

**Modulation of cytochrome P450 gene expression and arachidonic acid metabolism during
isoproterenol-induced cardiac hypertrophy in rats**

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Abbreviations: AA, Arachidonic Acid; ANP, Atrial Natriuretic Pept; BNP, Brain natriuretic Peptide; CYP, Cytochrome P450; DHET, Dihydroxyeicosatrienoic acid; EET, Epoxyeicosatrienoic acid; HETE, Hydroxyeicosatetraenoic acid; LC-ESI-MS, liquid chromatography-elelecron spray ionization-mass spectrometry; PCR, Polymerase chain reaction; SD, Sprague Dawley; SHHF, Spontaneously Hypertensive Heart Failure; SHR, Spontaneously Hypertensive Rats; sEH, Soluble epoxide hydrolase.

Abstract

Several CYP enzymes have been identified in the heart and their levels have been reported to be altered during cardiac hypertrophy. Moreover, there is a strong correlation between CYP-mediated arachidonic acid metabolites and the pathogenesis of cardiac hypertrophy. Therefore, we investigated the effect of isoproterenol-induced cardiac hypertrophy on the expression of several CYP genes and their associated CYP-derived metabolites of arachidonic acid. Cardiac hypertrophy was induced by seven daily intraperitoneal injections of 5 mg/kg isoproterenol. Thereafter, the heart, lung, liver, and kidney were harvested and the expression of different genes was determined by real time-PCR. Heart microsomal protein from control or isoproterenol treated rats was incubated with 50 μ M arachidonic acid, and arachidonic acid metabolites were determined by liquid chromatography-electron spray ionization-mass spectrometry. Our results show that isoproterenol treatment significantly increased the heart to body weight ratio as well as the hypertrophic markers. In addition, there was a significant induction of CYP1A1, CYP1B1, CYP4A3, and soluble epoxide hydrolase and a significant inhibition of CYP2C11 and CYP2E1 in the hypertrophied hearts as compared to the control. CYP1A1, CYP2E1, and CYP4A3 gene expression was induced in the kidney and CYP4A3 was induced in the liver of isoproterenol-treated rats. Isoproterenol treatment significantly reduced 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid formation and significantly increased their corresponding 8,9-, and 14,15-dihydroxyeicosatrienoic acid as well as the 20-hydroxyeicosatetraenoic acid metabolite. In conclusion, isoproterenol-induced cardiac hypertrophy alters arachidonic acid metabolism and its associated CYP enzymes, suggesting their role in the development and/or progression of cardiac hypertrophy.

Introduction

Heart failure affects more than five million people in North America with about half a million of new cases every year (Zordoky and El-Kadi, 2008). Cardiac hypertrophy and fibrosis precede heart failure; therefore, research into the molecular basis of hypertrophy can be considered as research into the initial steps of heart failure (Ritter and Neyses, 2003). Cardiac hypertrophy can be defined macroscopically as a thickening of the ventricular wall and/or septum leading to alterations in chamber size and geometry, collectively called remodeling (Braunwald and Bristow, 2000). Hypertrophy can be considered as a compensatory mechanism that transiently balances the biochemical stress and optimizes cardiac pump function; however, prolonged hypertrophy is a significant risk factor for heart failure (Carreno et al., 2006).

The role of cytochrome P450 (CYP) enzymes in cardiovascular health and disease is well established (Elbekai and El-Kadi, 2006; Zordoky and El-Kadi, 2008). CYP is a family of monooxygenases which is involved in the oxidative metabolism of a wide range of xenobiotics as well as endogenous substances (Elbekai and El-Kadi, 2006). Many studies have examined the expression of CYP enzymes in the heart (Elbekai and El-Kadi, 2006). *In vivo*, CYP enzymes have been reported in explanted human hearts (Thum and Borlak, 2000b; Delozier et al., 2007) and in the left ventricle of Sprague Dawley (SD) and spontaneously hypertensive rats (SHR) (Thum and Borlak, 2002). At *in vitro* level, the gene expression and protein activity of many CYP enzymes have been reported in cultured cardiomyocytes (Thum and Borlak, 2000a) and in the rat cardiomyoblast H9c2 cell line (Zordoky and El-Kadi, 2007).

CYP enzymes are considered one of the major metabolic pathways for the metabolism of arachidonic acid (AA) in addition to cyclooxygenases and lipoxygenases. In the presence of NADPH and oxygen, CYP metabolizes AA to epoxyeicosatrienoic acids (EETs) and hydroxy-

eicosatetraenoic acids (HETEs) metabolites. EETs can be either incorporated into membrane phospholipid pools or efficiently hydrolyzed by soluble epoxide hydrolase (sEH) to biologically less active dihydroxyeicosatrienoic acids (DHETs) or secreted into the extracellular space (Imig et al., 2002). CYP epoxygenases are known to metabolize AA to four regioisomeric metabolites 5,6-, 8,9-, 11,12-, and 14,15-EET and by CYP ω -hydroxylases to 20-HETE. The EETs and HETEs are regarded as important mediators in hypertension, cardiovascular disease, and inflammation (Roman, 2002).

The expression of several *CYP* genes identified in the heart have been reported to be altered during cardiac hypertrophy; however, there is a great discrepancy among various reports on CYP alterations during cardiac hypertrophy, likely due to differences in the etiology of hypertrophy, disease severity, species in question and other underlying conditions (Zordoky and El-Kadi, 2008). In the present study, we investigated the expression of multiple *CYP* genes in the heart, lung, liver, and kidney of male SD rats during isoproterenol-induced cardiac hypertrophy. Furthermore, we investigated the effect of isoproterenol-induced cardiac hypertrophy on the formation of AA metabolites and to determine whether the changes in CYP and sEH gene expression have led to changes in AA metabolism. Our findings provide the first evidence for the cardiac specific changes in *CYP* and sEH gene expression as well as AA metabolism during isoproterenol-induced cardiac hypertrophy.

Materials and Methods

Materials. TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences. AA, isoproterenol and 4-hydroxybenzophenone were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AA metabolite standards 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET and 20-HETE were obtained from Cayman Chemical (Ann Arbor, MI). Reagents used for liquid chromatographic-electron spray ionization-mass spectrometry (LC-ESI-MS) were at HPLC-grade. Acetonitrile and water (HPLC grade) were purchased from EM Scientific (Gibbstawn, NJ). Other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

Animals. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male SD rats weighing 300-350 g were obtained from Charles River Canada (St. Constant, QC, Canada). Animals were treated intraperitoneally (IP) with 5 mg/kg isoproterenol (dissolved in normal saline) daily for 7 days (n=6). Weight-matched controls received the same volume of normal saline daily for 7 days (n=6). Animals were euthanized 24 h following the last injection under isoflurane anesthesia. All animals were allowed free access to food and water throughout the treatment period. Heart, lung, kidney, and liver tissues were excised, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

RNA extraction and cDNA synthesis. Total RNA from the frozen tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first-strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μ g of total RNA from each sample was added to a mix of 2.0 μ l 10X RT buffer, 0.8 μ l 25X dNTP mix (100 mM), 2.0 μ l 10X RT random primers, 1.0 μ l MultiScribe™ reverse transcriptase, and 3.2 μ l nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 sec, and finally cooled to 4 °C.

Quantification by real time-PCR. Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). 25- μ l reaction mix contained 0.1 μ l of 10 μ M forward primer and 0.1 μ l of 10 μ M reverse primer (40 nM final concentration of each primer), 12.5 μ l of SYBR Green Universal Mastermix, 11.05 μ l of nuclease-free water, and 1.25 μ l of cDNA sample. The primers used in the current study were chosen from previously published studies (Bleicher et al., 2001; Kalsotra et al., 2002; Wang et al., 2003; Hirasawa et al., 2005; Rollin et al., 2005; Baldwin et al., 2006) and are listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Melting curve

(dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

Real time-PCR Data analysis. The real time-PCR data were analyzed using the relative gene expression i.e. ($\Delta\Delta CT$) method as described in Applied Biosystems User Bulletin No.2 and explained further by Livak et al (Livak and Schmittgen, 2001). Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by isoproterenol is being studied, whereas the *CYP* of lowest expression was used as a calibrator to compare the expression of different *CYP* genes.

Microsomal preparation and incubation. Microsomal protein was prepared from the heart tissue as described previously (Barakat et al., 2001). Briefly, hearts were washed in ice-cold potassium chloride (1.15% w/v), cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). Microsomal protein from homogenized tissues was separated by differential ultracentrifugation. The final pellet was reconstituted in cold sucrose and stored at -80°C . Heart microsomal protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951). Heart microsomes (1 mg protein/mL) 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 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. AA was added to a final concentration of 50 μM and incubated for 30 min. The reaction was terminated by the addition of 600 μL ice cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. AA metabolites were

extracted twice by 1 mL ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). The concentrations of these eicosanoids in the samples were calculated by comparing the ratios of peak heights to their corresponding standards.

Separation of different AA metabolites by liquid chromatography-electron spray ionization-mass spectrometry (LC-ESI-MS). Extracted AA metabolites were analyzed using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method as described previously (Nithipatikom et al., 2001). The mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The nebulizer gas was obtained 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 x 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 min, increased to 100% acetonitrile in 5 min, and held for 5 min. 4-hydroxybenzophenone was used as internal standard.

Statistical analysis. Data are presented as mean ± standard error of the mean. Control and treatment measurements were compared using student t-test. Comparative gene expression across tissues was analyzed using a one way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc comparison. A result was considered statistically significant where $p < 0.05$.

Results

Expression of *CYP* genes and sEH in the heart, lung, kidney, and liver

To examine the constitutive expression of various *CYP* genes and sEH in the heart, lung, liver, and kidney tissues of male SD rats, total RNA was isolated from different tissues and different genes were determined by real time-PCR. The gene expression profile of different *CYP* and sEH genes was found to be tissue specific. All the examined genes except CYP4A2 are found to be constitutively expressed in the heart at different levels (Fig. 1A). CYP4A1 was the lowest expressed gene and was considered as calibrator. CYP4F5, CYP2E1, CYP1B1, and sEH were the most highly expressed genes with about 10000 fold higher than the calibrator (Fig. 1A). CYP1A1 and CYP2J3 were also highly expressed with about 1000 fold higher than the calibrator (Fig. 1A). CYP2B1, CYP2B2, CYP2C11, and CYP4F4 were moderately expressed genes with about 100 fold higher than the calibrator. CYP4A3 was constitutively expressed at low level with about two fold higher than the calibrator (Fig. 1A).

Similar to the heart, not all the examined genes were expressed in the lung; CYP4A1, CYP4A2, and CYP4A3 mRNA were not detected in the lung tissue. CYP2B2 was the lowest expressed gene and was considered as calibrator. CYP2B1 was the most highly expressed gene with about 120000 fold higher than the calibrator (Fig. 1B). CYP1A1, CYP2E1, CYP4F5, and sEH were highly expressed with about 5000, 4600, 2200, and 1400 fold higher than the calibrator, respectively (Fig. 1B). CYP2J3, CYP1B1, CYP4F4, and CYP2C11 were moderate to low expressed genes with about 290, 65, 17, and 11 fold higher than the calibrator, respectively (Fig. 1B).

On the contrary to the heart and lung, all the examined genes were found to be constitutively expressed in the liver but at different levels. CYP1B1 was the lowest expressed

gene and was considered as calibrator. CYP2E1 and CYP2C11 were the most highly expressed genes with about 13000 and 11000 fold higher than the calibrator, respectively (Fig. 1C). sEH, CYP4A2, and CYP4A3 were also highly expressed with about 5000, 4600, and 2000 fold higher than the calibrator, respectively (Fig. 1C). CYP4F4, CYP2J3, CYP2B2, and CYP4A1 were moderately expressed genes with about 840, 800, 750, and 350 fold higher than the calibrator, respectively (Fig. 1C). The constitutive expression of CYP2B1, CYP1A1, and CYP4F5 was low with about 35, 10, and 10 fold higher than the calibrator, respectively (Fig. 1C).

Similar to the liver, all the examined genes were found to be constitutively expressed at different levels in the kidney. CYP2B2 was the lowest expressed gene and was considered as calibrator (Fig. 1D). CYP4A2 and CYP4A3 were the most highly expressed genes with about 50000 and 24000 fold higher than the calibrator, respectively. sEH and CYP2E1 were highly expressed genes with about 6000 fold higher than the calibrator (Fig. 1D). CYP2C11, CYP4A1, CYP4F5, CYP2J3, CYP1A1, and CYP1B1 were high to moderate expressed genes with about 750, 360, 210, 200, 150, and 60 fold higher than the calibrator, respectively (Fig. 1D). The constitutive expression of CYP4F4 and CYP2B1 was very low with about 2 and 1.5 fold higher than the calibrator, respectively (Fig. 1D).

Effect of isoproterenol treatment on hypertrophic markers and the heart to body weight ratio

In order to investigate whether isoproterenol treatment caused cardiac hypertrophy in the treated SD rats, we measured the cardiac gene expression of the hypertrophic markers, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) relative to control rats. Our results showed that isoproterenol treatment caused statistically significant induction of both

hypertrophic markers, ANP and BNP by 10 and 3 fold, respectively (Fig. 2A). In addition, isoproterenol treatment caused a statistically significant increase in the heart to body weight ratio by about 30% (Fig. 2B).

Effect of isoproterenol treatment on *CYP* gene expression

To examine the effect of isoproterenol-induced cardiac hypertrophy on the expression of several *CYP* genes as well as sEH in different tissues, total RNA was extracted from the heart, lung, liver, and kidney tissues of both control and isoproterenol-treated rats. Thereafter, the expression of different genes was measured using reverse transcription followed by real time-PCR as described under materials and methods.

Figure 3 shows the effect of isoproterenol-induced cardiac hypertrophy on CYP1 family gene expression. Our results demonstrate that CYP1A1 is highly expressed in the lung, moderately expressed in the liver and kidney, and low expressed in the heart (Fig. 3A). Similarly, CYP1B1 was highly expressed in the lung, moderately expressed in the heart and kidney, and low expressed in liver (Fig. 3B). Isoproterenol treatment caused a significant induction of CYP1A1 and CYP1B1 gene expression in the heart by about 2.8 and 4.2 fold, respectively (Fig. 3C and D). In addition, isoproterenol treatment caused a significant induction of CYP1A1, but not CYP1B1, in the kidney by 2.8 fold (Fig. 3C and D). However, there was no change in CYP1A1 and CYP1B1 gene expression in the other tissues (Fig. 3C and D).

With regard to the CYP2B subfamily, CYP2B1 was highly expressed in the lung with about 100000 fold higher than the heart (Fig. 4A). The liver expresses CYP2B1 to a moderate degree, while the constitutive expression of CYP2B1 in the heart and kidney was low (Fig 4.A). Unlike CYP2B1, CYP2B2 was highly expressed in the liver with about 10000 fold higher than its expression in the other organs (Fig 4B). Isoproterenol treatment did not cause any statistically

significant change of CYP2B1 or CYP2B2 gene expression in all the examined tissues (Fig 4C and D).

Figure 5 shows the relative expression of CYP2C11 and CYP2E1 in the different tissues as well as the changes in their expression during isoproterenol-induced cardiac hypertrophy. CYP2C11 is highly expressed in the liver, moderately expressed in the kidney, and low expressed in the lung and heart (Fig. 5A). Similarly, CYP2E1 is highly expressed in the liver, moderately expressed in the kidney and lung, and low expressed in the heart (Fig. 5B). Isoproterenol-induced cardiac hypertrophy caused a significant inhibition of CYP2C11 gene expression in the heart by 50% (Fig. 5C). Nevertheless, CYP2C11 gene expression did not change significantly in the other tissues (Fig. 5C). Similar to CYP2C11, isoproterenol treatment caused a significant inhibition of CYP2E1 gene expression in the heart by 80% (Fig. 5D). However, unlike CYP2C11, isoproterenol treatment caused a paradoxical induction of CYP2E1 in the kidney (Fig. 5D). There was no change in CYP2E1 gene expression in the liver or lung during isoproterenol-induced cardiac hypertrophy (Fig. 5D).

With regard to CYP2J3, it was found to be highly expressed in the liver, moderately expressed in the kidney and lung, and low expressed in the heart (Fig. 6A). CYP4A1 is also highly expressed in the liver and kidney, low expressed in heart, but not constitutively expressed in the lung (Fig. 6B). Isoproterenol-induced cardiac hypertrophy did not cause any changes in the gene expression of either CYP2J3 or CYP4A1 in all the examined tissues (Fig. 6C and D).

Figure 7 shows the relative gene expression of CYP4A2 and CYP4A3 enzymes. CYP4A2 was constitutively expressed in the liver and kidney but not in the heart or lung (Fig. 7A); however, CYP4A3 was constitutively expressed in the heart, liver, and kidney but not in the lung (Fig. 7B). Isoproterenol-induced cardiac hypertrophy did not cause any changes in the gene

expression of CYP4A2 in the liver and kidney (Fig. 7C); nevertheless, it caused a significant induction of CYP4A3 in the heart, liver, and kidney by 2.4, 1.6, and 1.7 fold, respectively (Fig. 7D).

With regard to CYP4F subfamily, CYP4F4 and CYP4F5 were found to be expressed in all the examined tissues. CYP4F4 was highly expressed in the liver compared to the other examined tissues (Fig. 8A). On the other hand, CYP4F5 was found to be highly expressed in the lung, moderately expressed in the heart and kidney, and of low expression in the liver (Fig. 8B). The gene expression of CYP4F4 and CYP4F5 was not altered during isoproterenol-induced cardiac hypertrophy in all the examined tissues (Fig. 8C and D).

Effect of isoproterenol treatment of sEH gene expression

sEH was found to be highly expressed in the liver, moderately expressed in the kidney, and low expressed in the lung and heart (Fig. 9A). Isoproterenol treatment caused a significant induction of sEH gene expression in the heart by 1.8 fold (Fig. 9B). However, the gene expression of sEH was not significantly altered in the other tissues (Fig. 9B).

Separation of AA metabolites using LC-ESI-MS selected ion chromatogram.

LC-ESI-MS has been used in this study for the separation and quantification of CYP-derived metabolites of AA. The mass spectrometer was operated in negative ionization mode with single ion recorder acquisition where the most abundant ion is corresponding to the $m/z = [M-1]^-$. All four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) exhibited the most abundant ions corresponding $m/z = 319$ ion, while their corresponding DHETs has $m/z = 337$ ion and 20-HETE has $m/z = 319$ ion. The fact of having functional groups

at different positions in the eicosanoids structure allowed successful separation which was achieved through the use of reverse phase C₁₈ HPLC column and linear gradient mobile phase. Using authentic standards, 14,15-, 11,12-, 8,9-, 5,6 -EET were found to be eluted at 26.33, 28.56, 29.38 and 30.09 min respectively, while that of 20-HETE was eluted at 15.07 min. The elution pattern of 14,15-, 11,12-, 8,9-, and 5,6-DHET were at 11.35, 12.58, 13.59 and 14.73 min, respectively.

Effect of isoproterenol treatment on AA metabolism.

To investigate the effect of isoproterenol treatment on the formation of AA metabolites, heart microsomes of either control or isoproterenol treated rats were incubated with 50 μ M AA for 30 min. Thereafter, AA metabolites were determined using LC-ESI-MS. Our results clearly show that, 5,6-EET was the major metabolite produced in both control and isoproterenol treated rats followed by 8,9-, 14,15- and 11,12-EET, respectively. In comparison to control animals, in microsomes of hypertrophied hearts, the formation of 5,6-, 8,9-, 11,12- and 14,15-EET were significantly lower by 34.20%, 44.34%, 50.84% and 44.17%, respectively (Fig. 10A).

We also measured levels of enzymatic hydroxylation of EETs products, DHETs. As shown in Figure 10B, the formation of 8,9- and 14,15-DHET were significantly higher by 122.69 % and 83.68 %, respectively compared to control. On the other hand, 5,6- and 11,12-DHET were not significantly altered.

To determine the effect of isoproterenol treatment on CYP ω -hydroxylases activity, we determined the formation of 20-HETE in microsomes from control and hypertrophied rats. Isoproterenol treatment significantly increased the 20-HETE formation by 192% in comparison to control group (Fig. 10C).

Discussion

Although cardiac hypertrophy is an important condition that usually precedes heart failure (Carreno et al., 2006), there are limited reports of altered expression of *CYP* genes during cardiac hypertrophy. The expression of *CYP* has been studied in SHRs as compared to SD rats (Thum and Borlak, 2002) and the expression of *sEH* has been studied in Spontaneously Hypertensive Heart Failure (SHHF) rats as compared to normal strains (Monti et al., 2008). Nevertheless, there are no data about the effect of experimentally induced cardiac hypertrophy on the expression of *CYP* and *sEH* genes and their associated *CYP*-derived metabolites of AA. To the best of our knowledge, this work demonstrates for the first time the effect of experimentally induced cardiac hypertrophy on the expression of several *CYP* genes as well as *CYP*-dependent AA metabolism in rats. In addition, the current study demonstrates the tissue specific expression of many *CYP* genes and *sEH* in male SD rats.

Tissue specific expression of *CYP* genes in the rat has been a subject of discrepancies (Zordoky and El-Kadi, 2007; Zordoky and El-Kadi, 2008). Several factors may have led to these discrepancies. The first factor is the use of conventional PCR technique in most of these studies which may be insensitive due to the low level of *CYP* expression especially in extra-hepatic tissues. Secondly, most of the previous studies focused on only one tissue without giving comparative information regarding the other tissues. Therefore, it was necessary to examine the expression of multiple *CYP* genes simultaneously in different organs by a sensitive technique such as real time-PCR technique. In the current study, we showed that *CYP1A1*, *CYP1B1*, *CYP2B1*, *CYP2B2*, *CYP2C11*, *CYP2E1*, *CYP2J3*, *CYP4F4*, *CYP4F5*, and *sEH* genes are constitutively expressed in all the examined tissues; whereas, *CYP4A1* and *CYP4A3* genes are expressed in the heart, kidney, and liver but not in the lung and *CYP4A2* gene was expressed in

the liver and kidney only. The lung has the highest constitutive expression of *CYP1A1*, *CYP1B1*, *CYP2B1* and *CYP4F5* genes; the liver and kidney have the highest expression of *CYP4A2* and *CYP4A3*; whereas, the liver has the highest expression level of all other *CYP* genes as well as sEH. Generally, the heart has the lowest level of constitutive *CYP* expression except for *CYP1B1* and *CYP4F5* which are expressed in the heart at higher levels than those in the liver.

To induce cardiac hypertrophy experimentally, we used a regimen of 7 daily IP injections of isoproterenol 5 mg/kg which is known to induce cardiac hypertrophy without heart failure or blood pressure elevation (Kralova et al., 2008). In the current study, isoproterenol treatment caused cardiac hypertrophy in SD rats as manifested by significant induction of the hypertrophic markers, ANP and BNP as well as the increase of the heart to body weight ratio. In agreement with our results, isoproterenol infusion of 4 mg/kg/day for seven days caused a significant increase of the heart to body weight and a significant induction of ANP (Masson et al., 1998). Similarly, BNP is known to be increased in various models of cardiac hypertrophy (Magga et al., 1998); however, there is limited information about the effect of isoproterenol-induced cardiac hypertrophy on BNP gene expression.

CYP1A1 and *CYP1B1* are found to be highly expressed in the heart at the constitutive level; therefore, their role in AA metabolism in the heart can not be ignored. *CYP1A1* has been shown to be involved in ω -terminal HETEs synthesis; whereas, *CYP1B1* can metabolize AA to both mid chain HETEs and EETs (Choudhary et al., 2004). Accordingly, it was very important to investigate the effect of isoproterenol-induced cardiac hypertrophy on the gene expression of *CYP1A1* and *CYP1B1* in the heart as well as in other tissues. In the current study, we found that *CYP1A1* gene expression is induced in the heart and kidney in isoproterenol-treated rats but not in the lung or liver; whereas, *CYP1B1* gene expression was induced only in the hypertrophied

heart. In agreement with our results, expression of CYP1A1 and CYP1B1 were significantly increased in left ventricular tissues of SHRs as compared to normotensive SD rats (Thum and Borlak, 2002).

On the contrary to CYP1A1 and CYP1B1, CYP2C11 gene expression was significantly lower in the hypertrophied heart than the control hearts; however, its gene expression was not altered in the other tissues. CYP2C11 is an important epoxygenase enzyme which is involved in AA metabolism and EETs synthesis (Ng et al., 2007). Unlike CYP2C11, CYP2E1 metabolizes AA to 18- and 19-HETEs (Laethem et al., 1993). Isoproterenol-induced cardiac hypertrophy caused a significant inhibition of CYP2E1 gene expression in the heart and a significant induction of its expression in kidney. On the contrary to this finding, CYP2E1 has been reported to be higher in SHR than in normotensive SD rats (Thum and Borlak, 2002).

In spite of previous reports that showed increased expression of CYP2B1/2 and CYP2J3 in SHR as compared to normotensive SD rats (Thum and Borlak, 2002), our results showed that CYP2B1, CYP2B2, and CYP2J3 gene expression were not altered during isoproterenol-induced cardiac hypertrophy. The discrepancy between our results and that of Thum and Borlak is likely due to the strain differences. CYP4A3 was significantly induced in the heart, liver, and kidney in isoproterenol-treated rats as compared to the control. The premise of this observation emerges from the fact that CYP4A3 is an important enzyme involved in AA metabolism to 20-HETE (Wang et al., 1996; Wang et al., 1999; Roman, 2002). Isoproterenol-treatment did not significantly alter the gene expression of CYP4A1, CYP4F4, and CYP4F5 in any of the examined tissues.

sEH enzyme is a crucial determinant of EETs level because it catalyzes the conversion of EETs to DHETs thus abolishing their biological activity (Imig et al., 2002). Therefore, any

change in AA metabolism due to alteration of CYP can be augmented or opposed by an altered level of sEH. Moreover, the gene encoding sEH was found to be a susceptibility factors for heart failure in SHHF rats (Monti et al., 2008). Therefore, it was necessary to investigate the effect of cardiac hypertrophy on sEH expression. In the current study, sEH gene expression in the heart was found to be increased during isoproterenol-induced cardiac hypertrophy. In agreement with our results, it has been shown that sEH activity is higher in SHHF rats (Monti et al., 2008); however, this is the first report to show an induction of sEH gene expression in experimentally-induced cardiac hypertrophy.

In order to investigate the effect of altered CYP gene expression on AA metabolism, we performed *in vitro* incubation of heart microsomes with AA. We found a significant decrease in 5,6-, 8,9-, 11,12-, 14,15-EET formation in microsomes of hypertrophied hearts in comparison to control. The decrease in EETs formation was accompanied by a significant increase in 8,9- and 14,15-DHET formation, whereas 5,6- and 11,12-DHET were not significantly altered. The decreased formation of EETs during isoproterenol-induced cardiac hypertrophy may be attributed to lower expression of CYP2C11 and higher expression of sEH. The significantly higher formation of 8,9- and 14,15- DHET is consistent with the higher expression of sEH because 14,15-EET is the best substrate for sEH followed by 8,9-EET (Karara et al., 1991).

Moreover, our results demonstrate that 20-HETE formation is significantly higher in microsomes of hypertrophied hearts in comparison to untreated rats. The increased formation of 20-HETE is suggestive of its role in cardiac hypertrophy. 20-HETE formation is mainly catalyzed by CYP ω -hydroxylases (Kroetz and Xu, 2005). CYP ω -hydroxylases involved in the formation of HETEs are CYP1A, CYP1B1, CYP4A and CYP4F (Wang et al., 1996; Wang et al., 1999; Roman, 2002; Choudhary et al., 2004; Elbekai and El-Kadi, 2006). Therefore, the increase

in 20-HETE formation in the present work could be attributed to the increased expression of CYP1A1, CYP1B1 and CYP4A3.

In conclusion, isoproterenol-induced cardiac hypertrophy caused significant changes of several CYP and sEH gene expression which is mostly specific to the heart. The overall balance of these changes has led to higher production of 20-HETE and lower production of EETs in the hypertrophied hearts. 20-HETE is known to be involved in many cardiovascular diseases; however, there is an indirect evidence about its role in the development of cardiac hypertrophy (Lee et al., 2004; Chabova et al., 2007). On the other hand, EETs are reported to have cardioprotective effects mainly by inhibiting the activation of nuclear factor kappa B (Campbell, 2000; Hirotsani et al., 2002; Xu et al., 2006). Increased expression of CYP1A1, CYP2E1, and CYP4A3 in the kidney during isoproterenol-induced cardiac hypertrophy could also lead to altered CYP-mediated AA metabolites which play a critical role in the kidney function (Elbekai and El-Kadi, 2006). Therefore, the cardiac and renal CYP may play an important role in the development and/or progression of cardiac hypertrophy. However, more studies are needed to explore the mechanisms by which cardiac hypertrophy modulates CYP gene expression.

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Footnotes

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Legends for figures:

Fig. 1 Constitutive expression of *CYP* genes and sEH in different tissues. Total RNA was isolated from different tissues and the relative expression of *CYP* genes and sEH in the heart (A), lung (B), liver (C), and kidney (D) was determined by reverse transcription followed by real time-PCR. The data were analyzed using the relative gene expression method. The data were normalized to the endogenous reference gene (GAPDH) and relative to a calibrator. The gene of lowest expression in each tissue was used as a calibrator as described under materials and methods. Results are presented as mean \pm SEM (n = 6).

Fig. 2 Effect of isoproterenol treatment on the hypertrophic markers. A: Hypertrophic markers gene expression was determined in the heart. Total RNA was isolated from control and isoproterenol-treated animals. ANP and BNP gene expression were determined by real time-PCR. B: Heart to body weight ratio (mg/g) were determined for each animal after 7 daily IP injections of isoproterenol or saline. Results are presented as mean \pm SEM (n = 6). * p < 0.05 compared with control.

Fig. 3 Expression of *CYP1A1* and *CYP1B1* in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of *CYP1A1* (A) and *CYP1B1* (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of *CYP1A1* and *CYP1B1* were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of *CYP1A1* (C) and *CYP1B1* (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney were

collected. Total RNA was isolated and gene expression of CYP1A1 and CYP1B1 were determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6). * p < 0.05 compared with control.

Fig. 4 Expression of CYP2B1 and CYP2B2 in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of CYP2B1 (A) and CYP2B2 (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of CYP2B1 and CYP2B2 were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of CYP2B1 (C) and CYP2B2 (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney were collected. Total RNA was isolated and gene expression of CYP2B1 and CYP2B2 were determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6).

Fig. 5 Expression of CYP2C11 and CYP2E1 in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of CYP2C11 (A) and CYP2E1 (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of CYP2C11 and CYP2E1 were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of CYP2C11 (C) and CYP2E1 (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney (n = 6) were collected. Total RNA was isolated and gene expression of CYP2C11 and

CYP2E1 were determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6). * p < 0.05 compared with control.

Fig. 6 Expression of CYP2J3 and CYP4A1 in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of CYP2J3 (A) and CYP4A1 (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of CYP2J3 and CYP4A1 were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of CYP2J3 (C) and CYP4A1 (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney (n = 6) were collected. Total RNA was isolated and gene expression of CYP2J3 and CYP4A1 were determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6).

Fig. 7 Expression of CYP4A2 and CYP4A3 in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of CYP4A2 (A) and CYP4A3 (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of CYP4A2 and CYP4A3 were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of CYP4A2 (C) and CYP4A3 (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney (n = 6) were collected. Total RNA was isolated and gene expression of CYP4A2 and CYP4A3 were

determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6). * p < 0.05 compared with control.

Fig. 8 Expression of CYP4F4 and CYP4F5 in different tissues and their modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of CYP4F4 (A) and CYP4F5 (B) in the lung, liver, and kidney relative to the heart. Total RNA was isolated from different tissues and the relative expression of CYP4F4 and CYP4F5 were determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of CYP4F4 (C) and CYP4F5 (D) during isoproterenol-induced cardiac hypertrophy. Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney (n = 6) were collected. Total RNA was isolated and gene expression of CYP4F4 and CYP4F5 were determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6).

Fig. 9 Expression of sEH in different tissues and its modulation during isoproterenol-induced cardiac hypertrophy. Gene expression of sEH in the lung, liver, and kidney relative to the heart (A). Total RNA was isolated from different tissues and the relative expression of sEH was determined by real time-PCR. * p < 0.05 compared with the heart. Gene expression of sEH during isoproterenol-induced cardiac hypertrophy (B). Cardiac hypertrophy was induced by seven daily IP injections of isoproterenol. 24 h after the last injection, the heart, lung, liver, and kidney (n = 6) were collected. Total RNA was isolated and gene expression of sEH was determined by real time-PCR in control and isoproterenol-treated rats. Results are presented as mean \pm SEM (n = 6). * p < 0.05 compared with control.

Fig. 10 Effect of isoproterenol treatment on (A) EETs, (B) DHETs, and (C) 20-HETE formation. Heart microsomes of control or isoproterenol treated rats were incubated with 50 μ M arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice cold acetonitrile. EETs, DHETs, and 20-HETE were extracted twice by 1 mL ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. Results are presented as mean \pm SEM (n=5). * p < 0.05 compared with control.

Table 1. Primers sequences used for real time-PCR reactions

Gene	Forward Primer	Reverse Primer
<i>CYP1A1</i>	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
<i>CYP1B1</i>	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
<i>CYP2B1</i>	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
<i>CYP2B2</i>	CCATCCCTTGATGATCGTACCA	AATTGGGGCAAGATCTGCAAA
<i>CYP2C11</i>	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
<i>CYP2E1</i>	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTAAAATGCTGCA
<i>CYP2J3</i>	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
<i>CYP4A1</i>	TTGAGCTACTGCCAGATCCCAC	CCCATTTTTGGACTTCAGCACA
<i>CYP4A2</i>	CTC GCC ATA GCC ATG CTT ATC	CCT TCA GCT CAT TCA TGG CAA TT
<i>CYP4A3</i>	CTC GCC ATA GCC ATG CTT ATC	CCT TCA GCT CAT TCA TGG CAA TC
<i>CYP4F4</i>	CAGGTCTGAAGCAGGTAACAAAGC	CCGTCAGGGTGGCACAGAGT
<i>CYP4F5</i>	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
<i>sEH</i>	CACATCCAAGCCACCAAGCC	CAGGCCTCCATCCTCCAG
<i>ANP</i>	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
<i>BNP</i>	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
<i>GAPDH</i>	CAAGGTCATCCATGACAACCTTG	GGGCCATCCACAGTCTTCTG

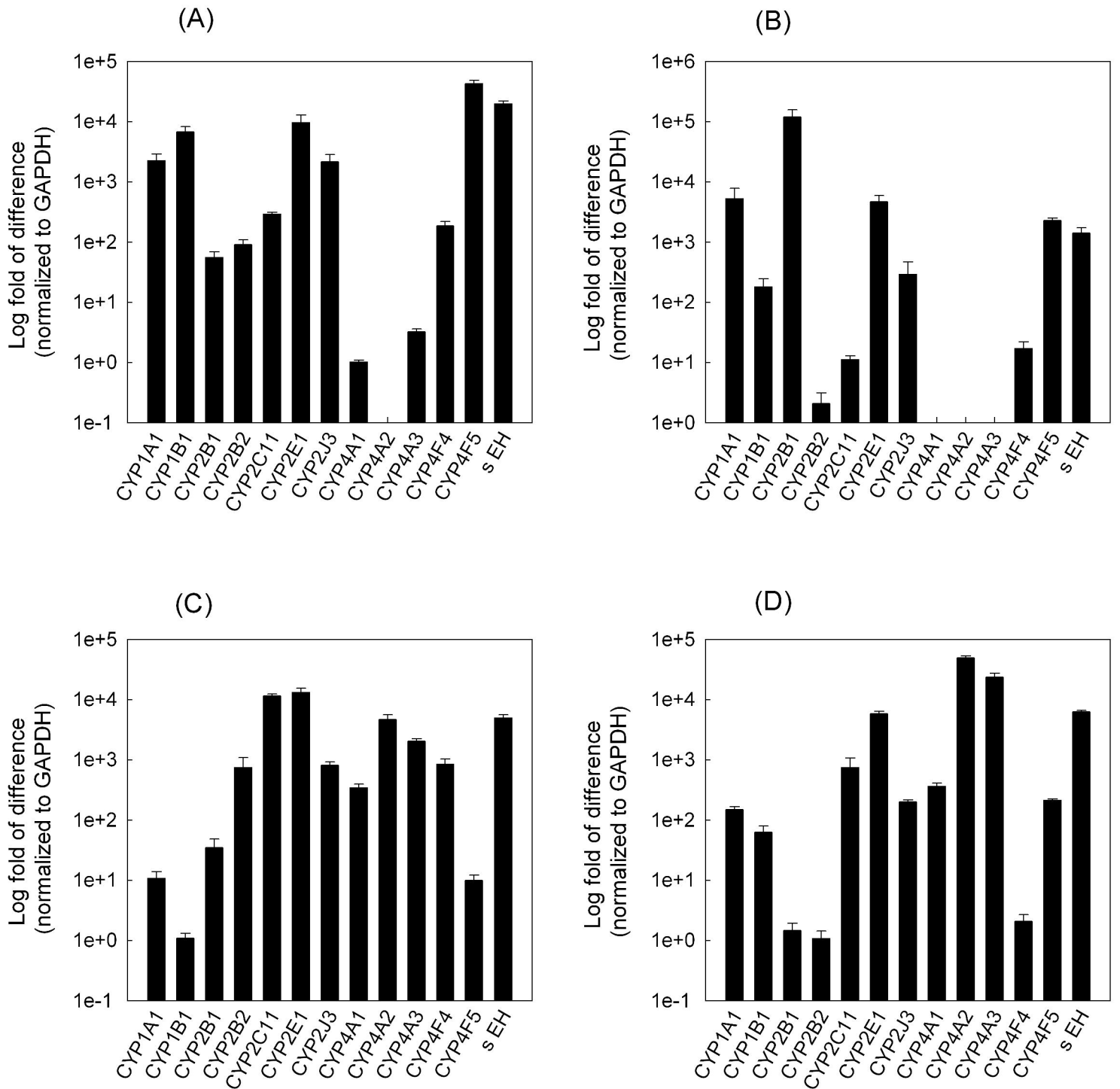


Figure (1)

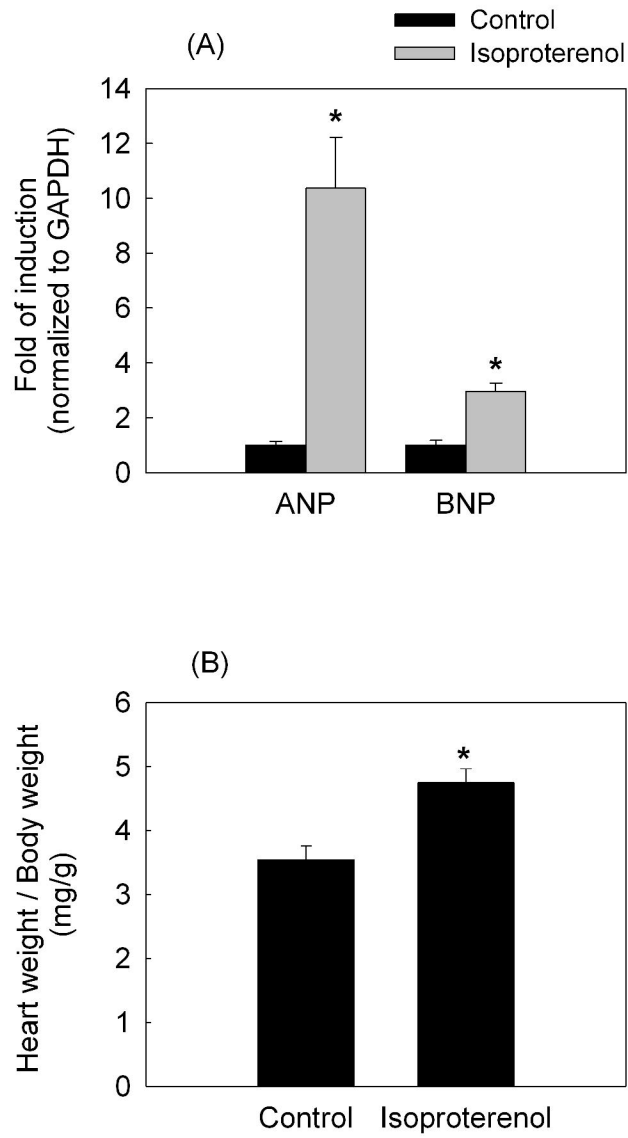


Figure (2)

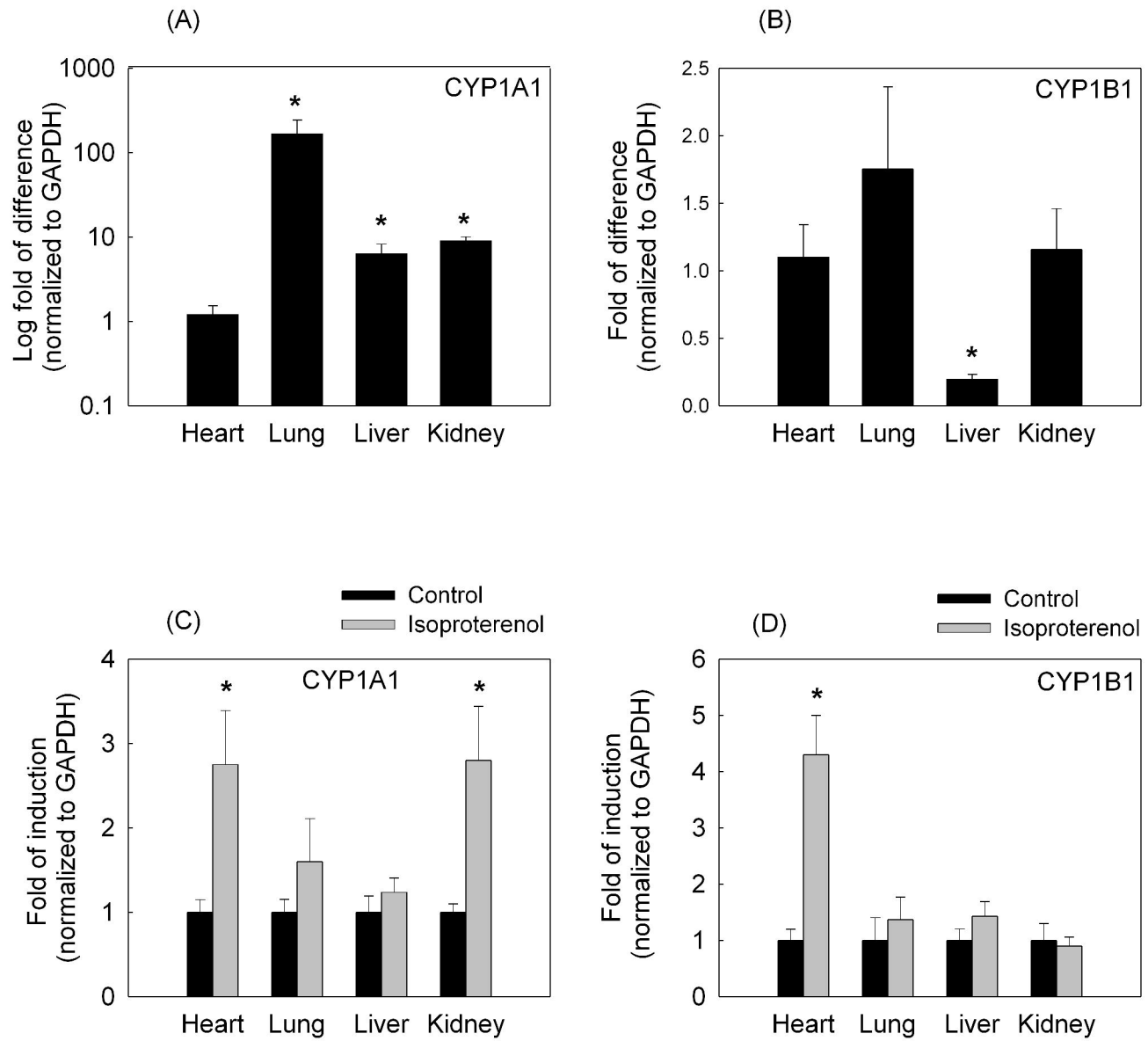


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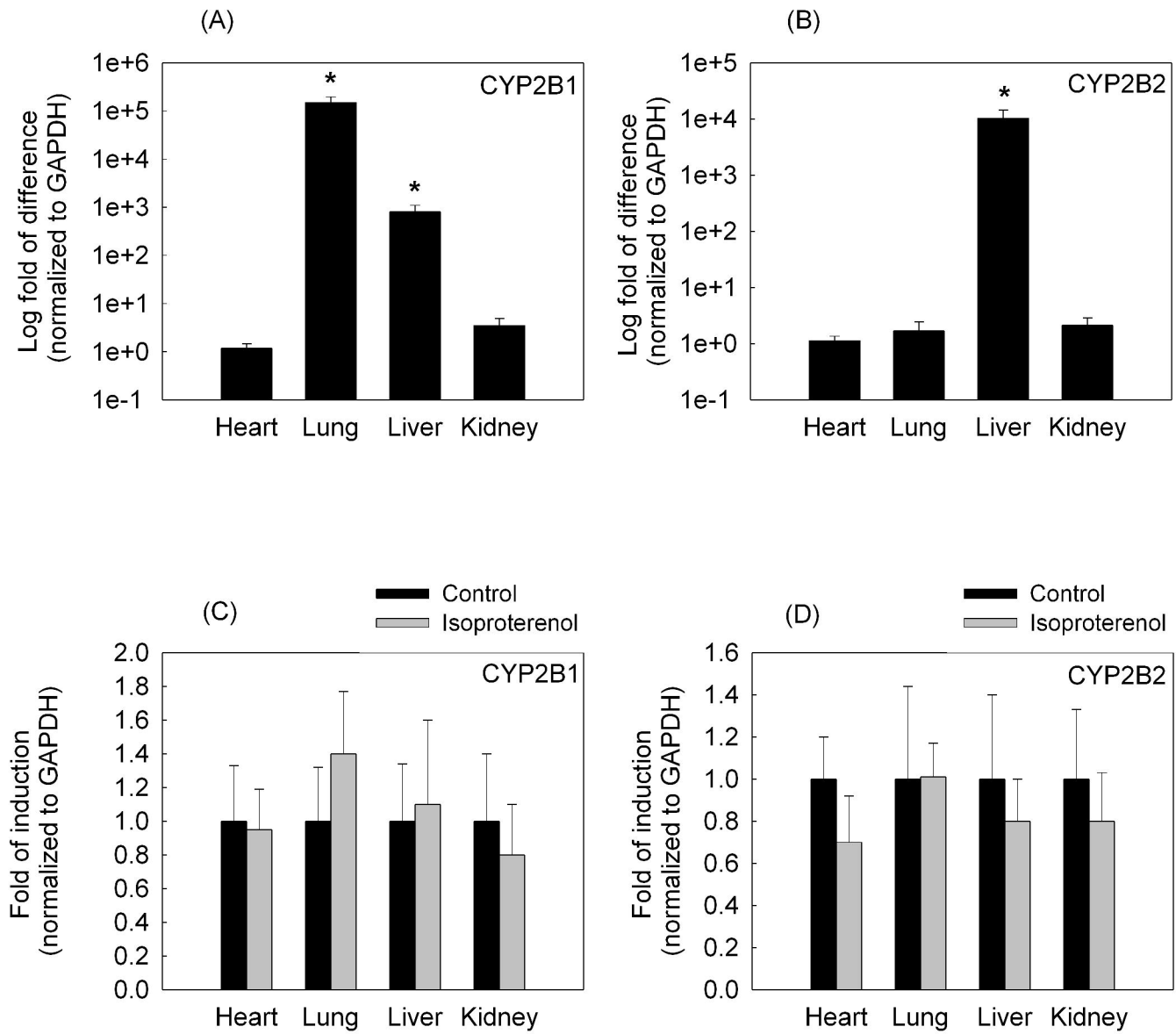


Figure (4)

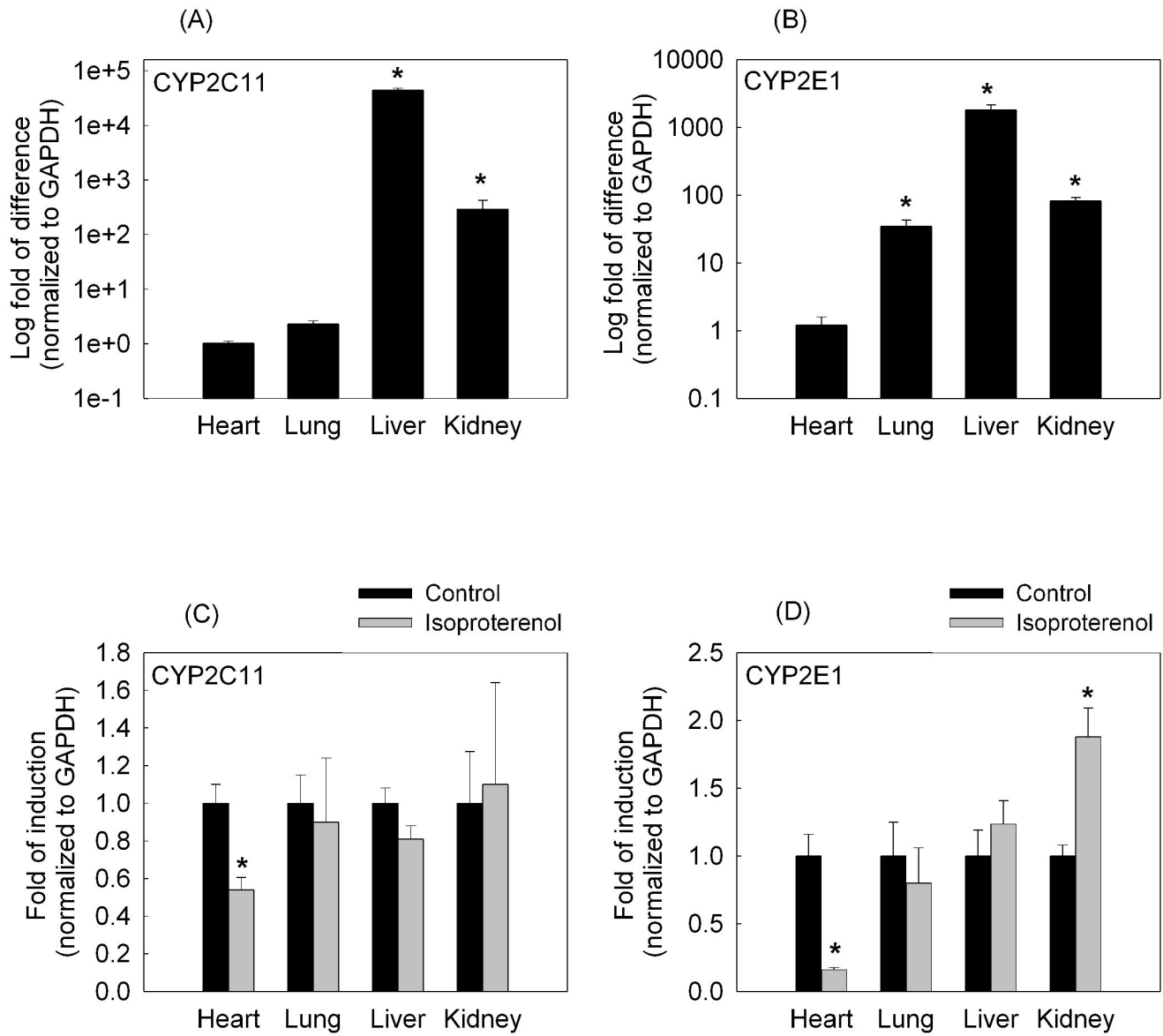


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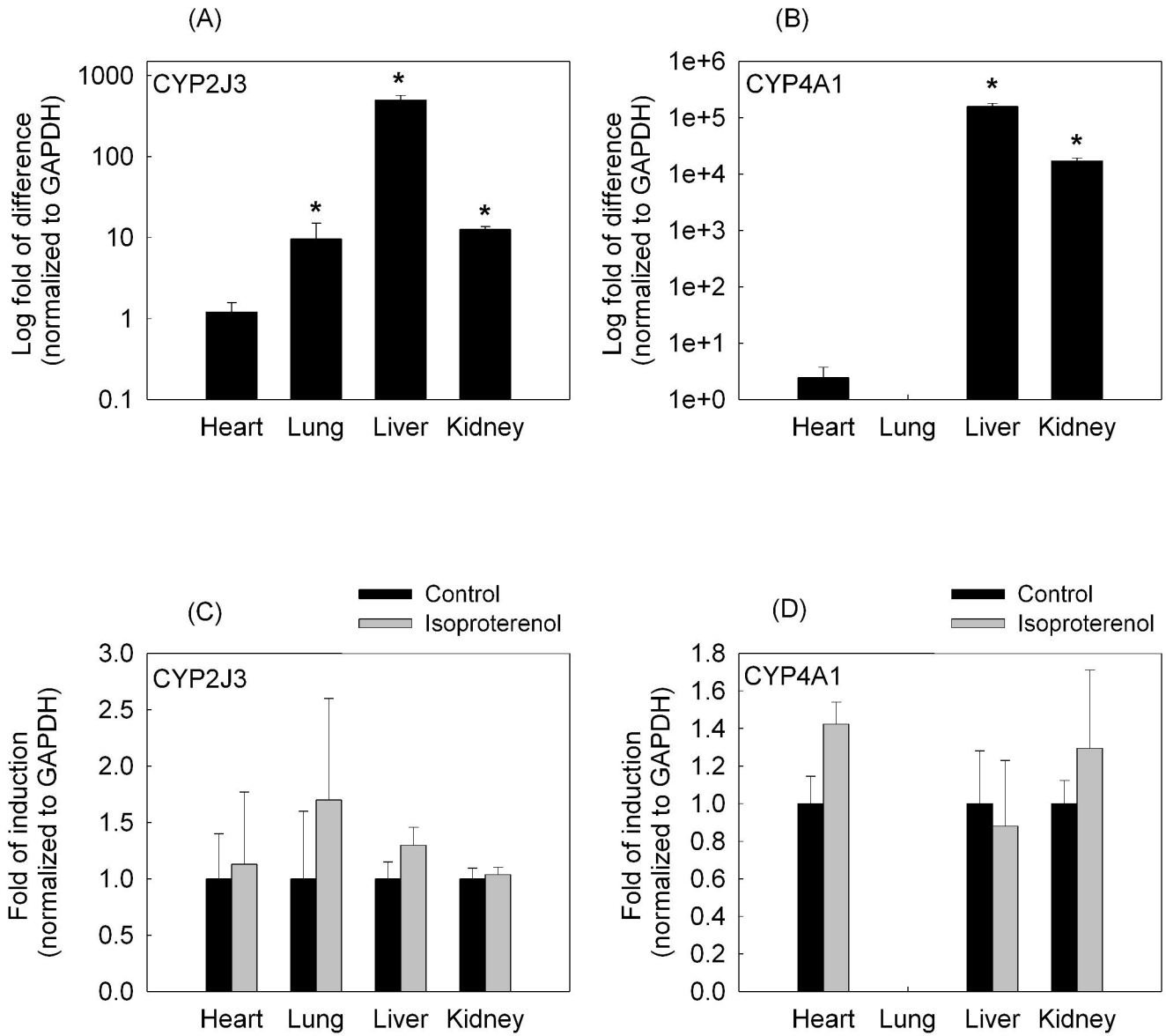


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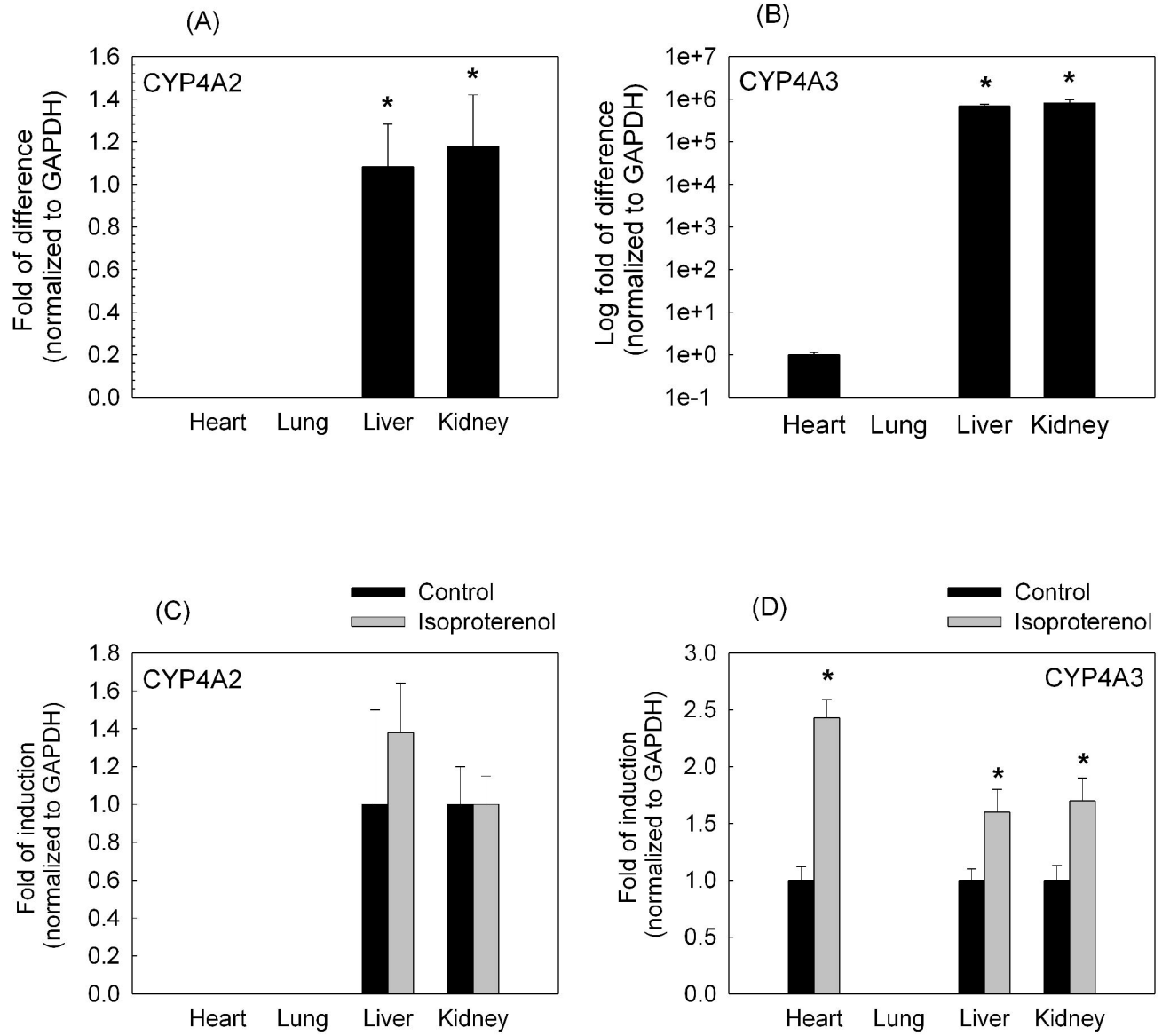


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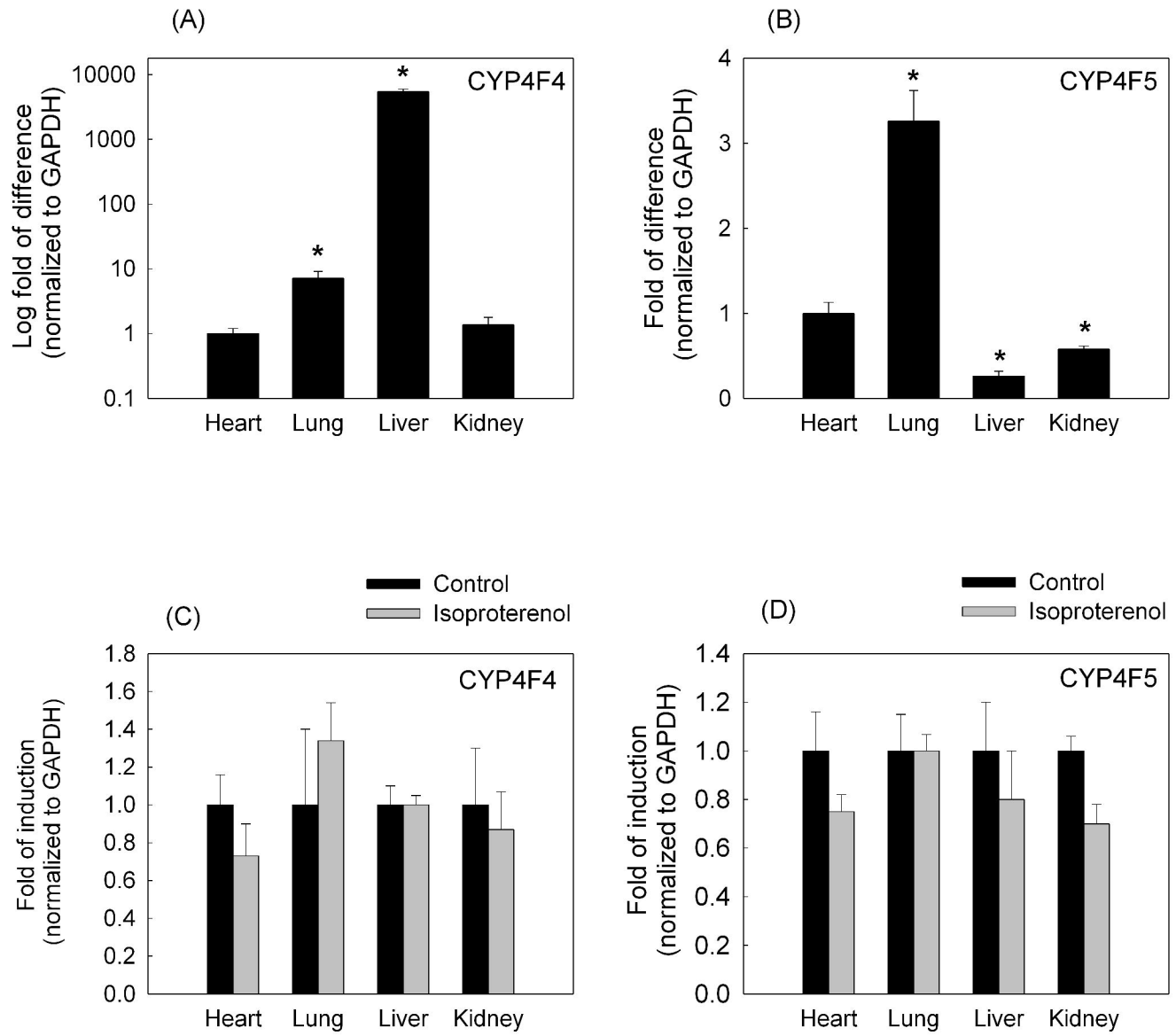


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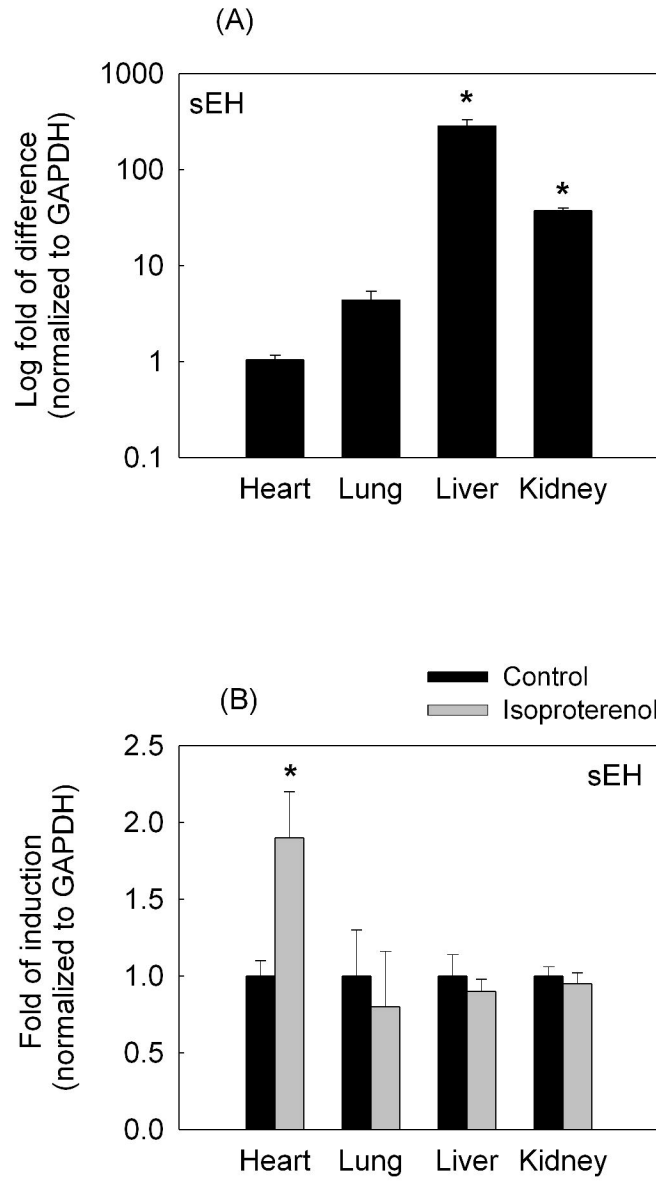


Figure (9)

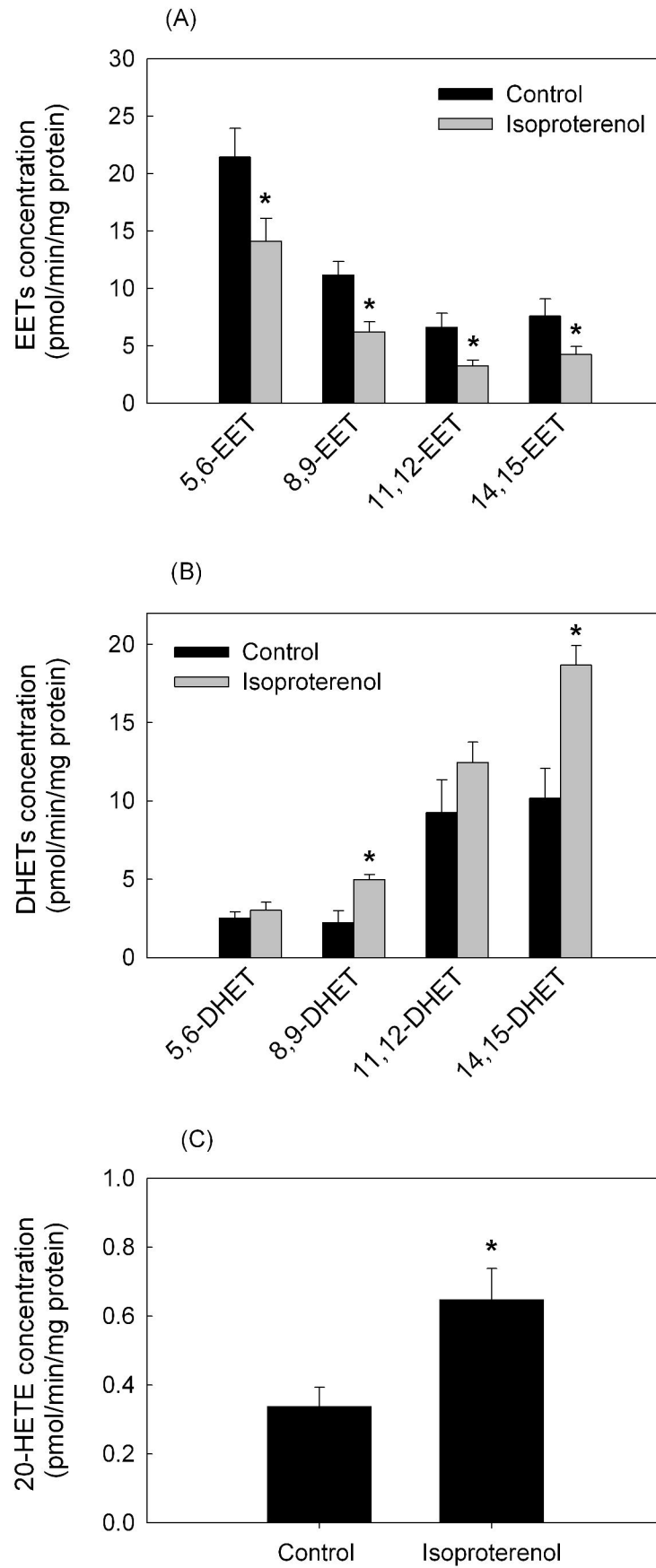


Figure (10)