Soluble Epoxide Hydrolase Inhibitors Reduce the Development of Atherosclerosis in Apolipoprotein E-Knockout Mouse Model

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Abstract: To determine whether sEH inhibitors influence atherosclerotic lesion formation, we used an established murine model of accelerated atherogenesis, ApoE knockout (-/-) mice. The sEH inhibitor, 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea (AEPU) was delivered in drinking water. All animals were fed an atherogenic diet while simultaneously infused with angiotensin II by osmotic minipump to induce atherosclerosis. In AEPU-treated animals, there was a 53% reduction in atherosclerotic lesions in the descending aortae as compared to control aortae. AEPU and its major metabolites were detected in the plasma of animals which received it. As expected from the inhibition of sEH, a significant increase in linoleic and arachidonic acid epoxides, as well as an increase in individual 11,12-EET/DHET and 14,15-EET/DHET ratios, were observed. The reduction in atherosclerotic lesion area was inversely correlated with 11,12- and 14,15- EET/DHET ratios, suggesting that the reduction corresponds to the inhibition of sEH. Our data suggest that orally-available sEH inhibitors may be useful in the treatment of patients with atherosclerotic cardiovascular disease.

Key Words: soluble epoxide hydrolase, 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea, atherosclerosis, Apo E knockout mice, epoxyeicosatrienoic acid.

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Atherosclerosis is the underlying cause of most cases of heart disease and stroke and as such is the major fatal disease in the western world.[1] In addition, many cases of chronic kidney disease are a result of the atherosclerotic process occurring in both small and large blood vessels, such that cardiovascular disease resulting from this process is the most common cause of death of patients on renal replacement therapies.[2,3] Although hypertension and hyperlipidemia have been known for decades to be causative factors in the development of arterial plaque,[4] data demonstrating the occurrence of severe atherosclerosis in a subset of patients without these risk factors suggest that inflammatory mediators are of prime importance in the pathogenesis of these lesions.[5] Thus, novel antihypertensive therapies, which target blood pressure as well as inflammation, are being sought.[6]

In this regard, epoxyeicosatrienoic acids (EETs) possess both antihypertensive[7] and anti-inflammatory[8] properties. Inhibitors of the major enzyme responsible for their degradation, the soluble epoxide hydrolase (sEH), can mimic these effects by increasing endogenous levels of EETs and other lipid epoxides.[8] Consistent with this finding, we and others have shown that a variety of sEH inhibitors can reduce blood pressure in hypertensive rats[7,9,10] and decrease inflammation in a murine sepsis model,[8] and that sEH inhibitors possess antinociceptive[11] properties in inflammatory pain models. Given that sEH is widely expressed in the kidney[12] and the heart and its inhibition is anti-inflammatory in a range of disease models, we tested the hypothesis that inhibition of sEH may attenuate atherosclerotic lesion formation. Because atherosclerosis is now considered to be an inflammatory disease[13] and is frequently associated with hypertension, we investigated the use of sEH inhibitors as potential pharmaceuticals for the treatment of this disease. In theory, such agents would have the capacity to modify both hypertensive and inflammatory arms of the atherosclerotic process, thus mounting a two-pronged attack on this disease.

In the pilot study described here, we asked whether administration of 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy) pentyl)urea (AEPU), a moderately water soluble, orally-available, highly potent and selective sEH inhibitor, leads to a decrease in aortic plaque formation in an atherosclerosis prone ApoE (-/-) murine model. We now show that the plasma signature expected by sEH inhibition by AEPU is inversely correlated with aortic plaque area. Our data suggest that

orally-available sEH inhibitors may be useful in the treatment of patients with atherosclerotic cardiovascular disease, particularly those with hypertension who have incipient or accelerated atherosclerosis.

MATERIALS AND METHODS

Animals and Induction of Atherosclerosis

All in vivo experiments were done following protocols approved by the Animal Use and Care Committee of University of California Davis. Homozygous ApoE-deficient (C57BL/6 background) male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Atherosclerosis was induced in 5 month old mice by infusion of angiotensin II and an atherogenic diet (Research Diets, Inc) as previously described.[14,15] Angiotensin II was delivered via subcutaneously implanted osmotic minipumps (Alzet, model 1002) for 4 weeks. Osmotic minipumps were inserted into a subcutaneous pocket under light anesthesia with a mixture of 80 mg/kg ketamine and 12 mg/kg xylazine (Sigma-Aldrich, St. Louis). The minipumps contained angiotensin II in sterile ddH_2O at a concentration calculated to deliver 1000 ng/min per kilogram. This dose of angiotensin II was selected on the basis of previous studies,[9] which demonstrated that this dose of angiotensin II increases mean arterial pressure (MAP) by approximately 30 mmHg which is relevant to clinical hypertension. Mice were fed an atherogenic diet for 4 weeks concurrent with angiotensin II infusion that consists of purified components designed to match the original Paigen's Atherogenic Rodent Diet The components list can be obtained from the manufacturer (Research Diets, Inc).

Drug Delivery

The highly potent soluble epoxide hydrolase inhibitor AEPU was chosen for this study[16] to overcome the limitations of previous high melting (mp 142°C) and lipophilic inhibitors of sEH such as the earlier generation sEH inhibitor AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid; Fig. 1A). In addition, AUDA is thought to not only be a potent transition state inhibitor of the sEH but also to be a weak mimic of 14,15-EET.[17] In contrast, AEPU has a low melting temperature (79°C) and is more water soluble such that can it be delivered orally through the drinking water without the need for hydroxypropyl-β-cyclodextrin to solubilize the compound. AEPU also has little structural resemblance to the EETs (Fig. 1B). Separate studies have shown AEPU to penetrate into cells much faster than AUDA and to have high oral availability. AEPU is also more potent on the murine sEH than AUDA (Table 1).

This improvement in physical properties was vital for this study, since the β-cyclodextrin used to solubilize AUDA sequesters cholesterol[18] and is itself capable of inhibiting atherosclerotic plaque formation. The sEH inhibitor AEPU was delivered at a dose of 90 μg/mL in drinking water for 8 weeks, starting 4 weeks prior to the initiation of the 4 week treatment with the atherogenic diet and angiotensin II infusion. This dose was selected based on water solubility data and previous pharmacokinetic studies in mice. Mice were observed to drink approximately 3 ml of water per day consistent with other published studies.[19,20] Parallel studies on other biological end points that have been carried out in our laboratory, indicate this procedure gives a dose of approximately 10 mg AEPU per kg per day.

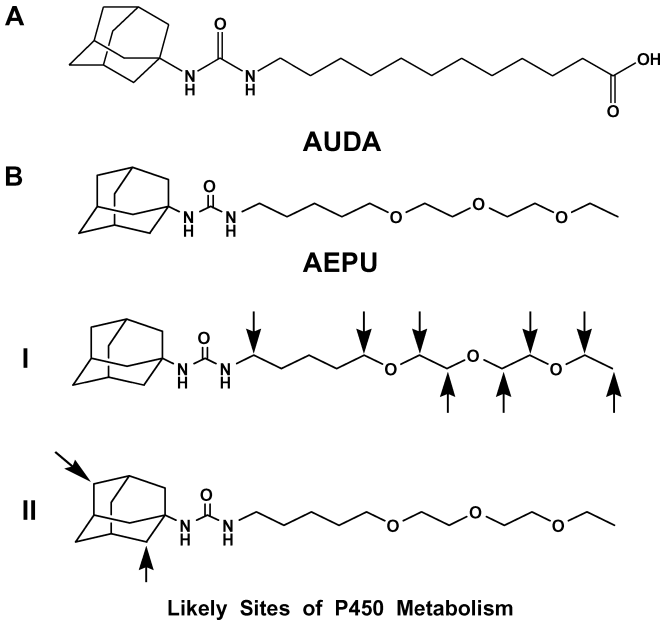

FIGURE 1. Structures of sEH inhibitors and metabolism by P450 enzymes. A, Structure of the first-generation sEH inhibitor AUDA. B, Structure of the sEH inhibitor AEPU and its possible metabolites, which can occur both on hydrocarbon chain (I) or adamantane secondary carbons (II) by P450 activity. In mice, the hydroxylation on the tertiary or β-carbon to the urea in AEPU was not detected based on a synthetic standard. Likely sites of P450 hydroxylation on the polyethylene glycol chain of AEPU are shown with the ↓ symbol. Hydroxylation alpha to a heteroatom of course leads to O-dealkylation. Although some of these metabolites were observed at low levels when AEPU was incubated with hepatic microsomes and NADPH, however the levels were very low and none of these metabolites were observed at the LOQ in vivo. Only the hydroxy adamantane derivatives were observed in addition to the parent AEPU. It is likely that these metabolites are relatively stable due to steric hindrance reducing conjugation and excretion.

Pharmacokinetic Profile of AEPU

For single oral dose pharmacokinetic studies, male CFW mice (Charles River Canada) between 25 to 35 g were used.

TABLE 1. Properties of the SEH Inhibitors AEPU and AUDA

sEH Inhibitor	Molecular Weight	IC_{50}*(nM)	IC_{50}†(nM)	Water Solubility (μg/mL)	Melting Point (°C)
AEPU§	396.5	14	3	120	79
AUDA	392.6	3	10	35	142

*Measured with fluorescent assay and purified human sEH recombinant enzyme.
†Measured with fluorescent assay and purified murine sEH recombinant enzyme.
§No symptoms of toxic effect were observed when AEPU was given orally to mice at 400 mg/kg body weight.

The animals were fed a standard rodent chow and allowed access to food and water ad libitum prior to experimentation. AEPU (4.5 mg) was dissolved in 3 mL of oleic ester rich triglyceride to a final concentration of 1.5 mg/mL. Each mouse was treated with 10 mg/kg of AEPU in 100 uL of triglyceride and 10 μL blood samples were collected from the tail vein using a heparinized pipet tip at 0, 0.5, 1, 2, 3, 4, 6, 24 hr after drug administration. After sample collection, each blood sample was diluted with 50 μL distilled water, extracted with ethyl acetate twice with 10 μL of surrogate solution (250 ng/mL of 1-(5-butoxypentyl)-3-adamantylurea in methanol) and reconstituted with 50 μL of internal standard solution (100 ng/mL of 1-adamantanyl-3-decylurea in methanol) following a drying step under nitrogen. The extracted samples were analyzed by liquid chromatography coupled with mass spectrometry (LC/MS-MS). Specifically, chromatographic separation was performed on an ACQUITY ultra performance liquid chromatography (UPLC) instrument equipped with a 2.1 X 50 mm ACQUITY UPLC BEH C18 1.7 μm column (Waters, Milford, MA) held at 25°C. Separation was done with two solvent systems A and B; A containing 0.1% formic acid and 10% acetonitrile and B containing 0.1% formic acid in acetonitrile. The gradient was begun at 30% solvent B and was increased to 100% solvent B in 5 minutes. This was maintained for 3 minutes, then returned to 30% solvent B in 2 minutes. The flow rate was 0.3 ml/min. The injection volume was 5 μL and the samples were kept at 4°C in the auto sampler.

Analytes were detected by positive mode electrospray ionization tandem quadrupole mass spectrometry in multiple reaction-monitoring mode (MRM) on a Quattro Premier Mass Spectrometer (Waters, Milford, MA). Nitrogen gas flow rates were fixed with the cone gas flow of 50 L/hr and the desolvation gas flow of 650 L/hr. Electrospray ionization was performed with a capillary voltage set at 1.0 kV and an extractor fixed at 5.0 V. The source temperature was set at 125°C and the desolvation temperature at 300°C, respectively. Collision gas argon was set at 3.0×10^{-3} Torr. Cone voltage and collision voltage were optimized by acquisition of precursor and production ions, respectively. The precursor and dominant daughter ions were used to set up the transition monitored in the MRM mode.

sEH Activity Assay: Obtaining IC_{50} Values

IC_{50} values were determined for the recombinant purified murine and human sEH using an α- cyanocarbonate epoxide (CMNPC) as the fluorescent substrate ($[S]_{final}$ = 5 μM) as described.[21] Assays were performed in triplicate. IC_{50} is a concentration of inhibitor, which reduces enzyme activity by 50%, and was determined by regression of at least five data points with a minimum of two points in the linear region of the curve on either side of the IC_{50}.

Plasma Cholesterol Levels

Cholesterol from mice plasma was extracted by isopropyl alcohol:acetonitrile water treatment followed by a derivatization reaction and was measured by gas chromatography mass spectrometry as previously described.[22]

Experiments on AEPU Metabolism: S9 Fraction Incubation

A rat liver S9 fraction diluted in phosphate buffer (100 uM, pH 7.4) was preincubated for 5 min in open glass tubes immersed in a shaking bath at 37°C. AEPU (10 μM) was then incubated with 2.5 mg/mL of this protein mixture. The reaction was initiated by adding 25 uL of NADPH generating system (NADP+ (2 mM), glucose 6-phosphate (57 mM), glucose 6-phosphate dehydrogenase (3.5 units), and magnesium chloride (50 mM) dissolved in 100 mM sodium phosphate buffer (pH 7.4) and terminated after 0, 30, and 60 minutes by adding 1 mL of cold ethanol. Half of this solution was used for the fluorescent assay[21] and the other half was used for inhibitor quantification by LC/MS-MS.

Evaluation of Atherosclerotic Lesions

At the end of the treatment, mice were anesthetized with a mixture of 80 mg/kg ketamine and 12 mg/kg xylazine and subjected to a lethal heart puncture blood draw. Plasma was separated from whole blood and frozen for subsequent oxylipin analysis. The heart and aorta were perfused with approximately 5 mL of phosphate-buffered saline (PBS) followed by perfusion with 5% formalin in PBS. The aorta was then removed, placed in 5% formalin for 5 min and transferred to PBS at 4°C to be analyzed within 24 hours. Adventitial fat was removed from the aortae, which were then opened longitudinally for en face analysis. Aortas were then stained with Sudan IV, and digital images of stained aortas were captured with a Kodak DC290 Zoom digital camera and were analyzed using Image J software by an individual blinded to AEPU treatment. The dependent measure used in subsequent analyses was obtained by calculating the ratio of the area of Sudan IV stained lesion divided by total area of the lumen of the descending aortic arch. The latter was defined as the portion of the aorta beginning at the point on the bottom of the arch adjacent to the left carotid artery branch and extending an equal distance distally. The aortic arch was photographed prior to perfusion with PBS.

AEPU Measurement in Drinking Water and Blood

Compounds 975, 1010, and AEPU (sEHI 950) are extracted quantitatively by the ethyl acetate procedure as are many more polar metabolites. However, conjugates would be expected to be poorly extracted. The measurement was done using MRM (multi reaction monitoring) in positive mode on a hybrid triple quadrupole/linear ion trap mass spectrometer, QTRAP 4000 LC/MS-MS mass spectrometry system (Applied Biosystems, CA). A Gemini C18 30 × 4.6 mm with 3 μm particle size column was used for this analysis (Phenomenex, Torrance, CA). Solvent A containing 0.1% formic acid and solvent B containing 0.1% formic acid in acetonitrile were used. The samples were run in isocratic mode with 90:10 organic:aqueous solvent mixture. The column oven temperature was 40°C and the flow rate was 0.5 mL/min. The run time was 3 min using 1-adamantanyl-3-decylurea as internal standard.

The values for nitrogen gas and desolvation gas flow rates were 50 L/hr and 650 L/hr; respectively. Electrospray

ionization, the source temperature and the desolvation temperature were the same as in the pharmokinetic (PK) study. Collision gas argon was set at 3.0×10^{-3} Torr. Cone voltage and collision voltage were optimized by acquisition of precursor and production ions, respectively. The precursor and dominant daughter ions were used to set up the transition monitored in the MRM mode.

Oxylipin Quantification and Statistics

The analysis of oxylipins was performed as described previously.[8] Ten microliters of anti-oxidant cocktail (0.2 mg/mL BHT, EDTA and 2.0 mg/mL triphenylphosphine, indomethacin in 2:1:1 MeOH:EtOH:H_20) was added to 250 μL of plasma. Surrogate mixtures were spiked into the samples at a final concentration of 2000 nM and passed through a solid phase extraction cartridge (Oasis HLB cartridges, Waters, Milford, MA). After a washing step with 2 ml of 2.5 mM phosphoric acid:20 % methanol, oxylipins were eluted by 2 mL of ethyl acetate. The samples were dried under nitrogen and resuspended in 50 μL of internal standard solution. The injection volume was 10 μL. The samples were then run in MRM (multireaction monitoring) mode in negative mode on a hybrid triple quadrupole/linear ion trap mass spectrometer, QTRAP 4000 LC/MS-MS mass spectrometry system (Applied Biosystems, CA) equipped with a 2.1 × 150 mm Pursuit XRs-C18 5 mm column (Varian Inc, Palo Alto, CA). The same values for nitrogen gas flow rates, desolvation gas, cone gas flow, capillary, cone, and collision gas voltages were followed as described.[8] All data are reported as mean ± SEM, normalized to same day controls. Statistical significance was determined by nonparametric univariate analysis of variance using SPSS software and Pearson correlation to test the correlation between the decrease in plaque area and the plasma epoxide to diol ratios with a significance threshold of $P < 0.05$.

RESULTS

An sEH Inhibitor Attenuates Aortic Plaque Formation

All animals in this study were fed an atherogenic diet[23] while receiving Ang II infusion through implanted osmotic minipumps, and at the end of the study, the aortic arch from each animal was examined after sacrifice. Striking differences were observed in visualized (unstained) plaque between AEPU-treated and control animals (Fig. 2A). Blinded analysis

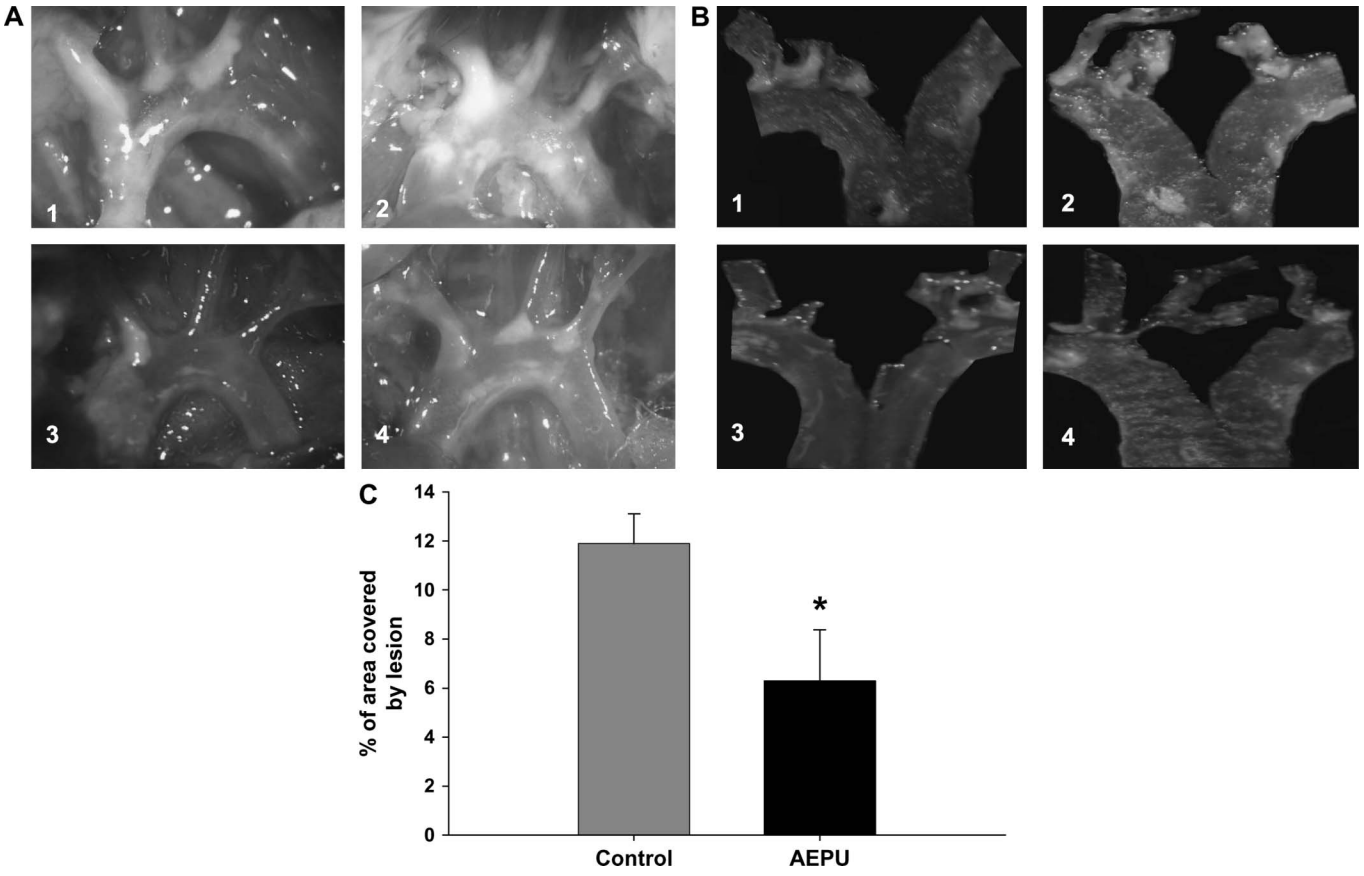

FIGURE 2. AEPU decreases plaque formation in ApoE knockout mice. A, Representative descending aorta in AEPU-treated and control mice visualized in situ prior to staining (1 and 2, control mice; 3 and 4, AEPU-treated mice) B, Representative descending aortae in AEPU-fed and control mice shown en face after staining with Sudan IV (1 and 2, control mice; 3 and 4, AEPU-treated mice) shows decreased lesion area. C, Quantitative measurement of data in B from all mice. Control group, n = 6; AEPU-treated group, n = 4. The data are shown as means ± SEM. *$P < 0.05$.

of Sudan IV stained sections of the descending aortic arches from all animals (Fig. 2B), quantitated using Image J software (Fig. 2C), demonstrated significantly decreased lesion area in AEPU-treated as compared to control animals.

Oral Delivery of AEPU Inhibits sEH In Vivo: Changes in Plasma Oxylipin Profile

AEPU is a very potent in vitro transition state inhibitor of the sEH, which is effective by oral delivery; however, the inhibition is rapidly reversible. Thus, inhibition cannot be directly measured in vivo. In order to confirm that administration of AEPU via the drinking water inhibited sEH, 55 oxylipins were assayed including the major sEH substrates (EpOMEs and EETs) and products (DiHOMEs and DHETs), of which 41 lipids were above our limit of quantitation. Significant increases in the plasma linoleic acid and arachidonic acid epoxides (EpOMEs and EETs; respectively) were observed (Fig. 3A), and an increase in the sum of EpOME to DiHOME ratio ($P = 0.02$) as well as the total epoxide to diol ratios ($P = 0.01$) in the treated group (Fig. 3B) were seen, implying robust inhibition of sEH in these animals. In addition, a statistically significant increase for 14,15-EET was detected in the plasma of the AEPU-treated group. This increase in the sum of anti-inflammatory 11,12- and 14,15-EET to DHET ratio inversely correlated with the amount of atherosclerosis (R = -0.79, Fig. 4) as expected. Furthermore, a moderate correlation was observed between the ratio of EpOME to DiHOME and the decrease in the atherosclerotic area (R = -0.39, Fig. 4).

Total epoxide to diol ratio was calculated using both the sum of arachidonic acid and the linoleic epoxides and diols (EETs/DiHOMEs and EpOMEs/DiHOMEs; respectively) whose epoxides were shown in Figure 3A. A strong correlation (R = -0.78) was found between the total epoxide/diol ratio and the decrease in the plaque area (Fig. 4). This implies that these oxylipins are valid biomarkers of atherosclerotic lesion formation, although it is unclear which regioisomer(s) contribute to the anti-inflammatory and anti-atherogenic effect due to sEH inhibition.

Plasma Concentrations of AEPU

Plasma was collected at the time of sacrifice and AEPU was measured by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). However, its concentration in the plasma was significantly lower than its IC_{50} (Table 2). The very low blood levels observed suggest that AEPU either distributes to the target tissues very rapidly to have therapeutic effects and/or the biological activity can be attributed to the hydroxylation and oxidation metabolites of AEPU. This is also supported by the pharmacokinetic profile which is shown in Figure 5. Upon detecting the low plasma levels of AEPU, we focused on the oxidation metabolites of AEPU. To determine whether AEPU metabolites possess sEH inhibitory activity, an in vitro experiment was performed to assess the association of inhibitory activity and quantity of AEPU and its major metabolites (Fig. 6). According to these data, while the amount of AEPU decreases with the time of incubation in the rat S9 fraction, the potency on the sEH decreased and the IC_{50} value increased less than anticipated. A control S9 fraction was also run to test the sEH enzyme activity. At 60 min, while there was less than 10% of AEPU remaining in the mixture, the IC_{50} value was still below 100 nM suggesting that at least part of inhibitory activity of AEPU can be attributed to its metabolites.

Metabolism of AEPU

While the total metabolite profile of AEPU in vivo is not known, a variety of likely metabolites based on known cytochrome P450 hydroxylation can be predicted. The predicted structures are shown in Figure 1BI and II. With the LC/MS-MS technique, the metabolites on the adamantane ring can be distinguished from metabolites arising from hydroxylation on the polyethylene glycol moiety of AEPU by the chromatogram and the fragmentation pattern (Fig. 7) in the rodent and human liver microsomal incubations.

One would expect that the metabolites on the polyethylene glycol chain, as sterically unhindered primary and secondary alcohols, would be rapidly metabolized by further oxidation and/or conjugation, and excreted. The major metabolites detected in the plasma were hydroxylated products

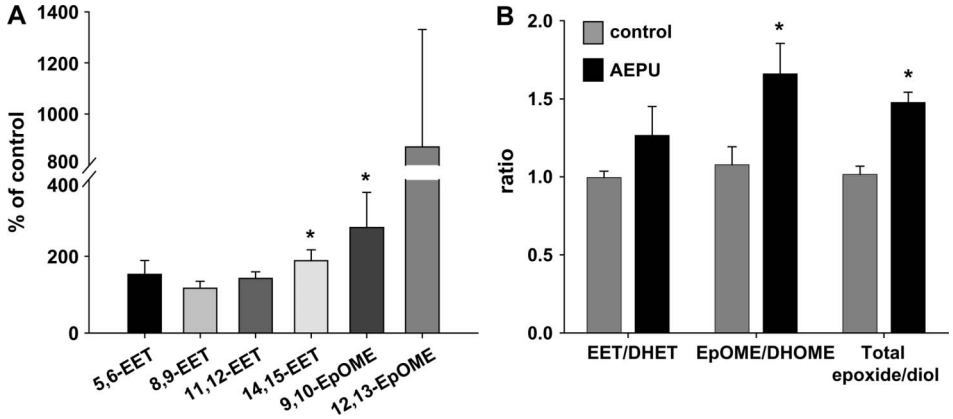

FIGURE 3. Mice treated with AEPU show increased plasma levels of P450 epoxygenase metabolites. A, Key epoxygenase metabolites. 14,15-EET and 9,10- EpOME were significantly higher in the AEPU-treated group, whereas 11,12-EET and 12,13-EpOME lost the significance due to the variation in the group. B, Ratios of lipid epoxides to their corresponding product diols showing the statistically significant differences of EpOME/DiHOME and total epoxide/diol ratios between the control and AEPU-treated groups. Levels of metabolites are expressed as percent of corresponding control animals; nonparametric univariate statistics was performed to compare experimental and control animals. The data are shown as means ± SEM. *$P < 0.05$.

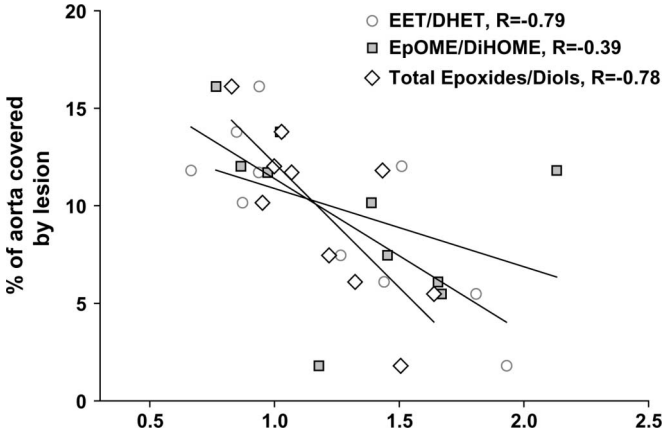

FIGURE 4. P450 epoxygenase metabolites correlate inversely with plaque area. Plasma levels of arachidonic acid (20:4) and linoleic acid (18:2) metabolites are correlated with aortic plaque area for each mouse. The correlation was calculated using Pearson correlation test. The epoxides arising from arachidonic acid (20:4), 11,12- and 14,15-EET/DHET (circles) showing a strong correlation, epoxides arising from linoleic acid (18:2), 9,10- and 12,13- EpOME/DiHOME (squares) showing a moderate correlation and the total epoxide/diol ratio (diamonds) showing a strong correlation with the aortic plaque area are shown.

on the adamantane moiety as distinguished by MRM. Because we have an authentic standard, we know that the 8.54 retention time peak is a hydroxylated product on the tertiary β-carbon. This compound is not further oxidized by chromate and it is only 28-fold less active than AEPU as an inhibitor on the murine sEH.

The peaks at retention times of 8.05 and 9.67 min are adamantane hydroxylation products on secondary carbons as indicated by oxidation by chromate to the corresponding ketone. We do not have standards to determine inhibitory potency or distinguish among α and γ positions or -*trans* or -*cis* geometry relative to one of the cyclohexane rings. Thus, to quantify the metabolites, standards including ω-hydroxylation on the polyethylene glycol chain (compound 975, 1-adamantan-1-yl-3-(5-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-pentyl)-urea) and β- hydroxylation on the adamantane ring (compound 1010, 1-{5-[2-(2-ethoxy-ethoxy)-ethoxy]-pentyl}-3-(3-hydroxy-adamantan-1-yl)-urea) were prepared.

TABLE 2. Plasma Concentration and IC_{50} Values of AEPU, Major Metabolites of AEPU, and Synthetic Standards

	Plasma Concentration (nM)	IC_{50} (nM)*
AEPU	0.06 ± 0.04	2.7 ± 0.4
Detected AEPU metabolites	716 ± 418	NA
Compound 975	<LOQ	4.7 ± 0.7
Compound 1010	565†	78 ± 19

NA, not available.

The values were obtained by subtracting the concentrations of the same day controls from each sample in the AEPU-treated group. The data are given as means ± SEM.

*Measured with fluorescent assay and purified murine sEH recombinant enzyme.

†The synthetic route precluded formation of bis-adamantyl ureas. The compound was 79 % pure based on reversed phase HPLC with total ion monitoring.

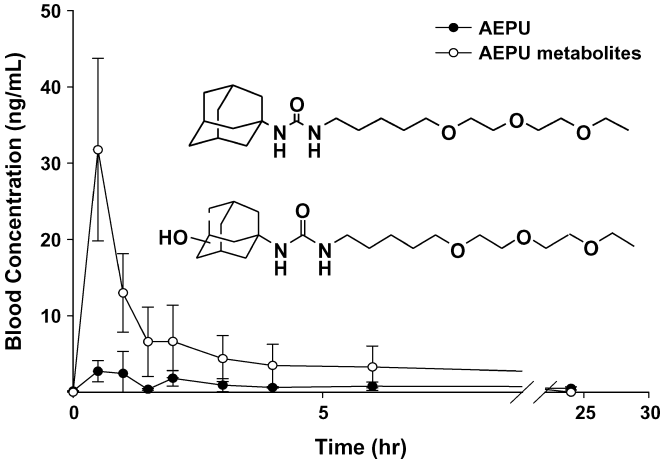

FIGURE 5. Pharmacokinetic profile of AEPU. Male CFW mice were treated with 10 mg/kg of AEPU in 100 μL of oleate rich triglyceride by oral gavage. Ten μL of blood was collected from tail vein at 0, 0.5, 1, 2, 3, 4, 6, and 24 h after the AEPU administration and placed in 50 μL of distilled water. Upon sample collection, each blood sample was extracted by liquid-liquid extraction method which was described previously in the methods section. The concentrations of AEPU and its metabolites were measured by LC-MS/MS. The LOQ and LOD for AEPU were 0.15 and 0.07 ng/mL, respectively. Only the putative α and γ hydroxylation products on the adamantane ring were detected above the LOQ.

Because we used only mass spectrometry, there are limitations in our investigation of the metabolites of AEPU. The major limitation is that, even with sophisticated software, one only can detect compounds for which the mass of the

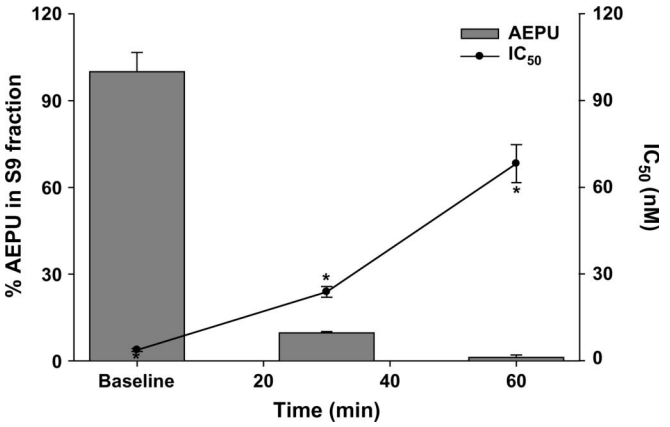

FIGURE 6. AEPU metabolism. AEPU was incubated with a rat S9 liver preparation with a NADPH-generating system (there was no metabolism in the absence of NADPH). At time points of 0, 20, 40, and 60 minutes, aliquots of the incubation were monitored for AEPU and major metabolites by LC-MS and an in vitro enzyme inhibition assay was run to determine the IC_{50} value of the material in units based on AEPU using the affinity purified recombinant murine enzyme. Theoretical IC_{50}s are also indicated with asterisks. These values were calculated by multiplying the initial rat IC_{50} for rat enzyme (IC_{50} rat: 6 nM) with the fold decrease in AEPU amount.

parent or a key fragment are known based on the anticipated to metabolism of AEPU. Also one can only quantitate compounds that are available as authentic standards with high accuracy. The parent compound can be detected at low levels in the plasma by positive ion LC-MS. However, one can estimate metabolite concentrations of similar concentration to a standard. Based on the knowledge of metabolism chemistry, a number of possible metabolites can be synthesized and their biological activity studied. However, many of the metabolites have very similar retention times and identical masses. One can reliably distinguish hydroxylation products on the adamantane ring from hydroxylation or dealkylation of the polyethylene glycol moiety of AEPU by the fragmentation pattern.

Based on mass spectrometry, we can exclude many possible metabolites of AEPU as undetectable in plasma by LC-MS. This does not mean these metabolites are not formed. Since we have the ϖ-hydroxylation product 975 as a synthetic standard, we can exclude it as a major metabolite in plasma down to the limit of quantification (LOQ).

Metabolite peaks of the appropriate retention time and fragmentation pattern are detected; however, other likely O-dealkylation products from the polyethylene glycol chain were not detected. However, this and other hydroxylation and O-dealkylation products may form and simply be rapidly further oxidized, conjugated and excreted. The major metabolites of AEPU detected in the plasma are hydroxylation products on the adamantane ring. These products all yield similar retention times and have identical masses. There are three metabolites extracted from plasma which give fragmentation patterns consistent with hydroxylation on the adamantane ring at retention times of 8.05, 8.54 and 9.67 minutes. When these metabolites were exposed to chromate, the products at 8.05 and 9.67 were oxidized to the corresponding ketone, but the 8.54 peak was unchanged. This indicates that the former compounds are secondary alcohols and the latter is a tertiary alcohol and thus is hydroxylated in the β- position on the adamantane. Compound 1010 was prepared as a synthetic standard. The standard co-chromatographs with the 8.54 metabolite peak and has similar fragmentation. With this synthetic standard the concentration of the 8.54 peak can be determined exactly.

It is difficult to prepare authentic standards for hydroxylation in the α and γ positions of the adamantane ring. The metabolites can have the hydroxyl group *cis* or *trans* across the cyclohexane and are potentially chiral. Thus, there are four possible adamantane metabolites hydroxylated on a secondary carbon but only two peaks detected. Estimates of amounts of the metabolites at retention times of 8.05 and 9.67 are based on the response factor of standard 1010. They clearly are isomers of AEPU hydroxylated in the α or γ position on the adamantane.

Mouse Pharmacokinetic Profile of AEPU

To determine if these potential metabolites are produced in vivo, AEPU was delivered to another group of animals via oral gavage and the parent compound and its metabolites were measured over 24 hours. The major metabolites detected arose from hydroxylation on the adamantane moiety instead of the hydroxy groups on the polyether chain or chain shorted products. The adamantane metabolites reached a higher plasma concentration than AEPU and remained higher throughout the experiment (Fig. 5). It is known that the adamantane metabolites have the hydroxy group on both secondary and tertiary carbons. Further, the potencies of those metabolites were determined in vitro by the sEH activity assay.

Although the ω-hydroxylation derivative on the polyethylene glycol chain (compound 975) has similar potency to AEPU (Table 2), it is far less abundant. Thus, it probably contributes little to overall biological activity. However, the β-hydroxy adamantane derivative (compound 1010) is only 28-fold less potent on the enzyme than AEPU, and it is roughly 5,000 times more abundant in the plasma. Thus, the β-hydroxy adamantane metabolite is likely to contribute a major component of the biological activity. The contribution of other isomers is not known.

Plasma concentrations of the two likely classes of AEPU metabolites in the ApoE -/- mice were assayed to determine if metabolites of AEPU are responsible for the observed sEH inhibition (Table 2). Similar to the pharmacokinetic study, the major metabolite detected arose from hydroxylation on the adamantane moiety, but no metabolites which were hydroxylated on the polyether chain were detected in these animals as well. The concentration of the hydroxy adamantane metabolites (β, α and/or γ) in the plasma were approximately 10,000-fold higher than that of AEPU and approximately 10-fold higher than the IC_{50} of the β-hydroxy adamantane derivative (compound 1010), suggesting decent inhibitory activity.

The IC_{50}s of compounds AEPU, 975, and 1010 were determined using the fluorimetric substrate α-cyanocarbonate epoxide (CMNPC) and the murine recombinant enzyme. The IC_{50}s of the compounds are shown in Table 2. For the murine enzyme, AEPU and compound 975 had very close IC_{50}s. Although compound 1010 is 28 times less active than AEPU and compound 975, the inhibitory activity was still below 80 nM. It is likely that compound 1010 is found at such high concentrations in the blood, because its overall polarity reduces rapid oxidation on the polyethylene glycol moiety and steric hindrance reduces conjugation on the tertiary hydroxy group on the adamantane. Its high plasma concentrations relative to AEPU suggests that it is a major contributor to biological activity. Based on our structure activity relationship experiments, we predict that the possible hydroxylated metabolites in the α position will have low inhibitory activity.[24] However, the putative metabolites in the gamma-position may be active.

Plasma Cholesterol Levels

There was no difference in the mean plasma cholesterol levels between the groups. The plasma concentrations were found to be 96.2 ± 23.1 mg/dL (2.5 ± 0.6 mM) and 96.2 ± 34.6 mg/dL (2.5 ± 0.9 mM) in control and AEPU-treated animal groups, respectively.

DISCUSSION

Arachidonic acid metabolism results in the generation of three broad classes of oxidative metabolites corresponding to the specific enzyme(s) involved. These metabolites, known as

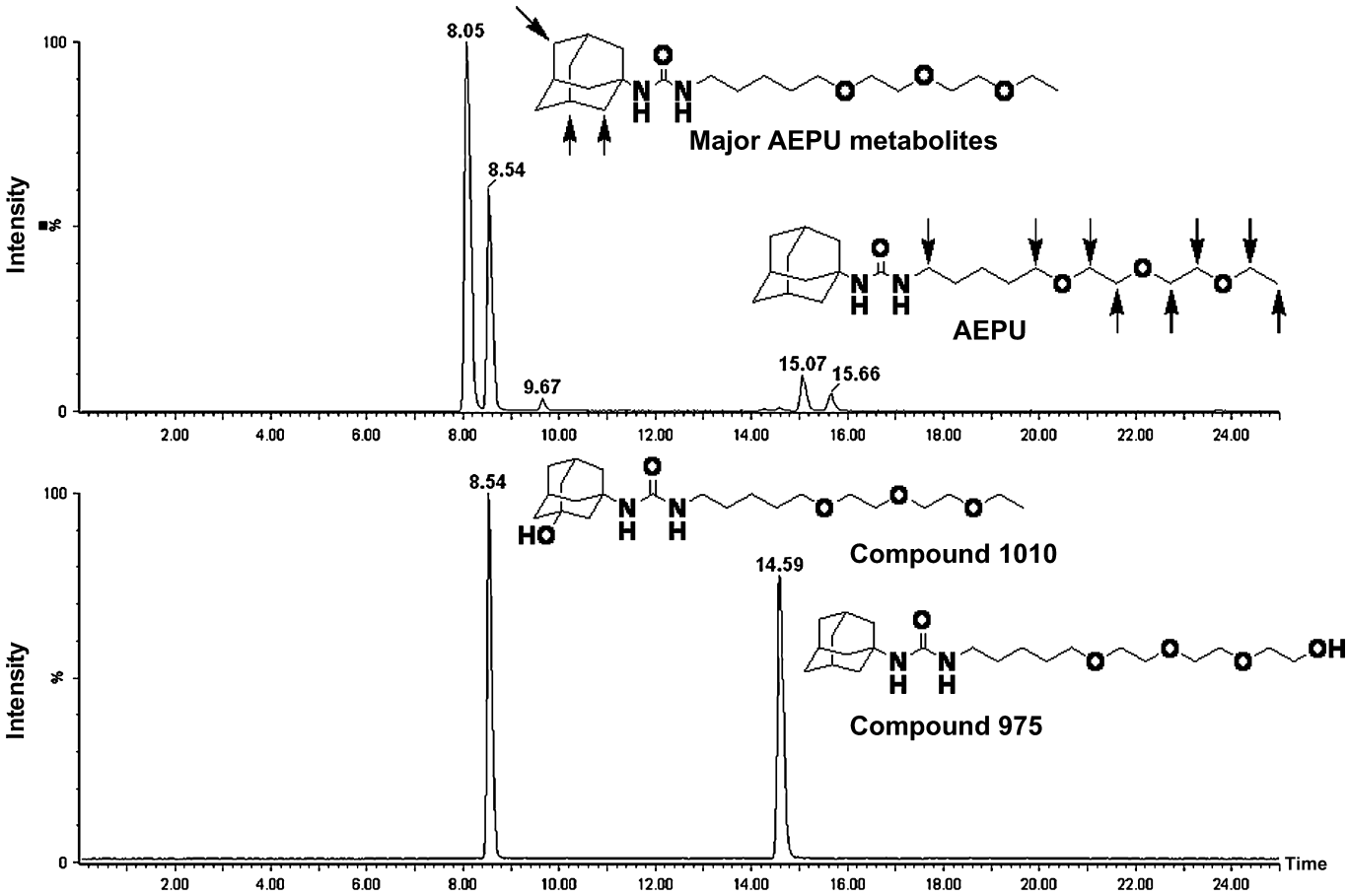

FIGURE 7. Schematic of MRM windows from LC-MS/MS. The chemical structures of AEPU and synthesized calibration standards with the corresponding retention times are indicated. The calibration standard, compound 975 (1-adamantan-1-yl-3-(5-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-pentyl)-urea) is the ϖ-hydroxylation standard (LOD = 0.13, LOQ = 0.34 ng/mL.). It is a possible metabolite arising from ϖ-hydroxylation of AEPU but could not be detected at the LOD. Shorter chain hydroxy compounds have low potency as inhibitors of the sEH. The calibration standard, Compound 1010 (1-{5-[2-(2-Ethoxy-ethoxy)ethoxy]-pentyl}-3-(3-hydroxy-adamantan-1-yl)-urea) has hydroxylation on the tertiary carbon or β to the urea carbon (LOD = 0.09, LOQ = 0.25 ng/mL). It is a possible metabolite arising from hydroxylation of AEPU on the tertiary carbon. AEPU in vivo (actual) metabolites are hydroxylated on the adamantane at positions α, γ, and β relative to carbon 1.

the eicosanoids, include (among others) prostaglandins, thromboxanes, lipoxins, leukotrienes, and epoxyeicosatrienoic acids, many of which have pleiotropic vasoactive, inflammatory, and nociceptive effects. Of the three arachidonate pathways, the metabolites of the cycloxygenase and lipoxygenase pathways have been studied most intensively. The epoxyeicosanoic acids (EETs), emanating from the cytochrome P450 branch of the arachidonate cascade, have been demonstrated to reduce inflammation and cause vasorelaxation. In several systems, the sEH has been demonstrated to be the major route of metabolism of EETs to more water soluble and in some cases less biologically active diols known as dihydroxyeicosatrienoic acids (DHETs).[9,25] As a result of extensive research on sEH inhibition in a variety of cell lines as well as in vivo manipulation of this pathway, sEH inhibitors have been proposed as a novel class of pharmaceuticals.

While it has been demonstrated that sEH inhibitors have a surprising variety of salutary effects on blood pressure and inflammation, it has not, prior to this study, been demonstrated whether they also are beneficial in the pathogenesis of atherosclerosis. We have now shown, in a pilot study using a murine atherosclerosis model, that 1) plaque area in the descending aorta is significantly decreased in animals fed an sEH inhibitor, and 2) these changes occur in parallel with metabolic profiles expected to arise from sEH enzyme inhibition.

It was not the purpose of this pilot study to determine the precise mechanism whereby sEH inhibition results in decreased aortic plaque area. However, the findings reported here suggest potential mechanisms. For example, the increased epoxide/diol ratio in AEPU-treated animals is consistent with previous reports showing that 11,12- and 14,15-EET have the strongest anti-inflammatory effects among the EET isoforms.[26] Moreover, a strong correlation was found for the sum of 11,12 and 14,15-EETs/ DHETs ratio, as well as a moderate correlation for the EpOME/ DiHOME ratio. Therefore, it is possible that sEH inhibitors reduce plaque formation via stabilization of the anti-inflammatory 11,12- and 14,15-EETs and other bioactive epoxy lipids by decreasing their metabolism.

It is unclear whether the linoleic acid epoxides were likely to contribute to the antiatherogenic effect due to the observed moderate correlation. It has been shown that the linoleic acid diols induce cellular toxicity and vascular permeability and these precursor EpOMEs are metabolized by sEH.[27] On one hand, pulmonary vasodilatory effects of EpOMEs have been shown in rats.[28] On the other hand, the toxicity of EpOMEs, as their diol products, have been associated with many pathological conditions including respiratory distress syndrome, severe burns, disruption of mitochondria, and cardiac failure.[29–32]

By contrast, a recent study has shown that the EpOMEs weakly activate the respiratory burst and that they are weak neutrophile chemoattractants.[33] The biological functions of EpOMEs are poorly understood and still unclear. Therefore, it is very difficult to make a statement on the contribution of linoleic acid epoxide/diol ratios on sEH inhibition resulting in antiatherogenic effect. However, we believe that total/epoxide ratio is a good biomarker of sEH inhibition. We do not know which regioisomer(s) of arachidonic and linoleic acid epoxides contribute to the observed biological effect. Our data also demonstrate that a prolonged but small increase in the plasma epoxide to diol ratio can result in profound biological effects. These anti-inflammatory eicosanoids in turn shift the profile of eicosanoids in the vasculature towards a profile more consistent with resolution of inflammation than its propagation.

Although we suspect that stabilization of 11,12- and 14,15-EETs are contributory to the observed decreased plaque formation, these compounds also display a variety of effects on vasomotor tone, inflammation, proliferation, and adhesion molecule expression. Thus, it is possible that there is an indirect influence of sEH inhibition on plaque formation through one of the processes enumerated above, a possibility which is being actively investigated in our laboratories. Whether a decrease in blood pressure from AEPU is contributory to the plaque attenuating effects seen in these animals was not addressed in this study; moreover, it is difficult to separate the proinflammatory effects from the hypertensive effects of angiotensin II. While blood pressure was not measured in these animals, it is likely that attenuation of blood pressure would need to be more chronic than the duration of this study to result in the observed effect on aortic plaque.

The plasma cholesterol levels were not different between the animal groups suggesting that AEPU treatment has no effect on cholesterol levels. The phosphatase domain of sEH[34] has been shown to metabolize the isoprenoid precursors in the cholesterol biosynthesis pathway suggesting a possible regulatory role for sEH.[35] Separate experiments with sEH gene knockout mice showed no difference in plasma cholesterol levels, whereas the chronic treatment with sEH inhibitors of wild-type animals showed an increase in plasma cholesterol levels (data not shown). Along with these findings, our data strongly suggest that AEPU does not reduce cholesterol levels and AEPU's observed antiatherogenic affect is not a result of attenuated plasma cholesterol.

Even though the plasma levels of AEPU detected is lower than its in vitro IC_{50}, the anti-atherogenic effect observed in these animals is likely due to sEH inhibition, which was substantiated with an increase in total epoxide/diol ratio. Previous studies with other animal models showed that AEPU has clear biological effects such as reduction of cardiac hypertrophy.[36] However, the blood levels of AEPU were found to be very low in this study as well. As mentioned in our results, it is possible that AEPU differentially partitions to reach the target organ or AEPU is not only an active inhibitor on its own but is also a prodrug for a series of active metabolites.

Based on the structure activity relationship, one can expect some of the metabolites of AEPU also have potent inhibitory activity. In this study, we showed that both metabolites with hydroxylation on the adamantane moiety and polyethylene glycol chain have potent inhibitory activity. There are more metabolites such as other likely O-dealkylation products from the polyethylene glycol chain which may have potent inhibitory activity. However, structure activity studies suggest that their potency will be low. We hypothesize that these hydroxylation and O-dealkylation products may form and simply be rapidly further oxidized, conjugated and excreted. It is very difficult to prepare authentic standard for hydroxylation in α and γ positions of the adamantane and these metabolites are also chiral compounds. Thus, estimates of amounts of α and γ products are based on the response factor of the β isomer.

Based on structure activity relationships published elsewhere[24] and the biological activity of the β-hydroxy adamantyl derivative 1010, we can anticipate that the γ hydroxylation products and the corresponding ketones will have similar inhibitory activity and that the α-hydroxylation products will be of low activity. The high blood level of these products may result from steric hindrance of the adamantane ring reducing further oxidation and conjugation.

Our data support the use of sEH inhibitors as a new class of antihypertensive drugs, which can be targeted to those patients with clinical findings of hypertension, elevated levels of inflammatory markers (such as C-reactive protein), as well as those individuals with incipient atherosclerosis as diagnosed clinically or by angiography. For example, patients with metabolic syndrome or those on renal replacement therapy are at high risk of developing atherosclerosis and may benefit most from this class of drug. Indeed, hemodialysis patients often have extremely high levels of inflammatory markers and, for unknown reasons, frequently die from cardiac disease. Drugs which target inflammation are likely to markedly reduce the exorbitant cardiovascular mortality in this patient population.

In this work, we show that inhibition of sEH in a mammalian model of atherosclerosis by an orally available agent reduces the formation of aortic plaque and augments the ratio of anti-inflammatory EETs to their metabolites. Comparative pharmacokinetic studies with AEPU suggest that much higher blood levels are obtained in canine and primate models. These data suggest an effective dose in primates of well under 1 mg/kg. The relative ease of synthesis of urea derivatives such as AEPU allows one to scale up sufficiently for large animal trials with these compounds. Given earlier work from our and other laboratories demonstrating antihypertensive and anti-inflammatory effects of the sEH inhibitors, the data in this study support eventual clinical

trials of these compounds in patients who have incipient or accelerated atherosclerosis, or who are at risk for this highly prevalent and morbid disease.

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