Soluble Epoxide Hydrolase Regulates Hydrolysis of Vasoactive Epoxyeicosatrienoic Acids

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Abstract—The cytochrome P450-derived epoxyeicosatrienoic acids (EETs) have potent effects on renal vascular reactivity and tubular sodium and water transport; however, the role of these eicosanoids in the pathogenesis of hypertension is controversial. The current study examined the hydrolysis of the EETs to the corresponding dihydroxyeicosatrienoic acids (DHETs) as a mechanism for regulation of EET activity and blood pressure. EET hydrolysis was increased 5- to 54-fold in renal cortical S9 fractions from the spontaneously hypertensive rat (SHR) relative to the normotensive Wistar-Kyoto (WKY) rat. This increase was most significant for the 14,15-EET regioisomer, and there was a clear preference for hydrolysis of 14,15-EET over the 8,9- and 11,12-EETs. Increased EET hydrolysis was consistent with increased expression of soluble epoxide hydrolase (sEH) in the SHR renal microsomes and cytosol relative to the WKY samples. The urinary excretion of 14,15-DHET was 2.6-fold higher in the SHR than in the WKY rat, confirming increased EET hydrolysis in the SHR in vivo. Blood pressure was decreased 22±4 mm Hg (P<0.01) 6 hours after treatment of SHRs with the selective sEH inhibitor N,N’-dicyclohexylurea; this treatment had no effect on blood pressure in the WKY rat. These studies identify sEH as a novel therapeutic target for control of blood pressure. The identification of a potent and selective inhibitor of EET hydrolysis will be invaluable in separating the vascular effects of the EET and DHET eicosanoids. (Circ Res. 2000;87:992-998.)

Key Words: epoxyeicosatrienoic acids ■ dihydroxyeicosatrienoic acids ■ cytochrome P450 ■ soluble epoxide hydrolase ■ hypertension

Eicosanoids produced by renal cytochromes P450 (CYPs) have potent effects on vascular tone and tubular ion and water transport and have been implicated in the control of blood pressure.1 The major products of CYP-catalyzed arachidonic acid metabolism are regio- and stereoisomeric epoxyeicosatrienoic acids (EETs) and 20-hydroxyicosatetraenoic acid (20-HETE). The EETs are further metabolized by soluble (sEH) and microsomal (mEH) epoxide hydrolases to form the corresponding dihydroxyeicosatrienoic acids (DHETs). CYP enzymes can also catalyze the formation of 19-HETE and other subterminal HETEs in smaller quantities.1

In the renal microcirculation 20-HETE produces potent vasoconstriction by inhibiting large-conductance, calcium-activated potassium channels leading to vascular smooth muscle depolarization.2 In contrast, both vasodilator and vasoconstrictor properties have been attributed to the EETs, depending on the vascular bed and EET regioisomer.3-5 Vasodilatory properties of the EETs have been associated with increased open-state probability of calcium-activated potassium channels and hyperpolarization of the vascular smooth muscle.4 Indeed, a CYP2C epoxygenase catalyzes the formation of 11,12-EET that acts as an endothelial-derived hyperpolarizing factor in coronary arteries.7 Recently, the EETs were shown to have anti-inflammatory effects in endothelial cells, suggesting that these eicosanoids also play an important role in vascular inflammation.8 Hydrolysis of the EETs to the corresponding DHETs generally is regarded as one mechanism whereby the biological effects of the EETs are attenuated or eliminated.9 However, the DHETs themselves have inherent effects on vascular tone,5,10 and their role in the regulation of blood pressure cannot be dismissed.

Alterations in renal vascular resistance are evident in the spontaneously hypertensive rat (SHR) and are proposed to play an important role in resetting the pressure-natriuresis relationship and the development of hypertension.11 The CYP eicosanoids are recognized as potent mediators of vascular tone and have been implicated in these vascular changes. Recent studies indicate that renal 20-HETE and EET forma-
tion and urinary excretion are increased in the SHR relative to the normotensive Wistar-Kyoto (WKY) rat. Whether the increase is causative or compensatory is unknown. The opposing effects of the EET and HETE eicosanoids on vascular tone make it difficult to predict their effect in vivo and emphasize the importance of understanding the factors that regulate their formation and degradation. Modulation of CYP enzyme activities in these rats is associated with changes in blood pressure; however, the utility of this approach has been limited by the ability of multiple CYP isoforms to catalyze these reactions and by the general lack of selectivity of available CYP inhibitors and inducers. A novel alternative for modulating the levels of the vasoactive EET and DHET eicosanoids is regulation of EET hydrolysis to the corresponding vicinal diols. The regulation of EET hydrolysis in the SHR kidney and the effect of modulation of EH activity on regulation of blood pressure are explored in the present study. Both in vitro and in vivo evidence demonstrate that increased blood pressure is associated with increased sEH expression and EET hydrolysis in the SHR. Furthermore, inhibition of EET hydrolysis with a tight binding sEH inhibitor can reverse the hypertensive phenotype in the SHR. These data also describe novel inhibitors for characterizing the distinct biological properties of the EET and DHET eicosanoids.

Materials and Methods

Animals

Male SHR and WKY rats 3 to 13 weeks of age were purchased from Charles River Laboratories (Wilmington, Mass). The University of California San Francisco Committee on Animal Research approved all animal use. Rats were anesthetized with methoxyflurane, and kidneys were dissected and removed as described previously. For the sEH inhibition studies, groups of 8-week-old male SHRs and WKY rats were treated daily for 1 to 4 days with a 3 mg/kg IP dose of N,N,N-trimethylhexylamine (DCU) in a 1:5:1 mixture of corn oil and DMSO. Systolic blood pressure was measured at room temperature using a photoelectric tail-cuff system (model 179, IITC, Inc) for up to 4 days after the dose of inhibitor. Blood pressures are reported as the average of 3 separate readings during a 30-minute period. Urine was collected for 24 hours immediately after a dose of DCU or vehicle for quantification of DHET and EET excretion. Similar inhibition studies were performed with equimolar doses of N-cyclohexyl-N’-dodecylurea, N-cyclohexyl-N’-ethylenurea, and dodecylamine.

Renal Microsomal Arachidonic Acid Metabolism and EET Hydrolysis

In vitro determination of arachidonic acid epoxygenase activity was performed as described previously. Hydrolysis of [1-13C]EETs was measured in WKY and SHR renal fractions as described. Detailed descriptions of these procedures are contained in the online data supplement.

Western Immunoblotting

Renal and hepatic microsomes and cytosol (4 to 10 μg) were separated on an 8% SDS-polyacrylamide gel transferred to nitrocellulose in Towbin’s buffer. Primary antibodies used in these studies were a rabbit anti-mouse sEH antiserum and a rabbit anti-rat mEH antiserum kindly provided by Drs Franz Oesch and Michael Arand (University of Mainz, Mainz, Germany). A second rabbit anti-mouse sEH antiserum was recently prepared in our laboratory against recombinant mouse sEH expressed in insect cell culture. Western blots were incubated with a 1:1000- (mEH) or 1:2000-fold (sEH) dilution of the primary antibody followed by a 1:2000-fold dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive proteins were visualized using an ECL detection kit (Amersham Life Science).

RNase Protection Assays

RNase protection assays for quantification of sEH mRNA levels were performed as described previously using a riboprobe corresponding to 383 to 775 bp of the cDNA.

DHET Urinary Excretion

Methods used to quantify endogenous EETs and DHETs present in rat urine were described previously and in detail in the online data supplement.

Other Enzyme Assays

Activities of mEH and sEH were determined using cis-stilbene oxide (cSO) and trans-1,3-diphenylpropene oxide (tDPPO), respectively, exactly as described. Inhibition of recombinant sEH by DCU was measured using tDPPO.

Statistics

Statistical significance of differences between mean values was evaluated by a one-way ANOVA or a Student’s t test. Significance was set at a P value of <0.05.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Renal EET Hydrolysis

A dramatic increase in DHET formation in incubations of arachidonic acid with SHR renal cortical microsomes relative to the WKY samples (data not shown) led us to hypothesize that EET hydrolysis may be altered in this experimental model of hypertension and that EH activity may be an important determinant of blood pressure regulation. Both mEH and sEH are expressed in most tissues and species, although constitutive levels of sEH in both hepatic and extrahepatic tissues are low in the rat. Higher rates of EET hydrolysis in cytosol versus microsomes and preference of sEH for endogenous arachidonate epoxide pools are consistent with the majority of EET hydrolysis being catalyzed by sEH. Direct hydrolysis of the regioisomeric EETs was measured in S9 fractions (containing both the soluble and microsomal forms of EH) from WKY and SHR renal cortex. The hydrolysis of 8,9-, 11,12-, and 14,15-EET in S9 fractions from both WKY and SHR kidneys was measurable (Figure 1), with a significant increase in hydrolysis in the SHR relative to the WKY. For example, 8,9- and 11,12-EET hydrolysis rates were 5- to 15-fold higher in the SHR than the WKY, and 14,15-EET hydrolysis was as much as 54-fold higher in the SHR. These data also showed a distinct preference of sEH for the 14,15-EET regioisomer. In the SHR kidney hydrolysis of 14,15-EET was 10-fold higher than that of 8,9- and 11,12-EET. Similar studies were also performed using a mixture of unlabeled EETs and detection by gas chromatography–mass spectrometry (GC-MS). The hydrolysis ratio from these studies was 20:7:1 for 14,15-EET:11,12-EET:8,9-EET (data not shown). This is consistent with the preference observed with the individual regioisomers (Figure 1). One explanation for an intermediate level of hydrolysis of the 11,12-EET when measured in the mixture and similar hydrolysis of the 11,12- and 8,9-EETs when measured
separately is that the relative concentrations of the isomers change during the course of the incubation with the EET mixture, thereby affecting the competition of these 3 substrates for the enzyme.

Epoxide Hydrolase Expression
To investigate the possibility that altered EH expression is responsible for the differences in EET hydrolysis in the SHR and WKY rat renal microsomes and S9 fractions, we measured EH protein levels in these samples. Control studies demonstrated that the rabbit anti-mouse sEH antisera cross-reacted with rat mEH but not mEH, and that the rabbit anti-rat mEH antisera did not cross-react with rat sEH (data not shown). mEH was abundantly expressed in the renal microsomes at relatively constant levels throughout development, and there was no evidence of altered expression of mEH in the SHR kidney (Figure 2A). sEH was detected easily in SHR cortical microsomes but not in the corresponding WKY samples (Figure 2A). Quantitation of the immunoreactive protein bands indicated that levels of sEH protein in the SHR microsomes were 6- to 90-fold higher than the corresponding levels in the WKY microsomes. This differential pattern of sEH expression in the WKY and SHR microsomes was confirmed using a second antibody against the enzyme (online Figure 1; available in the online data supplement at http://www.circresaha.org).

The high levels of expression of sEH in the SHR microsomes were limited to the renal cortex (Figure 2B). sEH was hardly detectable in SHR outer medulla and liver microsomes by Western blot. In contrast, relatively high levels of sEH were detected in SHR cortex, outer medulla, and liver cytosol (Figure 2B). In the WKY rats, the level of sEH protein was uniformly low in both microsomes and cytosol from the kidney and liver. sEH protein in the normotensive Sprague-Dawley rat kidney was also hardly detectable. Increased sEH expression in SHR versus WKY rats provides an explanation for the increased EET hydrolysis in the SHR kidney and the absence or very low levels of 14,15-EET hydrolysis, the preferred sEH substrate, in the WKY kidney.

The mechanistic basis for the dramatic difference in sEH protein levels between WKY and SHR kidneys was investigated with the use of RNase protection assays. sEH RNA was Figure 1. Hydrolysis of regioisomeric EETs is increased in the SHR kidney relative to the WKY. The hydrolysis of 11,12-EET (A), 8,9-EET (B), and 14,15-EET (C) was measured in incubations of WKY (○) and SHR (●) renal S9 fractions with [1-14C]EETs (50 μmol/L). Metabolites were extracted from the incubation mixture and quantified by HPLC with radiometric detection. Hydrolysis rates for each age and strain from 2 separate animals are shown, and the line is drawn through the average value.

Figure 2. Soluble epoxide hydrolase expression is increased in the SHR kidney and liver relative to the WKY. A, Microsomal proteins from WKY (W) and SHR (S) renal cortex were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with antisera against rat mEH (top) or mouse sEH (bottom). The age of the rats is indicated on the top of the blot. B, Microsomal (top) and cytosolic (bottom) proteins from WKY (W) and SHR (S) cortex, outer medulla and liver were separated and transferred as described above and blotted with antisera against mouse sEH. A renal cortex sample from a Sprague-Dawley (SD) rat and a purified recombinant sEH protein sample are also included on the blot. Immunoreactive proteins were detected by chemiluminescence. The blots are representative of the results from 3 to 6 animals per experimental group.

Figure 3. sEH RNA levels are increased in the SHR kidney relative to the WKY. Total cortical RNA was prepared from WKY (W) and SHR (S) kidneys and used for RNase protection assays. sEH RNA was hybridized with a rat sEH and a rat GAPDH probe. The autoradiograms are representative of 3 to 6 animals per experimental group. The age of the animals is indicated on the top of the gel. Autoradiograms were visualized with a Phosphor-Imager and analyzed using ImageQuant software. The sEH signal is expressed relative to the GAPDH signal for purposes of quantification.
Detected in both WKY and SHR kidneys with a 5- to 12-fold increase in expression in the SHR samples (Figure 3). This is consistent with the corresponding increase in sEH protein levels in the SHR kidney (Figure 2). In addition to the major protected fragment of predicted size, a slightly smaller fragment was detected in all the SHR samples. Additional smaller protected fragments were also found in the SHR but not in the WKY kidneys. This raises the possibility that a second mRNA with a structure very similar to the cloned sEH is also expressed in the SHR kidney at much higher levels than in the WKY kidney.

**Epoxide Hydrolase Activity**

Increased sEH activity in the SHR kidney was confirmed independently using the sEH substrate tDPPO. Consistent with the Western blots, there was a 26-fold increase in tDPPO hydrolysis in the SHR cortical cytosol relative to that in the WKY rat cortical cytosol (online Table 1; available in the online data supplement at http://www.circresaha.org). The corresponding difference in the microsomal fraction was 32-fold. Hydrolysis of tDPPO was also significantly higher in SHR versus WKY rat liver microsomes and cytosol. In contrast, mEH activity, as measured by cSO hydrolysis, was increased only slightly in SHR kidney cortex and liver microsomes and liver cytosol relative to the WKY rat (online Table 1). The sEH activity in microsomes as a fraction of the total cellular sEH activity was 2% to 9%, consistent with a low level of cytosolic contamination of microsomes.

**DHET Urinary Excretion**

Urinary excretion of DHETs was measured to evaluate whether increased sEH expression and EET hydrolysis in the SHR was also apparent in vivo. Urine was collected in untreated 4- and 8-week-old SHR and WKY rats, and their DHET excretion rates are shown in Figure 4. The excretion rates were similar for the 4- and 8-week-old animals, and the reported numbers are averages from all samples of a given strain. The excretion of 14,15-DHET was 2.6-fold higher in the SHR relative to the WKY rat, consistent with the increased EET hydrolysis and sEH expression in SHR kidney. In contrast, the 8,9- and 11,12-DHET urinary excretion in the SHR and WKY rats were similar.

**Inhibition of sEH and Effect on Blood Pressure**

A tight binding sEH specific inhibitor, DCU, was used to reduce sEH activity in vivo and to determine the effect of decreased EET hydrolysis on blood pressure. DCU was administered to 8-week-old SHRs daily for 4 days, and urinary DHET excretion was measured during the 24 hours immediately after the third dose. The dose of DCU was based on in vitro estimates of inhibitory potency and previous studies in the mouse. A significant 65% decrease in 14,15-DHET urinary excretion occurred in the DCU-treated rats, and a corresponding 30% increase in 14,15-EET urinary excretion relative to vehicle-treated controls (Figure 5), consistent with DCU-mediated inhibition of sEH in vivo. The excretion of total epoxygenase-derived products (EETs and DHETs) was decreased from 2020 pg/mg creatinine in the vehicle-treated animals to 1237 pg/mg creatinine in the DCU-treated rats (P < 0.05). This inhibition of 14,15-DHET excretion was accompanied by a significant decrease in blood pressure measured in conscious animals 3 to 5 hours after the fourth dose (data not shown). Systolic blood pressure decreased from 128±5 mm Hg in the vehicle-treated rats to 102±5 mm Hg (P < 0.01) in the DCU-treated animals.

A study of the time course of the effect of a single dose of DCU (3 mg/kg) demonstrated that the antihypertensive effect in the SHR was acute (Figure 6A). Blood pressure was decreased 22±4 mm Hg 6 hours after DCU treatment (P < 0.01) and returned to baseline levels by 24 hours after the dose. DCU had no effect on blood pressure in the WKY rats (Figure 6B). This is consistent with the very low levels of sEH protein in the WKY kidney. Several additional structurally related inhibitors were also studied in the SHR. N-Cyclohexyl-N'-dodecylurea is a sEH inhibitor with similar potency to DCU (IC50 with mouse sEH 0.05±0.01 compared to 0.0005).

**Figure 4.** Urinary DHET excretion in the WKY and SHR. Urine was collected for 24 hours from untreated WKY rats (solid bars) and SHR (hatched bars). DHETs were extracted from urine and quantified by GC-MS as described in Materials and Methods. The values shown are the mean±SE of 4 animals per strain. Significant differences between WKY and SHR are indicated (P < 0.0005).

**Figure 5.** Inhibition of DHET formation by DCU treatment in the SHR. Urine was collected for 24 hours after treatment of SHR with vehicle (solid bars) or DCU (hatched bars) daily for 3 days. EETs and DHETs were extracted from urine and quantified by GC-MS as described in Materials and Methods. The values shown are the mean±SE of 4 animals per strain. Significant differences between vehicle- and DCU-treated SHR are indicated (P < 0.05).
pared with 0.09±0.01 μmol/L for DCU; C. Morisseau and B. Hammock, unpublished data, 2000). A single dose of N-cyclohexyl-N9-dodecylurea significantly decreased systolic blood pressure 12±2 mm Hg 6 hours after the dose, and similar to DCU, blood pressure returned to normal by 24 hours after the dose (online Figure 2A). The N-cyclohexyl-N9-ethylurea analog is a weak sEH inhibitor (IC50 with mouse sEH 5±1.7±0.7 μmol/L; C. Morisseau and B. Hammock, unpublished data, 2000) and had no effect on blood pressure in the SHR (online Figure 2B). Likewise, the selective mEH inhibitor dodecylamine also had no effect on blood pressure (online Figure 2C). Collectively, these data suggest that the effect of DCU and N-cyclohexyl-N9-dodecylurea on blood pressure is related to their ability to inhibit sEH and EET hydrolysis in vivo.

The decreased 14,15-DHET excretion after treatment with DCU was consistent with a decrease in renal sEH activity. Inhibition of EET hydrolysis by DCU was confirmed in incubations of renal S9 fractions with the regioisomeric EETs (Figure 7). A dose-dependent inhibition of EET hydrolysis by DCU was apparent for all 3 regioisomers. DCU had the most significant effect on the hydrolysis of 8,9-EET, inhibiting this reaction with an IC50 of 0.086±0.014 μmol/L. The corresponding IC50 values for inhibition of 11,12- and 14,15-EET hydrolysis were 0.54±0.08 and 0.45±0.16 μmol/L, respectively. Because of the large difference in hydrolysis of the regioisomeric EETs by sEH, it was not possible to use the same amount of enzyme to measure an IC50 for DCU inhibition for these substrates. At a concentration of enzyme half of that used for 8,9- and 11,12-EET, the IC50 for 14,15-EET is similar to that for 11,12-EET and 5-fold higher than that for 8,9-EET. Thus, at a given concentration of DCU, sEH hydrolysis of 14,15-EET will be inhibited to a lesser degree than that of the other two regioisomers, consistent with the higher preference of the enzyme for this isomer. At concentrations up to 25 μmol/L, DCU had no effect on CYP epoxygenase or ω-hydroxylase activity (data not shown), and previous studies from our laboratory have shown that DCU does not inhibit mEH.21 The potent inhibition of sEH by DCU was confirmed with purified recombinant rat sEH. DCU inhibited sEH-catalyzed tDPPO hydrolysis with a Ki of 34 nmol/L (online Figure 3). This is similar to the Ki values for DCU with human (30 nmol/L) and murine (26 nmol/L) sEH.21

Discussion

EET and DHET eicosanoids are recognized as potent mediators of vascular tone and have been implicated in the pathogenesis of hypertension.1,5,11,23 The data presented herein provide substantial evidence of a primary defect in sEH expression in the SHR kidney, and of an antihypertensive effect of sEH inhibition. Accordingly, inhibition of EET hydrolysis increased EET levels and decreased DHET levels in vivo. Whether the antihypertensive effect is caused by a protective effect of EETs and/or a deleterious effect of the DHETs cannot be discerned. Indeed, the ratio of these eicosanoids may be the major determinant of their effect on vascular tone and blood pressure. The fact that DHETs are increased in SHR and that blood pressure is reduced coincident with decreased DHET formation suggests that the primary defect in renal sEH expression identified in the SHR
kidney is causal and that high levels of DHETs are detrimental. These data provide evidence that the DHETs may be important regulators of blood pressure and identify sEH as a novel therapeutic target for the treatment of hypertension. Whether increased levels of DHETs are sufficient to increase blood pressure or whether they modulate the response to other factors involved in vascular reactivity and blood pressure regulation remains to be determined.

Progress in understanding the role of the EET and DHET eicosanoids in the regulation of vascular tone is limited by the lack of selective and stable inhibitors and mimics. In many cases, in vitro and in situ studies do not separate the effects of the EETs from those of the corresponding DHETs, making it difficult to assign specific properties to these eicosanoids. EETs are generally considered antihypertensive because of their vasodilatory properties; however, vasoconstrictor effects have also been attributed to the EETs, depending on the vascular bed and species that are studied. After systemic administration to rats, both 5,6- and 8,9-EET caused a dose-dependent vasoconstriction and decrease in glomerular filtration rate which was cyclooxygenase-dependent. In contrast, studies using a rat juxtamedullary nephron preparation characterized 11(R),12(S)-EET as a potent vasodilator. Similar to the EETs, the effect of the DHETs on vascular tone is highly dependent on the vascular bed, species, and experimental system that is studied. For example, 11,12-DHET produces a vasoadilatory effect on porcine coronary artery rings and a vasoconstrictive effect on rat interlobular and afferent arterioles, whereas 5,6-DHET attenuates the vasoconstrictive effect of 5,6-EET. The novel sEH inhibitors described herein will be valuable reagents for further dissecting the vascular effects of the EETs and DHETs both in vivo and in vitro.

The hydrolysis of each of the major regioisomeric EETs was increased dramatically in the SHR relative to the WKY rat because of increased sEH expression in the SHR kidney. This increased hydrolysis results in altered levels of the vasoactive EET and DHET eicosanoids that are implicated in the control of blood pressure. Although these data suggest that increased EET hydrolysis is an important regulator of blood pressure, it is clearly not the sole mechanism for the SHR phenotype. In the SHR, blood pressure rises steadily between 4 and 13 weeks of age, in contrast to the relatively constant expression of sEH during this period. EET hydrolysis is also highest in prehypertensive SHR, suggesting that these changes in sEH expression might be most important in the early development of hypertension. Several lines of evidence support a major role for sEH in the hydrolysis of EETs in the SHR model of hypertension. First, studies with human and rabbit liver and rabbit lung indicate a 10-fold higher EET hydration rate in cytosol than in microsomes, and EET hydrolysis is catalyzed by purified sEH. Second, increased hydrolysis of EETs in SHR renal subcellular fractions compared with the WKY samples was consistent with the dramatic increase in sEH expression in the SHR kidney. Finally, the hydrolysis of 14,15-EET was absent or very low in the WKY microsomes and S9 fractions that have only minimal expression of sEH protein. This is consistent with the strong preference of purified sEH for the 14,15-EET regioisomer and the preference of the EH in renal S9 fractions for 14,15-EET. Although the hydrolysis of all the regioisomeric EETs was increased in SHR renal fractions, only 14,15-DHET urinary excretion was increased in vivo. This in vivo-in vitro discrepancy most likely reflects sequential metabolism of the DHETs or limiting efflux mechanisms that may operate only in the intact cell or organ.

These data may provide a link between the pathophysiological regulation of blood pressure in rats and humans. During pregnancy 8,9- and 11,12-DHET excretion is increased significantly, and excretion of 11,12- and 14,15-DHET is increased even further in patients with pregnancy-induced hypertension. This increase is most dramatic for 14,15-DHET, the major product of sEH-catalyzed EET hydrolysis. The present results in the SHR support the hypothesis that altered sEH expression may be responsible for the hypertension observed in pregnant women. In this regard, the substantial interindividual variation in sEH expression in humans is well established. Furthermore, we have recently discovered several single nucleotide polymorphisms within the human sEH gene that result in amino acid substitutions (D.C. Zeldin, unpublished data, 2000). The functional characteristics of these polymorphisms, and their frequency distribution in normal and hypertensive populations are currently being investigated.

The in vitro and in vivo evidence for increased EET formation and hydrolysis in the SHR kidney suggested that accelerated elimination of EETs may contribute to the hypertensive phenotype in the SHR, and it raised the question of whether sEH may be a novel therapeutic target for the treatment of hypertension. Initial studies with the selective tight binding sEH inhibitor DCU and several structural analogs support this approach. Inhibition of sEH by DCU in vivo was evident from urinary EET and DHET excretion measurements and was associated with a 15% reduction in systolic blood pressure. This antihypertensive effect was acute, with a maximal decline in blood pressure 6 hours after a dose and complete recovery to baseline levels by 24 hours after the dose. The antihypertensive effects of DCU are attributed to its inhibition of EET hydrolysis in vivo, because it has no effect in WKY rats with very low expression of sEH; another sEH inhibitor with similar potency also decreases blood pressure. Similarly, a weak sEH inhibitor and a selective mEH inhibitor have no effect on blood pressure. Recently, inhibition of arachidonic acid ω-hydroxylase (CYP4A) activity with a mechanism-based CYP inhibitor has effectively lowered blood pressure in the SHR. Combined inhibition of CYP4A, resulting in decreased levels of the vasoconstrictive 20-HETE eicosanoid, and sEH, leading to increased levels of the vasodilatory EETs, would be predicted to cause either an additive or synergistic reduction in blood pressure. Inhibition of related CYP isoforms is a potential limitation of ω-hydroxylase inhibition, but is of little concern with sEH inhibition in light of the recent development of potent and specific urea, carbamate, and amide inhibitors of sEH. The possibility of similar pharmacological changes in sEH activity in human hypertensive populations is compelling. Identification of individuals with elevated sEH activity
and lower EET levels may prove useful in designing the most effective antihypertensive therapy.

In summary, we have described an increased expression of sEH in the SHR kidney that leads to increased EET hydrolysis. Increased hydrolysis of the antihypertensive EETs is proposed as an important determinant of the hypertensive phenotype in this model. Consistent with this hypothesis is the reduction of blood pressure associated with significant inhibition of EET hydrolysis. These data suggest that modulation of sEH may have therapeutic uses for the treatment of hypertension. Selective sEH inhibitors may also have more general clinical applications in altering processes where in vivo levels of EETs and DHETs are involved, and will be invaluable in characterizing the vasoactive effects of the EETs and DHETs.

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References

10. Katoh T, Takahashi K, Capdevila J, Karara A, Falck JR, Badr KF. Glomerular stereospecific synthesis and hemodynamic actions of 8,9-

26. Fang X, Kaduce TL, Weintraub NL, VanRollins M, Spector AA. Functional implications of a newly characterized pathway of 11,12-
27. Catella F, Lawson JA, Fitzgerald DJ, Fitzgerald GA. Endogenous bio-
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MATERIALS AND METHODS

Renal microsomal arachidonic acid metabolism and EET hydrolysis

Microsomes, cytosol, and S9 fractions were prepared from the renal cortex as described previously (1,2). In vitro determination of arachidonic acid epoxygenase activity was performed under conditions in which product formation was linear with respect to protein and time (2). The $K_m$ for EET formation was estimated to be 8-10 $\mu$M and those for the hydrolysis of the regioisomeric EETs were similar (6-8 $\mu$M). Renal cortical arachidonic acid metabolism was measured in incubations containing [1-$^{14}$C]arachidonic acid (10 or 100 $\mu$M, 0.2 $\mu$Ci), microsomal protein (0.5 mg/ml), KCl (150 mM), MgCl$_2$ (10 mM), sodium isocitrate (8 mM), and isocitrate dehydrogenase (0.5 IU) in potassium phosphate buffer (100 mM, pH 7.4). The mixtures were preincubated for 5 min at 37°C and the reaction was started by addition of NADPH (1 mM). The incubation was carried out for 30 min at 37°C and the reaction was terminated by acidifying to pH 3.5 with HCl. Arachidonic acid and its metabolites were extracted twice with ethyl acetate and the combined organic phase was washed once with double distilled water. After evaporation of organic solvent under nitrogen the dry residue was stored at -80°C until HPLC analysis. Metabolites were separated on an Alltima C18 5 $\mu$m column (250 x 4 mm) with an Alltima C18 guard column and in-line filter (Alltech Associates, Deerfield, IL) and detected with a flow scintillation analyzer (Radiomatic 525TR, Packard Instrument Company, Downers, IL) exactly as described previously (1).

Racemic [1-$^{14}$C]EETs were synthesized and purified according to published methods from [1-$^{14}$C]arachidonic acid (56-57 $\mu$Ci/$\mu$mole) by nonselective epoxidation (3). Hydrolysis of [1-$^{14}$C]EETs was measured in WKY and SHR renal S9 fractions at 37°C as described previously.
Preliminary experiments established reaction conditions that were linear with respect to protein concentration and time. $K_m$ estimates for the individual EETs were 25-30 $\mu$M. The reaction mixture consisted of 50 $\mu$M EET (0.045-0.09 $\mu$Ci) and 1 mg/ml S9 protein (0.5 mg/ml SHR S9 protein for 14,15-EET hydrolysis) in 150 mM KCl, 10 mM MgCl$_2$, 50 mM potassium phosphate buffer pH 7.4. Reactions were carried out for 40 min (10 min for 14,15-EET hydrolysis in SHR samples) and the reaction products were extracted into ethyl acetate, evaporated under a blanket of nitrogen and detected by reverse phase HPLC with radiometric detection as described (2).

EET hydrolysis was also measured in a mixture of unlabeled 14,15-, 11,12- and 8,9-EET synthesized as described above. The reaction mixture consisted of SHR renal cytosol (4.3 mg/ml; 15 tDPPO hydrolysis units) and 50 $\mu$M of each EET in 0.1 M phosphate buffer (pH 7.4). Reactions were allowed to progress for 30 min at 30ºC, at which time they were stopped by the addition of MeOH. Samples were then spiked with ~50 $\mu$M 10-epoxyheptadecanoic acid and 10,11-dihydroxy nonadecanoic acid in MeOH, vortexed and incubated at room temperature for 5 min. Samples were extracted with chloroform, acidified with acetic acid, and extracted twice more with chloroform. The combined organic solvent was removed under dry nitrogen and the resulting residue was dissolved in 200 $\mu$L MeOH and treated with 200 $\mu$L of freshly prepared ethereal diazomethane. The solvent was removed under dry nitrogen, the residue dissolved in 200 $\mu$L EtOAc and the sample split. One half of each sample was then enriched with 15 $\mu$L of bis-silyl trifluoroacetamide (BSTFA; Supelco Inc; Belefonte Park, PA) in pyridine (80:20 v/v) incubated 1 hr at room temperature and stored at –25ºC until analysis. A 2 $\mu$L aliquot of each sample was analyzed on a Hewlet-Packard 6890 gas chromatograph equipped with a 5973 mass spectral detector. Analytes were separated with a constant helium flow of 0.8 mL/min on a 30m x 0.25mm x 0.25$\mu$m DB-17ms column (J&W Scientific, Folsom, CA) using the following oven program: Initial 100ºC (2 min hold), 15ºC/min to 240ºC (1min hold), 3ºC/min to 270ºC (7 min
hold), 15°C/min to 320°C (2 min hold). Under these conditions, ~30% to baseline resolution of 8,9- and 11,12-DHET-silylether, methyl esters were achieved while 5,6- and 14,15-DHET are fully resolved from this doublet. Similarly, the 14,15-EET methyl ester is resolved from the other three coeluting regioisomers. Peak retention time and characteristic ion abundances were used to positively identify silylated DHET isomers: 8,9-DHET me – t_R = 16.74 min, m/z 255, 243 (56%), 265 (21%); 11,12-DHET me – t_R = 16.8 min, m/z 295, 213 (62%); 14,15-DHET me – t_R = 17.06 min, m/z 173, 275 (60%), 294 (13%). Estimations of peak abundance were based on the intensity of the total ion current (m/z 50 – 315). Surrogate recoveries were used to correct epoxide (82 ± 11%) and diol (88 ± 18%) areas, respectively. The resolution of 14,15-EET me from the 8,9- and 11,12-EET pair allowed a simple assessment of the relative abundance of these isomeric groups and their shift during the reaction.

**DHET urinary excretion**

Urinary creatinine concentrations were measured by the Medical Center Clinical Laboratories at the University of California San Francisco. Methods used to quantify endogenous EETs and DHETs present in rat urine were similar to those described by Capdevila et al. (5). DHET and [1-14C]DHET internal standards were prepared by chemical hydration of EETs and [1-14C]EETs as described (4). All synthetic EETs and DHETs were purified by reverse-phase HPLC. EET quantifications were made by GC/MS analysis of their pentafluorobenzyl (PFB) esters with selected ion monitoring at m/z 319 (loss of PFB from endogenous EET-PFB) and m/z 321 (loss of PFB from [1-14C]EET-PFB internal standard). The EET-PFB/[1-14C]EET-PFB ratios were calculated from the integrated values of the corresponding ion current intensities. Quantifications of DHETs were made from GC/MS analysis of their PFB esters, trimethylsilyl (TMS) ethers with selected ion monitoring at m/z 481 (loss of PFB from endogenous DHET-PFB-TMS) and m/z 483 (loss of PFB from [1-14C]DHET-PFB-TMS internal standard). The DHET-PFB-TMS/[1-14C]DHET-PFB-TMS ratios were
calculated from the integrated values of the corresponding ion current intensities. Data were normalized for kidney function by expressing per mg creatinine. Control studies demonstrated that under the conditions used, artifactual EET or DHET formation was negligible.
**Table 1 Online**

**Soluble and Microsomal Epoxide Hydrolase Activity in WKY and SHR Kidneys and Livers**

<table>
<thead>
<tr>
<th>Tissue</th>
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<th>Microsomes</th>
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<td>Kidney Cortex</td>
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<tr>
<td>WKY</td>
<td>90 ± 28</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
<td>332 ± 28</td>
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<tr>
<td>SHR</td>
<td>2320 ± 29*</td>
<td>163 ± 5*</td>
<td>19 ± 1†</td>
<td>470 ± 23†</td>
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<td>Liver</td>
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<tr>
<td>WKY</td>
<td>690 ± 50</td>
<td>10 ± 1</td>
<td>988 ± 51</td>
<td>3250 ± 185</td>
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<tr>
<td>SHR</td>
<td>1920 ± 108†</td>
<td>150 ± 6*</td>
<td>1590 ± 58†</td>
<td>2660 ± 171†</td>
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</table>

sEH activity was measured in WKY and SHR kidney cortex and liver microsomes (2 mg/ml) and cytosol (0.1 mg/ml) using 5 nmol [2-³H]-tDPPO as substrate. mEH activity was measured in WKY and SHR kidney cortex and liver microsomes (0.2 mg/ml) and cytosol (2 mg/ml) using 5 nmol [2-³H]-cSO as substrate. Microsomes and cytosol were prepared from pooled tissue samples from four 9-16 wk old rats. Values shown are the mean ± SE from 3 determinations.

* Significantly different from WKY, p < 0.00005
† Significantly different from WKY, p < 0.0005
‡ Significantly different from WKY, p < 0.05
REFERENCES


FIGURE LEGENDS

Figure 1 Online. Increased expression of sEH in the SHR kidney relative to the WKY. Microsomal proteins from WKY (W) and SHR (S) renal cortex were separated on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with antisera against recombinant mouse sEH expressed in insect cell culture. The age of the rats is indicated on the top of the blot. Immunoreactive proteins were detected by chemiluminescence. The blots are representative of the results from three to six animals/experimental group.

Figure 2 Online. Effect of DCU structural analogs and a mEH inhibitor on blood pressure in the SHR. Male SHRs were treated with a single dose of vehicle (○) or inhibitor (●). The inhibitors used (equimolar to 3 mg/kg DCU) were N-cyclohexyl-N’-dodecylurea (A), N-cyclohexyl-N’-ethylurea (B) or dodecylamine (C). Systolic blood pressure was measured with a photoelectric tail cuff for 24 hr after the dose. The values shown are the mean ± SE from inhibitor- and vehicle-treated rats (n=5/group). Baseline systolic blood pressures were 135 ± 5 mm Hg in the N-cyclohexyl-N’-dodecylurea group, 142 ± 7 mm Hg in the N-cyclohexyl-N’-ethylurea, and 136 ± 9 mm Hg in the dodecylamine group. Blood pressure decreased an average of 12 mm Hg in the N-cyclohexyl-N’-dodecylurea-treated SHRs 6 hr after the dose (p < 0.01). N-cyclohexyl-N’-ethylurea and dodecylamine had no effect on blood pressure.

Figure 3 Online. Inhibition of recombinant rat sEH with DCU. The $K_{i,\text{app}}$ for DCU inhibition of recombinant rat sEH (1.5 nM) was measured using $[2-^{3}\text{H}]t\text{DPPO}$ as substrate. For each substrate concentration, the velocity is plotted as a function of the inhibitor concentration allowing the determination of an apparent inhibition constant ($K_{i,\text{app}}$). The $K_{i,\text{app}}$ values are plotted as a function of the substrate concentration (inset). For $[S] = 0$, a $K_i$ value of 33 nM is found.
<table>
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Figure 2 Online

A. BP Change (mm Hg)

Time after N-Cyclohexyl-N'-dodecylurea Administration (hr)

B. BP Change (mm Hg)

Time after N-Cyclohexyl-N'-ethyleurea Administration (hr)

C. BP Change (mm Hg)

Time after Dodecylamine Administration (hr)
Figure 3 Online

![Graph showing the relationship between v (pmol/min) and DCU (nM) with various [S] (mM) concentrations. The inset shows a plot of K_{app} (nM) versus [S] (mM) with a linear regression line and r^2 = 0.997. K_I = 33 nM.](image)