Inhibition of Soluble Epoxide Hydrolase Confers Cardioprotection and Prevents Cardiac Cytochrome P450 Induction by Benzo(a)pyrene

Mona E. Aboutabl, MSc,* Beshay N. M. Zordoky, MSc,*
Bruce D. Hammock, PhD,† and Ayman O. S. El-Kadi, PhD*

Abstract: We recently demonstrated that benzo(a)pyrene (BaP) causes cardiac hypertrophy by altering arachidonic acid metabolism through the induction of the expression of CYP ω-hydroxylases and soluble epoxide hydrolase (sEH) enzymes. The inhibition of CYP ω-hydroxylase enzymes partially reversed the BaP-induced cardiac hypertrophy. Therefore, it is important to examine whether the inhibition of sEH also confers cardioprotection. For this purpose, male Sprague-Dawley rats were injected intraperitoneally daily with either the sEH inhibitor 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS; 0.65 mg/kg), BaP (20 mg/kg), or the combination of BaP (20 mg/kg) and TUPS (0.65 mg/kg) for 7 days. Thereafter, the heart, liver, and kidney were harvested, and the heart to body weight ratio was measured. The expression of the hypertrophic markers, sEH, heme oxygenase-1, and CYP450 enzymes was determined. Our results demonstrate that BaP alone significantly induced the expression of sEH and CYP ω-hydroxylases in the heart, liver, and kidney tissues. Treatment with TUPS significantly reversed the BaP-mediated induction of the hypertrophic markers, completely prevented the increase in the heart to body weight ratio, and reduced the BaP-induced *CYP1A1, CYP1B1, CYP4F4,* and *CYP4F5* genes in the heart. The current study demonstrates the cardioprotective effect of sEH inhibitor, TUPS, against BaP-induced cardiac hypertrophy and further confirms the role of sEH and CYP450 enzymes in the development of cardiac hypertrophy.

Key Words: benzo(a)pyrene, cardiac hypertrophy, cytochrome P450, soluble epoxide hydrolase inhibitor, TUPS

(*J Cardiovasc Pharmacol*™ 2011;57:273–281)

INTRODUCTION

Polycyclic aromatic hydrocarbons are common environmental contaminants that have been highly associated with

the development of several cardiovascular diseases.[1] Human exposure to benzo(a)pyrene (BaP) may be continuous, where it exists in heavily polluted air, drinking water, smoked foods, and cigarette smoke.[2] It is also produced upon forest and rangeland fires, volcanic eruptions, aluminum smelting, waste incineration, motor vehicle operation, and coal tar distillation.[3,4] BaP is the prototypical example of the polycyclic aromatic hydrocarbons, which are known to activate the aryl hydrocarbon receptor (AhR).[5] Several epidemiological and clinical studies linked the exposure to AhR ligands and AhR activation to the pathogenesis of different cardiovascular diseases.[1,3] Moreover, activation of the AhR signaling pathways has been previously correlated with the development of cardiac hypertrophy.[1] The potent AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) caused cardiac hypertrophy in chicken embryo that was characterized by increase in heart size.[6] This was accompanied by induction in cardiac muscle proteins and hypertrophic genes.[1]

We previously demonstrated that AhR ligands, 3-methylcholanthrene (3-MC) and BaP, induced the expression of the hypertrophic markers, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) and the heart to body weight ratio, reflecting the induction of cardiac hypertrophy in male Sprague-Dawley rats.[7] In H9c2 cardiomyoblasts, AhR ligands, TCDD, and β-naphthoflavone caused a significant induction of ANP and BNP that was accompanied by an increase in the cells surface area, which further confirmed the hypertrophic effect of these AhR ligands.[8]

Different mechanisms have been proposed for the role of AhR in the development of cardiac hypertrophy.[1] The first mechanism involves the induction of reactive oxygen species. The second mechanism involves the induction of hypertrophic genes like ANP, BNP, and beta-myosin heavy chain by AhR ligands.[9] In addition, AhR ligands were found to alter the metabolism of arachidonic acid and to contribute in the pathogenesis of hypertrophy through increasing the 20-hydroxyeicosatetraenoic acid (20-HETE) to total epoxyeicosatrienoic acids (EETs) ratio.[7]

EETs are the major cardioprotective products of arachidonic acid metabolism by CYP450. Also, EETs are substrates of the soluble epoxide hydrolase enzyme (sEH), which is the enzyme responsible for the conversion of EETs to the biologically less active dihydroxyeicosatrienoic acids (DHETs) and thus reducing their beneficial cardiovascular effect.[10] Moreover, the gene encoding sEH enzyme, *EPHX2,* was found to be significantly induced in different models of

cardiac hypertrophy such as, 3-MC–induced and BaP-induced cardiac hypertrophy,[7] isoproterenol-induced cardiac hypertrophy, spontaneously hypertensive rats with heart failure,[11] and angiotensin II–induced hypertrophy.[12] The coincident upregulation of *EPHX2* together with the development of cardiac hypertrophy in different animal models suggested its involvement in the development of cardiac hypertrophy. Therefore, sEH inhibition is considered a new potential therapeutic target in the treatment of different cardiovascular diseases. The cardioprotective mechanism of sEH inhibitors involves inhibiting the degradation of EETs and hence blocking of nuclear factor kappa B (NF-κB) activation.[13,14]. Xu et al[13] demonstrated that sEH inhibitors prevented and reversed cardiac hypertrophy in murine model of chronic pressure overload–induced cardiac hypertrophy. In addition, treatment with an sEH inhibitor, 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS), decreased the left ventricular hypertrophy and prevented angiotensin II–induced cardiac hypertrophy in rats.[12]

Recently, we have demonstrated that BaP induces cardiac hypertrophy through the induction of CYP ω-hydroxylases, enzymes responsible for the formation of 20-HETE, such as CYP1A1, CYP1B1, CYP2E1, CYP4F4 and CYP4F5 and sEH enzyme in the hearts of male Sprague-Dawley rats.[7] This resulted in alteration in the arachidonic acid metabolism and significant increase in the 20-HETE to total EETs ratio.[7] Several studies demonstrated the association and the potential contribution of higher levels of the cardiotoxic metabolite 20-HETE and lower levels of the cardioprotective EETs in the development of cardiac hypertrophy.[7,15,16] Interestingly, we found that treatment with the CYP ω-hydroxylase inhibitor N-hydroxy-N′-(4-butyl-2-methylphenyl) formamidine (HET0016) partially reversed the BaP-induced cardiac hypertrophy through inhibition of 20-HETE formation.[7] Therefore, it is important to examine whether the inhibition of sEH confers cardioprotection against BaP-induced cardiac hypertrophy.

MATERIALS AND METHODS

Animals

The investigation follows the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996). All experimental animal procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague-Dawley rats weighing 300–350 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were allowed free access to food and water all over the treatment period.

Chemicals and Reagents

BaP, 4-hydroxybenzophenone, and anti-goat IgG with horseradish peroxidase secondary antibody were purchased from Sigma-Aldrich Chemical Co (St Louis, MO). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-capacity complementary DNA (cDNA) Reverse Transcription Kit along with SYBR Green SuperMix were purchased from Applied Biosystems (Foster City, CA). Real-time polymerase chain reaction (PCR) primers were synthesized by Integrated

DNA Technologies Incorporation (San Diego, CA) according to previously published sequences. 14,15-EET and 14,15-DHET were obtained from Cayman Chemical (Ann Arbor, MI). TUPS was synthesized by Paul Jones (University of California, Davis) as described in Tsai et al.[17] Acrylamide, N′N′-bis-methylene-acrylamide, β-mercaptoethanol, ammonium persulfate, glycine, pure nitrocellulose membrane (0.45 μm), and N,N,N′,N′-Tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescent Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Goat anti-rat CYP1A1, rabbit anti-rat CYP1B1, and rabbit anti-rat CYP2E1 polyclonal primary antibodies were purchased from Oxford Biomedical Research (Oxford, MI), BD Gentest (Woburn, MA), and Abcam (Cambridge, UK), respectively. Goat anti-rabbit IgG with horseradish peroxidase secondary antibody and actin goat polyclonal primary antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). All other chemicals were purchased from Fisher Scientific Co (Toronto, ON, Canada).

Experimental Design

Animals were injected intraperitoneally with 0.65 mg/kg of sEH inhibitor, TUPS, 20 mg/kg of BaP, or 20 mg/kg of BaP with 0.65 mg/kg of TUPS daily for 7 days (n = 4). Weight-matched controls received the same volume of corn oil and saline daily for 7 days (n = 4). BaP was dissolved in corn oil, whereas TUPS stock solution was dissolved in dimethyl sulfoxide, and then further diluted in saline to reach the desired concentration. Animals were euthanized under isoflurane anesthesia, 24 hours after the last injection. Heart, kidneys, and liver were excised, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA Extraction and cDNA Synthesis

Total RNA from the frozen heart, kidney, and liver tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and quantified by measuring the absorbance at 260 nm. The quality of the isolated RNA was determined by measuring the 260:280 ratio. Thereafter, first-strand cDNA was synthesized by using the High-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol.

Relative Gene Expression Analysis by Real-time PCR

The relative gene expression was determined by real-time PCR using the ABI Prism 7500 System (Applied Biosystems) according to the manufacturer's protocol. The primers used in the current study were previously published[18–23] and are listed in Table 1. A melting curve was determined at the end of each cycle to confirm the specificity of the primers and the purity of the PCR product. Thereafter, real-time PCR data were analyzed using the relative gene expression method as described previously.[24] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control and the untreated control was used as the calibrator when the change of gene expression by TUPS, BaP, and BaP + TUPS is being studied.

TABLE 1. Primer Sequences Used for Real-time PCRs

Gene	Forward Primer	Reverse Primer
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
CYP4F5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
EPHX2	CACATCCAAGCCACCAAGCC	CAGGCCTCCATCCTCCAG
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
HO-1	AGAGTTTCCGCCTCCAACCA	CGGGACTGGGCTAGTTCAGG
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

Microsomal Preparation and Western Blot Analysis

Preparation of heart microsomal protein was performed as described previously.[25] Lowry method was used for measuring heart microsomal protein concentration using bovine serum albumin as a standard.[26] Western blot analysis was carried out using a previously described method.[27] In brief, heart microsomal protein (20 μg) from each treatment group was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were electrophoretically transferred to pure nitrocellulose membrane. A blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (Tris-buffered saline), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween-20 was added to the protein blots for overnight blocking at 4°C. Thereafter, the blots were incubated with a primary polyclonal goat anti-rat CYP1A1 antibody, rabbit anti-rat CYP1B1, or rabbit anti-rat CYP2E1 for 4 hours at 4°C. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for CYP1A1 or goat anti-rabbit IgG secondary antibody for CYP1B1 and CYP2E1 was carried out for 2 hours at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences). The intensity of the protein bands was measured relative to the signals obtained for actin using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Microsomal Incubation and Measurement of sEH Activity

Heart microsomes (0.5 mg protein/mL) of control or animals treated with BaP for 7 days were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH 7.4) at 37°C in a shaking water bath (50 revolutions per minute). Heart microsomes were pre-equilibrated for 5 minutes. Thereafter, heart microsomes from both groups were incubated with 1 μM TUPS for 5 minutes followed by incubation with 10 μM 14,15-EET for 30 minutes. The reaction was stopped by the addition of 600 μL of ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. 14,15-EET and its diol metabolite, 14,15-DHET, were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). Extracted 14,15-EET and 14,15-DHET were analyzed using the liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (Waters Micromass ZQ 4000 spectrometer) method as described previously.[28] The mass spectrometer runs in negative ionization mode with single-ion recorder acquisition. The nebulizer gas was acquired from an in-house high-purity nitrogen source. The temperature of the source was set at 150°C, and the voltages of the capillary and the cone were 3.51 kV and 25 V, respectively. The samples (10 μL) were separated on reverse-phase C18 column (Kromasil, 250 × 3.2 mm) using linear gradient mobile phase system water/acetonitrile with 0.005% acetic acid as mobile phase at flow rate of 0.2 mL/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 minutes, increased to 100% acetonitrile in 5 minutes, and held for 5 minutes. sEH activity was determined by the 14,15-DHET:14,15-EET ratio.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Comparative gene and protein expression and metabolite formation across groups were analyzed using a 1-way analysis of variance followed by a Student–Newman–Keuls post hoc comparison. A result was considered statistically significant when $P < 0.05$.

RESULTS

Effect of the sEH Inhibitor, TUPS, Treatment on the Hypertrophic Markers and the Increase in Heart to Body Weight Ratio Induced by BaP

To investigate whether the inhibition of sEH confers cardioprotection in BaP-treated rats, we measured the cardiac gene expression of the hypertrophic markers, ANP and BNP relative to BaP-treated rats. BaP treatment caused a significant induction of both hypertrophic markers ANP and BNP by 2.3- and 2.5-fold, respectively (Fig. 1A). On the other hand, TUPS treatment significantly decreased the BaP-mediated induction of ANP and BNP by 4- and 1.4-fold, respectively (Fig. 1A). In addition, TUPS treatment alone did not alter the gene expression of ANP or BNP. Moreover, BaP significantly increased the heart weight to body weight ratio by 8.1%,
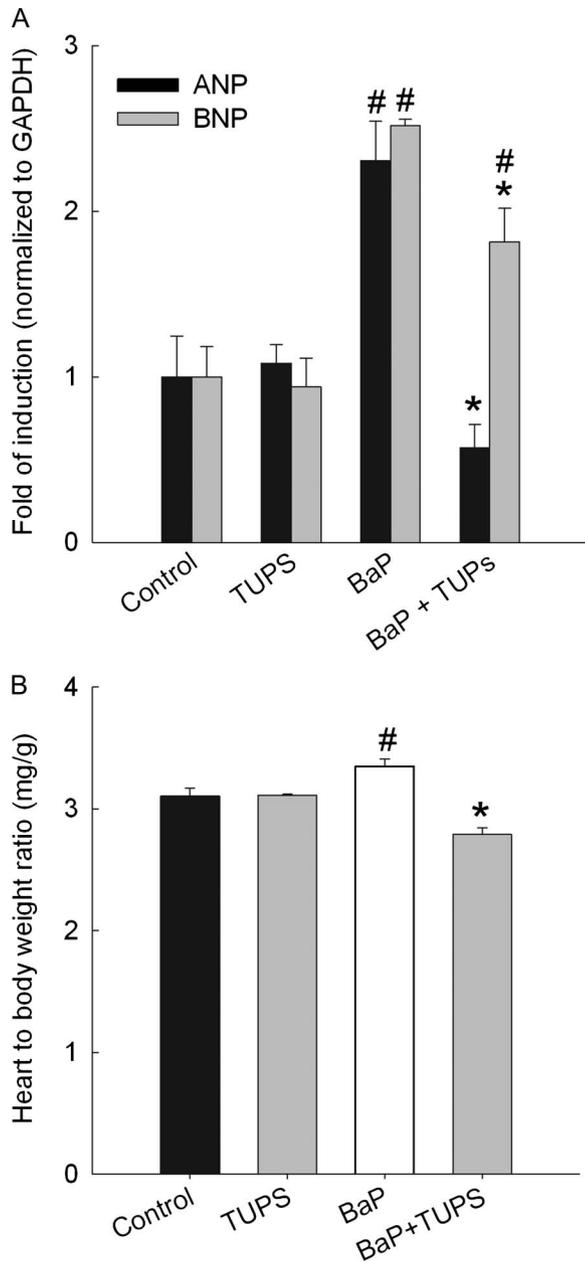
FIGURE 1. Effect of TUPS treatment on the hypertrophic markers induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. A, Gene expression of the hypertrophic genes, ANP and BNP was determined in the heart. B, Heart to body weight ratio (in milligrams per gram) was determined for each animal after 7 daily IP injections of TUPS, BaP, BaP + TUPS, or corn oil. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

FIGURE 2. Effect of TUPS treatment on gene expression of the *CYP1* family induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Total RNA was isolated from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP + TUPS–treated rats, and the relative gene expression of (A) *CYP1A1* and (B) *CYP1B1* was determined by real-time PCR. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

Effect of TUPS Treatment on the Changes in the *CYP450* Gene Expression Induced by BaP

To examine the effect of TUPS on BaP-mediated induction of *CYP450* genes, total RNA was extracted from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP + TUPS–treated rats. Thereafter, the expression of different *CYP450* genes was measured using reverse transcription followed by real-time PCR.

Figure 2A shows the effect of treatment with TUPS on BaP-induced *CYP1A1* gene expression. Our results demonstrate that BaP treatment caused a significant increase in *CYP1A1* gene expression in heart, kidney, and liver by about 400-, 440-, and 2240-fold respectively (Fig. 2A). On the other hand, TUPS treatment caused significant inhibition of BaP-induced *CYP1A1* gene by 27.8% in the heart compared with the BaP treatment. In the kidney and liver, CYP1A1 messenger RNA (mRNA) levels

whereas treatment with TUPS completely prevented the BaP-mediated increase in the heart weight to body weight ratio. Furthermore, no significant difference was observed between the control and the TUPS treatment alone (Fig. 1B).
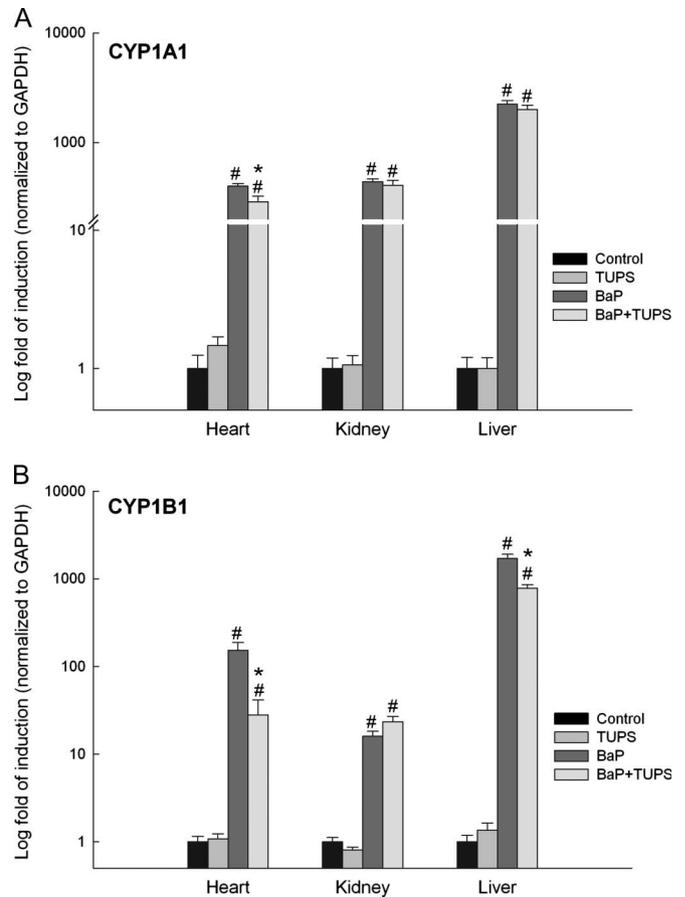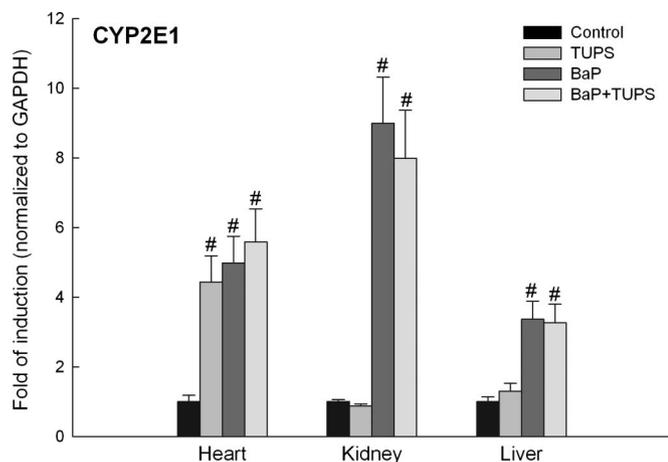
FIGURE 3. Effect of TUPS treatment on gene expression of the CYP2E1 induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Total RNA was isolated from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP+TUPS–treated rats, and the relative gene expression of CYP2E1 was determined by real-time PCR. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

were not altered in response to TUPS treatment when compared with the BaP treatment (Fig. 2A).

Figure 2B shows that BaP treatment significantly induced CYP1B1 gene expression in heart, kidney, and liver by 153-, 16-, and 2000-fold, respectively. Interestingly, CYP1B1 mRNA level was significantly decreased in both the heart and liver after treatment with TUPS by 82% and 54.2%, respectively, compared with the BaP treatment. With respect to the kidney, CYP1B1 mRNA levels were not significantly altered compared with the BaP-treated rats (Fig. 2B).

CYP2E1 mRNA levels were significantly increased in heart, kidney, and liver of rats treated with BaP by 5-, 9-, 3.4-fold, respectively. In the heart, treatment with TUPS alone resulted in a significant increase in CYP2E1 gene expression by 4.4-fold. However, TUPS treatment did not significantly alter the gene expression of CYP2E1 induced by BaP in all the tissues tested (Fig. 3).

With regard to CYP4 family, CYP4F4 gene expression was significantly increased by 1.7-, 7.5-, and 23.8- fold in the heart, kidney, and liver of BaP-treated rats. Interestingly, the gene expression of CYP4F4 was significantly reduced by 63% in the heart, but not the kidney or liver of rats treated with TUPS (Fig. 4A). Similar to CYP4F4, CYP4F5 gene expression was induced in the heart, kidney, and liver of BaP-treated rats by 2.3-, 3.4, and 1.9-fold, respectively. However, TUPS treatment significantly reduced the BaP-mediated induction of CYP4F5 mRNA by 54% in the heart and 51% in the kidney but not the liver (Fig. 4B).

Effect of TUPS Treatment on BaP-induced EPHX2 Gene Expression

The enzyme sEH is a major determinant of EET level; therefore, we determined the effect of TUPS treatment on the
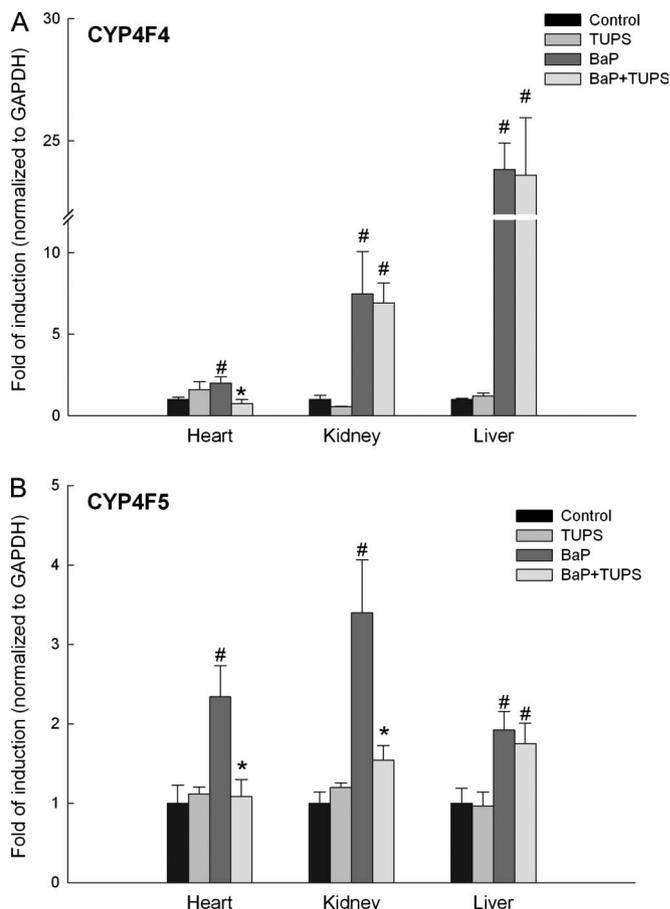

FIGURE 4. Effect of TUPS treatment on gene expression of the CYP4 family induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Total RNA was isolated from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP+TUPS–treated rats, and the relative gene expression of (A) CYP4F4 and (B) CYP4F5 was determined by real-time PCR. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

expression of EPHX2 gene. Total RNA was extracted from the heart, kidney, and liver of control, BaP-treated, and BaP + TUPS–treated rats. Thereafter, the expression of EPHX2 gene was measured using reverse transcription followed by real-time PCR. Our results show that BaP treatments caused a significant induction of EPHX2 gene expression in the heart, kidney, and liver by 3.9-, 3.1-, 2.9-fold, respectively (Fig. 5). On the other hand, treatment with TUPS did not alter the BaP-induced EPHX2 gene expression in all tissues tested.

Effect of TUPS Treatment on BaP-induced Heme Oxygenase-1 Gene Expression

The gene expression of heme oxygenase-1 (HO-1) was significantly induced by 2.4-, 2.2-, and 2.3-fold in the heart, kidney, and liver of BaP-treated rats. Interestingly, treatment
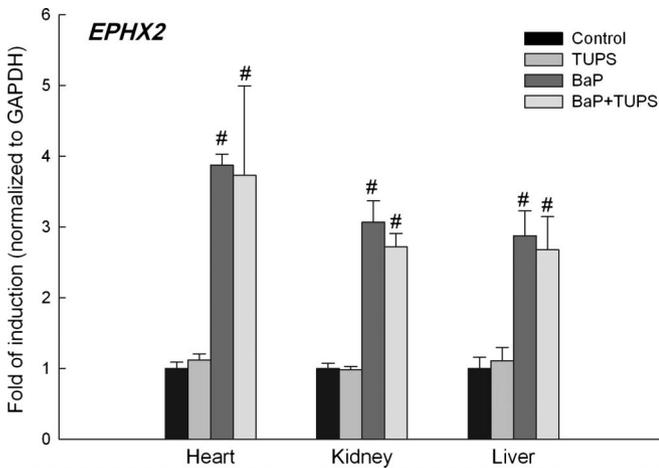
FIGURE 5. Effect of TUPS treatment on gene expression of *EPHX2*-induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Total RNA was isolated from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP+TUPS–treated rats, and the relative gene expression of *EPHX2* was determined by real-time PCR. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

with TUPS alone significantly reduced HO-1 gene expression in the kidney by 40% fold. In addition, cotreatment of TUPS with BaP significantly decreased the BaP-mediated increase of HO-1 by 62%, 48%, and 37% in the heart, kidney, and liver, respectively (Fig. 6).
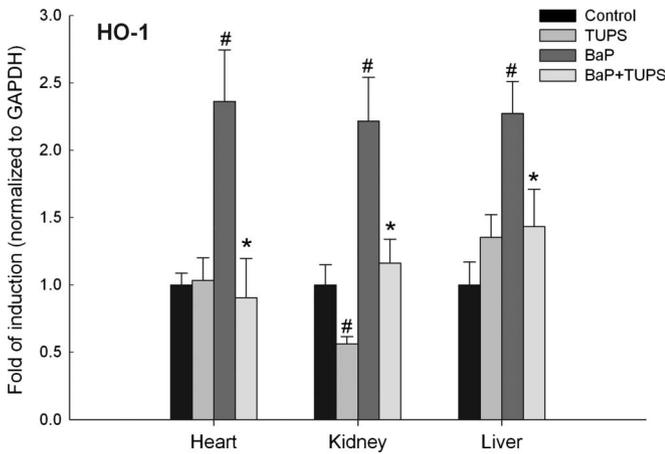

FIGURE 6. Effect of TUPS treatment on gene expression of HO-1 induced by BaP. Sprague-Dawley rats received daily injections of TUPS (0.65 mg/kg), BaP (20 mg/kg), or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Total RNA was isolated from the heart, kidney, and liver of control, TUPS-treated, BaP-treated, and BaP + TUPS–treated rats, and the relative gene expression of HO-1 was determined by real-time PCR. Results are presented as mean ± standard error of the mean (n = 4). #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.
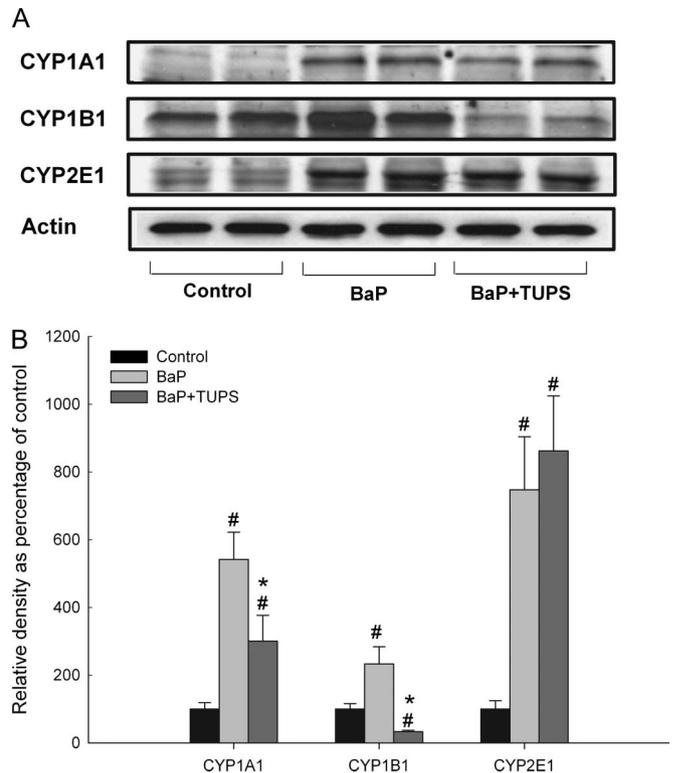

FIGURE 7. Effect of TUPS treatment on the protein level of CYP450 induced by BaP. Sprague-Dawley rats received daily injections of BaP (20 mg/kg) or BaP (20 mg/kg) plus TUPS (0.65 mg/kg) for 7 days while weight-matched controls received the same volume of corn oil and saline. Heart microsomal protein was isolated from the hearts of control, BaP-treated, TUPS + BaP–treated rats. Thereafter, 20 μg of microsomal protein was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. CYP1A1, CYP1B1, and CYP2E1 proteins were detected using the enhanced chemiluminescence method. This experiment was repeated 3 times; 1 representative result is shown. The graph represents the relative normalized amount of CYP450 protein (mean ± standard error of the mean, n = 3), which was calculated by dividing the levels of CYP450 by the level of actin in the same sample, and the results are expressed as percentage of the control values taken as 100%. #$P < 0.05$ compared with control. *$P < 0.05$ compared with BaP-treated rats.

Effect of TUPS Treatment on the Changes in the CYP450 Protein Expression Mediated by BaP

To investigate whether the induction of *CYP450* gene expression by BaP is further translated to functional protein, microsomal protein was prepared from the hearts of control, BaP-treated, and BaP + TUPS–treated rats. Thereafter, CYP1A1, CYP1B1, and CYP2E1 protein levels were determined using Western blot analysis. Our results showed that BaP treatment significantly increased the protein level of CYP1A1, CYP1B1, and CYP2E1 by 542%, 233%, and 747%, respectively (Fig. 7). On the other hand, TUPS treatment caused a significant inhibition of BaP-mediated induction of CYP1A1 and CYP1B1 protein expression relative to the BaP group (Fig. 7). However, TUPS treatment did not alter
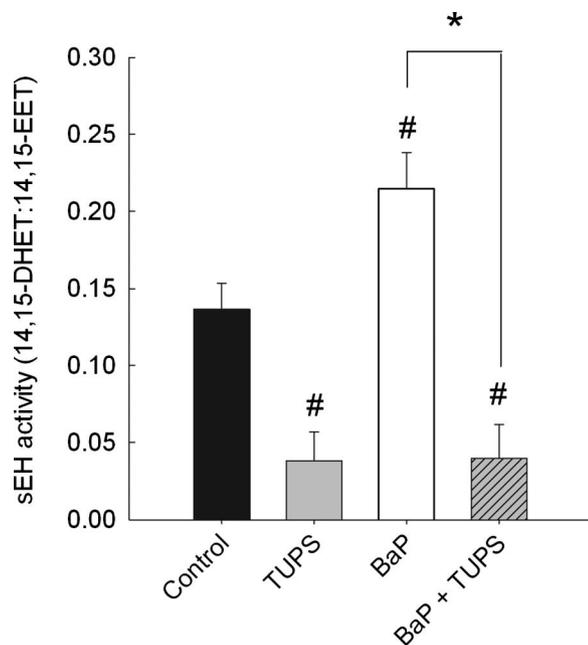
FIGURE 8. Effect of TUPS treatment on sEH activity. Heart microsomes from control or BaP-treated rats were incubated with 1 μM TUPS for 5 minutes followed by incubation with 10 μM 14,15-EET. The reaction lasted for 30 minutes and was terminated by the addition of ice-cold acetonitrile. 14,15-EET and 14,15-DHET were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. sEH activity was calculated as the ratio of 14,15-DHET:14,15-EETs. Results are presented as mean ± standard error of the mean (n = 4). *$P < 0.05$ compared with control, #$P < 0.05$ compared with heart microsomes from animals treated with BaP in the absence of TUPS.

BaP-induced CYP2E1 protein expression compared with BaP-treated group. Unfortunately, we were unable to measure the protein level of any of the CYP4F enzymes because the antibodies for rat CYP4F were not commercially available.

Effect of TUPS on the Changes in the sEH Activity Mediated by BaP

To investigate the effect of sEH inhibitor TUPS on BaP-induced sEH activity, heart microsomes from control and BaP-treated rats were incubated with 1 μM TUPS for 5 minutes followed by incubation with 14,15-EET as described under Materials and Methods. Thereafter, sEH activity was assessed by calculating the ratio of the 14,15-DHET to the 14,15-EET formed in the heart microsomes of control and BaP-treated rats. Our results show that sEH activity was induced by 157% in the heart microsomes of BaP-treated rats (Fig. 8). In addition, TUPS significantly decreased the 14,15-DHET:14,15-EET ratio from 0.136 to 0.038 in the control group. Similarly, in BaP-treated group, TUPS significantly decreased the 14,15-DHET:14,15-EET ratio from 0.215 to 0.039 (Fig. 8).

DISCUSSION

The cardioprotective effect of sEH inhibitors emerges from their potential inhibition of the degradation of EETs and other epoxy fatty acids and hence enhancing the cardioprotective effect of EETs. In this context, several sEH inhibitors have been synthesized, among which the newly discovered, 1,3-disubstituted ureas, carbamates, and amides are considered cutting-edge discoveries of sEH inhibitors. They are potent and stable transition-state inhibitors of sEH that act through inhibition of the carboxy-terminal domain that possess the epoxide hydrolase activity of the sEH enzyme.[29] Among the different sEH pharmacophores discovered, the urea pharmacophore seemed to be the most potent, competitive, and tight-binding inhibitors of sEH.[30] The sEH of choice in this study is TUPS, which comprises a highly potent urea pharmacophore.[31]

In the current study, we investigated the effect of treatment of TUPS in the protection of BaP-induced cardiac hypertrophy. Our results demonstrated that TUPS significantly decreased the BaP-mediated induction of the hypertrophic markers ANP and BNP and the increase in the heart to body weight ratio. In agreement with our results, it has been previously demonstrated that TUPS decreases the left ventricular hypertrophy, cardiomyocyte size, heart to body weight ratio, and the hypertrophic markers ANP and beta-myosin heavy chain in angiotensin II–induced cardiac hypertrophy, reflecting its cardioprotective effect.[12] Furthermore, sEH inhibitors were reported to prevent the development of cardiac hypertrophy in thoracic aortic constriction murine model.[13] Therefore, our study provides a new model in which TUPS is shown to protect against cardiac hypertrophy.

TUPS exerts its cardioprotective effect mainly through inhibiting the degradation of EETs to the less biologically active DHETs, which results in higher EETs level.[12] EETs play an important role in the inhibition of NF-κB, which is a downstream target of several signaling pathways implicated in cardiac hypertrophy such as angiotensin II, α-adrenergic stimulation, PI3K/Akt, ras, P38, MEKK1/4, PKC, and gp130 pathways. In addition to NF-κB inhibition, EETs have several other downstream targets that may explain their cardioprotective effect; EETs activate the p42/p44 MAPK pathway, ATP-sensitive potassium channels, and protein kinase A (PKA)-dependent signaling pathway.[32–35]

We have previously demonstrated that the induction of cardiac hypertrophy in BaP-treated rats is due to the increased formation of the cardiotoxic metabolite 20-HETE compared with the total EETs formation.[7] The relative increase in 20-HETE to total EETs ratio was attributed to the increased expression of several CYP ω-hydroxylases and the induction of sEH.[7] Interestingly, we have previously demonstrated that the CYP ω-hydroxylase inhibitor HET0016 partially reversed the BaP-induced cardiac hypertrophy through inhibition of 20-HETE formation. In the current study, we investigated the other possible mechanism of cardioprotection through inhibiting the degradation of the cardioprotective EETs by the use of sEH inhibitor. We believe that increasing the EETs and decreasing the 20-HETE may share common downstream targets, which include the NF-κB signaling pathway as EETs inhibit NF-κB, whereas 20-HETE activates it.[36]

Due to the importance of CYP450 in the pathogenesis of cardiac hypertrophy, in the current study we investigated the effect of sEH inhibition on the expression of different CYP genes involved in BaP-induced cardiac hypertrophy. Our results

demonstrated that, BaP significantly induced the gene expression of *CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP4F4*, and *CYP4F5* in all the examined tissues. We previously demonstrated the induction of these enzymes in the heart tissue.[7] In addition, Denison and Whitlock[37] reported the significant induction of *CYP1A1* in response to exposure to 3-MC and BaP compared with its constitutively very low expression in most tissues. In agreement with our results, we recently demonstrated that treatment of H9c2 cells with TCDD and β-naphthoflavone for 48 hours caused a significant induction of *CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP2J3*, and *CYP4F4* gene expression.[8] In the current study, treatment with TUPS significantly reduced the BaP-induced CYP ω-hydroxylase enzymes including CYP1A1, CYP1B1, CYP4F4, and CYP4F5 in the heart tissue.

To investigate whether the alterations of *CYP450* gene expression is further translated into functional protein, we determined the protein expression of CYP1A1, CYP1B1, and CYP2E1. Our results show that the protein levels of CYP1A1, CYP1B1, and CYP2E1 were significantly increased in the heart of BaP-treated rats, whereas TUPS treatment significantly decreased the BaP-mediated induction of CYP1A1 and CYP1B1 proteins only. On the other hand, TUPS treatment did not alter the BaP-mediated induction of CYP2E1 protein expression. This could be attributed to the finding that TUPS alone caused a significant induction of CYP2E1 in the heart and the transcriptional and posttranscriptional regulation of CYP2E1.[38]

We have previously demonstrated the induction of *EPHX2* gene expression in BaP-mediated cardiac hypertrophy[7] and isoproterenol-induced cardiac hypertrophy.[16] Moreover, *EPHX2* gene expression was found to be induced in spontaneously hypertensive rats with heart failure.[11] In the current study, we assessed the gene expression of *EPHX2* in the heart, kidney, and liver of BaP-treated rats. Our results demonstrated that BaP significantly induced the gene expression of *EPHX2* in all the tissues tested. However, treatment with TUPS did not alter the induction of gene expression caused by BaP treatment.

In addition to the induction of the CYP ω-hydroxylases and sEH, treatment with BaP significantly induced HO-1 mRNA level in the heart, kidney, and liver. We previously demonstrated that treatment with BaP significantly induced the gene expression of HO-1 in the rat heart.[7] Several studies have shown that exposure to TCDD and other AhR ligands induces oxidative stress in different organs.[39] Moreover, subchronic treatment of C57BL6 mice with TCDD resulted in a significant increase in superoxide production in the kidney, heart, and aorta.[39] This could be explained by the fact that AhR ligands are potent inducers of oxidative stress,[40] which in turn results in the induction of HO enzyme to protect against oxidative insult and injury.[41] Interestingly, in the current study treatment with TUPS significantly attenuated the BaP-mediated increase in HO-1 in the heart, kidney, and liver through inhibiting the EETs degradation, which is known to possess antioxidant properties.[42] We have previously reported that induction of HO-1 is associated with the disruption of arachidonic acid metabolism due to its potential role in the degradation of the heme component of the CYP epoxygenases and CYP ω-hydroxylases. Therefore, it is expected that TUPS will restore the derailed arachidonic acid metabolism through inhibiting the BaP-induced HO-1.

To further confirm the effect of TUPS as sEH inhibitor, TUPS was incubated with heart microsomes from both control and BaP-treated rats in the presence of 14,15-EET, which is the best substrate for sEH. Our results show that BaP alone caused a significant increase in the 14,15-DHET:14,15-EET ratio, which is in accordance with its effect on sEH mRNA level. On the other hand, incubation with TUPS caused a significant inhibition of 14,15-DHET:14,15-EET ratio. Interestingly, TUPS did not completely abolish the DHET formation because there was around 30% remaining DHETs formed in heart microsomes of both control and BaP-treated rats. This is in agreement with our previously published results demonstrating that sEH inhibitor, trans-4-[4-(3-Adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (tAUCB), inhibits 70% of the conversion of EETs to DHETs in rat heart microsomes.[43] This could be attributed to the involvement of other pathways like nonenzymatic conversion of EETs to DHETs. Taken together, these results confirm the role of TUPS in reversing the increased activity of sEH mediated by BaP treatment. In addition, TUPS did not change the gene expression of *EPHX2*, which further confirms its mechanism as a competitive inhibitor of the sEH enzyme.

In conclusion, in the current study we demonstrated for the first time that treatment with the sEH inhibitor TUPS significantly attenuated the BaP-induced cardiac hypertrophy. In addition, TUPS effects on BaP-induced CYP450 enzymes show some degree of cardioselectivity, which is thought to be secondary to its cardioprotective effect. Taking into account the accumulating evidence of AhR ligands–induced heart disease, sEH inhibition will provide a new therapeutic tool to protect the heart from their deleterious effects. However, more research is needed to further demonstrate the protective effect of sEH inhibitors against AhR ligands–mediated toxicity, especially in other organs such as the kidney and the liver.

REFERENCES

1. Korashy HM, El-Kadi AO. The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases. *Drug Metab Rev.* 2006;38:411–450.
2. Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo[a]pyrene and related hydrocarbons. *Drug Metab Rev.* 2001;33:1–35.
3. Burstyn I, Kromhout H, Partanen T, et al. Polycyclic aromatic hydrocarbons and fatal ischemic heart disease. *Epidemiology.* 2005;16:744–750.
4. Haritash AK, Kaushik CP. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J Hazard Mater.* 2009;169:1–15.
5. Ding YS, Trommel JS, Yan XJ, et al. Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. *Environ Sci Technol.* 2005;39:471–478.
6. Kanzawa N, Kondo M, Okushima T, et al. Biochemical and molecular biological analysis of different responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin in chick embryo heart and liver. *Arch Biochem Biophys.* 2004;427:58–67.
7. Aboutabl ME, Zordoky BN, El-Kadi AO. 3-Methylcholanthrene and benzo(a)pyrene modulate cardiac cytochrome P450 gene expression and arachidonic acid metabolism in male Sprague Dawley rats. *Br J Pharmacol.* 2009;158:1808–1819.
8. Zordoky BN, El-Kadi AO. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and beta-naphthoflavone induce cellular hypertrophy in H9c2 cells by an aryl hydrocarbon receptor-dependant mechanism. *Toxicol In Vitro.* 2010;24:863–871.

9. Borlak J, Thum T. PCBs alter gene expression of nuclear transcription factors and other heart-specific genes in cultures of primary cardiomyocytes: possible implications for cardiotoxicity. *Xenobiotica*. 2002;32:1173–1183.

10. Spector AA, Fang X, Snyder GD, et al. Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function. *Prog Lipid Res*. 2004;43:55–90.

11. Monti J, Fischer J, Paskas S, et al. Soluble epoxide hydrolase is a susceptibility factor for heart failure in a rat model of human disease. *Nat Genet*. 2008;40:529–537.

12. Ai D, Pang W, Li N, et al. Soluble epoxide hydrolase plays an essential role in angiotensin II-induced cardiac hypertrophy. *Proc Natl Acad Sci U S A*. 2009;106:564–569.

13. Xu D, Li N, He Y, et al. Prevention and reversal of cardiac hypertrophy by soluble epoxide hydrolase inhibitors. *Proc Natl Acad Sci U S A*. 2006;103:18733–18738.

14. Imig JD, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov*. 2009;8:794–805.

15. Chabova VC, Kramer HJ, Vaneckova I, et al. Effects of chronic cytochrome P-450 inhibition on the course of hypertension and end-organ damage in Ren-2 transgenic rats. *Vascul Pharmacol*. 2007;47:145–159.

16. Zordoky BN, Aboutabl ME, El-Kadi AO. Modulation of cytochrome P450 gene expression and arachidonic acid metabolism during isoproterenol-induced cardiac hypertrophy in rats. *Drug Metab Dispos*. 2008;36:2277–2286.

17. Tsai HJ, Hwang SH, Morisseau C, et al. Pharmacokinetic screening of soluble epoxide hydrolase inhibitors in dogs. *Eur J Pharm Sci*. 2010;40:222–238.

18. Bleicher KB, Pippert TR, Glaab WE, et al. Use of real-time gene-specific polymerase chain reaction to measure RNA expression of three family members of rat cytochrome P450 4A. *J Biochem Mol Toxicol*. 2001;15:133–142.

19. Kalsotra A, Anakk S, Boehme CL, et al. Sexual dimorphism and tissue specificity in the expression of CYP4F forms in Sprague Dawley rats. *Drug Metab Dispos*. 2002;30:1022–1028.

20. Hirasawa F, Kawagoe M, Arany S, et al. Styrene monomer primarily induces CYP2B1 mRNA in rat liver. *Xenobiotica*. 2005;35:1089–1099.

21. Rollin R, Mediero A, Fernandez-Cruz A, et al. Downregulation of the atrial natriuretic peptide/natriuretic peptide receptor-C system in the early stages of diabetic retinopathy in the rat. *Mol Vis*. 2005;11:216–224.

22. Baldwin SJ, Bramhall JL, Ashby CA, et al. Cytochrome P450 gene induction in rats ex vivo assessed by quantitative real-time reverse transcriptase-polymerase chain reaction (TaqMan). *Drug Metab Dispos*. 2006;34:1063–1069.

23. Jin L, Foss CE, Zhao X, et al. Cytochrome P450 epoxygenases provide a novel mechanism for penile erection. *FASEB J*. 2006;20:539–541.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–408.

25. Barakat MM, El-Kadi AO, du Souich P. L-NAME prevents in vivo the inactivation but not the down-regulation of hepatic cytochrome P450 caused by an acute inflammatory reaction. *Life Sci*. 2001;69:1559–1571.

26. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951;193:265–275.

27. Gharavi N, El-Kadi AO. Tert-butylhydroquinone is a novel aryl hydrocarbon receptor ligand. *Drug Metab Dispos*. 2005;33:365–372.

28. Nithipatikom K, Grall AJ, Holmes BB, et al. Liquid chromatographic-electrospray ionization-mass spectrometric analysis of cytochrome P450 metabolites of arachidonic acid. *Anal Biochem*. 2001;298:327–336.

29. Morisseau C, Hammock BD. Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol*. 2005;45:311–333.

30. Morisseau C, Goodrow MH, Dowdy D, et al. Potent urea and carbamate inhibitors of soluble epoxide hydrolases. *Proc Natl Acad Sci U S A*. 1999;96:8849–8854.

31. Chiamvimonvat N, Ho CM, Tsai HJ, et al. The soluble epoxide hydrolase as a pharmaceutical target for hypertension. *J Cardiovasc Pharmacol*. 2007;50:225–237.

32. Lu T, Ye D, Wang X, et al. Cardiac and vascular KATP channels in rats are activated by endogenous epoxyeicosatrienoic acids through different mechanisms. *J Physiol*. 2006;575(pt 2):627–644.

33. Seubert J, Yang B, Bradbury JA, et al. Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+ channels and p42/p44 MAPK pathway. *Circ Res*. 2004;95:506–514.

34. Batchu SN, Law E, Brocks DR, et al. Epoxyeicosatrienoic acid prevents postischemic electrocardiogram abnormalities in an isolated heart model. *J Mol Cell Cardiol*. 2009;46:67–74.

35. Zordoky BN, El-Kadi AO. Effect of cytochrome P450 polymorphism on arachidonic acid metabolism and their impact on cardiovascular diseases. *Pharmacol Ther*. 2010;125:446–463.

36. Ishizuka T, Cheng J, Singh H, et al. 20-Hydroxyeicosatetraenoic acid stimulates nuclear factor-kappaB activation and the production of inflammatory cytokines in human endothelial cells. *J Pharmacol Exp Ther*. 2008;324:103–110.

37. Denison MS, Whitlock JP Jr. Xenobiotic-inducible transcription of cytochrome P450 genes. *J Biol Chem*. 1995;270:18175–18178.

38. Hu Y, Ingelman-Sundberg M, Lindros KO. Induction mechanisms of cytochrome P450 2E1 in liver: interplay between ethanol treatment and starvation. *Biochem Pharmacol*. 1995;50:155–161.

39. Kopf PG, Huwe JK, Walker MK. Hypertension, cardiac hypertrophy, and impaired vascular relaxation induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin are associated with increased superoxide. *Cardiovasc Toxicol*. 2008;8:181–193.

40. Reichard JF, Dalton TP, Shertzer HG, et al. Induction of oxidative stress responses by dioxin and other ligands of the aryl hydrocarbon receptor. *Dose Response*. 2005;3:306–331.

41. Peterson SJ, Frishman WH, Abraham NG. Targeting heme oxygenase: therapeutic implications for diseases of the cardiovascular system. *Cardiol Rev*. 2009;17:99–111.

42. Iliff JJ, Alkayed NJ. Soluble epoxide hydrolase inhibition: targeting multiple mechanisms of ischemic brain injury with a single agent. *Future Neurol*. 2009;4:179–199.

43. Zordoky BN, Anwar-Mohamed A, Aboutabl ME, et al. Acute doxorubicin cardiotoxicity alters cardiac cytochrome P450 expression and arachidonic acid metabolism in rats. *Toxicol Appl Pharmacol*. 2010;242:38–46.