for the human sEH enzyme and also had the least amount of vasoactivity. AUHA had an intermediate IC_{50} for the human sEH enzyme, and although the responses look attenuated compared with those measured with AUDA, they are not statistically different. Each σ bond that comprises the aliphatic carbon side chain can freely rotate giving the side chain significant flexibility. Therefore, it is possible that the compounds with the 12-carbon (AUDA) and six-carbon (AUHA) side chains are able to assume a configuration that allows them to induce the observed relaxation. Conversely, these configurations might not be possible in the eight-carbon (AUOA) or four-carbon (AUBA) side chains. In addition, 950, which has a carbon side chain that is significantly more sterically hindered by the addition of oxygen atoms to the carbon side chain and therefore cannot assume conformations that are available to AUDA, does not induce changes in vessel diameter. Taken together, these data suggest that the length of the aliphatic carbon chain and its flexibility are important structural features of adamantyl-urea inhibitors that are responsible for the observed vasoreactive properties.

Inhibitors of the sEH enzyme increase endogenous EET levels by decreasing the metabolism of the EETs to less active diols. If the mechanism by which adamantyl-urea inhibitors induce vasorelaxation is by increasing endogenous EET levels, then it would be expected that inhibiting EET synthesis would attenuate the AUDA-induced response. However, pretreatment of mesenteric resistance vessels with the epoxygenase inhibitor MS-PPOH did not alter AUDA-induced increases in vessel diameter. One interpretation of these data is that the mechanism by which AUDA induces vascular reactivity is not only due to increasing EET levels but may have effects independent of sEH inhibition.

One mechanism by which the EETs exert their renal and cardiovascular effects is via activation of calcium-activated potassium channels in the endothelium and smooth muscle cells, resulting in a decrease in the membrane potential (Chataigneau et al., 1998; Wu et al., 2000). Neither TEA, a nonspecific potassium channel blocker, nor IbTX, a specific calcium-activated potassium channel blocker, attenuated the observed AUDA-induced dilation (Fig. 4). These data suggest that a possible mechanism by which adamantyl-urea inhibitors exert their vasoactive effects is not through their action on these specific potassium channels. In addition, these data suggest that the dilation observed with AUDA may not due to the inhibition of sEH and the resulting increase in vascular EET levels.

The EETs have also been reported to alter the activity of other channels that might participate in the observed vasoreactivity, the Na^+/K^+-ATPase and the K_{ATP} channel (Pratt et al., 2001; Yang et al., 2005; Ye et al., 2005, 2006). The observed AUDA-induced vasorelaxation was not altered with ouabain treatment; however, glibenclamide did tend to attenuate the AUDA-induced response, although this decrease did not reach statistical significance. Therefore, the mechanism by which adamantyl-urea inhibitors induce increases in vessel diameter is not by their actions on the Na^+/K^+-ATPase or the K_{ATP} channel.

Denuded mesenteric resistance vessels were used to determine whether adamantyl-urea inhibitors are acting on the endothelium or directly on the smooth muscle to induce the observed vasodilation. Disrupting the endothelium did not affect AUDA-induced relaxation at the lower concentrations

tested. However, disrupting the endothelium did result in a more robust relaxation in response to 100 μM AUDA compared with vessels where the endothelium remained intact. These data provide evidence that AUDA acts directly on the vascular smooth muscle in an endothelium-independent manner to induce its vascular reactivity. In addition, AUDA-induced relaxation was completely abolished when mesenteric resistance vessels were pretreated with KCl. This finding provides additional support that AUDA acts on a vascular smooth muscle cell membrane channel. Therefore, it is possible that AUDA is perturbing the function of ligand- and/or ion-gated channel or channels present in the smooth muscle membrane.

Taken together, these data provide support for the hypothesis that the adamantyl-urea inhibitors of the sEH enzyme have unique vasodilator actions compared with other sEH inhibitors and that these actions depend on specific structural elements, the adamantyl group, and carbon chain length. AUDA does not act on potassium channels, the Na^+/K^+-ATPase, or the K_{ATP} channel, known EET effector proteins; however, the effects of AUDA could still be mediated by other membrane potential-sensitive mechanisms. In addition, AUDA was found to act directly on the vascular smooth muscle to induce vasodilation, although this mechanism requires further investigation.

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Address correspondence to: Dr. Jeffrey J. Olearczyk, The Medical College of Georgia, Vascular Biology Center, 1120 15th Street, Augusta, GA 30912. E-mail: jolearczyk@mail.mcg.edu