

# Polymorphism in Soluble Epoxide Hydrolase and Blood Pressure in Spontaneously Hypertensive Rats

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**Abstract**—We measured soluble epoxide hydrolase (sEH) renal gene expression in prehypertensive (4 to 5 weeks old) spontaneously hypertensive rats of the Heidelberg SP substrain (SHR [Heid]) and when blood pressure levels entered the hypertensive plateau (17 to 18 weeks old) and compared expression with matched Wistar-Kyoto (WKY [Heid]) rats. Less expression of the gene encoding sEH (EPHX2) was observed in SHR (Heid) than in WKY (Heid). Analysis of sEH protein abundance showed a similar difference. However, no correlation between sEH abundance and blood pressure was observed in the F<sub>2</sub> progeny of a parental strain cross. Measurement of protein abundance in SHR and WKY obtained from Charles River confirmed a recent report that abundance of sEH was greater in SHR (CRiv) than WKY (CRiv) strains. Polymorphisms were detected in EPHX2. Resequencing revealed that 2 alleles of EPHX2 exist in these 4 rat strains, differing by 4 single nucleotide polymorphisms, of which 3 produce nonsynonymous amino acid substitutions. The ancestral allele was shared by SHR (Heid) and WKY (CRiv), and the variant allele was shared by WKY (Heid) and SHR (CRiv). Activity of sEH was greater in animals carrying the variant allele. However, inheritance of this allele was not correlated with blood pressure in the F<sub>2</sub> progeny of a cross between SHR (Heid) and WKY (Heid). These data indicate that sequence variation determining functional alterations in EPHX2 is not likely to contribute to blood pressure levels in SHR. (*Hypertension*. 2002;40:485-490.)

**Key Words:** hypertension, genetic ■ polymorphism ■ genetics ■ metabolism ■ rats, inbred SHR

There is substantial evidence that alteration in renal eicosanoid metabolism participates in the pathogenesis of hypertension in the spontaneously hypertensive rat (SHR). Studies of differential gene expression in kidney identified cytochrome p450 4A2 (CYP450 4A2) as upregulated in SHR.<sup>1</sup> This enzyme acts on arachidonic acid to generate regio-isomeric hydroxyeicosatetraenoic acids (HETEs), such as 20-HETE, which have been shown to be potent vasoactive agents in the renal vasculature.<sup>2</sup> Production of 20-HETE is elevated in SHR kidney during the development phase of hypertension.<sup>3</sup>

Additional attention has focused on the role in blood pressure regulation and renal function of pathways of arachidonic acid metabolism that result in the formation of epoxyeicosatrienoic acids (EETs).<sup>4</sup> In addition to its effect of generating HETE, CYP450 4A2 can generate EET.<sup>5</sup> Upregulation of renal cytochrome p450 mono-oxygenase enzymes of the 2C group, which also generate EETs, has been reported during salt loading.<sup>6</sup> Various EET regio-isomers can antagonize renal tubular sodium reabsorption and may have both vasoconstrictive and vasodilatory effects on the renal vasculature, depending on the prevailing level of cyclooxygenase

activity.<sup>7,8</sup> Recently, EET has been identified as an endothelium-derived hyperpolarizing factor and has been shown to operate through vascular smooth muscle Ca<sup>++</sup>-activated potassium channels.<sup>9</sup> This suggests a possible role of EETs in integrating vascular and tubular mechanisms of renal sodium balance.

EETs are metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols,<sup>10,11</sup> which lack the renal vasodilatory effects of their progenitor EETs.<sup>12</sup> Targeted disruption of the sEH gene results in modestly reduced blood pressure in male, though not in female, mice.<sup>13</sup> A selective antagonist of sEH has been developed,<sup>14</sup> which has been reported to have a hypotensive effect in SHR<sup>15</sup> and in angiotensin II-induced hypertension.<sup>16</sup> Increased sEH gene expression and protein abundance have been reported in SHR suggesting overactive conversion of EETs, which may contribute to hypertension.<sup>15</sup>

The studies reported here have examined further the association between sEH and blood pressure regulation in SHR. We find that inbred rat strains, including substrains of SHR and of its control WKY strain, differ profoundly in their level of sEH gene expression and protein abundance. We have identified several coding sequence single nucleotide

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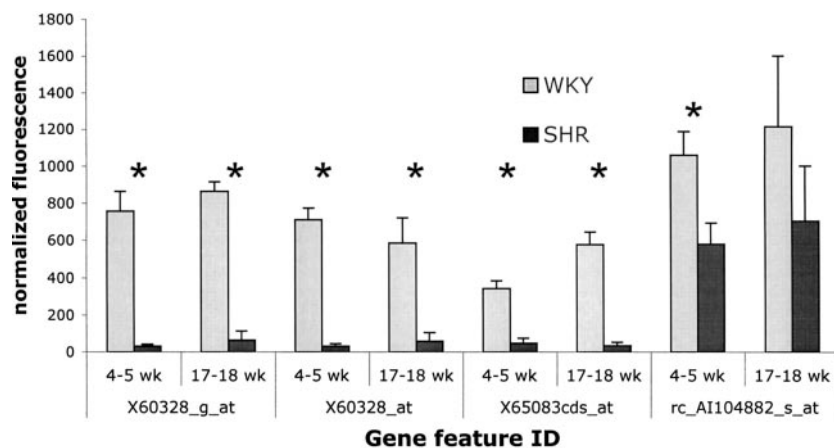
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**Figure 1.** Average fluorescence intensity ( $\pm$ SD) of 4 gene features, each comprising 16 unique oligonucleotide probes that report expression of sEH in RNA extracted from kidneys of 4- to 5-week-old and 17- to 18-week-old male SHR (Heid) and WKY (Heid) rats. Studies were performed on individual RNA samples from 3 animals per strain at each time point. Statistical analysis was performed using the Affymetrix Data Mining Tool. Because of the small sample number, we were unable to determine whether the data were normally distributed and therefore used the Mann-Whitney test to calculate the *t* statistic with a significance threshold of  $P < 0.05$  (indicated by asterisk on chart).

polymorphisms (SNPs) that may provide the mechanism underlying these changes. The SNPs create 2 soluble epoxide hydrolase (EPHX2) alleles. The inheritance of these alleles correlates with gene expression, protein abundance, and sEH activity, but not with blood pressure. SHR strains from 2 sources are homozygous for ancestral and variant alleles, respectively. Similarly, WKY controls from the same 2 sources also possess both alleles. Finally, no association between blood pressure level and inheritance of EPHX2 alleles was observed in the  $F_2$  progeny of a cross between SHR (Heid) and WKY (Heid) animals. Thus, sequence variation in this gene and its associated level of gene expression, protein abundance, and activity do not determine the presence of hypertension in these spontaneously hypertensive rats.

### Methods

Studies were performed on male SHR and WKY rats of the Heidelberg SP substrain (originally obtained from Dr Klaus Lindpaintner, Brigham and Women's Hospital, Boston, Mass), hereafter referred to as SHR (Heid) and WKY (Heid), and on the male  $F_2$  progeny of a cross between these strains. SHR/NCrIBR and WKY/NCrIBR male animals were obtained from Charles River Laboratories (Wilmington, Mass) and are hereafter referred to as SHR (CRiv) and WKY (CRiv). Blood pressure was determined by implanted radiotelemetry devices (Data Sciences). Renal gene expression was compared in kidney total RNA preparations from male 4- to 5-week old and 17- to 18-week-old SHR (Heid) and WKY (Heid) using the Affymetrix rat U34A array following the manufacturer's recommended protocols. Samples from multiple animals ( $n=3$  per group) were analyzed, and differences in mean level of expression between the 2 groups were assessed for each probe set using the Mann-Whitney test.

Western blotting of sEH was performed using an antibody previously described.<sup>15</sup> Activity of sEH in subcellular fractions of renal cortical homogenates was determined using  $^3\text{H}$ -*trans* stilbene oxide (tSO) as previously described.<sup>17</sup> Studies examining the effect of pharmacological inhibition of sEH activity used male SHR (CRiv) and WKY (CRiv) animals. Blood pressure recordings were made for 24 hours before intraperitoneal administration (3 mg/kg) of 1,3-dicyclohexylurea in a 1.5:1 mixture of corn oil and dimethyl sulfoxide (DMSO) and were continued on the same schedule for 4 days after initiation of treatment.

EPHX2 polymorphism screening was performed by denaturing high-performance liquid chromatography (HPLC; Wave, Transgenomic Inc) analysis of EPHX2 reverse transcription-polymerase chain reaction (RT-PCR) products. Amplicons that indicated heteroduplex formation were resequenced. Genotyping of EPHX2 poly-

morphisms in  $F_2$  progeny used PCR amplification of genomic DNA combined with oligo extension and mass spectrometry for genotype calling.

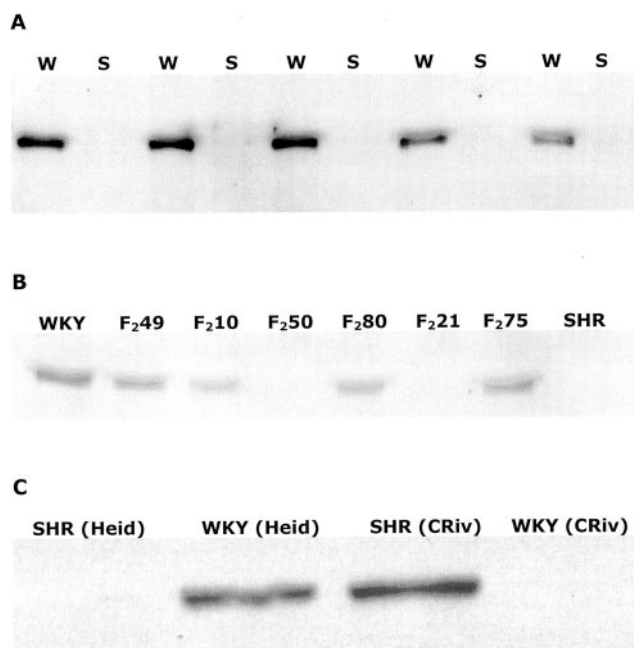
### Results

Studies of renal gene expression using Affymetrix rat U34A expression arrays indicated that renal expression of EPHX2 across SHR (Heid) and WKY (Heid) at both 4 to 5 weeks and 17 to 18 weeks of age was more strongly discordant than that of any other differentially expressed gene detected by this system (Figure 1). The Affymetrix rat U34A chip contains 4 probe sets (64 unique oligonucleotides) that report EPHX2 gene expression. For the 3 cDNA-derived EPHX2 probe sets, the difference in expression between SHR (Heid) and WKY (Heid) ranged from 6.7- to 23.7-fold in 4-week-old animals to 9.6- to 15.5-fold in 18-week-old animals. These differences were all statistically significant. Cross-strain differences in the signals derived from the EST-based probe set were approximately 1.8-fold and were significant only in 4-week-old animals.

To determine whether this gene expression difference resulted in altered levels of specific protein abundance, we performed semiquantitative Western blot analysis. Signals were always readily detected in preparations from WKY (Heid) renal cortex (predominantly proximal convoluted tubules). Under identical loading and exposure conditions, signals were almost completely absent in all corresponding SHR (Heid) samples (Figure 2A).

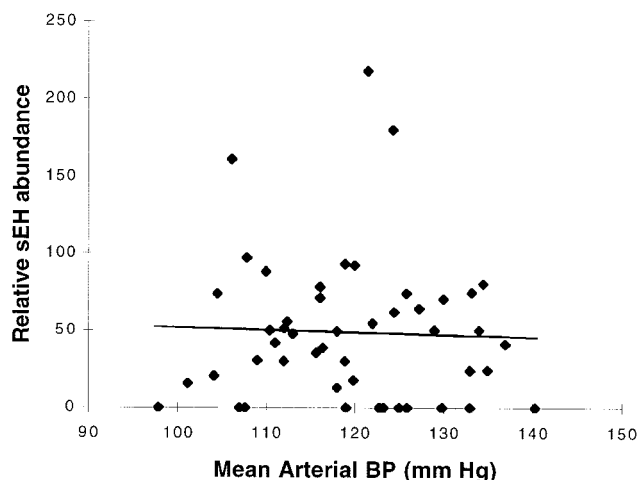
To determine whether the difference in soluble epoxide hydrolase protein abundance between these strains was related to altered blood pressure regulation, we examined the relationship between blood pressure and renal soluble epoxide hydrolase abundance measured by semiquantitative Western blot analysis in the  $F_2$  progeny ( $n=47$ ) of a cross derived from SHR (Heid) and WKY (Heid). A Western blot representing such an analysis is shown in Figure 2B. To determine whether sEH abundance and blood pressure were correlated in  $F_2$  animals, we performed regression analysis of the relative abundance of sEH and blood pressure. No relationship between soluble epoxide hydrolase abundance and blood pressure (systolic, mean, or diastolic) was observed ( $R^2=0.001$ ,  $P=NS$ , Figure 3).

Contemporaneous with these observations, published reports appeared that indicated an involvement of EPHX2 in



**Figure 2.** A, Western blot of sEH in postnuclear supernatants from renal cortex (principally proximal convoluted tubules) of 4- to 5-week-old male rats (5 WKY [Heid] and 5 SHR [Heid] individuals, W and S, respectively). Each lane was loaded with 1  $\mu$ g of total protein from an individual animal. Longer film exposure indicated that signal was detectable in S, but at very low levels compared with W. B, Western blot of sEH in postnuclear supernatants from renal cortex of 16-week-old male rats (1 WKY [Heid] sample pooled from multiple individuals, W; 1 SHR [Heid] sample pooled from multiple individuals, S; and 6 male F<sub>2</sub> individuals identified by numbers). Each lane was loaded with 5  $\mu$ g of total protein. Relative quantitation was obtained by bracketing individual F<sub>2</sub> samples (n=47) on each gel with samples derived from WKY (Heid) and SHR (Heid) animals. The densitometry value for the WKY (Heid) pool was set at 100%, whereas that of the SHR (Heid) pool was set at 0%, and the density of the F<sub>2</sub> individuals (all males) was determined with reference to the parental strain levels present on each blot. C, sEH abundance determined by Western blot from renal cortical cell lysate (20  $\mu$ g protein loaded per lane) of male Heidelberg and Charles River substrains of SHR and WKY.

blood pressure regulation.<sup>13,15</sup> In addition, levels of message and protein abundance of sEH measured in SHR and WKY rats supplied from Charles River Laboratories were the inverse of our observations on Heidelberg substrains of SHR and WKY.<sup>15</sup> Furthermore, treatment of these animals with a selective potent inhibitor of sEH was reported to lower blood pressure measured by tail cuff in SHR, but not in WKY. To investigate this apparent anomaly, we purchased rats from Charles River Laboratories and performed semiquantitative Western blot analysis of renal cortical preparations. We observed that WKY (CRiv) and SHR (Heid) had similar low levels of protein abundance, whereas SHR (CRiv) and WKY (Heid) had similarly abundant levels of expression of this protein (Figure 2C). The similarity in relative abundance of sEH in SHR (Heid) and WKY (CRiv), as well as the similarity between WKY (Heid) and SHR (CRiv), suggests that these differences might be explained by the action of as few as 2 alleles across the 4 strains. However, a wider range of sEH abundance than expected by simple diallelic control



**Figure 3.** No relationship was observed ( $r=0.03$ ,  $P=NS$ ,  $n=47$ ) between renal sEH determined by Western blot and blood pressure in the male F<sub>2</sub> progeny of a cross between SHR (Heid) and WKY (Heid).

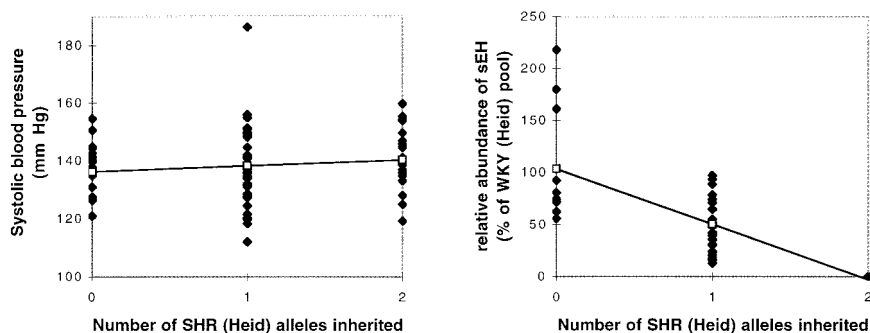
was observed in the F<sub>2</sub> animals (Figures 2B and 3), suggesting that multiple factors influence sEH, including measurement variation.

We assessed the enzymatic activity of renal soluble epoxide hydrolase across the rat strains. In WKY (Heid) animals, we observed that greater protein abundance was accompanied by greater cytosolic sEH activity than in SHR (Heid) (Table 1). We made similar measurements in SHR and WKY animals obtained from Charles River and found that the difference in activity was reversed between SHR and WKY animals from this source.

To determine whether the altered relationship between protein abundance and enzyme activity was associated with EPHX2 coding sequence variation, we examined SHR (Heid) and WKY (Heid) EPHX2 coding sequence for polymorphism by denaturing HPLC. RT-PCR primer design constraints allowed us to interrogate 1527 bases of the 1665 base coding sequence of EPHX2. Sequence variation was indicated by denaturing HPLC (dHPLC) in each of the 3 RT-PCR fragments used to provide coverage of the EPHX2 coding sequence. These amplicons were resequenced, and 4 single nucleotide polymorphisms (SNPs) were identified in the

**TABLE 1. Activity of sEH in Subcellular Fractions of Rat Kidney Cortex Determined by <sup>3</sup>H-Trans Stilbene Oxide Hydrolysis**

Cell Fraction	Strain (Each: n=4)	sEH Activity (pmol/min/mg)	SEM	P
Cytosol	SHR (Heid)	81.03	3.53	<0.001
	WKY (Heid)	642.77	28.68	
	WKY (CRiv)	135.20	7.24	<0.001
	SHR (CRiv)	329.29	30.32	
Peroxisome	SHR (Heid)	19.94	5.51	<0.01
	WKY (Heid)	136.13	22.87	
	WKY (CRiv)	74.15	11.39	NS
	SHR (CRiv)	84.69	10.61	



**Figure 4.** Association of EPHX2 alleles with blood pressure and sEH protein abundance in the male F<sub>2</sub> progeny of a cross between SHR (Heid) and WKY (Heid). No relationship was observed between inheritance of SHR (Heid) alleles of EPHX2 and systolic, mean, or diastolic blood pressure (systolic BP is shown,  $r=0.03$ ,  $P=NS$ ). A significant negative correlation was observed between inheritance of SHR (Heid) alleles of EPHX2 and relative abundance of sEH protein in kidney ( $r=0.76$ ,  $P<0.0001$ ).

WKY (Heid) sequence (For details, see supplemental material in an online supplement available at <http://www.hypertensionaha.org>). The SHR (Heid) sequence was identical to the National Center for Biotechnology Information (NCBI) reference sequence (NM\_022936) derived from the Sprague-Dawley rat. The variant bases, using NM\_022936 as a reference, were as follows: 405 g/a, 560 g/a, 780 c/t, and 1465 g/a. Three of the SNPs (405, 780, and 1465) were nonsynonymous base substitutions (see supplemental material). The rat reference sequence shares identical base residues at the polymorphic loci as the NCBI mouse EPHX2 reference sequence (NM\_007940), and consequently we consider the rat reference sequence to represent the ancestral allele. Resequencing of Charles River strains indicated that SHR (Heid) and WKY (CRiv) possessed identical alleles of EPHX2, whereas WKY (Heid) and SHR (CRiv) shared identical alleles containing the 4 SNPs. Thus, a close association exists in the inbred parental strains between gene sequence, protein abundance, and enzyme activity, regardless of the presence of hypertension.

To further investigate the possible association between sEH and hypertension, we examined the inheritance of alleles of the EPHX2 gene in the F<sub>2</sub> progeny ( $n=83$ ) of a cross between SHR (Heid) and WKY (Heid). Our results indicate that there was no relationship between either diastolic or systolic blood pressure and inheritance of 0, 1, or 2 variant alleles (Figure 4, left). In contrast, a strong inverse relationship between inheritance of the ancestral allele and sEH protein abundance in kidney was observed (Figure 4, right).

A blood pressure-lowering effect of the selective inhibitor of sEH activity, 1,3-dicyclohexylurea (DCU),<sup>14</sup> was previously detected by tail cuff manometry in SHR (CRiv), but not in WKY (CRiv).<sup>15</sup> We reanalyzed the effect of administration of this inhibitor using SHR (CRiv) and WKY (CRiv) animals chronically implanted with telemetry instrumentation, which allowed continuous, highly precise measurement capable of revealing small effects of drug treatment on blood pressure in freely behaving animals housed in their home cages. Table 2 indicates the observed effects on blood pressure and heart rate as a result of each of the 2 treatment regimens tested (daily drug treatment and single dose treatment). We found that treatment of SHR (CRiv) with DCU resulted in a modest reduction in systolic blood pressure from  $166.5 \pm 1.7$  to  $157.3 \pm 0.7$  mm Hg ( $P<0.001$ , single drug treatment). Similarly treated WKY (CRiv) animals also responded with a small reduction in systolic blood pressure from  $111.3 \pm 0.6$  to  $108.7 \pm 0.4$  mm Hg ( $P<0.001$ ). Although the effect of DCU

on blood pressure in SHR (CRiv) was the largest effect observed, it was substantially less than previously reported by tail-cuff manometry.<sup>15</sup>

## Discussion

EETs can have powerful effects on renal vascular regulation<sup>18,19</sup> by acting as an endothelium-dependent hyperpolarizing factor<sup>9</sup> and on renal tubular function by modifying transepithelial ion flux.<sup>20–22</sup> This suggests that the enzyme involved in clearance of EETs,<sup>10</sup> sEH, may have an important role in regulating EET levels and may therefore be an important mediator of cardiorenal mechanisms integrating blood pressure regulation. Our observation of marked difference between SHR (Heid) and WKY (Heid) in renal expression of the RNA encoding this protein sparked interest in pursuing the possible relevance of this gene to the contrasting renal function that determines hypertension status across these 2 strains. Our initial results confirmed that gene expression differences were sustained at the level of protein abundance. However, around the time this work was completed, a report emerged that sEH message and protein abundance was the inverse of the observations we had made when SHR and WKY animals from Charles River were examined.<sup>15</sup> Our present results indicate that this cross-strain difference is not erroneous. The most likely explanation appears to be that, during the process of inbreeding from the outbred progenitor stocks of Wistar rats from which SHR and WKY are derived, several lines were distributed after various levels of inbreeding and in which variant alleles affecting EPHX2 expression became differentially fixed.<sup>23</sup>

It is unclear which and how many loci affect EPHX2 expression. Our results showing association between sequence variation in this gene and abundance and activity of the gene product suggest that the EPHX2 locus influences the level of gene expression. Studies in F<sub>2</sub> animals show a broad range of expression level in animals inheriting one or more of the variant EPHX2 alleles, suggesting that other loci may influence the level of expression of EPHX2. Indeed, there is evidence that activators of peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) can strongly induce sEH activity.<sup>24</sup> Thus any differential influence of this transcription factor across the rat strains could influence sEH gene expression and activity. Further studies of how polymorphism affects the kinetics of enzyme activity and its substrate preferences may be required to fully interpret the implications of the sequence variation identified. Regulation of expression does not appear to be strongly influenced by feedback mechanisms respond-

**TABLE 2. Effects of Single Dose or Daily DCU Treatment on Blood Pressure and Heart Rate in Male SHR and WKY Rats Obtained From Charles River**

	Injection	WKY		SHR	
		Control (1 Day)	Injection (4 Days)	Control (1 Day)	Injection (4 Days)
Daily treatment (n=1 per group)					
Heart rate, bpm	Vehicle	317.6±1.1	319.0±0.5	330.1±1.3	317.8±0.4
	DCU	350.1±2.6	337.4±0.5	328.5±2.1	333.5±0.5
Diastolic pressure, mm Hg	Vehicle	80.8±0.3	81.6±0.1	113.3±0.3	110.1±0.1
	DCU	82.4±0.6	80.4±0.1	109.0±0.7	107.8±0.2
Mean pressure, mm Hg	Vehicle	92.6±0.3	93.0±0.1	144.0±0.4	141.3±0.2
	DCU	95.8±0.6	93.7±0.2	144.0±0.7	138.0±0.2
Systolic pressure, mm Hg	Vehicle	102.8±0.3	102.7±0.1	174.4±0.4	172.5±0.5
	DCU	110.4±2.5	106.1±0.5	170.8±0.8	168.0±0.5
Single treatment (n=4 per group)					
Heart rate, bpm	Vehicle	326.1±0.5	329.9±0.3	330.9±0.5	334.9±0.3
	DCU	337.7±0.6	333.9±0.3	322.4±0.5	325.9±0.3
Diastolic pressure, mm Hg	Vehicle	87.3±0.2	84.6±0.1	106.0±0.2	109.2±0.1
	DCU	88.0±0.2	86.1±0.1	104.7±0.3	102.5±0.1
Mean pressure, mm Hg	Vehicle	97.8±0.2	96.1±0.1	133.1±0.4	134.8±0.2
	DCU	99.4±0.2	96.8±0.2	133.9±0.6	129.6±0.3
Systolic pressure, mm Hg	Vehicle	109.9±0.7	109.7±0.4	159.5±0.9	159.4±0.4
	DCU	111.3±0.6	108.7±0.4	166.5±1.7	157.3±0.7

Measurements were made for 1 day prior to drug administration (Control) and for 4 days following (Injection).

All values are mean±SEM.

ing to the effect of polymorphism on function of the enzyme. Our observations that both expression level and activity are increased in animals inheriting the variant allele strongly indicate that negative feedback inhibition resulting from increased enzyme activity is ineffective in adjusting expression to achieve activity of sEH at the same levels as present in animals inheriting 2 ancestral alleles.

Our results indicate that sequence variation in EPHX2 and the associated phenotypes we report here are unlikely to be primary determinants of blood pressure levels in SHR. The existence of contrasting alleles in SHR substrains that share similarly elevated blood pressure levels argues against such a role. The absence in the F<sub>2</sub> progeny of any association between EPHX2 alleles inherited from the hypertensive strain and blood pressure is further evidence against such a role. The absence of correlation between sEH abundance and blood pressure in the F<sub>2</sub> progeny further extends this evidence. It is possible that there are other differences in the genetic background between SHR (Heid) and SHR (CRiv) that may permit a greater role for sEH activity in hypertension than is evident from our studies. For example, if the genetic background of SHR (CRiv) includes other variation that tends to enhance an effect of increased sEH activity on blood pressure, then this effect might be discernible in an F<sub>2</sub> progeny derived from a SHR (CRiv) parent. In outbred Sprague-Dawley rats 38-fold differences in sEH activity have been observed.<sup>25</sup> However, blood pressure measurements in outbred Sprague-Dawley rats have been made in numerous laboratories without evidence coming to light of broad

differences in interindividual blood pressure. Substantial differences in renal and hepatic sEH abundance in control Sprague-Dawley rats in the absence of significant variation in blood pressure has also been observed.<sup>16</sup> This evidence suggests that outbred Sprague-Dawley rats, in addition to SHR substrains, may contain more than one allele influencing sEH abundance without influence on basal blood pressure.

Our use of the highly precise, direct intra-arterial radiotelemetry measurement of blood pressure in unrestrained Charles River SHR animals (expressing high levels of sEH activity) in experiments to examine the effect of pharmacological inhibition of sEH suggests a modest role of sEH in determining blood pressure in these animals. However, several important caveats must be considered. First, DCU is an early lead compound in the development of sEH inhibitors. It is poorly soluble in DMSO, oil, and water, and efficacy may be influenced by physical properties of the compound determined during manufacture (eg, crystal size). Second, although we have identified interesting sequence variation and associated phenotypes, there may be still further properties of sEH relevant to blood pressure regulation and endogenous eicosanoid metabolism that we are unaware of and that are not influenced by the sequence variation. Such properties may still permit useful antihypertensive effects to result from the pharmacological antagonism of this enzyme on epoxyeicosanoid metabolism.

### Perspectives

The broad involvement of eicosanoids in cardiovascular function suggests that major biological consequences may

result from the gene polymorphism and associated alteration in tissue enzyme activity levels reported here. Among the additional phenotypes examined for association with EPHX2 variation in our F<sub>2</sub> progeny (heart rate, heart weight, body weight, and diastolic and mean arterial pressure), none were found to be affected by inheritance of the variant allele. Conceivably, the overlapping and redundant functions of the various pathways and products of arachidonic acid metabolism may permit the consequences of the sequence variation we have described to be tolerated without creation of obvious phenotypes. Further work examining the metabolism of eicosanoids, their role in cardiovascular function, and the effects on characteristics of recombinant proteins encoded by the ancestral and variant EPHX2 alleles may be required to gain further insight into the biological implications of the sequence variation reported here.

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