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The role of cytochrome P450 and the protective effect of EETs against isoproterenol-induced cellular hypertrophy in rat H9c2 cell line

by

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To my beloved parents,

My brother and all of my friends,

For their endless love, patience and support,

Without whom none of my success would be possible.

Abstract

Cytochrome P450 (CYP) enzymes have been identified in the heart and their levels have been reported to be altered during cardiac hypertrophy and heart failure. Furthermore, CYP enzymes have been shown to metabolize arachidonic acid to cardioprotective epoxyeicosatrienoic acids (EETs) and cardiotoxic 20-hydroxyeicosatetraenoic acid (20-HETE). Therefore, the objective of this study was to investigate the protective effect of EETs and the role of CYPs and soluble epoxide hydrolase (sEH) in the development of cardiac hypertrophy. Our results showed that isoproterenol-induced cellular hypertrophy caused a significant induction in the mRNA expression of CYP1A1, CYP1B1, CYP2J3, CYP4F4, CYP4F5 and *EPHX2* in H9c2 cells. Also, we demonstrated that 11,12- and 14,15-EETs significantly attenuated the isoproterenol-mediated induction of hypertrophic markers, ANP and BNP, as well as CYP1A1, CYP2J3, CYP4F4, CYP4F5 and *EPHX2*. Furthermore, we showed that the inhibition of sEH by TUPS significantly decreased the isoproterenol-mediated induction of ANP, BNP, CYP1A1, CYP2J3, CYP4F4, CYP4F5 and *EPHX2*.

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3-MC	3-methylcholanthrene
AC	Adenylate cyclase
ACTH	Adrenocorticotropic hormone
ADU	N-adamantyl-N′-dodecylurea
AEPU	1-adamantan-3-(5-(2-(2- ethylethoxy)ethoxy)pentyl) urea
AhR	Aryl hydrocarbon receptor
AR	Adrenergic receptor
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine-5’-triphosphate
AUDA	12-(3-adamantan-1-yl-ureido)-dode-canoic acid
AUDA-BE	AUDA butyl ester
BaP	Benzo(a)pyrene
BNP	B-type natriuretic peptide
CaMK	Ca^{2+}/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CYP	Cytochrome P450
CDU	1-cyclohexyl-3-dodecyl-urea
DCU	N,N'-dicyclohexyl urea
DHET	Dihydroxyeicosatrienoic acid
DMEM	Dulbecco’s modified Eagle’s medium
DOCA	Deoxycorticosterone acetate
EET	Epoxyeicosatrienoic acid
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GPCR	G protein-coupled receptors
GSK 3b	Glycogen synthase kinase 3b
HETE	Hydroxyeicosatetraenoic acid
IKB	Inhibitor of nuclear factor Kappa B
IKK	Inhibitor of nuclear factor Kappa B kinase
IGF	Insulin-like growth factor
JNK	c-Jun N-terminal kinase
K_{ca}	Calcium-activated potassium channel
LOX	Lipooxygenase
MAPK	Mitogen-activated protein kinases
MCIP	Myocyte-enriched calcineurin-interacting protein 1
MEF2	Myocyte enhancer factor 2
MEK	MAPK kinase
MEKK	MAPK kinase kinase
MKP-1	MAPK phophatase 1
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate tetrasodium
NE	Norepinephrine
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
P38	P38 mitogen-activated protein kinases
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phenylephrine
PG	Prostaglandin
PGI_2	Prostacycline I_2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A

PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PPAR	Peroxisome proliferator activated receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
sEH	Soluble epoxide hydrolase
SERCA2a	Sarcoplasmic reticulum Ca^{2+}-ATPase 2a
SHHF	Spontaneously hypertensive heart failure
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat
STAT	Signal transducer and activator of transcription
t-AUCB	Trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxyl]-benzoic acid
TAK1	Transforming growth factor β–activated kinase 1
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α
TRPV4	Transient receptor potential Ca^{2+} channels
TUPS	[1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea]
TxA_2	Thromboxane A_2
VSMC	Vascular smooth muscle cell
α-MHC	α- myosin heavy chain
β-ARK1	β- adrenergic receptor kinase 1
β-MHC	β-myosin heavy chain
βNF	β-naphthoflavon

1. Introduction

1.1 Cardiac hypertrophy

Cardiac hypertrophy is a major pathological event leading to heart failure and sudden death (Rame and Dries 2007). It is the enlargement of ventricles and can be defined as an increase in heart mass in response to stress stimuli (Bernardo, Weeks et al. 2010). Initially, cardiac hypertrophy is an adaptive cellular response to an increase in cardiac overload. In response to the chronic increase in wall stress, the heart adapts to the higher demands for cardiac work by enlarging the cardiac muscle cells, leading to an increase in size and mass (Cooper 1987; Sugden and Clerk 1998; Hunter and Chien 1999). However, it becomes maladaptive if left untreated and prolonged hypertrophy has been known as an important risk factor for cardiovascular mortality (Muiesan, Salvetti et al. 1995; Verdecchia, Schillaci et al. 1998). This hypertrophic response can be broadly classified as either physiological or pathological. Physiological and pathological cardiac hypertrophies are caused by different stimuli, which are functionally distinguishable and associated with distinct structural and molecular features (McMullen and Jennings 2007).

1.1.1 Classifications of cardiac hypertrophy

Physiological cardiac hypertrophy occurs in response to normal postnatal development, pregnancy and chronic exercise training (Fagard 1997; Eghbali, Deva et al. 2005). Normally physiological growth is associated with normal cardiac structure and enhanced cardiac function (Fagard 1997). In contrast, pathological cardiac hypertrophy occurs in response to a variety of mechanical, hemodynamic, hormonal and pathologic stimuli, including hypertension, valve disease, myocardial infarction and genetic mutations in cardiac contractile protein genes (Kannel 1974; Levy, Anderson et al. 1988; Braunwald and Bristow 2000; Seidman and Seidman 2001; McMullen and Jennings 2007). Pathological hypertrophy is typically associated with loss of myocytes and fibrotic replacement, cardiac dysfunction and increased risk of heart failure and sudden

death (Levy, Garrison et al. 1990; Weber, Brilla et al. 1993; Cohn, Bristow et al. 1997).

Physiological and pathological hypertrophy can be sub-classified as concentric or eccentric based on the changes in shape that are dependent on the initiating stimulus (Grossman, Jones et al. 1975; Pluim, Zwinderman et al. 2000). Concentric hypertrophy is the hypertrophic growth of the heart without an overall enlargement, in which the ventricular walls thicken with a reduction of heart cavity volume. It is characterized by the addition of new sarcomeres in a parallel pattern, leading to an increase in myocyte cell width (Smith and Bishop 1985; Campbell, Rakusan et al. 1989; Campbell, Korecky et al. 1991). Concentric hypertrophy is related to the increased pressure load of the heart. A pathological stimulus causing pressure overload, often due to hypertension or aortic stenosis produces an increase in systolic wall stress, which results in concentric hypertrophy (Grossman, Jones et al. 1975). Similarly, a physiological stimulus can also produce concentric hypertrophy. Strength training such as weight lifting, wrestling and throwing heavy objects can result in a pressure overload on the heart, leading to concentric hypertrophy (Pluim, Zwinderman et al. 2000). In contrast, eccentric hypertrophy refers to an increase in cardiac mass with increased chamber volume, i.e. dilated chambers. In eccentric hypertrophy, the relative wall thickness may be normal, decreased, or increased depending on the initial stimulus. However in general, eccentric hypertrophy is characterized by the addition of new sarcomeres in series, leadings to an increase in myocyte cell length (Grossman, Jones et al. 1975). A pathological stimulus causing volume overload, such as aortic regurgitation or arteriovenous fistulas can produce an increase in diastolic wall stress and results in eccentric hypertrophy (Grossman, Jones et al. 1975; Pluim, Zwinderman et al. 2000). The diseased setting of eccentric hypertrophy is generally associated with thinning of the ventricular walls (Gerdes, Campbell et al. 1988). Also, physiological stimuli including pregnancy and aerobic exercise (also known as endurance training) such as running and swimming increase venous return to the heart, resulting in volume overload and eccentric hypertrophy (Pluim, Zwinderman et al. 2000; Eghbali,

Deva et al. 2005). This type of eccentric hypertrophy is usually characterized by chamber enlargement and a proportional change in wall thickness.

1.1.2 Characteristic distinctions

The increase in heart size is a common feature of physiological and pathological hypertrophy; however, each is associated with distinct structural and molecular bases. Cardiac hypertrophy is associated with structural remodeling of the ventricular walls to accommodate increases in myocyte size. In physiological hypertrophy, a network of collagen fibers provides structural integrity of adjoining myocytes and facilitates myocyte shortening, which supports an efficient cardiac pump function (Bernardo, Weeks et al. 2010). Pathological hypertrophy is associated with cell death and the loss of myocytes is replaced with excessive collagen, known as fibrosis. The cardiac fibroblasts and extracellular matrix proteins accumulate disproportionately and excessively. This leads to mechanical stiffening of the ventricles, which impairs contraction and relaxation and impairs the electrical coupling capillary density. As a result, it increases oxygen diffusion distances, leading to myocardial ischemia, and is likely to progress from hypertrophy to heart failure (Brower, Gardner et al. 2006; Bernardo, Weeks et al. 2010). In the normal healthy heart, fatty acid oxidation is the main metabolic pathway responsible for generating 70% of adenosine-5'-triphosphate (ATP) energy, with glucose and lactate metabolism accounting for the remaining 30% (van der Vusse, Glatz et al. 1992). Physiological hypertrophy induced by exercise training is characterized by enhanced fatty acid and glucose oxidation (Gertz, Wisneski et al. 1988). With more ATP available, it is also associated with elevations in myosin ATPase activity and enhancement of contractility. In contrast, pathological hypertrophy is associated with decreased fatty acid oxidation and increased glucose metabolism (Allard, Schonekess et al. 1994; Christe and Rodgers 1994; Davila-Roman, Vedala et al. 2002). Correspondingly, it has a lower myosin ATPase activity and depressed contractile function (Wikman-Coffelt, Parmley et al. 1979; Rupp 1981). It is believed that this alternation in substrate utilization may be a protective mechanism, enabling

the heart to produce more ATP per molecule of oxygen consumed (van der Vusse, Glatz et al. 1992). Moreover, models of pathological cardiac hypertrophy are often associated with up-regulation of fetal genes, including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and genes for fetal isoforms of contractile proteins, such as skeletal α-actin and β-myosin heavy chain (MHC). In comparison, this is accompanied by the down-regulation of genes normally expressed at higher levels in the adult than in embryonic ventricles, such as α-MHC and sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (Iemitsu, Miyauchi et al. 2001; Bernardo, Weeks et al. 2010). The re-expression of the fetal gene program genes does not usually occur in models of physiological hypertrophy induced by exercise training (McMullen, Shioi et al. 2003).

1.1.2.1 Natriuretic Peptides

Natriuretic peptides are a family of hormones that exhibit important autocrine and paracrine effects in regulating cardiovascular homeostasis, fat metabolism and bone growth (Potter, Abbey-Hosch et al. 2006). Within the myocardium and coronary circulation, they are involved in the regulation of myocyte growth, inhibition of fibroblast proliferation and extracellular matrix deposition, a cytoprotective anti-ischemic function, and influences on coronary endothelium and vascular smooth muscle proliferation and contractility (D'Souza, Davis et al. 2004). ANP, BNP and C-type natriuretic peptides (CNP) are three main natriuretic peptides. Other members of the natriuretic peptide family includes Dendroaspis natriuretic peptides (DNP), urodilatin, guanylin and uroguanylin (D'Souza, Davis et al. 2004). The most known effects of ANP, BNP and CNP on the cardiovascular system are cardioprotective, natriuretic, diuretic and vasorelasent effects (Nishikimi, Maeda et al. 2006). The ANP and BNP coding genes are expressed in almost all tissues, however the heart is the organ in which the expression in the highest (Clerico and Emdin c2006). Effects of natriuretic peptides are mediated through natriuretic peptide receptors (NPR), in which three types have been identified; NPR-A, NPR-B and NPR-C. NPR-A and NPR-B are membrane associated guanylate cyclase receptors, while NPR-C is the

clearance receptor (Maack 1992; Misono 2002; D'Souza, Davis et al. 2004). NPRs are expressed on cardiomyocytes, endocardial endothelial cells and coronary vascular smooth muscle cells (Singh, Kuc et al. 2006).

ANP is a 28-amino acid polypeptide secreted largely from the atria in a prompt response to stretch (de Bold, Borenstein et al. 2001). ANP is normally expressed under physiological conditions primarily in the atrium, however the induction of left ventricular ANP gene expression is seen in many cardiovascular disorders, as well as in experimental models with pressure or volume overload (D'Souza, Davis et al. 2004). BNP is a 32-amino acid polypeptide secreted predominantly by cardiac atria and ventricles (D'Souza, Davis et al. 2004). The levels of BNP are markedly elevated under the pathophysiological conditions in cardiac ventricles from patients or animals undergoing cardiac stress such as diastolic dysfunction, congestive heart failure, myocardial infarction and cardiac hypertrophy (Mukoyama, Nakao et al. 1991). As a result, the upregulation of ANP and BNP expression is widely used as a clinical diagnostic marker for left ventricular hypertrophy, diastolic dysfunction and heart failure.

1.1.3 Mechanisms of Cardiac hypertrophy

Several signaling pathways have been shown to play a critical role in the signal transduction of growth and hypertrophic response in the heart. The best characterized signaling cascade responsible for pathological cardiac hypertrophy is the G protein-coupled receptors (GPCR), $G\alpha_q$ signaling. Other signaling pathways implicated in mediating pathological cardiac growth are those involved with phosphoinositide 3-kinase (PI3K, p110γ), mitogen activated protein kinases (MAPKs), protein kinase C (PKC) and PKD, calcineurin/nuclear factor of activated T-cells (NFAT) and nuclear factor kappa B (NF-κB).

1.1.3.1 G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) play a critical role in the regulation of cardiac functions. The most important myocardiam GPCRs include the adrenergic (α- and β-adrenergic) receptors and muscarinic receptors. There are

two forms of signal transducing G proteins, the heterotrimeric G proteins and the small monomeric G proteins. Heterotrimeric G proteins consist of three subunits (α, β and γ) and couple to GPCRs. Binding of an agonist to the GPCR leads to dissociation of the Gα and Gβγ subunits, followed by activation of downstream signaling pathways (Gutkind 1998a; Gutkind 1998b; Rockman, Koch et al. 2002). Isoforms of the heterotrimeric G proteins are largely determined by the isoform of the α subunits, which fall into three subfamilies; G_s, G_i, and G_q/G_{11} (Simon, Strathmann et al. 1991; Neer 1995; Frey and Olson 2003).

In pathological hypertrophy, stress stimuli such as pressure or volume overload induce the release of humoral factors including angiotensin II (Ang II), endothelin 1 (ET-1) and norepinephrine (NE) (Schunkert, Dzau et al. 1990; Arai, Yoguchi et al. 1995; Yamazaki, Komuro et al. 1999; Rapacciuolo, Esposito et al. 2001; Yayama, Horii et al. 2004). These ligands bind to their GPCRs, i.e. Ang II receptors type 1 (AT_1 receptors), endothelin receptors (ET_A and ET_B) and α_1-adrenergic receptors (ARs), respectively. This causes the activation of $G\alpha_q/_{11}$, which in turn activates downstream signaling proteins, including phospholipase C (PLC), MAPKs, and PKC. Transgenic over-expression of these receptors (Koch, Lefkowitz et al. 2000; Paradis, Dali-Youcef et al. 2000) and their downstream mediator $G\alpha_q$ (D'Angelo, Sakata et al. 1997; Sakata, Hoit et al. 1998) results in cardiac hypertrophy, and subsequently leads to cardiomyopathy. Stimulation with α-adrenergic agonists coupled to $G\alpha_q/_{11}$ has been shown to mediate cardiomyocyte hypertrophy (Nicol, Frey et al. 2000). Furthermore, transgenic mice over-expressing $G\alpha_q$ developed cardiac hypertrophy and were associated with cardiac dysfunction and premature death (D'Angelo, Sakata et al. 1997; Mende, Kagen et al. 1998). In contrast, transgenic mice lacking the $G\alpha_q/_{11}$ proteins displayed no hypertrophy or a significantly blunted response to pressure overload (Akhter, Luttrell et al. 1998; Wettschureck, Rutten et al. 2001).

On the other hand, the most abundant adrenergic receptor in cardiac tissue is the β_1-AR, which couples to the $G\alpha_s$ protein. It activates downstream adenylate cyclase (AC), resulting in the production of cAMP which in turn activates protein kinase A (PKA). Over-expression of the β_1-AR and $G\alpha_s$ proteins in transgenic

mice increase the responsiveness to isoproterenol, and eventually leads to progressive deterioration of cardiac performance, cardiomyocyte hypertrophy and heart failure (Gaudin, Ishikawa et al. 1995; Engelhardt, Hein et al. 1999; Bisognano, Weinberger et al. 2000; Engelhardt, Hein et al. 2002). Similar findings were obtained with over-expression of $G\alpha_s$ in transgenic animals, but surprisingly were not dependent on the activation of AC (Gaudin, Ishikawa et al. 1995). In contrast, over-expression of AC type VI does not appear to exert adverse effects on cardiac function (Roth, Gao et al. 1999; Roth, Bayat et al. 2002). However, transgenic over-expression of PKA results in dilated cardiomyopathy associated with cardiomyocyte hypertrophy and fibrosis (Antos, Frey et al. 2001). It has been reported that the β_1-AR coupling also activates the calcium/calmodulin-dependent protein kinases (CaMKs) signaling (Zhu, Wang et al. 2003). Studies suggest that the cardiac hypertrophic effect of β_1-AR is mainly attributed to the activation of calcium/CaMK and Akt glycogen synthase kinase 3b (GSK 3b)-GATA4 signaling pathways rather than the cAMP/PKA pathway. Sustained β_1-AR stimulation is able to increase the Ca^{2+}/CaMK II activity in a PKA-independent manner (Zhu, Wang et al. 2003; Zheng, Han et al. 2004) and to induce myocyte hypertrophy (Morisco, Zebrowski et al. 2000).

The cardiac muscarinic and β_2-adrenergic receptors are both coupled through the $G\alpha_i$ protein. The expression of $G\alpha_i$ is up-regulated in human hypertrophic and failing hearts (Neumann, Schmitz et al. 1988; Eschenhagen, Mende et al. 1992; Bohm, Gierschik et al. 1993). Conditional over-expression of $G\alpha_i$-coupled GPCR resulted in cardiomyopathy and arrhythmias (Redfern, Degtyarev et al. 2000). Altogether, these findings indicate that G protein-dependent signaling is sufficient to cause hypertrophy and heart failure.

The family of small monomeric G proteins can be divided into 5 subfamilies, and these are Ras, Rho, ADP ribosylation factors, Rab and Ran. Small G proteins act as molecular switches which link receptors to downstream signaling cascades (Clerk and Sugden 2000; Frey and Olson 2003). Various small G proteins such as Ras, Rho and Rac are activated by $G\alpha_q/_{11}$ agonists and each is sufficient to induce cardiac hypertrophy (Proud 2004; Lezoualc'h, Metrich et al.

2008). Several *in vitro* studies have shown that the over-expression of Ras induced hypertrophic gene expression and increased cell size (Thorburn, Thorburn et al. 1993; Abdellatif and Schneider 1997; Fuller, Finn et al. 1998). Cardiospecifically over-expressing a constitutively active form of Ras induced cardiac hypertrophy in transgenic mice (Hunter, Tanaka et al. 1995; Gottshall, Hunter et al. 1997). In contrast, inhibition of Ras prevented phenylephrine (PE)-induced hypertrophic responses (Thorburn, Thorburn et al. 1993). Similarly, expressing an activated Rho also stimulated ANF expression (Sah, Hoshijima et al. 1996; Thorburn, Xu et al. 1997; Hoshijima, Sah et al. 1998). A dominant inhibitory mutant of Rho prevented PE-stimulated, $G\alpha_q$-stimulated, and Ras-induced models of hypertrophy (Sah, Hoshijima et al. 1996; Hines and Thorburn 1998). Furthermore, using a Rho-kinase inhibitor, one study showed that Rho-kinase was critical for pressure overload-induced pathological hypertrophy, but not swimming-induced physiological cardiac hypertrophy in rats (Balakumar and Singh 2006). Over-expression of Rab1a was able to cause pathological cardiac hypertrophy which progressed to heart failure (Wu, Yussman et al. 2001).

1.1.3.2 Phosphoinositide 3-kinase (PI3K, p110γ) signaling

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that release inositol lipid products from the plasma membrane, which in turn mediate intracellular signaling pathways (Toker and Cantley 1997; Vanhaesebroeck, Leevers et al. 1997). Activation of PI3Ks is coupled to both GPCRs and several receptor tyrosine kinases, such as insulin-like growth factor (IGF), fibroblast growth factor (FGF) and transforming growth factor (TGF) (Chesley, Lundberg et al. 2000; Zhu, Zheng et al. 2001). There are three classes of PI3Ks, classes I, II, and III. Class I PI3Ks are heterodimers and further divided into the subclasses I_A and I_B. Class I_A PI3Ks consist of a p110 catalytic subunit (α, β or δ) and a p85 or p55 regulatory subunit. The only Class I_B PI3K is p110γ, which is regulated by the p101 regulatory subunit (Vanhaesebroeck, Leevers et al. 1997). Of the Class I PI3Ks, p110α and p110γ are abundantly expressed in the heart. PI3K (p110γ) is coupled to GPCRs and has a detrimental effect in the heart (Oudit, Sun et al.

2004). PI3K (p110γ) is a negative regulator of cardiac contractility, and PI3K (p110γ) knockout mice displayed enhanced contractile function, less hypertrophy and fibrosis, and were protected from heart failure induced by the activation of β-adrenergic receptors (Crackower, Oudit et al. 2002; Oudit, Crackower et al. 2003). PI3K (p110γ) contributes to cardiac dysfunction by its effect on β-AR internalization and regulation of phosphodiesterases (Oudit and Kassiri 2007; Pretorius, Owen et al. 2009). Binding of p110γ to β-AR kinase 1 (β-ARK1) lead to the down-regulation and desensitization of β-ARs, which is a hallmark of heart failure. Expression of an inactive p110γ mutant disrupted the interaction between p110γ and β-ARK1, and restored β-AR signaling in transgenic mice subjected to chronic β-AR stimulation (Nienaber, Tachibana et al. 2003; Perrino, Naga Prasad et al. 2005; Perrino, Schroder et al. 2007).

1.1.3.3 Mitogen activated protein kinase (MAPK) pathways

Mitogen activated protein kinases (MAPKs) can be categorized into three subfamilies, extracellular-signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and P38 MAPKs (Clerk and Sugden 1999; Widmann, Gibson et al. 1999; Pearson, Robinson et al. 2001). All three types of MAPKs are activated in response to GPCR agonists (couple to $G\alpha_q$), and mechanical stress, as well as in pressure overloaded hearts and failing human hearts (Yamazaki, Tobe et al. 1993; Sadoshima, Qiu et al. 1995; Komuro, Kudo et al. 1996; Sugden and Clerk 1998; Cook, Sugden et al. 1999; Esposito, Prasad et al. 2001; Pearson, Robinson et al. 2001; Takeishi, Huang et al. 2001; Purcell, Wilkins et al. 2007). Interestingly, over-expression of MAPK phosphatase 1 (MKP-1), which inhibits the three MAPK signaling pathways, prevented both agonist-induced *in vitro* hypertrophy and pressure overload-induced *in vivo* hypertrophy (Bueno, De Windt et al. 2000). This demonstrates the significant role of these pathways in hypertrophic signaling.

ERK1/2 are ubiquitously expressed and their activation has been reported in various settings of cardiac hypertrophy and heart failure (Boulton, Nye et al. 1991; Muslin 2008). ERK1/2 was activated in response to agonists that induce pathological hypertrophy such as Ang II, ET-1 and NE, but not to physiological

hypertrophic agonist, IGF1 (Clerk, Aggeli et al. 2006). Stimulation with hypertrophic agonists that signal the $G\alpha_q$ protein coupled receptors also activated ERK1/2 (Wang and Proud 2002). The major activators of ERK1/2 are MAPK kinase (MEK) 1 and MEK2, and inhibition of these activators attenuated the hypertrophic response to agonist stimulation in cultured cardiomyocytes (Glennon, Kaddoura et al. 1996; Clerk, Michael et al. 1998). Transgenic over-expression of MEK1 activated ERK1/2, and resulted in hypertrophic phenotypes (Bueno, De Windt et al. 2000). On the other hand, JNK has been found to be activated in failing human hearts (Cook, Sugden et al. 1999) and in the myocardium of infarcted rat hearts (Li, Zaheer et al. 1998). Several *in vitro* studies have demonstrated that the expression of JNKs was activated and that they may be important regulators of pathological hypertrophy (Bogoyevitch, Gillespie-Brown et al. 1996; Ramirez, Sah et al. 1997; Choukroun, Hajjar et al. 1998; Wang, Su et al. 1998; Choukroun, Hajjar et al. 1999). JNK is phosphorylated and activated by MEK4 and MEK7, which in turn are regulated by MAPK kinase kinase 1 (MEKK1). Stimulation of mechanical stress or agonists by ET-1 or PE induced a rapid phosphorylation of JNK (Komuro, Kudo et al. 1996; Ramirez, Zhao et al. 1997; Choukroun, Hajjar et al. 1998). MEKK1/JNK has been shown to be involved in the hypertrophic response of cardiomyocytes subjected to $G\alpha_q$ coupled receptor stimulation (Bogoyevitch, Andersson et al. 1996). Additionally, over-expression of MEK7 has been found to induce hypertrophy in cultured cardiomyocytes (Wang, Su et al. 1998). Transgenic mice with constitutive activation of MEK7 died prematurely from congestive heart failure (Petrich, Molkentin et al. 2003). Contrarily, expression of a MEK4 mutant attenuated the hypertrophic response to ET-1 stimulation (Choukroun, Hajjar et al. 1998), along with pressure overload-induced hypertrophy (Choukroun, Hajjar et al. 1999). Finally, the major activators of p38 MAPKs are MEK3 and MEK6. Similarly, p38 activity has been shown to be increased in pressure overload, and in ET-1 and PE-induced cardiac hypertrophy (Clerk, Michael et al. 1998; Ueyama, Kawashima et al. 1999; Takeishi, Huang et al. 2001). Over-expression of MEK3 and MEK6 mediators has also demonstrated an induction of hypertrophy in cardiomyocytes

(Wang, Huang et al. 1998). Moreover, the upstream regulator of MEK3/6, transforming growth factor β–activated kinase 1 (TAK1) was up-regulated in hypertrophy, and a constitutively active TAK1 mutant also resulted in severe hypertrophy and subsequently cardiac failure in transgenic mice (Zhang, Gaussin et al. 2000). Of interest, p38 activates several transcriptional factors implicated in the hypertrophic response such as MEF2 and NFATs (Frey and Olson 2003).

1.1.3.4 Protein Kinases

Extracellular stimuli such as pressure overload activate PKC and PKD via GPCRs to trigger hypertrophic responses (Dorn and Force 2005; Harrison, Kim et al. 2006). PKC is one of the critical signal transducers downstream of $G\alpha_q$. There are 12 isoforms of PKC and four of these isoforms have been implicated in the induction of cardiac hypertrophy, these are the α, β, δ, and ε isoforms (Dorn and Force 2005). Transgenic mice over expressing PKCβ developed cardiac hypertrophy associated with cardiac dysfunction, fibrosis and premature death (Bowman, Steinberg et al. 1997; Wakasaki, Koya et al. 1997; Chen, Hahn et al. 2001). However, PKCβ knockout mice displayed a typical hypertrophic response to a GPCR agonist, PE or aortic banding-induced hypertrophy (Roman, Geenen et al. 2001). Thus, these findings suggest that PKCβ is not necessary for the pathological hypertrophic response. PKCε has been shown to cause compensated cardiac hypertrophy *in vivo* (Takeishi, Ping et al. 2000), and a similar study reported to induce hypertrophy with rapid progression to heart failure (Pass, Zheng et al. 2001). Transgenic mice over-expressing PKCδ displayed a mild hypertrophy, but in response to a cardiac insult, such as ischemia-induced damage, PKCδ exacerbated the damage (Chen, Hahn et al. 2001). PKCα appears to be critical for regulating cardiac contractility (Braz, Bueno et al. 2002; Hahn, Marreez et al. 2003; Braz, Gregory et al. 2004). Over-expression of PKCα in transgenic mice resulted in diminished cardiac contractility, while PKCα knockout mice had improved cardiac contractility (Braz, Gregory et al. 2004). Furthermore, inhibition of PKCα activity in $G\alpha_q$-mediated hypertrophy showed an improvement of cardiac contractility, whereas activation of PKCα resulted in a

lethal cardiomyopathy (Hahn, Marreez et al. 2003). Similarly, transgenic mice expressing a constitutively active PKD1 developed pathological hypertrophy and died prematurely (Harrison, Kim et al. 2006). In contrast, conditional deletion of PKD1 resulted in a blunted hypertrophic response in various pathological models, including pressure overload, Ang II and isoproterenol-induced hypertrophy (Fielitz, Kim et al. 2008).

1.1.3.5 Calcineurin/nuclear factor of activated T-cells (NFAT)

Calcium is central to the control of contractile function and cardiac growth. Calcium/calmodulin is an important second messenger for GPCR agonists and biomechanical stress (Frey, McKinsey et al. 2000; Sugden 2001). The best described calcium-dependent signaling proteins are the calcineurin and calcium/calmodulin-dependent protein kinases (CaMKs). Calcineurin is a calmodulin-dependent phosphatase that dephosphorylates nuclear factor of activated T-cells (NFAT) transcription factors. It unmasks the nuclear localization signals, which results in the translocation of NFAT proteins to the nucleus and the activation of gene transcription. Stimulation with GPCR hypertrophic agonists such as Ang II and PE increased the calcineurin activity in cultured cardiomyocytes (Taigen, De Windt et al. 2000). Calcineurin activity was also increased in human hypertrophied and failing hearts (Haq, Choukroun et al. 2001), as well as in the ventricular muscle of hearts exposed to Ang II and ET-1(Li, Wang et al. 2005). Furthermore, calcineurin activity was up-regulated in hypertrophied hearts following aortic banding in rodents (Shimoyama, Hayashi et al. 1999; Lim, De Windt et al. 2000; De Windt, Lim et al. 2001; Zou, Hiroi et al. 2001; Saito, Fukuzawa et al. 2003). Finally, expression of an activated form of calcineurin in transgenic mice produced profound cardiac hypertrophy, which rapidly progressed to dilated cardiomyopathy and heart failure (Molkentin, Lu et al. 1998). Contrarily, targeted inactivation of calcineurin resulted in an impaired hypertrophic response to GPCR agonists and pressure overload-induced hypertrophy in transgenic mice (Bueno, Wilkins et al. 2002). In addition, transgenic expression of a dominant negative form of the calcineurin A produced

an attenuated hypertrophic response to pressure overload stimulus (Zou, Hiroi et al. 2001). Pharmacological inhibition of calcineurin activity also attenuated cardiac hypertrophy in mouse models of transgenic constitutively active calcineurin A and pressure overload hypertrophy (Molkentin, Lu et al. 1998; Meguro, Hong et al. 1999), as well as in the cultured cardiomyocytes in response to Ang II and PE stimulation (Lattion, Michel et al. 1986; Obata, Nagata et al. 2005). Consistent with the idea that calcineurin/NFAT coupling induces pathological cardiac hypertrophy, calcineurin regulates the hypertrophic response by dephosphorylation of NFAT (Olson and Williams 2000). NFAT translocates to the nucleus, where it associates with other transcription factors such as myocyte enhancer factor 2 (MEF2), to regulate cardiac genes (Wilkins, De Windt et al. 2002; Frey and Olson 2003). NFAT-luciferase reporter mice were subjected to both physiological and pathological stimuli, and NFAT luciferase reporter activity was induced only in the pathological models, but not in the physiological models (Wilkins, Dai et al. 2004). Moreover, constitutive activation of a NFAT3 mutant in transgenic mice resulted in cardiac hypertrophy and heart failure, whereas the control mice did not develop hypertrophy (Molkentin, Lu et al. 1998). To further examine the role of calcineurin/NFAT signaling in mediating cardiac hypertrophy, other mediators in this pathway have been investigated. The myocyte-enriched calcineurin-interacting protein 1 (MCIP) is able to inhibit calcineurin signaling by binding directly to the catalytic subunit, which inactivates its ability to dephosphorylate NFAT and MEF2. Over-expression of MCIP1 in transgenic mice attenuated hypertrophy and prevented the progression to dilated cardiomyopathy in response to aortic-banding and the β-AR agonist isoproterenol (Rothermel, McKinsey et al. 2001; Hill, Rothermel et al. 2002).

CaMK II is a protein kinase that has been implicated in cardiac hypertrophy and heart failure. Up-regulation of CaMKII has been found in human failing hearts and animal models of heart failure (Hoch, Meyer et al. 1999; Kirchhefer, Schmitz et al. 1999; Bossuyt, Helmstadter et al. 2008). CaMKII was also found to be induced in hypertrophy-inducing stimuli *in vitro* and pressure overload-induced hypertrophy in mice (Kato, Sano et al. 2000; Colomer, Mao et

al. 2003). Over-expression of CaMKII in transgenic mice induced cardiac hypertrophy, dilated cardiomyopathy and heart failure (Zhang, Johnson et al. 2002; Zhang, Maier et al. 2003). Several studies have reported that the hypertrophic response in cultured cardiomyocytes was decreased by inhibiting CaMKs (Kato, Sano et al. 2000; Zhu, Zou et al. 2000). Further supporting this role, transgenic inhibition of CaMKII prevented cardiac dilation and dysfunction resulting from myocardial infarction and β-AR stimulation in mice (Zhang, Khoo et al. 2005). Recently, CaMKII-null mice were shown to be protected against hypertrophy and fibrosis in response to pressure overload stimulus (Backs, Backs et al. 2009). Furthermore, the activation of CaMKII in both cultured cardiomyocytes and *in vivo* models lead to the induction of hypertrophic markers ANP, BNP and β-MHC (Ramirez, Zhao et al. 1997; Colomer and Means 2000).

1.1.3.6 Nuclear factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is a transcription factor involved in the regulation of a variety of cellular processes, including cytokines, growth factors, immunoreceptors, cell adhesion molecules, acute phase response proteins and cell surface receptors (Zordoky and El-Kadi 2009). The NF-κB family is comprised of five hetero- or homo-dimers, i.e. p50, p52, p65 (RelA), RelB, and c-Rel (Kumar, Takada et al. 2004; Pereira and Oakley 2008). Each subunit has its own biological activity and different dimeric combinations of these subunits have different effects on cell function (Pereira and Oakley 2008). NF-κB can be activated by many stimuli including cytokines, viruses, oxidative stress and chemical agents (Guijarro and Egido 2001). Recently, NF-κB has been recognized as an important mediator in the development of cardiac hypertrophy and heart failure. Activation of NF-κB has been observed in the myocardium of patients with congestive heart failure (Wong, Fukuchi et al. 1998; Grabellus, Levkau et al. 2002). Similarly, NF-κB has been found to be activated in myotrophin-induced hypertrophy in neonatal rat caridomyocytes (Gupta, Purcell et al. 2002). GPCR agonist stimulation by Ang II, ET-1 and PE also resulted in an activation of NF-κB in hypertrophic cardiomyocytes (Purcell, Tang et al. 2001; Hirotani, Otsu et al. 2002).

Additionally, it has been shown that reactive oxygen species-mediated activation of NF-κB is involved in tumor necrosis factor-α (TNF-α)-induced cardiomyocyte hypertrophy (Higuchi, Otsu et al. 2002). Furthermore, activation of NF-κB itself induces cellular hypertrophy in neonatal rat caridomyocytes (Purcell, Tang et al. 2001). Conversely, expression of a dominant-negative NF-κB mutant significantly attenuated the hypertrophic response of aortic banding, Ang II and PE stimulation (Purcell, Tang et al. 2001; Li, Ha et al. 2004; Kawano, Kubota et al. 2005). Recently, it has been shown that NF-κB inhibition attenuated the hypertrophic response, but not left ventricular remodeling, suggesting that NF-κB is necessary for adaptive cardiac hypertrophy (Zelarayan, Renger et al. 2009).

1.1.3.7 Other Signaling pathways

Recently, several other pathways have been also implicated in cardiac hypertrophy, and these are pathways involving MEF2, Na^+/H^+ exchanger, peroxisome proliferator activated receptor (PPAR), and NADPH oxidase (Frey and Olson 2003; Rohini, Agrawal et al. 2010). Over-expression of CaMKII in transgenic mice induced cardiac hypertrophy and was associated with an induction of the MEF2 activity (Zhang, Johnson et al. 2002). The activity of Na^+/H^+ exchanger is increased in several animal models of cardiac hypertrophy, including pressure overload (Takewaki, Kuro-o et al. 1995) and post infarction remodeling (Yoshida and Karmazyn 2000), as well as in cultured cardiomyocytes subjected to mechanical stress (Yamazaki, Komuro et al. 1998). Enhanced Na^+/H^+ exchanger activity reduces the transmembrane Na^+ gradient, which leads to increased intracellular Ca^{2+} levels, and triggesr cardiomyocyte hypertrophy via several pathways including calcium/calmodulin, calcineurin and MAPK. Inhibition of the Na^+/H^+ exchanger decreased the stretch-induced activation of the ERK pathway and protein synthesis in cultured cardiomyocytes (Takano, Komuro et al. 1996), which suggests that the Na^+/H^+ exchanger may also associated with the ERK-dependent pathway. It has been shown that agonist-induced PPARα activation progresses to contractile dysfunction in pressure overload-induced hypertrophied rat hearts (Young, Laws et al. 2001). Furthermore, cardiac over-

expression of PPARα caused cardiomyopathy with contractile dysfunction (Czubryt, McAnally et al. 2003). With regard to NADPH oxidase, a study has shown that induction of cardiac hypertrophy by Ang II is dependent on NADPH oxidase activation (Hingtgen, Tian et al. 2006).

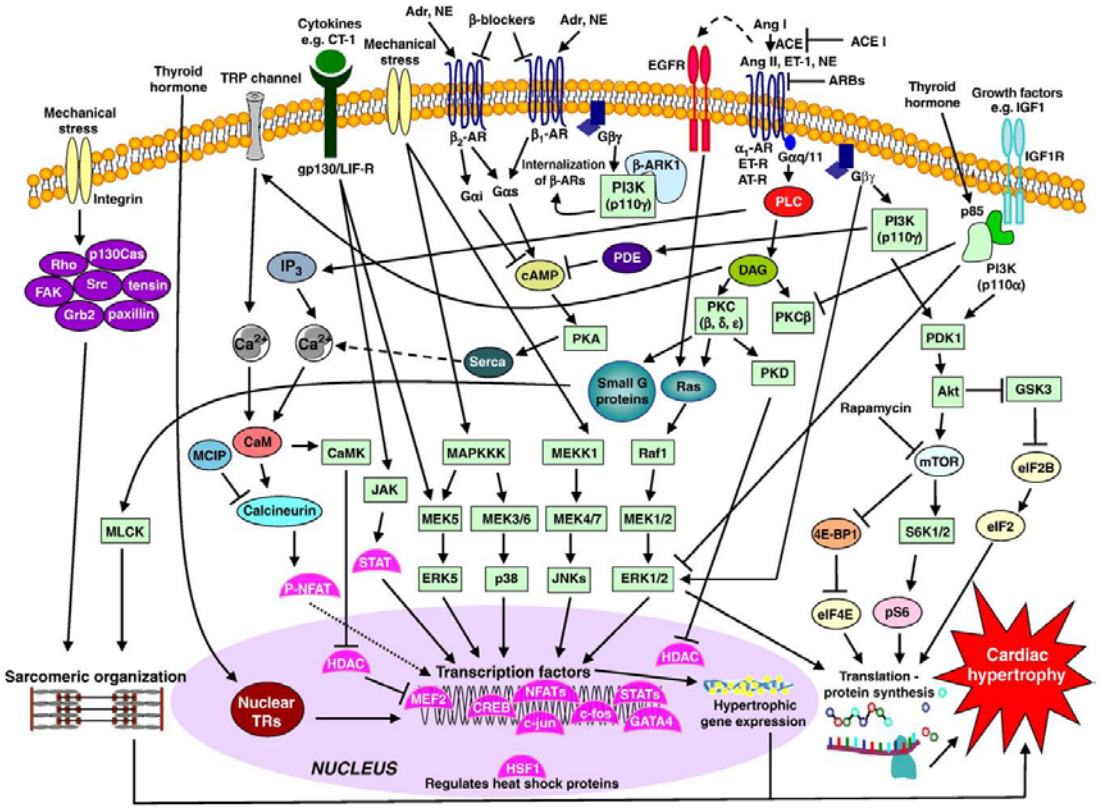

Figure 1.1 Schematic diagram of the major intracellular signaling pathways involved in cardiac hypertrophic response. There is a degree of complexity between the signaling pathways, therefore a highlight for the central pathways and effectors that have been shown to play a critical role in the signal transduction of hypertrophic and growth response in the heart. Those signaling pathways include: G protein-coupled receptors (GPCRs) activated by angiotensin II (Ang II), endothelin-1 (ET-1) and catecholamines, three various mitogen activated protein kinases (MAPKs) [i.e. extracellular signal regulated kinase (ERKs), c-Jun N-terminal kinase (JNKs) and p38 MAPKs], protein kinase C, calcineurin/nuclear factor of activated T-cells (NFAT), phosphoinositide 3-kinase (PI3K, p110γ). Adopted from (Bernardo, Weeks et al. 2010)

1.2 Cytochrome P450 (CYP)

Cytochrome P450 (CYP) is a superfamily of hemeproteins that are involved in the oxidative metabolism of a wide range of endogenous and exogenous compounds (Nelson, Koymans et al. 1996). Some of the CYP substrates include steroids, bile acids, fatty acids, prostaglandins, environmental chemicals, pollutants and drugs (Nebert and Russell 2002). CYP can be classified into different families and subfamilies according to their amino acid sequences (Gonzalez and Nebert 1990; Guengerich 2003).They are phase I drug metabolism enzymes and are mainly expressed in the liver and to a varying degree in other extrahepatic tissues, including the lungs, kidneys, brain, gastrointestinal tract, and heart (Rushmore and Kong 2002; Imaoka, Hashizume et al. 2005). Within the superfamily, CYP1, CYP2 and CYP3 families have been identified as the major contributors in hepatic and extra-hepatic drug metabolism (Lewis 2003). Other CYP families are more involved in the biosynthesis and/or metabolism of endogenous substances (Meyer 1996; Danielson 2002).

1.2.1 CYP expression in the heart

The liver is known to express a vast majority of CYP enzymes; however, early studies detected the expression of CYP enzymes in cardiac tissues of various *in vivo* animal models (Geetha, Marar et al. 1991; Yamada, Kaneko et al. 1992; McCallum, Horton et al. 1993; Fulton, Mahboubi et al. 1995). Recently, several studies have identified the expression of many CYPs in human heart tissues (Thum and Borlak 2000a; Delozier, Kissling et al. 2007). *In vitro* studies have also indicated that CYP enzymes are expressed in cultured rat cardiomyocytes and the rat cardiomyoblast H9c2 cell line (Thum and Borlak 2000b; Zordoky and El-Kadi 2007). The expression of CYPs in the cardiovascular tissues is summarized in Table 1.1.

1.2.1.1 CYP1 family

The CYP1 family consists of three members, CYP1A1, CYP1A2 and CYP1B1. CYP1A1 and CYP1B1 are expressed in the hepatic and extra-hepatic tissues, whereas, CYP1A2 is mainly expressed in the liver (Whitlock 1986; Gonzalez 1990; Murray, Melvin et al. 2001; Danielson 2002). CYP1A1 and CYP1B1 are responsible for the oxidative metabolism of exogenous chemicals, such as polycyclic aromatic hydrocarbons (PAHs) (Fujii-Kuriyama, Imataka et al. 1992; Murray, Melvin et al. 2001). CYP1A2 is involved in the metabolism of exogenous chemicals and drugs, such as caffeine and warfarin (Danielson 2002).

The expression of CYP1 family has been detected in both humans and animals. In humans, the mRNA expression of CYP1A1 has been detected in the left ventricle of healthy subjects and in the right ventricle, aorta and left atrium of patients with dilated cardiomyopathy (Thum and Borlak 2000a; Thum and Borlak 2002). Other studies also found that CYP1A1 was expressed in the left ventricular tissues of explanted human failing hearts and cardiac fibroblasts (Dubey, Jackson et al. 2005; Michaud, Frappier et al. 2010). CYP1A1 mRNA was also reported in rat left ventricular tissues (Thum and Borlak 2002). The inducibility of CYP1A1 has been reported *in vitro* by the treatment of Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and β-naphthoflavone (βNF) in rat cardiomyocytes and H9c2 cells (Thum and Borlak 2000b; Zordoky and El-Kadi 2010). *In vivo* studies showed that the treatment of βNF induced CYP1A1 in hearts of mice and guinea pigs (McCallum, Horton et al. 1993; Brittebo 1994; Granberg, Brunstrom et al. 2000). Moreover, CYP1A1 protein was detected in human coronary artery smooth muscle cells pretreated with 3-methylcholantherene (3-MC) (Dubey, Jackson et al. 2004). On the other hand, CYP1A2 mRNA has been found in pig hearts (Messina, Chirulli et al. 2008), but not in human hearts (Thum and Borlak 2000a; Thum and Borlak 2002). In explanted human hearts, CYP1B1 has been reported to be the second most abundantly expressed *CYP* gene (Bieche, Narjoz et al. 2007). Furthermore, CYP1B1 was found to be expressed at a higher basal level than CYP1A1 in human cardiac fibroblast (Dubey, Jackson et al. 2005). The expression of

CYP1B1 was also detected in rat left ventricle (Thum and Borlak 2000b). In contrast to CYP1A1, CYP1B1 expression is expressed under constitutive and inducible conditions in vascular smooth muscle cells (VSMC) (Kerzee and Ramos 2001). CYP1B1 protein was detected in mouse endothelial cells and aortic SMC following the treatment of TCDD and benzo(a)pyrene (BaP), respectively (Moorthy, Miller et al. 2003; Filbrandt, Wu et al. 2004). AhR ligands, TCDD and βNF have been shown to induce the expression of CYP1B1 in H9c2 cells (Zordoky and El-Kadi 2010). Similarly, it has been reported that 3-MC and BaP induced CYP1B1 in rat hearts (Aboutabl, Zordoky et al. 2009). Another study also indicated concentrated ambient particles such as oxygenated compounds and PAHs were able to induce CYP1B1 in rats (Ito, Suzuki et al. 2008).

1.2.1.2 CYP2 family

The CYP2 family is the largest family of CYP in humans (Lewis 2004). CYP2A6 is primarily expressed in the liver and mediates oxidative metabolism of several procarcinogens and drugs including aflatoxin and coumarin (Yun, Shimada et al. 1991; Salonpaa, Hakkola et al. 1993; Koskela, Hakkola et al. 1999). CYP2A7 is also found in the liver, while CYP2A13 is expressed at a very low level (Koskela, Hakkola et al. 1999). Little is known about these two isoforms; however, CYP2A13 has been observed to metabolize coumarin as well (Lewis 2004). The CYP2B subfamily in humans only consists of CYP2B6, which accounts for less than 1% of the hepatic CYP content (Danielson 2002). It is involved in the metabolism of some clinically important drugs such as selegiline (Hidestrand, Oscarson et al. 2001). However, CYP2B subfamily enzymes are abundantly expressed in rodent hepatic tissues and are inducible by bartbiturates (Danielson 2002). The CYP2C subfamily constitutes about 20% of the hepatic CYP content in humans and metabolizes 20% of all clinically used drugs (Imaoka, Yamada et al. 1996; Guengerich 2006). CYP2D6 represents only 2% of the total hepatic CYP content in humans, yet it metabolizes more than 70 different drugs (Danielson 2002). Similarly, CYP2E1 is the only member of the CYP2E subfamily in humans and it catalyzes the metabolism of 70 substrates, which are

mainly small hydrophobic compounds (Hakkak, Korourian et al. 1996). Lastly, CYP2J2 is primarily expressed in extrahepatic tissues, with a high expression in the heart. It is involved in the metabolism of arachidonic acid to epoxyeicosatrienoic acids (EETs), which has significant biological implications (Wu, Moomaw et al. 1996; Zeldin, Foley et al. 1996).

Like the CYP1 family, the CYP2 family is also found in the cardiovascular system. In humans, CYP2A6/7, CYP2B6/7, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP2J2 have all been identified in heart tissues (Thum and Borlak 2000a; Delozier, Kissling et al. 2007). The major CYP isoforms in the human heart are CYP2C8 and CYP2J2, with the CYP2J2 being highly and constitutively expressed (Thum and Borlak 2002). However, the expression of CYP2C8, CYP2C9 and CYP2C11 were also found in the coronary arteries under basal conditions (Mancy, Dijols et al. 1996; Bolz, Fisslthaler et al. 2000). On the other hand, the expression of CYP2B6/7, CYP2C8-19, and CYP2D6 were also predominantly expressed in the right ventricle of patients with dilated cardiomyopathy (Thum and Borlak 2000a). Furthermore, CYP2E1 mRNA was detected in various parts of the heart including the right and left atria, right and left ventricle, and the ventricular septum (Thum and Borlak 2000a). CYP2J2 has been shown to be the main CYP isoform highly expressed in normal human hearts (Bieche, Narjoz et al. 2007; Delozier, Kissling et al. 2007). Recently, CYP2S1 has been found to be expressed at very low levels in fetal and adult human hearts (Choudhary, Jansson et al. 2005). In rats, CYP2A1/2, CYP2B1/2, CYP2C23, CYP2E1 and CYP2J3 were identified in the ventricular tissue (Thum and Borlak 2002; Imaoka, Hashizume et al. 2005). Contrarily, CYP2C11 mRNA was only observed in isolated rat cardiomyocytes (Thum and Borlak 2000b), but not in heart tissues (Thum and Borlak 2002). Evidence for the expression of CYP2B1/2 in the heart is still limited, however one study detected CYP2B1/2 in fetal rat heart at the protein level (Czekaj, Wiaderkiewicz et al. 2000). Similar to human hearts, CYP2S1 mRNA was detected at a very low level in the mouse hearts (Choudhary, Jansson et al. 2003). Moreover, *in vitro* studies showed that

CYP2B1/2, CYP2E1 and CYP2J3 were also expressed in H9c2 cells at comparable levels to those in rat heart tissues (Zordoky and El-Kadi 2007).

1.2.1.3 CYP3 family

In humans, the CYP3 subfamily comprises 4 members, CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (Gellner, Eiselt et al. 2001). The most important CYP enzyme is CYP3A4, which is predominantly expressed in the liver and is responsible for 60% of the hepatic CYP-mediated metabolism of drugs (Martinez-Jimenez, Jover et al. 2007). CYP3A4 is involved in the metabolism of more than 30% of clinically administered drugs including dihydropyridine calcium channel blockers and macrolide antibiotics (Anzenbacher and Anzenbacherova 2001). The level of CYP3A5 in the human liver is approximate to that of CYP3A4, whereas CYP3A7 and CYP3A43 are expressed at much lower levels (Gibson, Plant et al. 2002). CYP3A5 appears to be more limited in its metabolic capacity than CYP3A4, but it actively metabolizes nifedipine, testosterone and cortisol. On the other hand, CYP3A7 seems to be involved in the metabolism of endogenous steroids (Gibson, Plant et al. 2002).

The expression of the CYP3 family in cardiovascular tissues still remains to be elucidated. CYP3A4, CYP3A5, and CYP3A7 mRNA were not detected in human heart tissues; however, the expression of CYP3A4 was found in human endocardium and coronary vessels (Minamiyama, Takemura et al. 1999; Thum and Borlak 2002). Similarly, expression of CYP3A1/2 isoforms was not found in rat cardiovascular tissues, and only CYP3A1 mRNA was detected in isolated cardiomyocytes (Thum and Borlak 2000b).

1.2.1.4 CYP4 family

The CYP4 family is abundantly expressed in the kidney and to a lesser extent in the liver (Theken, Deng et al. 2011). The CYP4A isoforms are involved in the metabolism of endogenous compounds, namely long chain fatty acids and some prostaglandins, together with eicosanoids such as arachidonic acid. They

metabolize these medium and long chain fatty acids at their ω-carbon (Okita and Okita 2001; Lewis 2004).

In humans, CYP4A11 has been detected in failing hearts (Michaud, Frappier et al. 2010), whereas CYP4B1 is predominantly expressed in the right ventricle of explanted human hearts (Thum and Borlak 2000a). In addition, CYP4F11 and CYP4F12 have been reported in human hearts as well (Cui, Nelson et al. 2000; Bylund, Bylund et al. 2001). CYP4A1, CYP4A3 and CYP4F4 were found to be expressed in rat heart tissues, while CYP4A2 was not detected (Engels, van Bilsen et al. 1999; Zordoky, Aboutabl et al. 2008). Similarly, CYP 4A1 was also detected in the isolated rat cardiomyocytes (Thum and Borlak 2000b). On the other hand, Cyp4a12 mRNA was found in mice hearts (Theken, Deng et al. 2011). In dogs, CYP4A1, CYP4A2 and CYP4F were all found in the heart tissues (Nithipatikom, Gross et al. 2004).

1.2.1.5 Other CYP families

Other CYP families are involved in the biosynthesis and/or metabolism of endogenous compounds, which includes thromboxane A_2, bile acid, prostacyclin, and steroid biosynthesis (Elbekai and El-Kadi 2006). The expression of these CYP families has been less characterized in the heart tissues. In humans, CYP11A mRNA was identified in the normal and failing hearts (Young, Clyne et al. 2001). However, CYP11B1 and CYP11B2 were only found in some chambers of the human failing hearts but not in the normal hearts (Young, Clyne et al. 2001). Also, the expression of CYP11B2 mRNA was detected in rat hearts (Silvestre, Robert et al. 1998).

Table 1.1 The expression of CYP enzymes in cardiovascular tissues

CYP	Level	Tissues	Species	References
CYP1A1	mRNA	heart	human	(Thum and Borlak 2000a; Walles, Thum et al. 2002; Choudhary, Jansson et al. 2003; Michaud, Frappier et al. 2010)
	mRNA	heart	human, rat	(Thum and Borlak 2002)
	protein	SMC	human	(Dubey, Jackson et al. 2004)
	mRNA, protein	heart	rat	(Thum and Borlak 2000b)
	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
	mRNA, protein, activity	VSMC	mouse	(Kerzee and Ramos 2001)
	activity	endothelial cells	mouse	(Granberg, Brunstrom et al. 2000)
CYP1A2	protein	endothelial cells	human	(Minamiyama, Takemura et al. 1999)
CYP1B1	mRNA	heart	human	(Choudhary, Jansson et al. 2003)
	mRNA	veins	human	(Bertrand-Thiebault, Ferrari et al. 2004)
	protein	SMC	human	(Dubey, Jackson et al. 2004)
	mRNA	heart	rat	(Thum and Borlak 2002)
	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
	mRNA, protein, activity	VSMC	mouse	(Kerzee and Ramos 2001)
	protein	SMC	mouse	(Moorthy, Miller et al. 2003)
	protein	endothelial cells	mouse	(Filbrandt, Wu et al. 2004)
CYP2A1/2	mRNA	heart	rat	(Thum and Borlak 2002)
CYP2A6/7	mRNA	heart	human	(Thum and Borlak 2002)
CYP2B1	mRNA	endothelial cells	human	(Hoebel, Steyrer et al. 1998)
CYP2B1/2	mRNA	heart	rat	(Thum and Borlak 2000b; Thum and Borlak 2002)
	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
	protein, activity	heart	rat	(Czekaj, Wiaderkiewicz et al. 2000)
CYP2B6/7	mRNA	heart	human	(Thum and Borlak 2000a; Thum and Borlak 2002; Walles, Thum et al. 2002; Michaud, Frappier et al. 2010)
CYP2B23	mRNA	heart	mouse	(Renaud, Cui et al. 2011)

CYP2C	mRNA	veins	human	(Bertrand-Thiebault, Ferrari et al. 2004)
CYP2C8-19	mRNA	heart	human	(Thum and Borlak 2000a; Thum and Borlak 2002; Walles, Thum et al. 2002)
CYP2C8/9	mRNA, protein	heart	human	(Delozier, Kissling et al. 2007; Michaud, Frappier et al. 2010)
CYP2C8/19	mRNA	endothelial cells	human	(Fisslthaler, Popp et al. 1999)
CYP2C11	mRNA	heart	rat	(Thum and Borlak 2000b; Zordoky, Aboutabl et al. 2008)
CYP2C11/13	mRNA	heart	rat	(Aboutabl, Zordoky et al. 2009)
CYP2C29	mRNA	heart	human	(Choudhary, Jansson et al. 2003)
CYP2E1	mRNA	heart	human	(Thum and Borlak 2000a; Walles, Thum et al. 2002; Michaud, Frappier et al. 2010)
	mRNA	veins	human	(Bertrand-Thiebault, Ferrari et al. 2004)
	mRNA	heart	human, rat	(Thum and Borlak 2002)
	protein	coronary vessels, endocardium	human, rat	(Minamiyama, Takemura et al. 1999)
	mRNA	heart	rat	(Thum and Borlak 2000b)
	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
CYP2J2/3	mRNA	heart	human	(Walles, Thum et al. 2002; Delozier, Kissling et al. 2007)
	mRNA, protein	heart	human	(Wu, Moomaw et al. 1996; Michaud, Frappier et al. 2010)
	mRNA	heart	human, rat	(Thum and Borlak 2002)
	mRNA, protein	heart	rat	(Wu, Chen et al. 1997)
	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
CYP2S1	mRNA	heart	mouse	(Choudhary, Jansson et al. 2003)
	mRNA	heart	human, mouse	(Choudhary, Jansson et al. 2005)
CYP2U1	mRNA	heart	human	(Karlgren, Backlund et al. 2004; Bieche, Narjoz et al. 2007)
	mRNA	heart	mouse	(Renaud, Cui et al. 2011)
	mRNA	heart	human, mouse	(Choudhary, Jansson et al. 2003; Choudhary, Jansson et al. 2005)
CYP3A1	mRNA	heart	rat	(Thum and Borlak 2000b)
CYP3A4	protein	endothelium, endocardium,	human	(Minamiyama, Takemura et al. 1999)

		coronary vessels		
CYP4A	protein	arteries	rat	(Kunert, Roman et al. 2001)
CYP4A1	mRNA	heart	rat	(Thum and Borlak 2000b; Zordoky, Aboutabl et al. 2008)
CYP4A3	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009)
CYP4A1/2	protein	heart	dog	(Nithipatikom, Gross et al. 2004)
CYP4A1/2/3	activity	heart	rat	(Engels, van Bilsen et al. 1999)
	Protein	arteries (VSMC)	cat	(Harder, Gebremedhin et al. 1994)
CYP4A1/2/3/8	mRNA	arteries	rat	(Gebremedhin, Lange et al. 2000)
CYP4A11	mRNA	heart	human	(Thum and Borlak 2002; Michaud, Frappier et al. 2010)
	mRNA	veins	human	(Bertrand-Thiebault, Ferrari et al. 2004)
CYP4A29	mRNA	heart	mouse	(Renaud, Cui et al. 2011)
CYP4B1	mRNA	heart	human	(Thum and Borlak 2000a; Walles, Thum et al. 2002)
CYP4F	activity	heart	dog	(Nithipatikom, Gross et al. 2004)
CYP4F3	mRNA	PMN	human	(Kikuta, Kusunose et al. 1993)
CYP4F1/4/5/6	mRNA	heart	rat	(Aboutabl, Zordoky et al. 2009)
CYP4F4/5	mRNA	heart	rat	(Zordoky, Aboutabl et al. 2008)
CYP4F11	mRNA	heart	human	(Cui, Nelson et al. 2000)
CYP4F12	mRNA	heart	human	(Bylund, Bylund et al. 2001)
CYP4F13	mRNA	heart	mouse	(Choudhary, Jansson et al. 2003)
CYP4F13/16	mRNA	heart	mouse	(Theken, Deng et al. 2011)
CYP4F37	mRNA	heart	mouse	(Renaud, Cui et al. 2011)
CYP11A	mRNA	heart, arteries, veins	human, mouse	(Young, Clyne et al. 2001)
CYP11B2	mRNA	heart	rat	(Silvestre, Robert et al. 1998)

1.2.2 CYP expression in cardiac hypertrophy and heart failure

The expression of several CYP isoforms has been associated with the development of cardiac hypertrophy and heart failure in human and rat hearts. In humans, it has been demonstrated that the induction of CYP2A6/7 and CYP4A11 expression was found in hypertrophied left ventricle (Thum and Borlak 2002). CYP2E1 protein level was significantly increased in the left ventricular myocardium of patients with dilated cardiomyopathy (Sidorik, Kyyamova et al. 2005). In addition, an upregulation of CYP1B1, CYP2E1, CYP2J2, CYP2F2 and CYP4A10 was observed in human failing hearts (Tan, Moravec et al. 2002; Elbekai and El-Kadi 2006). Furthermore, CYP11B1 and CYP11B2 were found to be expressed in the vascular wall and left atria of human failing hearts, while not detected in normal hearts (Young, Clyne et al. 2001). The elevated levels of CYP11B2 mRNA were found to be associated with increased myocardial fibrosis and left ventricular dysfunction in chronic heart failure patients (Satoh, Nakamura et al. 2002). In rats, the expression of CYP1B1, CYP2A1/2, CYP2B1/2, CYP2E1 and CYP2J3 was significantly increased in the left ventricle of spontaneously hypertensive rats (SHRs) compared to normotensive rats (Thum and Borlak 2002). Furthermore, a significant induction in the mRNA expression of CYP1A1, CYP1B1 and CYP4A3, with an inhibition of CYP2C11 and CYP2E1 were found in isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008).

1.3 Arachidonic acid metabolism

Arachidonic acid is a polyunsaturated fatty acid found in the phospholipids of cell membranes. It is released into the cytosol by the activation of phospholipase A_2 in response to stress stimuli (Mukherjee, Miele et al. 1994; Jenkins, Cedars et al. 2009). The free arachidonic acid can be metabolized via three major enzymatic pathways, namely those involving cyclooxygenases (COXs), lipoxygenases (LOXs), and CYP enzymes (Roman 2002).

1.3.1 CYP-mediated arachidonic acid metabolism

In the first pathway, COXs metabolize arachidonic acid to prostaglandin (PG) G_2 and PGH_2. PGH_2 is further metabolized by CYP5A1 (thromboxane A_2 synthase) to thromboxane A_2 and by CYP8A1 (prostacyline I_2 synthase) to prostacyclin I_2 (PGI_2). In the second pathway, LOX enzymes metabolize arachidonic acid to hydroxyeicosatetraenoic acids (HETEs) and dihydroxyeicosatetraenoic acids (DiHETEs), which are subsequently converted to hydroxyeicosatetraenoic acids (HETEs), leukotrienes, or lipoxins. In the third pathway, CYP enzymes can metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs) by CYP epoxygenases and hydroxyeicosatetraenoic acids (HETEs) by CYP ω-hydroxylases (Roman 2002). The metabolism of arachidonic acid by the COXs and LOXs pathways has been well established. However, the role of the CYP pathway in arachidonic acid metabolism has recently gained scientific attention. Several studies have demonstrated the involvement of CYP-mediated arachidonic acid metabolites in different physiological functions. Moreover, other studies have investigated the ability of specific CYP isoforms to metabolize arachidonic acid to certain metabolites.

1.3.1.1 Epoxyeicosatrienoic acids (EETs)

The enzymatic reaction involving CYP epoxygenases involves epoxidation at the 5-, 8-, 11-, and 14-positions of arachidonic acid, resulting in the formation of four regioisomers, 5,6-, 8,9-, 11,12- and 14,15-EETs. Several CYP enzymes can be considered as CYP epoxygenases and metabolize arachidonic acid into different EET regioisomers. These mainly include the CYP1A, CYP2B, CYP2C, CYP2E and CYP2J subfamilies (Imig 2000; Zeldin 2001). Nevertheless, members of the CYP2C and CYP2J subfamilies are considered as the major enzymes involved in the synthesis of EETs (Kroetz and Zeldin 2002; Jenkins, Cedars et al. 2009).

One of the metabolic fates of EETs is the hydrolysis by soluble epoxide hydrolase (sEH) into a less biologically active form, 5,6-, 8,9-, 11,12- and 14,15-

dihydroxyeicosatrienoic acids (DHETs) (Zeldin, Kobayashi et al. 1993; Imig 2000; Spector and Norris 2007). Alterations in the sEH activity would result in an accumulation of EETs, and predominantly metabolize by β-oxidation or chain elongation. β-Oxidation would result in the formation of a 16 carbon epoxy fatty acid, while chain elongation would form the 22 carbon epoxy fatty acid (Fang, Kaduce et al. 2001; Fang, Weintraub et al. 2002). Other pathways in the metabolism of EETs involve the CYP ω-oxidases, which result in the insertion of a hydroxyl group at the ω terminal of EETs. Additionally, 5,6- and 8,9-EETs are also substrates for COX enzymes, which converts them to 5,6-epoxy-PGE1 and 11-hydroxy-8,9-EET, respectively (Zhang, Prakash et al. 1992; Carroll, Balazy et al. 1993). If EETs are not further metabolized, they are generally incorporated into the phospholipid pools of cell membranes, which are the storage sites for these molecules in many cell types (VanRollins, Kaduce et al. 1993; Weintraub, Fang et al. 1997; Roman 2002). EETs can then be released from the membrane phospholipids in response to vasoactive hormones and other stimuli that activate phospholipases (Carroll, Balazy et al. 1997; Weintraub, Fang et al. 1997). About 90% of the total EETs are incorporated back into the membrane phospholipids (Kaspera and Totah 2009).

1.3.1.2 20-Hydroxyeicosatetraenoic acid (20-HETE)

20-HETE is the major product in the metabolism of arachidonic acid by CYP ω-hydroxylases. The metabolic reaction by CYP ω-hydroxylases is the hydroxylation of arachidonic acid at the ω terminal, forming the 20-HETE. Members of the CYP4A and CYP4F subfamilies are the major CYP ω-hydroxylase enzymes responsible for 20-HETE formation. However, CYP1A1, CYP1B1 and CYP2E1 have been reported to produce different regioisomers of HETEs (Laethem, Balazy et al. 1993; Elbekai and El-Kadi 2006). Similarly, 20-HETE can be metabolized by β-oxidation to 16- or 18-carbon derivatives, which are less biologically active (Fang, Kaduce et al. 2001; Roman 2002). 20-HETE can also be metabolized by COXs to vasodilator prostaglandin or prostacyclin-like derivatives, such as 20-hydroxy-prostaglandin G2 and H2 (Schwartzman,

Falck et al. 1989). Likewise, if 20-HETE is not further metabolized, it can be re-incorporated into membrane phospholipid pools, where it can be released again through the actions of agents that activate phospholipases.

1.3.2 The role of CYP-mediated arachidonic acid metabolites in cardiovascular diseases

1.3.2.1 Epoxyeicosatrienoic acids (EETs)

EETs exhibit a variety of diverse actions in different types of tissues and cells. The role of EETs in cardiovascular physiology has been the focus of several studies (Imig 2000; Zeldin 2001; Kroetz and Zeldin 2002; Roman 2002; Jenkins, Cedars et al. 2009). EETs have been reported to possess vasodilating (Pomposiello, Carroll et al. 2001; Pratt, Li et al. 2001; Zhang, Oltman et al. 2001), fibrinolytic (Node, Ruan et al. 2001), anti-inflammatory (Node, Huo et al. 1999; Campbell 2000), anti-apoptotic, (Chen, Capdevila et al. 2001) and potential anti-fibrotic effects (Levick, Loch et al. 2007).

EETs display a potent vasodilating property in small resistance vessels of heart, brain, kidney, skeletal muscle and intestine (Fang, Kaduce et al. 2001). EETs activate the opening of calcium-activated K^+ channels (K_{ca}) and transient receptor potential Ca^{2+} channels (TRPV4) (Hu and Kim 1993; Earley, Heppner et al. 2005), resulting in hyperpolarization of the VSMCs (Gebremedhin, Ma et al. 1992) and thereby producing vasorelaxation. The vasodilating effect of EETs is regioisomer-selective and organ-specific. Generally, EETs are vasodilators, however, 5,6- and 8,9-EETs can be metabolized by COXs to vasoconstrictor metabolites (Imig, Navar et al. 1996; Zhu, Bousamra et al. 2000; Roman 2002). Vasoconstriction effects of EETs were found *in vitro* to constrict pulmonary arteries of rabbits (Zhu, Bousamra et al. 2000). Similarly, EETs were found to increase acute hypoxic vasoconstriction in mouse lungs (Pokreisz, Fleming et al. 2006; Keseru, Barbosa-Sicard et al. 2008). However, a recent report has demonstrated that sEH inhibitors, which increase the endogenous levels of EETs,

prevented monocrotaline-induced pulmonary hypertension in rats (Revermann, Barbosa-Sicard et al. 2009).

In addition to their vasodilating effect, EETs have potent anti-inflammatory properties (Node, Huo et al. 1999). It has been reported that EET decreased cytokine-induced endothelial cell adhesion molecule expression, and prevented leukocyte adhesion to the vascular wall by inhibiting NF-κB and IκB kinase (Node, Huo et al. 1999). Also, activation of the tyrosine kinase and the MAPK signaling pathway may be involved in mediating the anti-inflammatory effect of EETs (Roman 2002). In vascular endothelial cells, EETs displayed fibrinolytic properties through the induction of plasminogen activator gene expression, and antithrombotic properties through the inhibition of platelet aggregation and platelet adhesion to endothelial cells (Node, Ruan et al. 2001; Jiang, McGiff et al. 2004; Krotz, Riexinger et al. 2004). Similarly, studies have shown that EETs increased endothelial cell growth and angiogenesis (Munzenmaier and Harder 2000; Wang, Wei et al. 2005; Michaelis and Fleming 2006). On the other hand, EETs has been reported to inhibit the migration and proliferation of VSMCs through the cAMP/PKA signaling pathway (Davis, Thompson et al. 2002; Sun, Sui et al. 2002).

In the kidney, EETs have been found to be important regulators of glomerular filtration by activating Na^+/H^+ exchanger (Harris, Munger et al. 1990). Additionally, EETs was shown to mediate pressure natriuresis and control blood pressure (Dos Santos, Dahly-Vernon et al. 2004). In cerebral circulation, EETs are important regulator of cerebral blood flow (Alkayed, Birks et al. 1996). Astrocytes have been reported to produce EETs upon receptor stimulation with excitatory neurotransmitters (Alkayed, Birks et al. 1997); it suggests EETs are acting on the cerebral VSMC to dilate cerebral arteries which allow more blood flow to the active regions of the brain.

In the heart, numerous studies have demonstrated that EETs have cardioprotective effects. Studies demonstrated that exogenous administration of 11,12-EET improved the recovery of contractile function, increased cell shortening and increased intracellular Ca^{2+} concentrations after ischemia-

reperfusion injury in rat hearts (Moffat, Ward et al. 1993; Wu, Chen et al. 1997). Exogenous administration of 11,12- and 14,15-EETs into the ischemic hearts of canines and mice produced a remarkable decrease in myocardial infarct size (Nithipatikom, Moore et al. 2006; Motoki, Merkel et al. 2008). EETs also exert protective effects to attenuate myocardial stunning and block the loss in membrane potential in rat cardiomyocytes and H9c2 myocytes (Javadov, Karmazyn et al. 2009; Katragadda, Batchu et al. 2009). Furthermore, transgenic over-expression of CYP2J2, a CYP epoxygenase enzyme responsible for the endogenous production of EETs, demonstrated an improved recovery of left ventricular developed pressure after ischemia-reperfusion injury in mice (Seubert, Yang et al. 2004). Similarly, increasing the intracellular concentration of EETs in a knockout mouse model of the *Ephx2* gene to abolish the formation of sEH also showed an improved recovery of left ventricular developed pressure and less infarction after ischemia-reperfusion (Seubert, Sinal et al. 2006). Consistently, sEH-null mice were found to be protected from pressure overload-induced cardiac arrhythmia and heart failure (Monti, Fischer et al. 2008). Moreover, inhibiting the sEH with selective inhibitors has been shown to attenuate left ventricular hypertrophy in mice with pressure overload-induced myocardial failure (Xu, Li et al. 2006) and in Ang II-induced cardiac hypertrophy (Ai, Pang et al. 2009). These cardioprotecitve effects of EETs have been attributed to the activation of ATP-sensitive K^+ channels, a p42/p44 MAPK pathway, and a cAMP/PKA-dependent signaling pathway, as well as inhibition of NF-κB (Seubert, Yang et al. 2004; Lu, Ye et al. 2006; Xu, Li et al. 2006; Batchu, Law et al. 2009). In addition, EETs modulate the activation of several ion channels. It has been reported that EETs inhibited cardiac Na^+ channels (Lee, Lu et al. 1999) and activated ATP-sensitive K^+ channels (Lu, Hoshi et al. 2001; Lu, VanRollins et al. 2002; Lu, Ye et al. 2006). Furthermore, studies have shown that EETs increased cardiac Ca^{2+} currents by enhancing phosphorylation of the L-type Ca^{2+} channels (Xiao, Huang et al. 1998; Xiao, Ke et al. 2004). It also increased the maximal peak transient outward K^+ currents and activated the voltage-dependent K^+ channels by phosphorylation

of α-subunit via cAMP/PKA-dependent pathway (Anderson, Adams et al. 2000; Ke, Xiao et al. 2007).

1.3.2.2 20-Hydroxyeicosatetraenoic acid (20-HETE)

In contrast to EETs, 20-HETE has been reported to be a potent vasoconstrictor in the renal, cerebral, pulmonary, mesenteric and skeletal muscle arterioles (Imig, Navar et al. 1996; Alonso-Galicia, Falck et al. 1999; Gebremedhin, Lange et al. 2000; Zhu, Bousamra et al. 2000; Kunert, Roman et al. 2001; Wang, Zhang et al. 2001). The vasoconstriction effect of 20-HETE is through the blocking of calcium-activated K^+ channels, resulting in depolarization of the VSMCs, and leading to an increase of intracellular K^+ levels with subsequent activation of voltage-gated Ca^{2+} channels (Harder, Gebremedhin et al. 1994; Imig, Zou et al. 1996; Zou, Fleming et al. 1996; Roman 2002). 20-HETE also contributes to the vasoconstriction effects of the ET-1-induced changes in the kidney (Oyekan and McGiff 1998). In addition, it has been found that 20-HETE mediates the proliferation of VSMCs in response to Ang II and NE stimulation through the activation of a Ras MAPK signaling pathway (Muthalif, Benter et al. 1998; Uddin, Muthalif et al. 1998; Muthalif, Parmentier et al. 2000). In the cerebral circulation, cerebral microvessels have been found to produce 20-HETE when incubated with arachidonic acid (Harder, Gebremedhin et al. 1994; Gebremedhin, Lange et al. 2000). 20-HETE also plays an important role in the heart. Inhibition of 20-HETE formation was found to improve the cardiac function and to reduce cardiomyocyte apoptosis following ischemia-reperfusion injury in rats (Lv, Wan et al. 2008; Yousif, Benter et al. 2009). Furthermore, it has been shown that 20-HETE formation was increased in isoproterenol-induced hypertrophied rat hearts (Zordoky, Aboutabl et al. 2008).

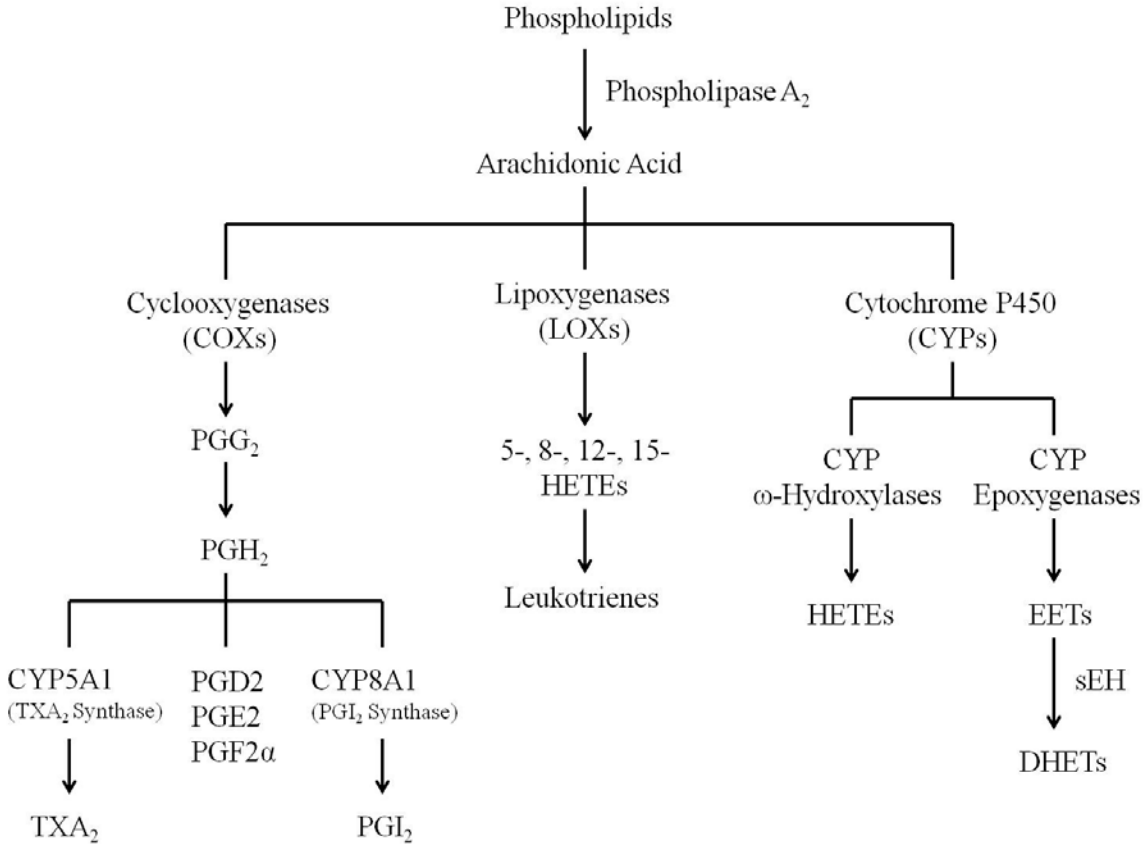

Figure 1.2 Pathways of arachidonic acid metabolism. Arachidonic acid is metabolized by cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochromes P450 (CYPs). COXs metabolize arachidonic acid into PGH_2, which is further metabolized by CYP5A1 and CYP8A1 to TXA_2 and PGI_2, respectively. LOXs metabolize arachidonic acid to HETEs and subsequently to leukotrienes. The third metabolic pathway is mediated by the CYP enzymes, which metabolize arachidonic by ω-hydroxylases and epoxygenases to HETEs and EETs, respectively (Roman 2002).

1.4 Soluble epoxide hydrolase (sEH) as a therapeutic target

EETs exert a variety of cardioprotective effects including vasodilation, anti-inflammatory and anti-migratory actions on VSMCs, anti-hypertensive effect and protection against ischemic stroke and vascular disease. However, EETs can be metabolized by soluble epoxide hydrolase (sEH) into their corresponding diols (DHETs), diminishing their beneficial cardiovascular properties. Therefore, inhibition of sEH is a promising therapeutic strategy to enhance the endogenous levels of EETs by preventing their metabolism (Imig and Hammock 2009).

1.4.1 Soluble epoxide hydrolase (sEH)

The *EPHX2* gene encodes the sEH enzyme, which primarily exists as a homodimer with monomers arranged in an anti-parallel form of the two (Oesch, Schladt et al. 1986; Newman, Morisseau et al. 2005; Morisseau and Hammock 2008). Each monomer is a 62.5kDa protein and composed of two domains, a C-terminal epoxide hydrolase activity which acts on epoxy fatty acids, and an N-terminal phosphatase activity which acts on lipid phosphates (Newman, Morisseau et al. 2003; Morisseau and Hammock 2005; EnayetAllah, Luria et al. 2008). The epoxide hydrolase activity is well defined; however, the biological role of the phosphatase activity remains to be elucidated (Tran, Aronov et al. 2005). sEH is a highly conserved enzyme that is widely distributed in numerous tissues, including lungs, heart, brain, spleen, adrenal, intestine, bladder, vascular endothelium, smooth muscle, placenta, skin, mammary glands and other tissue (Enayetallah, French et al. 2004). However, sEH has demonstrated to be highly expressed in the liver and kidney (Yu, Davis et al. 2004; Newman, Morisseau et al. 2005). Within the cell, sEH is mainly localized in the cytosolic faction; it has also been found to localize in the peroxisomes (Enayetallah, French et al. 2006).

1.4.1.1 sEH expression in the heart

The expression of she has been found in the human myocardium and blood vessels (Enayetallah, French et al. 2004). Other studies showed that the

activity of sEH was highly expressed in the human coronary endothelial cells (VanRollins, Kaduce et al. 1993), and lower levels were detected in arteries and vascular smooth muscle cells (Yu, Davis et al. 2004). In rats, a recent study detected the mRNA expression of sEH in the heart of Sprague-Dawley rats (Zordoky, Aboutabl et al. 2008). Several studies also determined a high level of sEH activity in the heart of these rats (Oesch, Schladt et al. 1986; Schladt, Worner et al. 1986). Furthermore, the mRNA expression of sEH was identified in the heart of mice at a moderate level (Johansson, Stark et al. 1995). The protein and activity levels were also reported in mouse heart by another study (Waechter, Bentley et al. 1988).

1.4.1.2 sEH expression in the cardiac hypertrophy and heart failure

The expression of sEH has been found to be altered in several cardiovascular conditions. One study has identified *EPHX2*, the gene encoding sEH, as a susceptibility factor for heart failure in spontaneously hypertensive heart failure (SHHF) rats. They found an increase in the expression of the transcript, protein, and enzymatic activity levels in these rats, which lead to a more rapid hydrolysis of cardioprotective EETs. The role of sEH in heart failure was confirmed using *Ephx2* knockout mice, where *Ephx2* gene ablation protected from pressure overload-induced heart failure and cardiac arrhythmias (Monti, Fischer et al. 2008). Other studies have shown that increased the mRNA and protein expression of sEH in both human umbilical vein ECs and bovine aortic ECs. Similarly, the protein level of sEH was induced in the aortic intima of spontaneous hypertensive rats and by Ang II in normotensive rats (Ai, Fu et al. 2007). To further confirm the role of sEH in cardiac hypertrophy, Ai and colleagues have detected an increased level of sEH protein in the rat myocardium of Ang II-induced cardiac hypertrophy (Ai, Pang et al. 2009). Correspondingly, an *in vitro* study showed that Ang II induced sEH protein expression and cardiac hypertrophy in rat neonatal cardiomyocytes (NCMs). Interestingly, the authors further showed that adenoviral over-expression of sEH in cultured NCMs increased the cell area and expression of hypertrophic markers. These results

suggest sEH as a mediator for the hypertrophic effect of Ang II, but also sufficient for the induction of cardiac hypertrophy (Ai, Pang et al. 2009). Recently, a study has demonstrated that isoproterenol-induced cardiac hypertrophy significantly increased the mRNA expression of sEH in rat hearts (Zordoky, Aboutabl et al. 2008). Moreover, the mRNA and protein expression of sEH were also found to be induced in AhR ligands; 3-MC- and BaP-mediated cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2009).

1.4.2 Soluble epoxide hydrolase (sEH) inhibitors

Several sEH inhibitors have been synthesized, targeting compounds with high potency, solubility and stability. The first-generation of sEH inhibitors were potent competitive inhibitors, which included chalcone oxides and glycidols (Mullin and Hammock 1982; Spector, Fang et al. 2004; Morisseau and Hammock 2005). However, instability was a concern as these compounds are rapidly inactivated by glutathione and glutathione transfereases (Spector, Fang et al. 2004; Morisseau and Hammock 2005). Amides, ureas and carbamate sEH inhibitors were discovered later; they are potent, competitive tight-binding and stable transition state inhibitors of sEH. Among the different sEH inhibitors, urea is the central pharmacophore that binds to the sEH active site through the formation of hydrogen bond-stabilized salt bridges between the urea moiety and residues of the C-terminal of sEH (Morisseau, Goodrow et al. 1999; Morisseau and Hammock 2005). This suggests that this interaction imitates the transient intermediates during the enzymatic reaction that involves the opening of epoxide ring by sEH (Argiriadi, Morisseau et al. 2000; Morisseau, Goodrow et al. 2002).

sEH inhibitors with the urea pharmacophore include 1-cyclohexyl-3-dodecyl-urea (CDU) and 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA). Although these are highly potent enzyme inhibitors, they seem to have poor solubility in water (Fang, Weintraub et al. 2004). The solubility of AUDA is higher than that of cyclohexyl ureas, however, AUDA requires a considerable amount of 2-hydroxylpropyl β-cyclodextrin to increase its solubility and bioavailability. Several structural modifications were made to increase the water

solubility of the inhibitor without reducing its potency (Kim, Heirtzler et al. 2005; Hwang, Tsai et al. 2007; Ghosh, Chiang et al. 2008; Morisseau and Hammock 2008). Finally, 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea (AEPU) demonstrated improved water solubility, good oral bioavailability and high potency, but a decline in plasma concentration as it was rapidly metabolized. Recently, several compounds have been shown excellent potency and efficacy in many species, and these include *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxyl]-benzoic acid (*t*-AUCB), and 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxyphenyl)-urea (TUPS) (Chiamvimonvat, Ho et al. 2007). TUPS has demonstrated a high potency on human sEH and appropriate oral bioavailability and pharmacokinetic in a canine model (Tsai, Hwang et al. 2010).

1.4.3 Role of sEH inhibitors in cardiovascular diseases

Inhibition of sEH has emerged as a novel approach for the treatment of some cardiovascular diseases. Several studies have shown that sEH inhibitors exert a significant effect in reducing blood pressure in numerous animal models of hypertension (Imig and Hammock 2009). It has been found that the N,N'-dicyclohexyl urea (DCU) lowered blood pressure and decreased DHET excretion in SHRs (Yu, Xu et al. 2000). DCU decreased blood pressure in a rat model of Ang II-induced hypertension (Imig, Zhao et al. 2002). AUDA was the first sEH inhibitor administered orally and found to attenuate blood pressure and increase the plasma EET to DHET ratio in rat and mouse models of Ang II-induced hypertension (Imig, Zhao et al. 2005; Jung, Brandes et al. 2005; Loch, Hoey et al. 2007), as well as in stroke-prone spontaneously hypertensive rats (SHRSP) (Li, Carroll et al. 2008). Similarly, treatment with N-adamantyl-N'-dodecylurea (ADU) and *t*-AUDA decreased systolic blood pressure and normalized endothelial function in deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Loch, Hoey et al. 2007; Manhiani, Quigley et al. 2009). Furthermore, a significant reduction of the systolic blood pressure was found in male sEH knockout mice as compared to the wild-type mice in dietary salt loading and DOCA-salt hypertension (Sinal, Miyata et al. 2000; Manhiani, Quigley et al. 2009). The

mechanism by which sEH inhibitors lower blood pressure is thought to depend on decreased vascular resistance and enhanced Na^+ excretion (Zhao, Yamamoto et al. 2004; Imig, Zhao et al. 2005; Jung, Brandes et al. 2005). These findings are consistent with the biological actions of EETs to dilate blood vessels and inhibit renal tubular Na^+ reabsorption (Spector, Fang et al. 2004; Imig 2005; Fleming 2008).

sEH inhibitors also exert cardioprotective effects against ischemia-reperfusion. Administration of AUDA showed an improved recovery of left ventricular developed pressure and a smaller myocardial infarct size in canine hearts (Gross, Gauthier et al. 2008). Similarly, a sEH inhibitor, AUDA butyl ester (AUDA-BE) significantly reduced the infarct size after myocardial ischemia-reperfusion injury in mice (Motoki, Merkel et al. 2008). Furthermore, AEPU and *t*-AUCB showed an improved cardiac function and prevented the progression of cardiac remodeling post myocardial infarction in mice (Li, Liu et al. 2009). With regard to its role in cardiac hypertrophy, AUDA has been shown to attenuate cardiac hypertrophy in SHRSP rats (Li, Carroll et al. 2008). Moreover, the sEH inhibitors AEPU and AUDA were found to decrease left ventricular hypertrophy in a mouse model of pressure overload-induced cardiac hypertrophy (Xu, Li et al. 2006). Administration of TUPS also prevented the left ventricular hypertrophy of Ang II-induced cardiac hypertrophy (Ai, Pang et al. 2009). More recently, it has been shown that inhibition of sEH with TUPS protects against BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011).

The effect of sEH inhibitors in the protection of cardiac hypertrophy was attributed to their ability to prevent metabolism of EETs, thereby prolonging their effects, which in turn blocks the activation of NF-κB (Xu, Li et al. 2006). Generally, NF-κB is inactive when bound to its inhibitor protein, IKB. Once IKB is phosphorylated by IKB kinase (IKK), NF-κB translocates to the nucleus and mediates gene transcription. EETs can inhibit IKK, which prevents the activation of NF-κB and ultimately the NF-κB-mediated gene transcription (Campbell 2000). NF-κB is a downstream target of several signaling pathways that are

involved in cardiac hypertrophy and heart failure; these include the α-AR, ras, PI3K/Akt, PKC, p38 and MEKK1/4-MAPK pathways (Harris, Li et al. 2008).

1.5 Rationale, Hypotheses, and Objectives

1.5.1 Rationale

Cardiovascular disease is the leading cause of death in North America. While the rate of deaths from cardiovascular disease has been declining over the past few decades, it remains as one of the major health problems of modern times. It accounts for more than 30% of all deaths in 2008 and costs more than $298 billion in the United States and $21 billion in Canada. Heart failure alone affects more than 5.8 million patients and is the main cause of mortality and morbidity resulting from cardiovascular diseases in North America. (Roger, Go et al. 2012). Cardiac hypertrophy is a major pathological event and prolonged hypertrophy is known as a significant risk factor for heart failure and sudden death. Therefore, it is important to identify the molecular basis of cardiac hypertrophy and develop treatment approaches to prevent or reverse the hypertrophic phenotype and subsequently heart failure at an early stage.

In searching for the pathogenesis of cardiovascular disease, increasing evidence has suggested a role of the CYP superfamily in the onset, progression and prognosis of cardiovascular disease (Roman 2002). The expression of many CYP enzymes has been identified in the heart and their levels have been reported to be altered during cardiac hypertrophy and heart failure. The CYP enzymes are considered as one of the major metabolic factors for the metabolism of arachidonic acid. Arachidonic acid is metabolized by CYP epoxygenases to cardioprotective metabolites, EETs and by CYP ω-hydroxylases to cardiotoxic metabolites, HETEs. These metabolites have a significant biological role in the regulation of the cardiovascular system (Roman 2002). The opposing effects of these metabolites suggest the maintenance of a balanced formation of EETs and 20-HETE is critical for normal homeostasis.

EETs have been shown to have numerous beneficial effects in the cardiovascular system. Studies have demonstrated that exogenous administration of EETs improved the recovery of contractile function, increased cell shortening and intracellular Ca^{2+} concentrations, and decreased myocardial infarct size in ischemic hearts (Moffat, Ward et al. 1993; Wu, Chen et al. 1997; Nithipatikom, Moore et al. 2006; Motoki, Merkel et al. 2008). Furthermore, EETs exert protective effects to attenuate myocardial stunning and block the loss in membrane potential in rat cardiomyocytes and H9c2 myocytes (Javadov, Karmazyn et al. 2009; Katragadda, Batchu et al. 2009). Recently, EETs have been considered as a new therapeutic target, as increased EET levels protect the overall health of cardiovascular physiology. Therefore, several studies have demonstrated a novel therapeutic approach using sEH inhibitors in the treatment of cardiovascular diseases. The beneficial effect of sEH inhibitors is attributed to their ability to inhibit the sEH enzyme and increase the endogenous levels of EETs by preventing their metabolism. As a result, EETs are preserved to exhibit several cardioprotective effects, including vasodilation, anti-inflammatory and anti-hypertensive effects, preventing the development of cardiac hypertrophy and protecting against ischemic stroke and vascular disease (Imig and Hammock 2009). Among the sEH inhibitors, TUPS has been shown to prevent Ang II and BaP-induced cardiac hypertrophy in rats (Ai, Pang et al. 2009; Aboutabl, Zordoky et al. 2011).

The importance of studying the role of CYP in the heart has been of great interest in recent years. Previous studies examining the expression and role of CYPs in the heart employed *in vivo* models (Thum and Borlak 2002; Imaoka, Hashizume et al. 2005) or primary cultures of isolated cardiomyocytes (Thum and Borlak 2000b). Each model has its difficulties and limitations. For instance, *in vivo* models may limit the use of chemicals due to the administration route and bioavailability of the compounds. On the other hand, the isolation and cultivation technique of cardiomyocytes is a difficult multi-step process, and isolated cardiomyoctyes are rather fragile (Schluter and Schreiber 2005; Louch, Sheehan et al. 2011). Therefore, the H9c2 cell line is commonly use as an *in vitro* model

for studying the cellular mechanisms and signaling pathways in the heart. The H9c2 cell line is a clonal cardiomyoblast cell line derived from embryonic rat heart tissue (Kimes and Brandt 1976). These cells maintain many molecular features of cardiomyocytes and exert morphological characteristics of immature embryonic cardiomyocytes (Hescheler, Meyer et al. 1991). Furthermore, the H9c2 cell line can be used to study the role of CYPs in the pathogenesis of cellular hypertrophy as multiple CYPs were expressed at a comparable level to those expressed in the rat heart (Zordoky and El-Kadi 2007).

1.5.2 Hypotheses

1) Isoproterenol induces cellular hypertrophy through the alteration of the expression of CYPs and sEH in the H9c2 cell line.
2) Administration of 11,12- and 14,15-EETs exert a protective effect against isoproterenol-induced cellular hypertrophy through modification of the expression of CYPs and sEH.
3) Inhibition of sEH using a sEH inhibitor, TUPS protects against isoproterenol-induced cellular hypertrophy through modification of the expression of CYPs and sEH.

1.5.3 Objectives

The specific objectives of the present work are:

1) To investigate the hypertrophic effect of isoproterenol and its modulation of the expression of CYPs and *EPHX2* genes in rat cardiomyoblast H9c2 cells and rat hepatoma H4IIE cells.
2) To examine the effect of 11,12- and 14,15-EETs against the isoproterenol-induced cellular hypertrophy and their modulations of the expression of CYPs and *EPHX2* genes.
3) To determine the role of sEH in cardiac hypertrophy using a sEH inhibitor, TUPS and its protective effect against isoproterenol-induced cellular hypertrophy through the modulation of the expression of CYPs and *EPHX2* genes.

2. Materials and Methods

2.1 Materials

1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxyphenyl)-urea, TUPS was a generous gift from Dr. Bruce Hammock (University of California, Davis), and synthesized by Paul Jones (University of California, Davis) as described previously (Tsai, Hwang et al. 2010). Isoproterenol and anti-goat IgG with horseradish peroxidase secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) base, fetal bovine serum, L-glutamine, penicillin–streptomycin, TRIzol reagent and UltraPure distilled water were purchased from Invitrogen (Carlsbad, CA). Amphotericin B was purchased from ICN Biomedicals Canada (Montreal, QC, Canada). Amikacin Sulfate injection USP was purchased from Sandoz Canada Inc. (Boucherville, QC, Canada). For real time-polymerase chain reaction (PCR), a 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, USA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) according to previously published sequences. Arachidonic acid metabolite standards, 11, 12-, 14, 15-EETs were obtained from Cayman Chemical (Ann Arbor, MI, USA). Acrylamide, N' N'-bis-methylene-acrylamide, ammonium persulphate, β-mercaptoethanol, glycine, nitrocellulose membrand (0.45μm) and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). CYP1A1 goat anti-rat, CYP1B1 rabbit anti-rat, sEH goat anti-rat, ANP goat anti-rat and actin rabbit anti-rat primary antibodies were purchased from Cruz Biotechnology Inc. (Santa Cruz, CA, USA). CYP2J primary antibody was obtained as a generous gift from Dr. Darryl Zeldin (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA). The pGL3-Basic vector, pRL-CMV Rluc control vector, and

Luciferase Reporter Assay System were purchased from Promega (Mannheim, Germany). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

2.2 Cell culture and treatments

The H9c2(2-1) cell line is a subclone of the original clonal H9 cell line derived from a 13 day embryonic BD1X rat heart. The mononucleate myoblasts of H9c2 cells resemble morphological characteristics of cardiac muscle myoblasts (Kimes and Brandt 1976). H9c2 cells (American Type Culture Collection, Manassas, VA) were maintained in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4mg/ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 62.5 μg/ml amikacin. The H4IIE cell line is an established cell line derived from rat hepatoma. H4IIE cells (American Type Culture Collection, Manassas, VA) were maintained in standard DMEM supplemented with 10% fetal bovine serum, 4mg/ml L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The H9c2 cells and H4IIE cells were grown in 75-cm^2 tissue culture flasks at 37 °C in a 5% CO_2 humidified incubator. For analysis of mRNA, H9c2 cells and H4IIE cells were grown at a density of $1–1.5 \times 10^6$ cells per well in a 6-well tissue culture plate. On 60–80% confluence (2–3 days), H9c2 cells and H4IIE cells were incubated with appropriate stock solutions of isoproterenol to reach final concentrations of 1, 10, 50 and 100 μM for 24 and 48 h. For the EETs studies, 100 μM of isoproterenol was incubated in the presence or absence of either 11, 12-EET or 14, 15-EET at a concentration of 1 μM, every 8 h for 24 h in H9c2 cells. For the TUPS study, 100 μM of isoproterenol was incubated in the presence or absence of 10 μM TUPS for 24 h in H9c2 cells. All treatments were performed in serum-free medium (DMEM). Cell passage around 15 to 21 were used in these experiments.

2.3 Measurement of cell viability

The effects of isoproterenol and TUPS on cell viability were determined by measuring the capacity of reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals. After incubating the H9c2 cells and H4IIE cells with isoproterenol or TUPS for 24 and 48 h in a 96-well plate at 37°C under a 5% CO_2 humidified incubator. The medium was replaced with 100 µl of serum free medium containing 1.2 mM of MTT dissolved in PBS, pH 7.2. The plate was then incubated at 37°C in a 5% CO_2 humidified incubator for 2 h. The medium was then decanted off by inverting the plate, and 100 µl of isopropyl alcohol was added to each well with shaking for 1 h to dissolve the formazan crystals. The color intensity in each well was measured at wavelength of 550 ηm using a BIO-TEK Instruments EL 312e microplate reader. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.4 RNA extraction and cDNA synthesis

Total RNA was isolated from H9c2 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 0.3 ml of TRIzol reagent was added to each six-well cell culture plate to lyse the cells. Cell lysates in TRIzol were then collected into 1.5 ml tubes and mixed with 120 ul chloroform, followed by centrifugation at 12,000 x g for 15 min at 4°C. The aqueous phase which contains RNA was then transferred to a new tube and 300 µl of isopropyl alcohol was added to each tube to precipitate the RNA by freezing the samples at -20°C overnight. The tubes were centrifuged once more at 12,000 x g for 10 min at 4°C. The isopropyl alcohol was aspirated out and the RNA pellet was washed once with 500 µl of 75% ethanol in UltraPure distilled water. The tubes were centrifuged for the last time at 12,000 x g for 5 min at 4°C to precipitate the pellets. The supernatant was collected and discarded, and the pellet was dried, then dissolved in 20-100 µl of UltraPure distilled water depending on the size of the pellet. The tubes were placed in a water bath at 65°C for 10-12 min

to ensure that the pellet was completely dissolved. Total RNA was 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 using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 µg of total RNA from each sample was added to a mix of 2.0 µl 10× reverse transcription buffer, 0.8 µl of 25× dNTP mix (100 mM), 2.0 µl of 10× reverse transcription random primers, 1.0 µl of MultiScribe reverse transcription, and 3.2 µl of 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 s, and finally cooled to 4°C.

2.5 Quantification by real time polymerase chain reaction (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) according to the manufacturer's instructions. The 25 µl reaction mix contained 0.1 µl of 10 µM forward primer, 0.1 µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. The primers used in the current study were chosen from previously published studies and are listed in Table 2.1. (Bleicher, Pippert et al. 2001; Kalsotra, Anakk et al. 2002; Kuwahara, Kai et al. 2002; Grygielko, Martin et al. 2005; Hirasawa, Kawagoe et al. 2005; Rollin, Mediero et al. 2005; Sellers, Sun et al. 2005; Baldwin, Bramhall et al. 2006; Soppa, Lee et al. 2008; Zordoky, Anwar-Mohamed et al. 2011). 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 s, and annealing/extension at 60°C for 1 min.

2.6 Real time-PCR data analysis

The real time-PCR data were analyzed using the relative gene expression, that is ΔΔCT method as described in Applied Biosystems User Bulletin No. 2 and as described previously (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Briefly, the data are presented as the -fold change in gene expression normalized to the endogenous reference gene, glyceraldehyde-3-phophate dehydrogenase (GAPDH) and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by the treatments was being studied.

2.7 Cellular protein extraction and Western blot analysis

H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50, 100 μM) for 48 h and cells were collected in lysis buffer (50mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% glycerol (v/v), 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail). The total cellular proteins were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min. The lysates were centrifuged at 12,000 x g for 10 min at 4°C. The protein concentrations in the H9c2 cells were determined by the Lowry method using bovine serum albumin as a standard (Lowry, Rosebrough et al. 1951).

Western blot analysis was performed using a previously described method (Gharavi and El-Kadi 2005). Briefly, 35-90 μg of cellular protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in blocking solution containing 0.15 M sodium chloride, 3 mM KCl, 25 mM Tris-base (TBS), 5% skimmed milk, 2% bovine serum albumin and 0.5% Tween-20.

After blocking, the blots were incubated with primary polyclonal goat anti-rat CYP1A1 antibody, rabbit anti-rat CYP1B1, rabbit anti-rat CYP2E1, rabbit anti-rat CYP2C11, rabbit anti-mouse CYP2J, goat anti-rat sEH and rabbit anti-rat

actin and were incubated overnight at 4°C. The primary antibodies were prepared in TBS solution containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody for CYP1A1 and sEH or goat anti-rabbit IgG secondary antibody for CYP1B1, CYP2C11, CYP2E1, CYP2J, and actin was carried out for 4 h at room temperate. The bands were visualized using an enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensities of the protein bands were quantified relative to the signals obtained for actin, using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

2.8 Luciferase reporter gene assay

H9c2 cells were seeded on 12-well culture plates, allowed to attach and recover for 24 h, and then transfected using Lipfectamine 2000 (Invitrogen). All of the transfections were carried out according to the manufacturer's instructions. The *EPHX2* promoter was cloned upstream of the firefly luciferase gene in the pGL3-Basic reporter plasmid (Promega). For each transfection, 1.6 μg of luciferase promoter constructs were contransfected with 0.1 μg of renilla luciferase control plasmid (pRL-CMV, Promega, Mannheim, Germany) using 4 μl of Lipfectamine 2000 per well. Thereafter, 100 μl of the complexes were added to each well with 900 μl of media to achieve the final transfection volume of 1ml/well. All transfections were performed in serum-free medium (DMEM). After a 24 h long incubation at 37°C, the cells were treated with different concentrations of isoproterenol (1, 10, 50, 100 μM) for 24 and 48 h. For the effect of EETs on *EPHX2* promoter activity, 100 μM of isoproterenol was incubated in the presence or absence of either 11, 12-EET or 14, 15-EET at a concentration of 1 μM, every 8 h for 24 h. For the effect of TUPS on *EPHX2* promoter activity, 100 μM of isoproterenol was incubated in the presence or absence of 10 μM TUPS for 24 h. The cells were lysed with 250μl/well of Passive Lysis Buffer. The luciferase assay was carried by means of the Dual Luciferase Reporter Assay System (Promega) using 20 μl of the cell lysate. Luciferase activities were

determined using a Spectra Fluor Plus microplate reader (TECAN). Firefly luciferase activity was normalized with respect to renilla luciferase activity, and shown as relative luciferase activity (relative luciferase activity = firefly luciferase activity/renilla luciferase activity).

2.9 Statistical analysis

Data are presented as mean $\pm$ S.E.M. Control and treatment measurements were compared using the Student's *t* test. The two-way analysis of variance (ANOVA) was used for comparison across groups in the isoproterenol treatment. A result was considered statistically significant where $p < 0.05$.

Table 2.1 Primer oligonucleotides for quantitative real-time PCR for rat genes

Gene	Forward Primer	Reverse Primer
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2B1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
CYP2J3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
CYP4A1	TCACCTCCCTTCCACTGGTT	TCCACACATGTCATAATTTGCT
CYP4A2	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATT
CYP4A3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATC
CYP4F1	CCCCCAAGGCTTTTTGATG	GAGCGCAACGGCAGCT
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
CYP4F5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
EPHX2	GATTCTCATCAAGTGGCTGAAGAC	GGACACGCCACTGGCTAAAT
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
β-MHC	AGCTCCTAAGTAATCTGTTTGCCAA	AAAGGATGAGCCTTTCTTTGCT
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
β-actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA

3. Results

3.1 Isoproterenol induces cellular hypertrophy and modulates the gene expression of cytochromes P450 and *EPHX2* in H9c2 cells

3.1.1 Effect of isoproterenol on cell viability

To determine the cytotoxic effect of isoproterenol, H9c2 cells and H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50, 100 μM) for 24 and 48 h. Thereafter, cell viability was evaluated by the MTT assay as described in materials and methods. The MTT assay showed that the isoproterenol concentrations ranging from 1-100 μM did not significantly affect cell viability as compared to the control in H9c2 cells and H4IIE cells (Fig. 3.1A-B). Therefore, the observed changes in the gene expression are not due to the decreased cell viability or toxicity.

3.1.2 The expression of *ADRB1* in H9c2 cells and H4IIE cells

In order to determine whether isoproterenol can have an effect on the H9c2 cells and H4IIE cells, we measured the gene expression of the β_1-adrenergic receptor. Isoproterenol is a β-adrenergic agonist, which activates the β_1-receptor in the heart and induces hypertrophy. Our results showed that both H9c2 cells and H4IIE cells expressed *ADRB1* and the treatment of isoproterenol did not cause any significant changes to the *ADRB1* gene expression (Fig. 3.2).

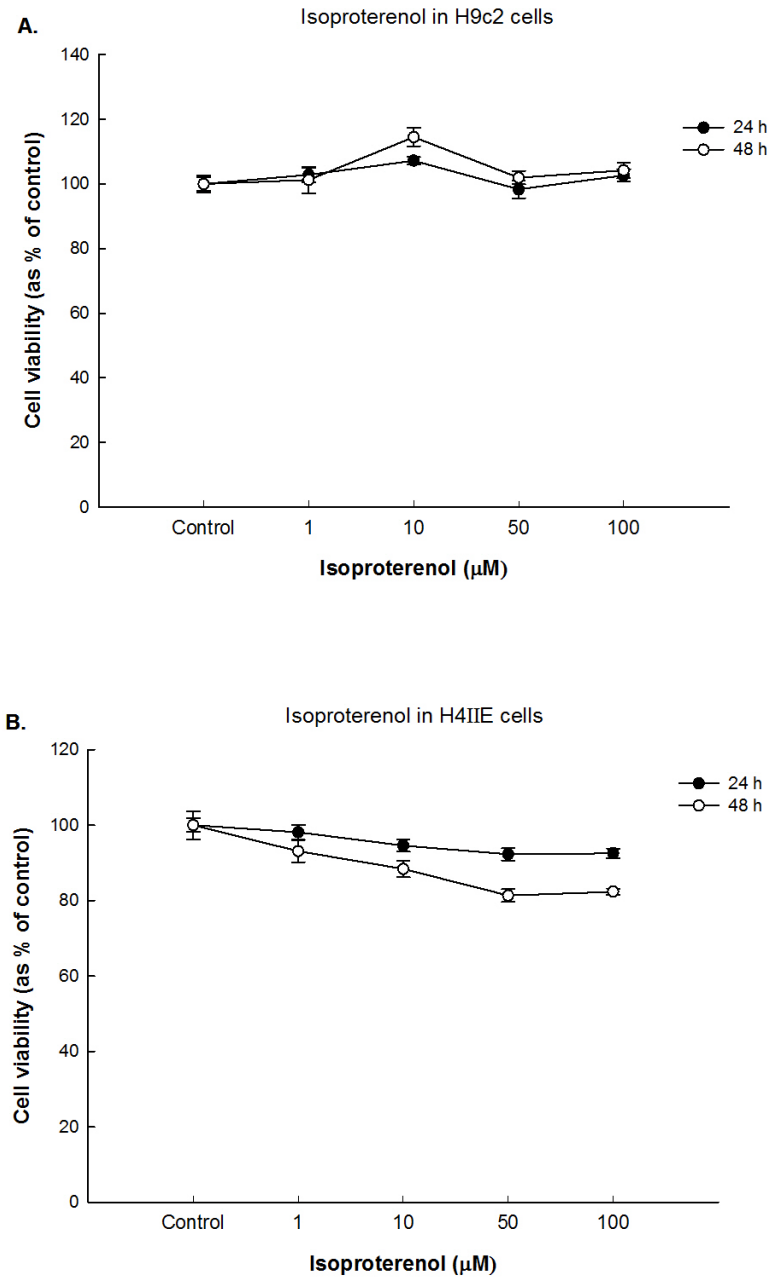

Figure 3.1 Effect of isoproterenol on cell viability. H9c2 cells and H4IIE cells were incubated with increasing concentration of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. The cell viability was measured by the MTT assay as described in materials and methods. Data are presented as a percentage of control (mean ± S.E.M, n=8). * $p < 0.05$ compared to control.

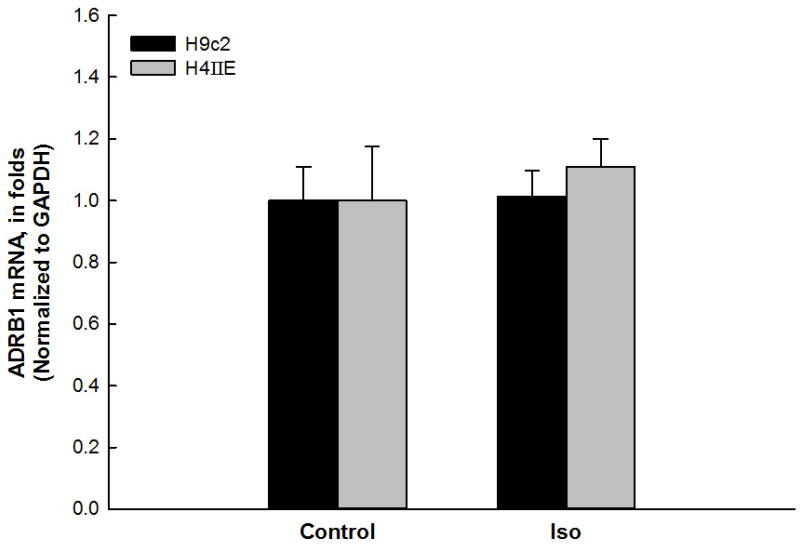

Figure 3.2 The expression of *ADRB1* in H9c2 cells and H4IIE cells. H9c2 cells and H4IIE cells were incubated with 100 μM of isoproterenol for 24 h. Total RNA was isolated and the expression of *ADRB1* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control.

3.1.3 Effect of isoproterenol on the expression of hypertrophic markers in H9c2 cells

To investigate whether isoproterenol causes cellular hypertrophy in H9c2 cells, we determined the mRNA expression of the hypertrophic markers, ANP, BNP and β-MHC relative to the untreated cells following 24 and 48 h of incubation with isoproterenol at different concentrations (1, 10, 50, 100 μM). At the 24 h time point, isoproterenol at 50 and 100 μM caused a significant induction of ANP by 2 and 2.2-fold, respectively. At 48 h, isoproterenol at 50 and 100 μM also caused a significant up-regulation of ANP by 1.5 and 1.3-fold, respectively (Fig. 3.3A). Similarly, our results showed that isoproterenol at 10, 50 and 100 μM caused a significant induction of BNP at 24 h by about 1.5, 1.4 and 2.8-fold, respectively. At 48 h, isoproterenol at 50 and 100 μM caused a significant increase in the BNP levels by 2.7 and 2.3-fold, respectively (Fig. 3.3B). On the other hand, there were no significant changes in the expression of β-MHC with the isoproterenol treatment at both 24 and 48 h (Fig. 3.3C).

3.1.4 Effect of isoproterenol on the expression of CYPs in H9c2 cells

To examine the effect of isoproterenol on the expression of CYPs, H9c2 cells were treated with isoproterenol for 24 and 48 h. Following, the mRNA expression of different CYPs was analyzed using real-time PCR. After 24 h of treatment, we observed a significant increase in the CYP1A1 expression at 10, 50 and 100 μM of isoproterenol by 2.2, 2.9 and 5.6-fold, respectively. Correspondingly at 48 h, isoproterenol at these concentrations caused a significant induction in the CYP1A1 expression by 1.4, 5.3 and 8.9-fold, respectively (Fig. 3.4A). Similarly, a concentration-dependent induction was found in the expression of CYP1B1 at 24 h with 1, 10, 50 and 100 μM of isoproterenol by 1.6, 3.4, 4.6 and 5.9-fold, respectively. These changes were also seen at 48 h with the same concentrations by 1.7, 3.9, 7.1 and 4.5-fold, respectively (Fig. 3.4B).

With regard to the expression of the CYP2 family, there were no significant changes in the mRNA expression of CYP2B1, CYP2C11 and CYP2E1

by isoproterenol at both 24 and 48 h (Figs. 3.5A-B, 3.6A). However, the expression of CYP2J3 at 10, 50 and 100 μM of isoproterenol was significantly increased at 24 h by 3, 3.7 and 5.2-fold, respectively (Fig. 3.6B). Comparable changes were also observed at 48 h with 10, 50 and 100 μM by 2.5, 2.3 and 4.5-fold of induction, respectively (Fig. 3.6B). Fig. 3.7A shows the effect of isoproterenol on the mRNA expression of CYP4F1. There were no significant changes in the expression of CYP4F1 produced by the treatment with isoproterenol for 24 or 48 h. On the other hand, isoproterenol significantly increased the expression of CYP4F4 at 24 h at concentrations of 10, 50 and 100 μM by 2.4, 2.6 and 2.7-fold, respectively. At 48 h, the expression of CYP4F4 was also significantly increased at 10, 50 and 100 μM by 1.7, 2.4 and 1.9-fold, respectively (Fig. 3.7B). Likewise, treatment with isoproterenol at 10, 50 and 100 μM significantly induced the expression of CYP4F5 by 1.6, 1.6 and 2-fold, respectively. Similar changes were observed at 48 h with the concentrations of 10, 50 and 100 μM by approximately 1.6, 1.6 and 1.5-fold, respectively (Fig. 3.7C). Furthermore, we also examined the expression of CYP4A1, CYP4A2 and CYP4A3, but the mRNA levels of these CYPs were not detectable in H9c2 cells.

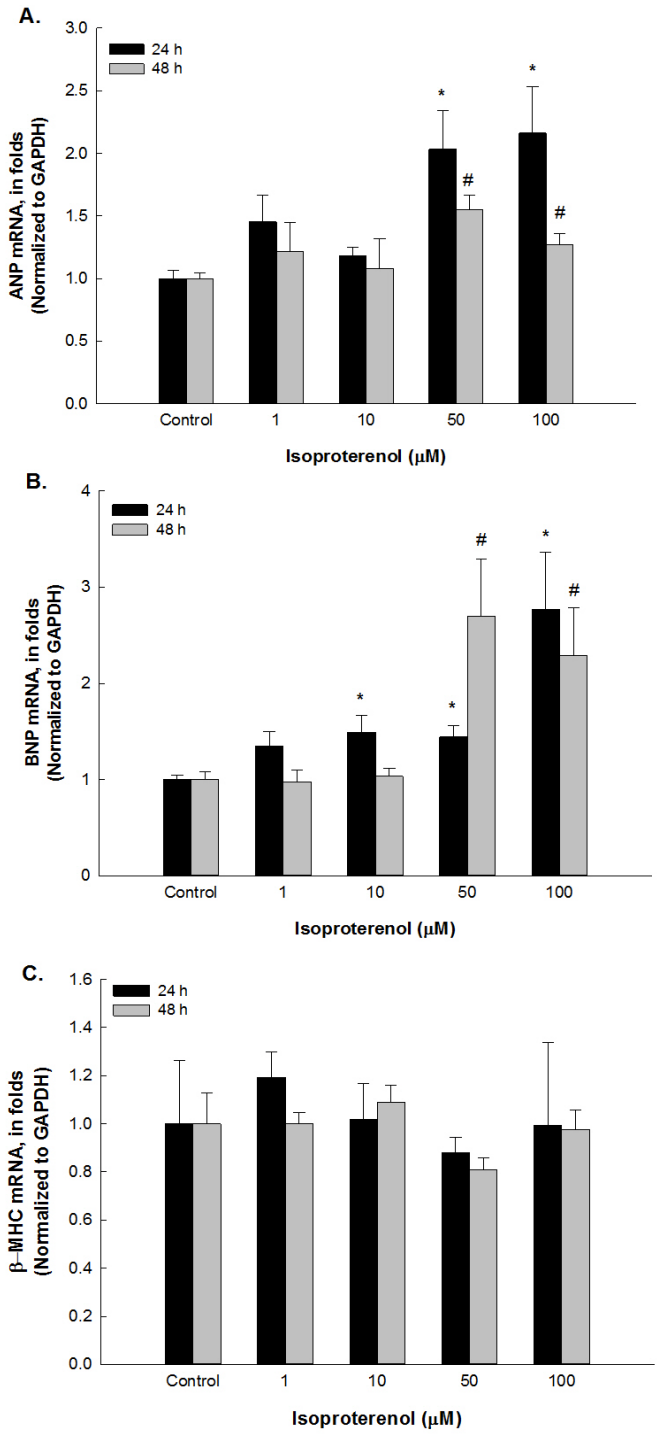

Figure 3.3 Effect of isoproterenol on the expression of hypertrophic markers, ANP (A), BNP (B) and β-MHC (C) in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of the hypertrophic markers, ANP, BNP and β-MHC was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

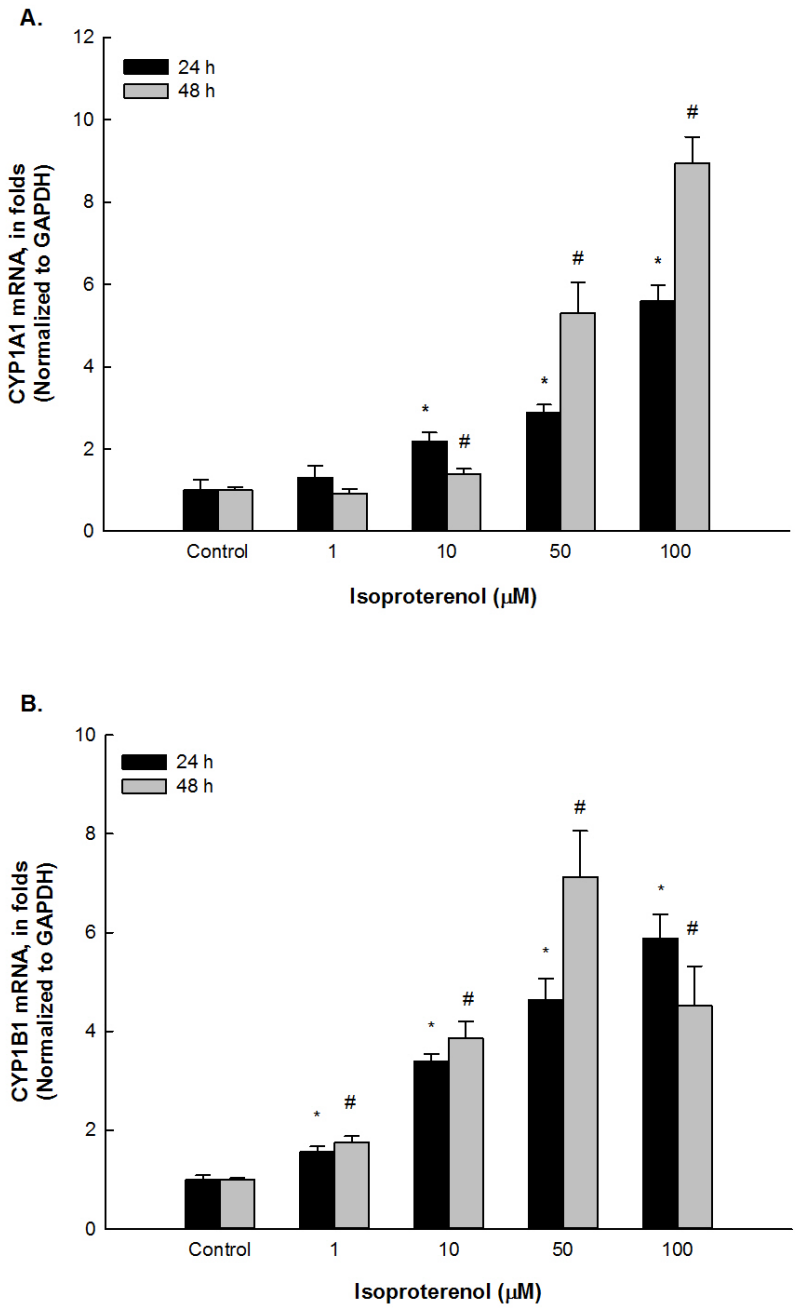

Figure 3.4 Effect of isoproterenol on the expression of CYP1A1 (A) and CYP1B1 (B) in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP1A1 and CYP1B1 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

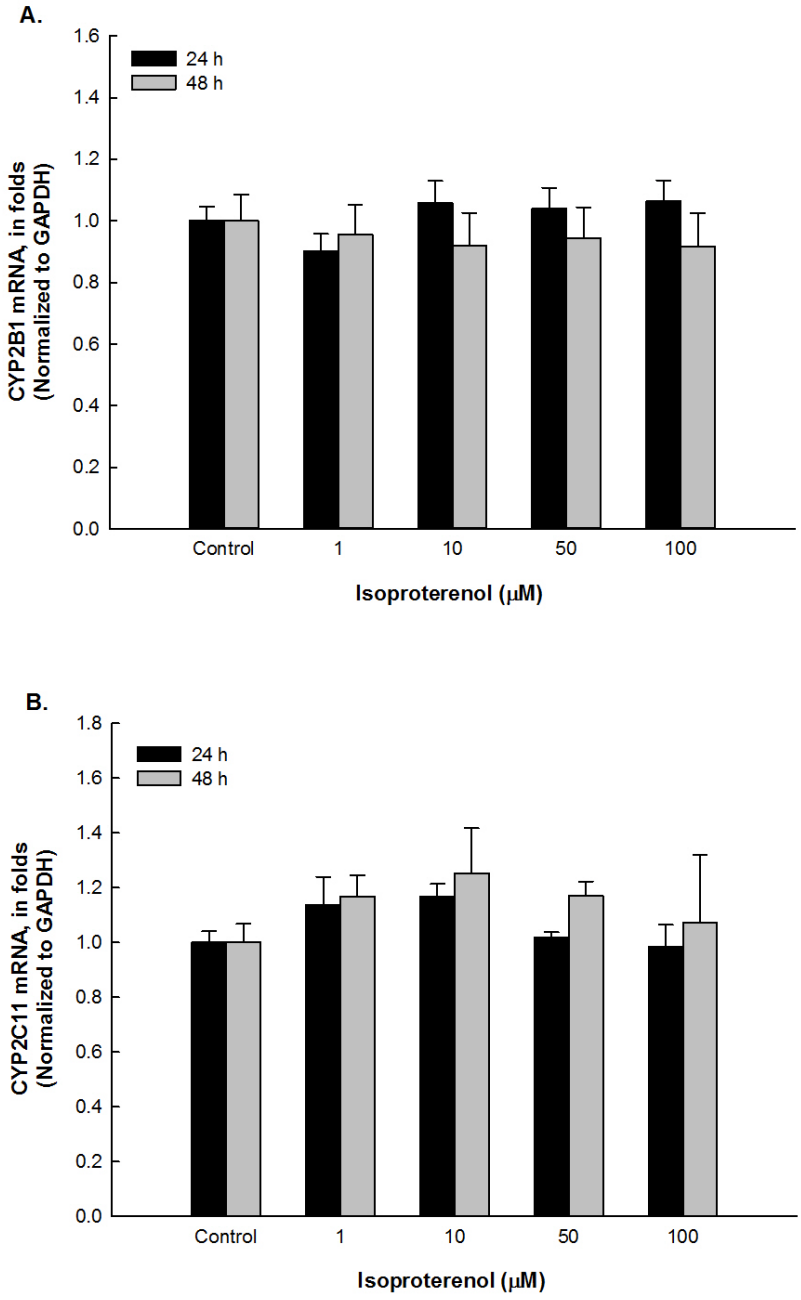

Figure 3.5 Effect of isoproterenol on the expression of CYP2B1 (A) and CYP2C11 (B) in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP2B1 and CYP2C11 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

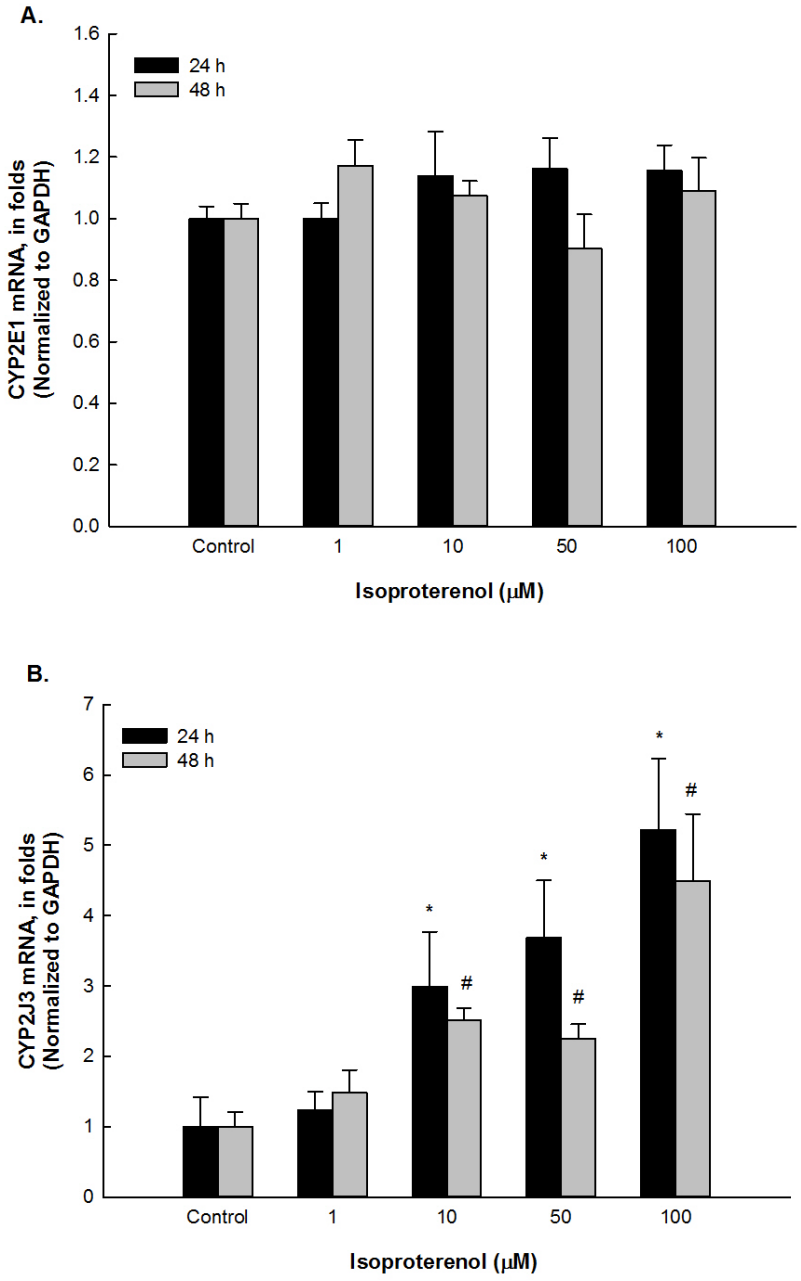

Figure 3.6 Effect of isoproterenol on the expression of CYP2E1 (A) and CYP2J3 (B) in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP2E1 and CYP2J3 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

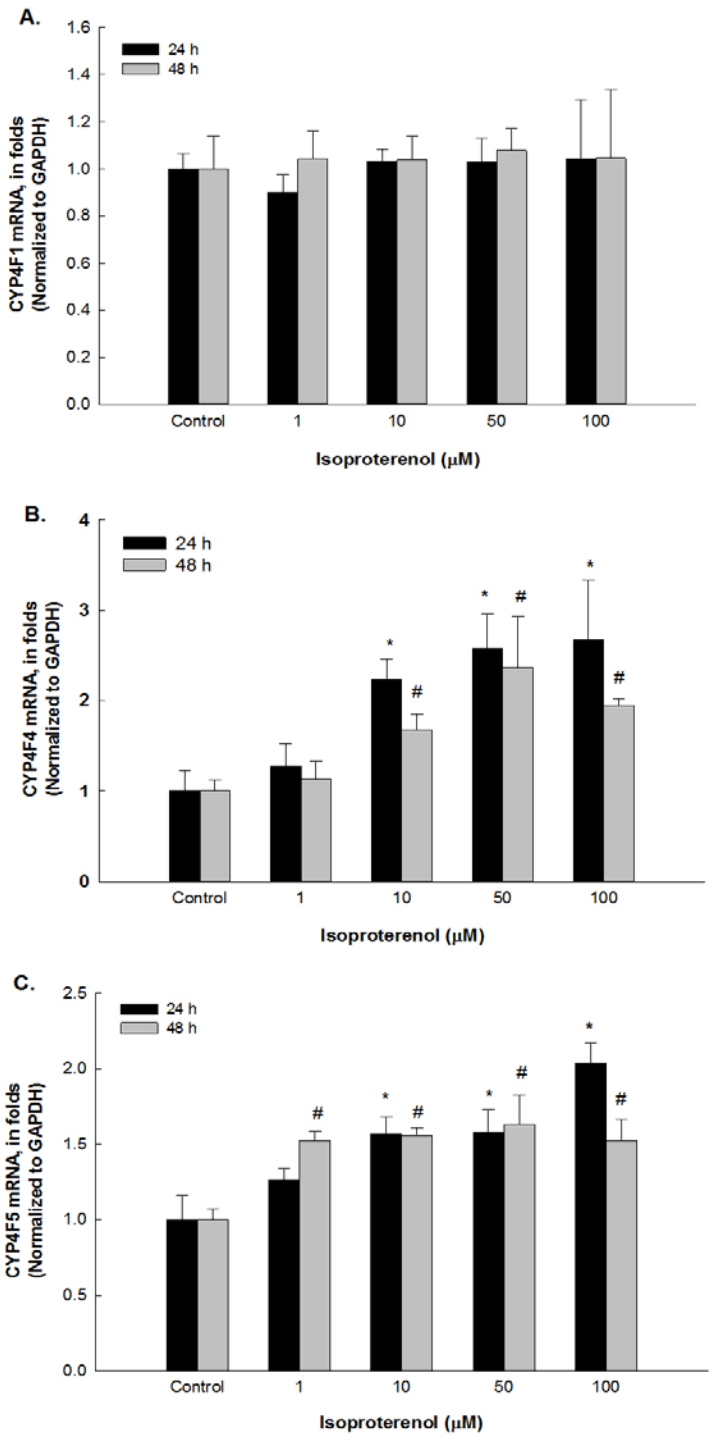

Figure 3.7 Effect of isoproterenol on the expression of CYP4F1 (A), CYP4F4 (B) and CYP4F5 (C) in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP4F1, CYP4F4 and CYP4F5 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

3.1.5 Effect of isoproterenol on the expression of *EPHX2* and the luciferase transcriptional activity of *EPHX2* in H9c2 cells

As depicted in Fig. 3.8A, isoproterenol caused a significant induction of *EPHX2* at 24 h at 1, 10, 50 and 100 μM by 1.3, 1.7, 2 and 1.8-fold, respectively. At 48 h, a prominent increase was found in the *EPHX2* expression at 1, 10, 50 and 100 μM by 1.4, 1.9, 3 and 3.9-fold of induction, respectively (Fig. 3.8A). Furthermore, we examined the effect of isoproterenol on the transcriptional activity of *EPHX2*, a transient expression assay based on a reporter firefly luciferase gene was established. Our findings demonstrated that the luciferase expression resulted in a significant induction at 24 h with 10, 50 and 100 μM of isoproterenol by 1.2, 1.3 and 1.6-fold, respectively. Similarly, treatment with isoproterenol for 48 h caused a significant increase at 10, 50 and 100 μM by 1.3, 1.8 and 1.8-fold, respectively (Fig. 3.8B).

3.1.6 Effect of isoproterenol on the protein expression of CYPs and sEH

To investigate whether isoproterenol-mediated induction of CYPs and *EPHX2* gene expression is further translated into functional proteins, cellular proteins were extracted from H9c2 cells treated with increasing concentrations of isoproterenol (1, 10, 50, 100 μM) for 48 h. Thereafter, CYP1A1, CYP1B1, CYP2C11, CYP2E1, CYP2J3 and sEH protein levels were determined using Western blot analysis. Our results demonstrated that isoproterenol significantly increased the protein levels of sEH at 1, 10, 50 and 100 μM to about 267%, 265%, 270% and 210% of control, respectively (Fig. 3.9A). Protein levels of the CYPs were not found at a detectable level in H9c2 cells.

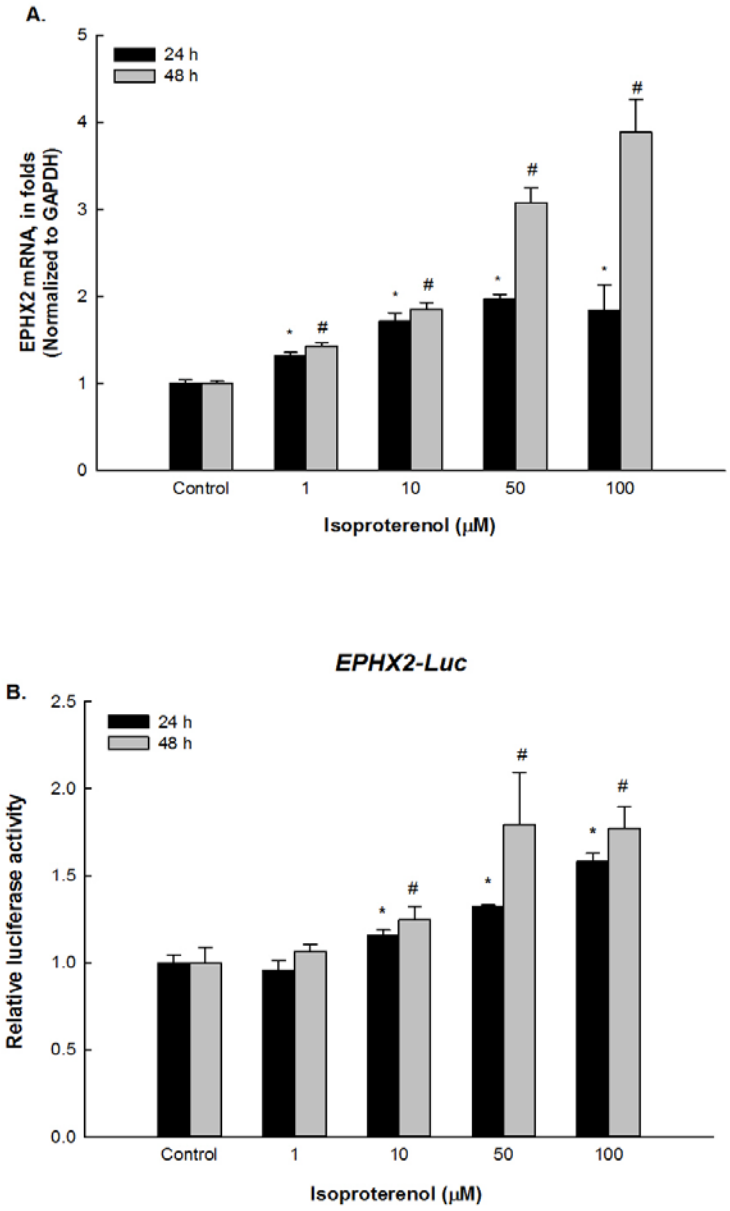

Figure 3.8 Effect of isoproterenol on the expression of *EPHX2* (A) and the luciferase transcriptional activity of *EPHX2* (B) in H9c2 cells. (A) H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of *EPHX2* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=5-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h. (B) H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. H9c2 cells were lysed and luciferase activities were determined using a Spectra Fluor Plus microplate reader. Fold of induction was calculated as a relative luciferase activity (firefly luciferase activity divided by the control renilla luciferase activity). Data are presented in fold of control (mean ± S.E.M, n=3/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

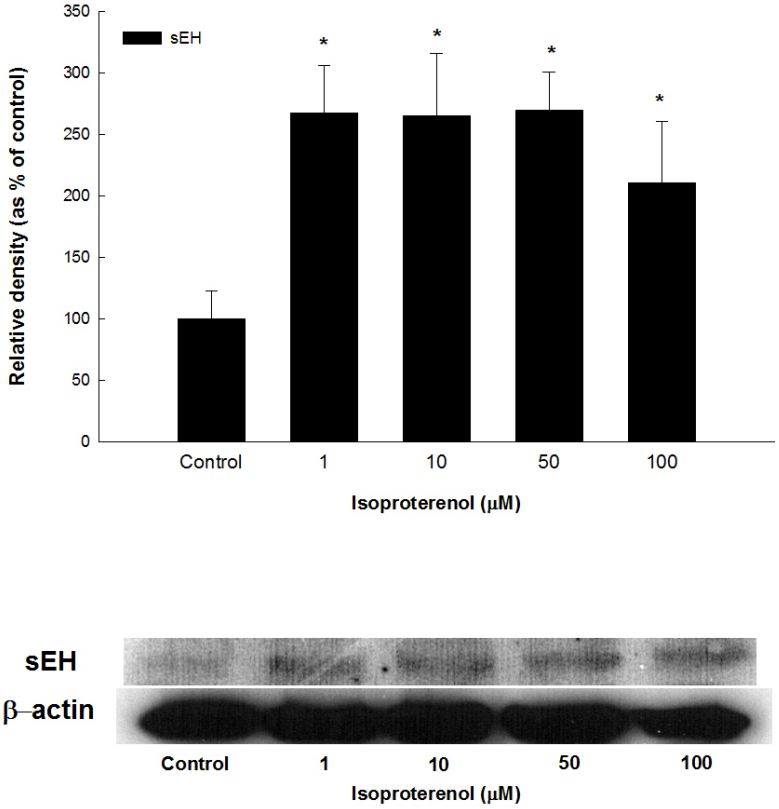

Figure 3.9 Effect of isoproterenol on the protein level of sEH in H9c2 cells. H9c2 cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 48 h. Cellular protein was isolated and 35 μg of protein was separated on a 10% SDS-PAGE. The protein level of sEH was detected using the enhanced chemiluminescence method. The graph represents the relative normalized amount of sEH protein as percentage of control (mean + S.E.M, n=3/group). Representative Western blots are shown for each group, control, isoproterenol (1, 10, 50 and 100 μM). * $p < 0.05$ compared to control.

3.1.7 Effect of isoproterenol on the expression of hypertrophic markers in H4IIE cells

To investigate whether the hypertrophic effect of isoproterenol was specific to H9c2 cells, we examined the effect of isoproterenol in H4IIE cells. The expression of ANP and BNP were not detectable in H4IIE cells. Although β-MHC was expressed in the H4IIE cells, but no significant changes were observed with any of the isoproterenol concentrations at either 24 or 48 h (Fig. 3.10).

3.1.8 Effect of isoproterenol on the expression of CYPs in H4IIE cells

To further examine whether the modulation of CYP expression in isoproterenol-mediated cellular hypertrophy was specific to cardiac cells, rat hepatoma H4IIE cells were also treated with isoproterenol at increasing concentrations for 24 and 48 h. The mRNA expression of CYP1A1 was not significantly changed with any of the isoproterenol concentrations at 24 or 48 h (Fig. 3.11A). Similarly, CYP1B1 was not significantly affected by the isoproterenol treatment at either 24 or 48 h (Fig. 3.11B).

As for the CYP2 family, there were no significant changes in the gene expression of CYP2B1, CYP2C11 or CYP2E1 after treating H4IIE cells with isoproterenol for 24 or 48 h (Fig. 3.12A-C). For CYP2J3, it was found at a very low or undetectable level in the H4IIE cells. In contrast to the H9c2 cells, CYP4A1, CYP4A2 and CYP4A3 were detectable in the H4IIE cells; however, isoproterenol treatment did not caused a significant change in the expression of these CYPs at either time point (Fig. 3.13A-C). Furthermore, the expression of CYP4F1, CYP4F4 and CYP4F5 was not significantly changed in H4IIE cells treated with isoproterenol at 24 and 48 h (Fig.3.14A-C).

3.1.9 Effect of isoproterenol on expression of *EPHX2* in H4IIE cells

Figure 3.15 shows the effect of isoproterenol on the expression of *EPHX2* in H4IIE cells. At 24 and 48 h, isoproterenol caused no significant changes in the mRNA levels of *EPHX2* at any of the isoproterenol concentrations (Fig. 3.15).

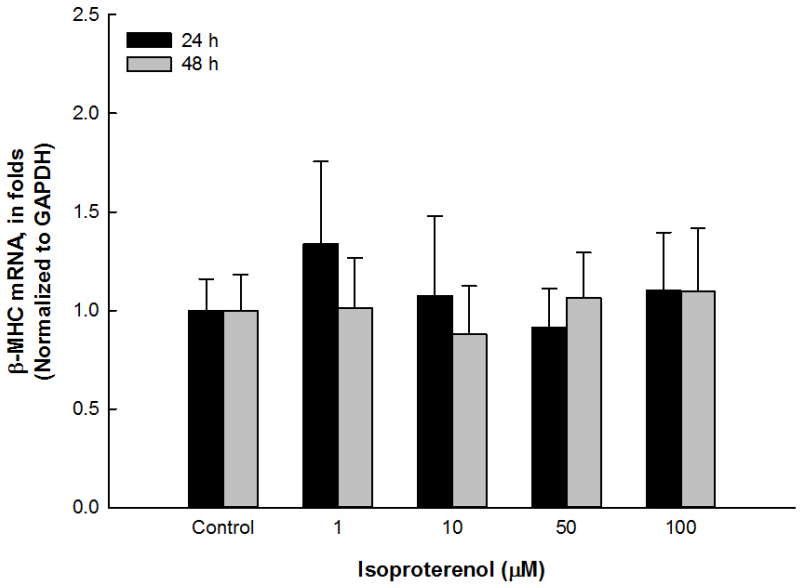

Figure 3.10 Effect of isoproterenol on the expression of hypertrophic marker, β-MHC in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of the hypertrophic markers ANP, BNP and β-MHC were determined by real-time PCR. The expression of ANP and BNP was not detectable in the H4IIE cells. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

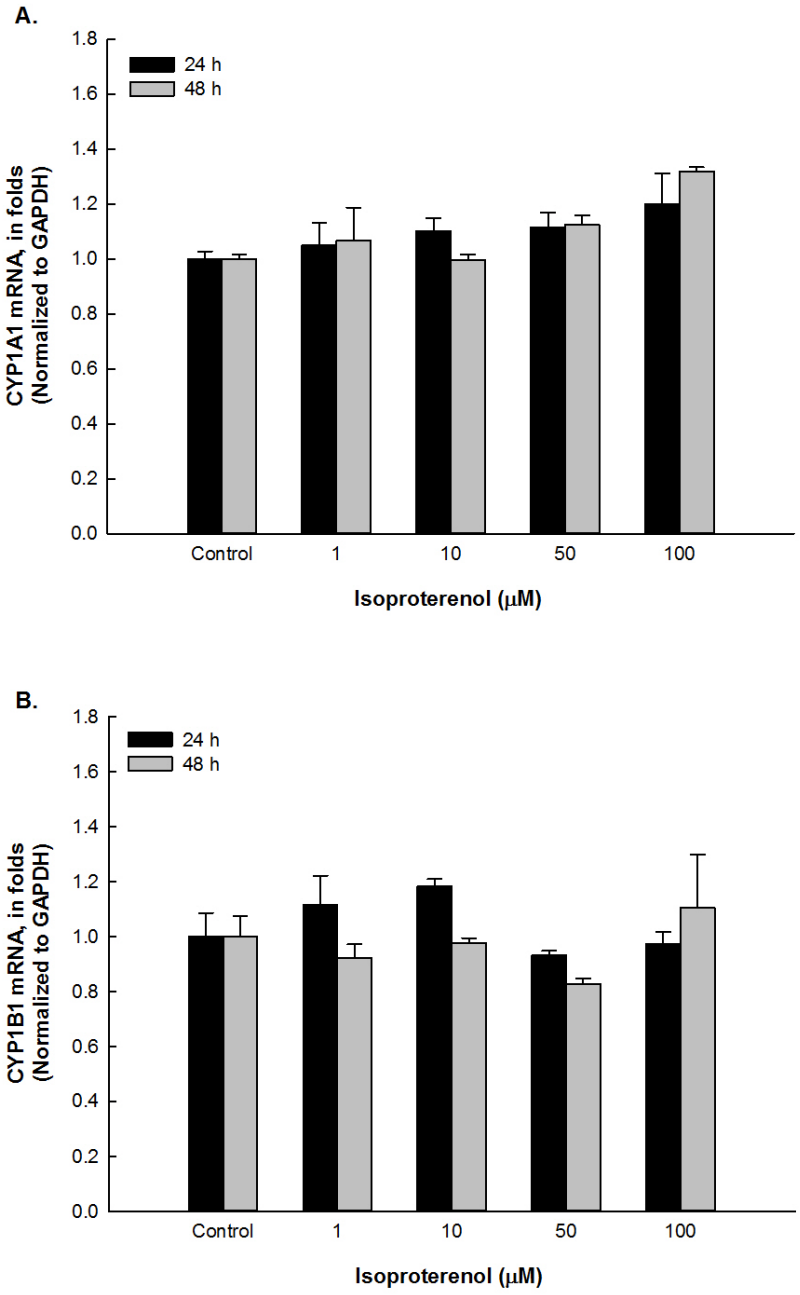

Figure 3.11 Effect of isoproterenol on the expression of CYP1A1 (A) and CYP1B1 (B) in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP1A1 and CYP1B1 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

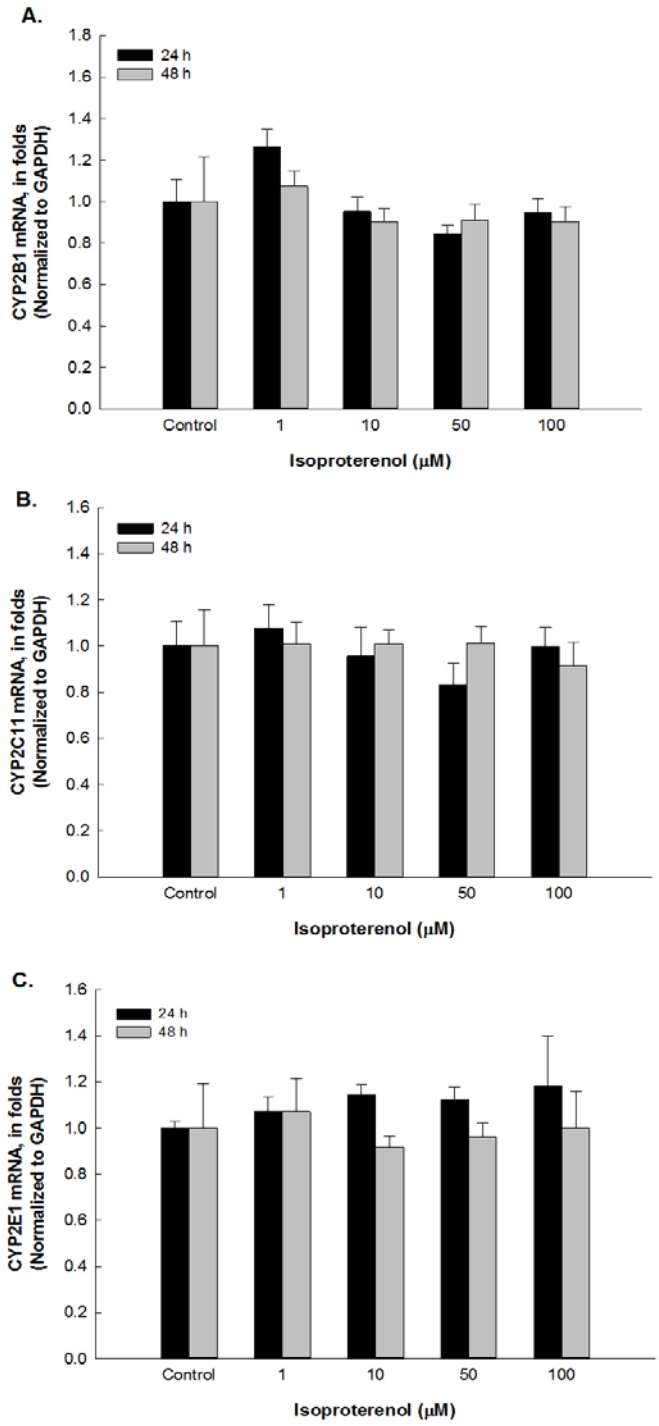

Figure 3.12 Effect of isoproterenol on the expression of CYP2B1 (A), CYP2C11 (B) and CYP2E1 (C) in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP2B1, CYP2C11, CYP2E1 and CYP2J3 was determined by real-time PCR. The expression of CYP2J3 was not detectable level in the H4IIE cells. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

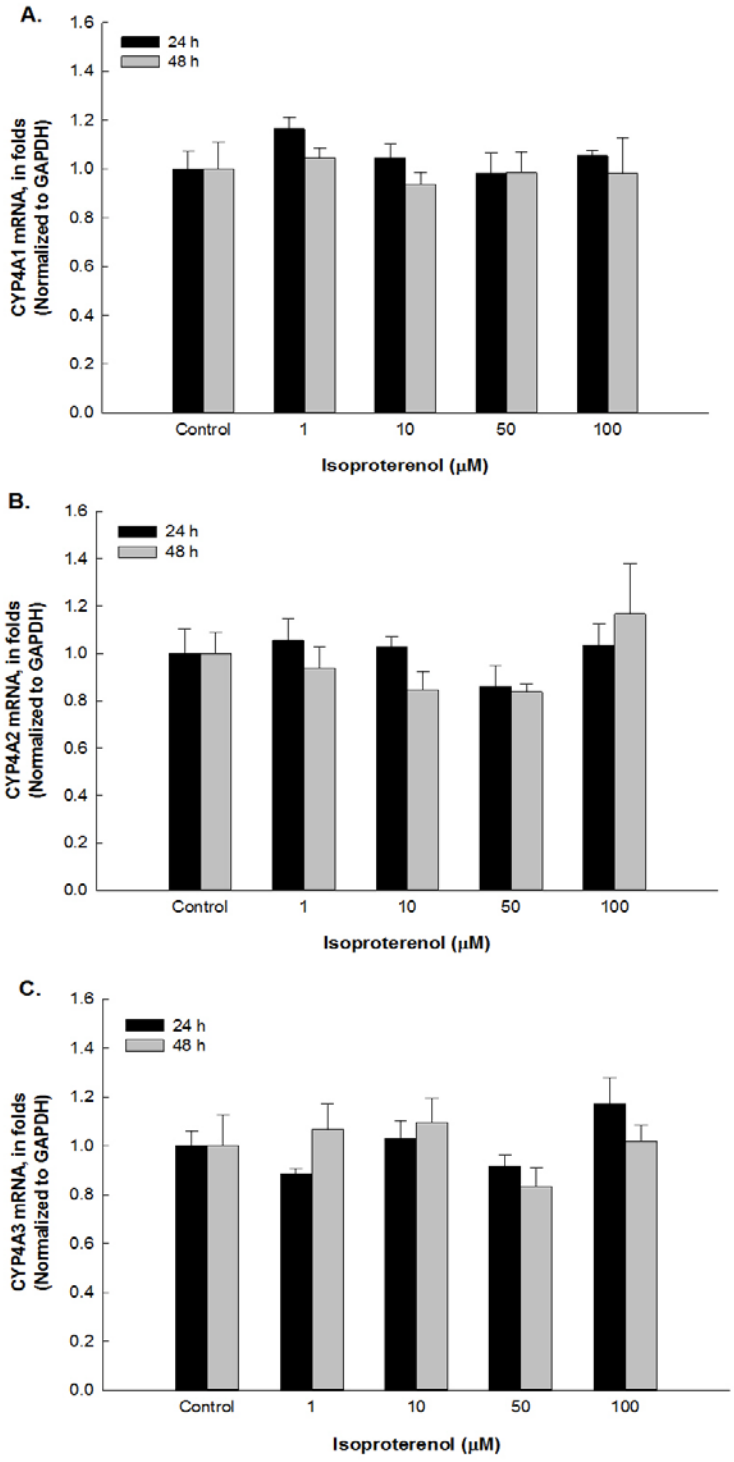

Figure 3.13 Effect of isoproterenol on the expression of CYP4A1 (A), CYP4A2 (B) and CYP4A3 (C) in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP4A1, CYP4A2 and CYP4A3 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

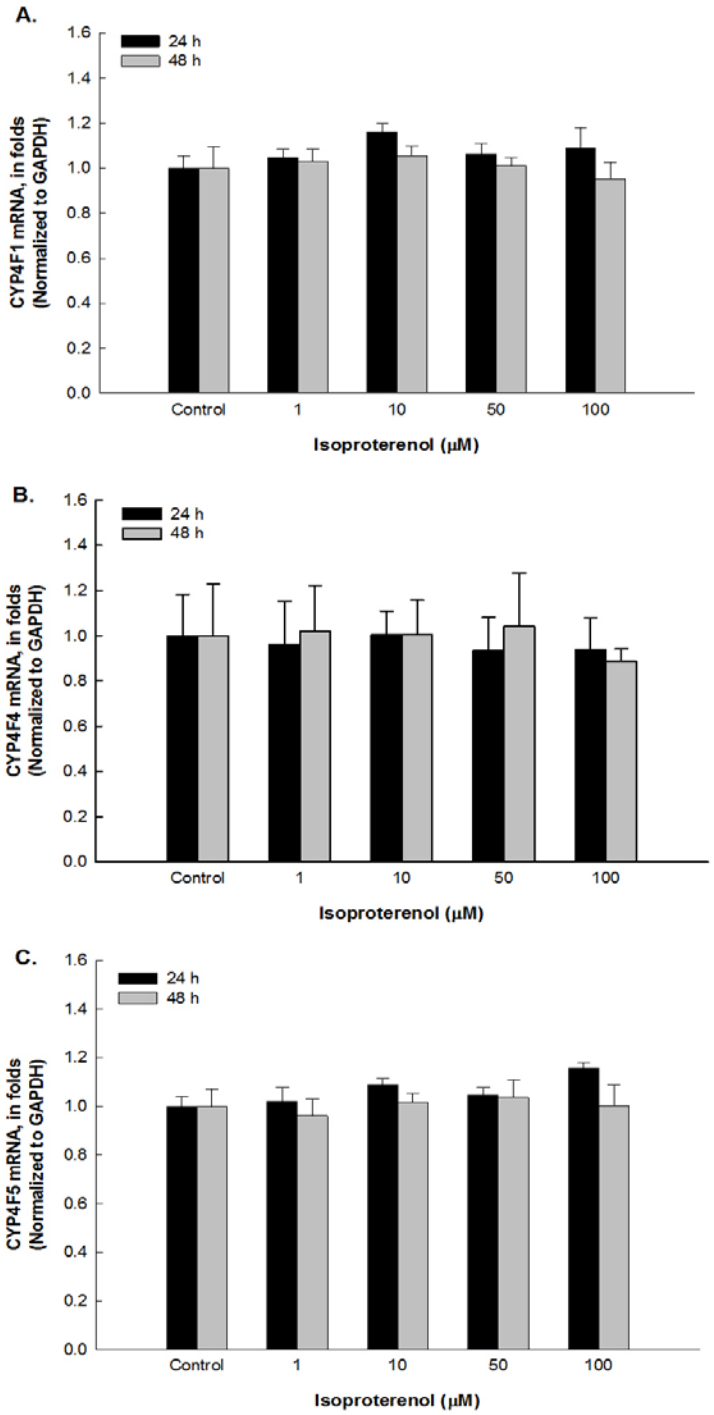

Figure 3.14 Effect of isoproterenol on the expression of CYP4F1 (A), CYP4F4 (B) and CYP4F5 (C) in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of CYP4F1, CYP4F4 and CYP4F5 was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

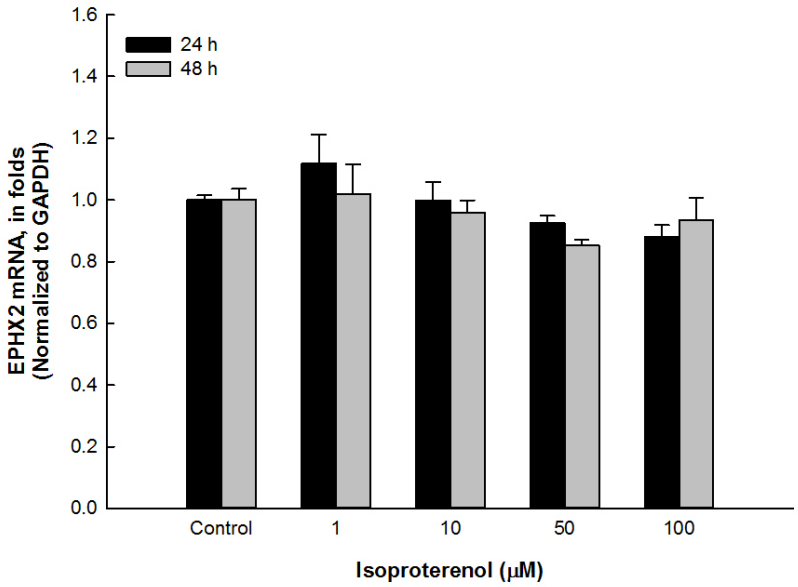

Figure 3.15 Effect of isoproterenol on the expression of *EPHX2* in H4IIE cells. H4IIE cells were incubated with increasing concentrations of isoproterenol (1, 10, 50 and 100 μM) for 24 and 48 h. Total RNA was isolated and the expression of *EPHX2* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control at 24 h. # $p < 0.05$ compared to control at 48 h.

3.2 11,12- and 14,15-EETs protect against isoproterenol-induced cellular hypertrophy

3.2.1 Effect of 11,12-EET on the changes of hypertrophic markers in isoproterenol-mediated hypertrophy

To study the protective effect of 11,12-EET against isoproterenol-induced cellular hypertrophy, H9c2 cells were pretreated with 1 μM of 11,12-EET for 2 h and then 100 μM of isoproterenol, or isoproterenol alone for an additional 24 h. Thereafter, the mRNA expression of the hypertrophic markers ANP, BNP and β-MHC was measured using real-time PCR. Isoproterenol alone caused a significant induction of ANP and BNP. Pre-treatment with 11,12-EET significantly decreased the isoproterenol-mediated effect by 72% and 94% for the expression of ANP and BNP, respectively (Fig. 3.16A-B). On the other hand, isoproterenol did not affect the expression of β-MHC. In addition, 11,12-EET treatment also did not alter the expression of β-MHC (Fig. 3.16C).

3.2.2 Effect of 11,12-EET on the changes of CYPs expression in isoproterenol-mediated hypertrophy

To explore the protective effect of 11,12-EET on the isoproterenol-mediated alterations of CYPs, the mRNA expression was assessed using real-time PCR. Consistent with our earlier findings, isoproterenol alone significantly induced the mRNA expression of CYP1A1 and CYP1B1. Pre-treatment with 11,12-EET significantly attenuated the isoproterenol-mediated induction of CYP1A1 by 77% (Fig. 3.17A). However, treatment of 11,12-EET did not decrease the isoproterenol-mediated induction of CYP1B1. Interestingly, 11,12-EET alone caused a significant increase in the expression of CYP1B1 to about 2-fold of control (Fig. 3.17B).

Within the CYP2 family, CYP2J3 was the only CYP that was significantly induced by the isoproterenol treatment. Treatment of 11,12-EET resulted in a significant reduction in the CYP2J3 expression by 74% (Fig.3.18C). Interestingly, expression of several CYPs were altered by the treatment of 11,12-EET, but not

by the treatment of isoproterenol. As shown in Fig. 3.18A, we observed a significant decrease in the mRNA levels of CYP2C11 in the 11,12-EET alone and isoproterenol plus 11,12-EET groups to about 0.6-fold of control. In contrast, 11,12-EET caused a significant induction of CYP2E1 in the 11,12-EET alone and isoproterenol plus 11,12-EET groups to about 1.6 and 1.3 -fold, respectively (Fig. 3.18B). Furthermore, treatment with 11,12-EET significantly increased the expression of CYP4F1 in the 11,12-EET alone and isoproterenol plus 11.12-EET groups to about 2.5 and 2.2-fold, respectively (Fig. 3.19A). However, isoproterenol alone did not change the expression of these CYPs, i.e. CYP2C11, CYP2E1 and CYP4F1. On the other hand, isoproterenol significantly increased the expression of CYP4F4 and CYP4F5, as we observed earlier. The treatment with 11,12-EET caused a significant reduction in the expression of CYP4F4 that was mediated by isoproterenol by about 116% (Fig. 3.19B). Similarly, 11,12-EET also decreased the isoproterenol-mediated induction of CYP4F5 by 83% (Fig. 3.19C).

3.2.3 Effect of 11,12-EET on the changes of *EPHX2* expression and the luciferase transcriptional activity of *EPHX2* in isoproterenol-mediated hypertrophy

With regard to the expression of *EPHX2*, our results demonstrated a similar induction caused by isoproterenol as mentioned earlier, and 11,12-EET treatment significantly inhibited the isoproterenol-mediated induction of *EPHX2* by about 125% (Fig. 3.20A). Moreover, we examined the effect of isoproterenol on the transcriptional activity of *EPHX2*; a transient expression assay based on a reporter firefly luciferase gene was used in the study. Our results showed that treatment of 11,12-EET caused a significant inhibition in the isoproterenol-induced luciferase activity by 147% (Fig. 3.20B).

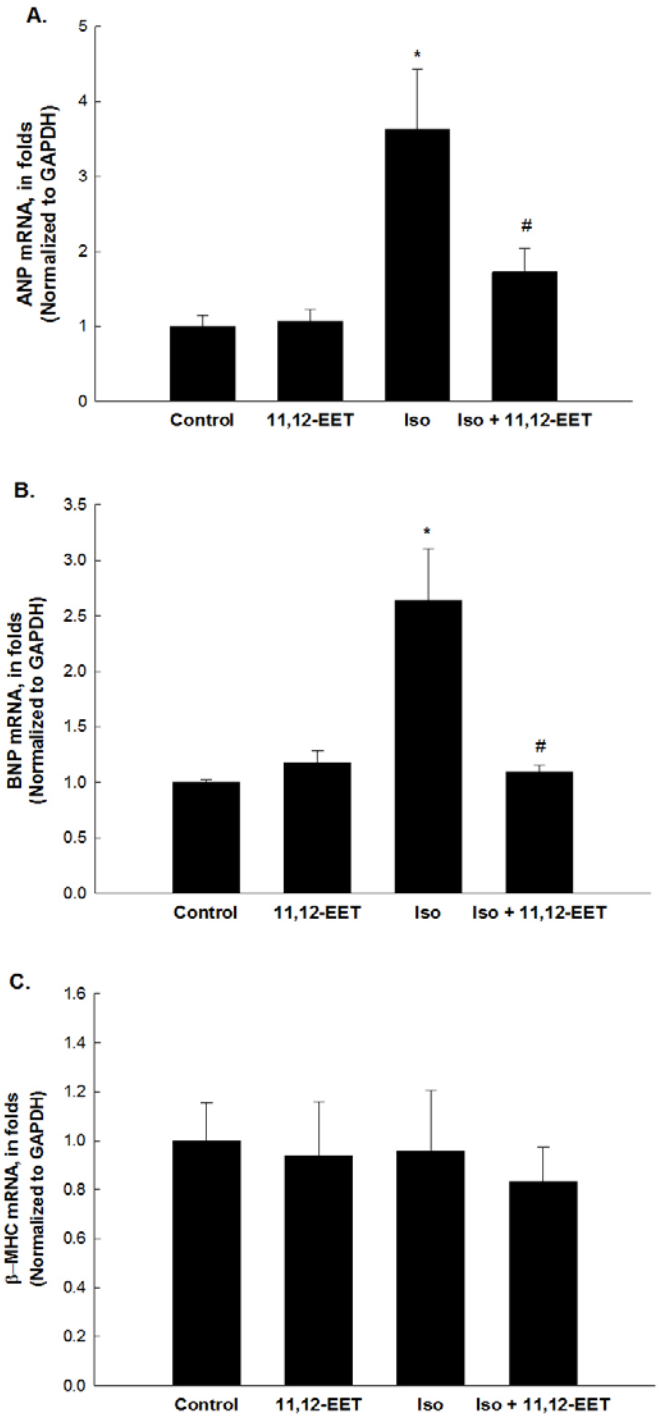

Figure 3.16 Effect of 11,12-EET on the expression of hypertrophic markers, ANP (A) and BNP (B) and β-MHC (C). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. Total RNA was isolated and the expression of the hypertrophic markers ANP, BNP and β-MHC were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

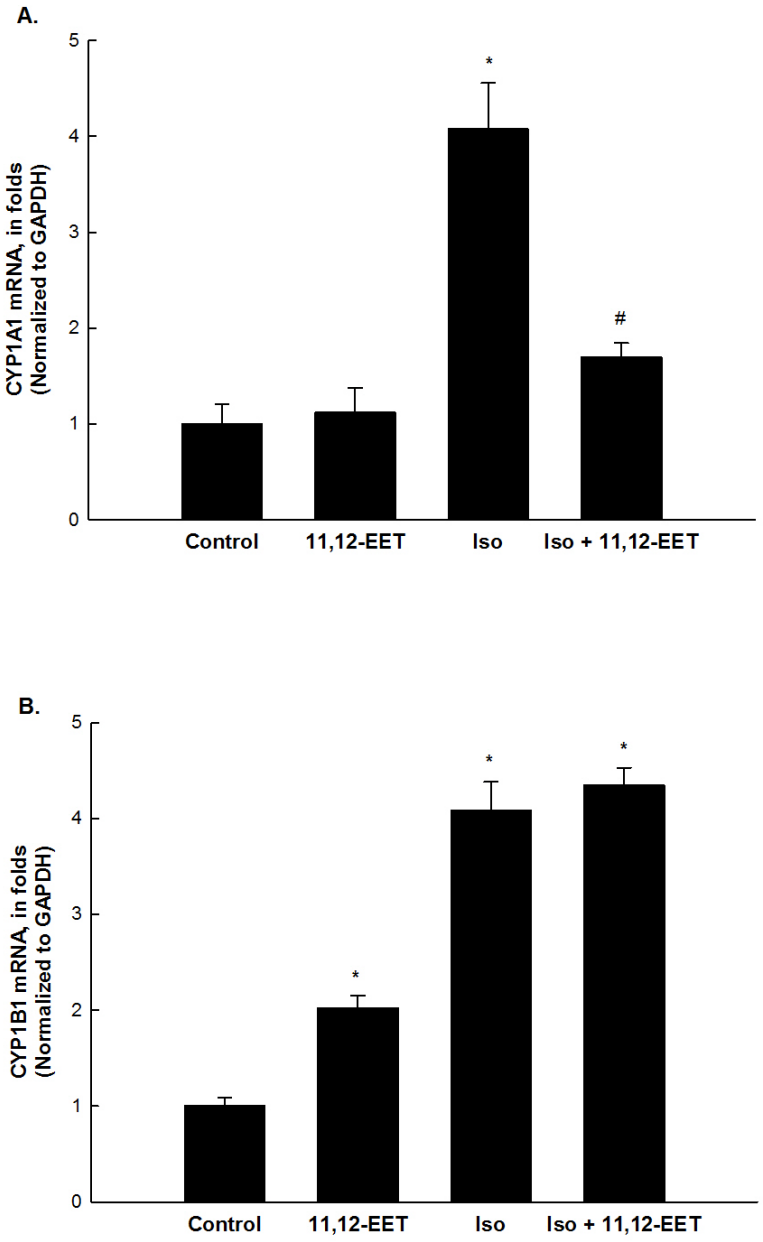

Figure 3.17 Effect of 11,12-EET on the expression of CYP1A1 (A) and CYP1B1 (B). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. Total RNA was isolated and the expression of CYP1A1 and CYP1B1 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

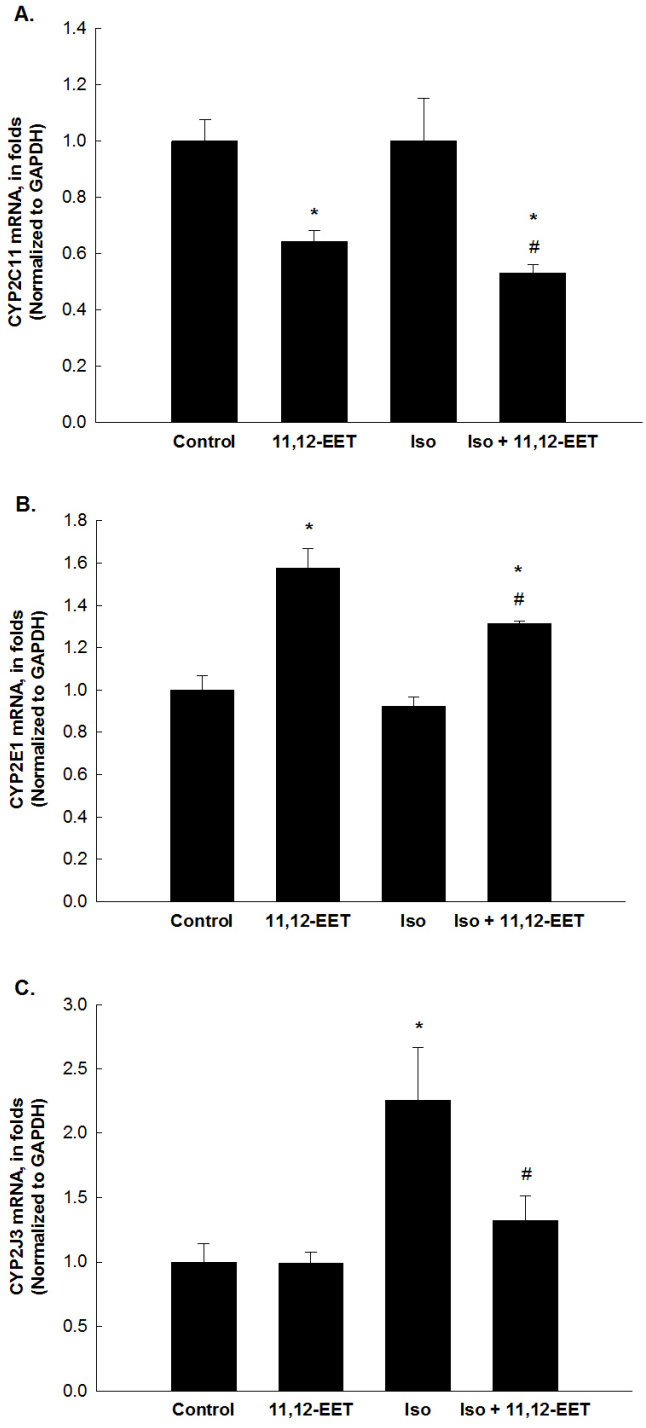

Figure 3.18 Effect of 11,12-EET on the expression of CYP2C11 (A), CYP2E1 (B) and CYP2J3 (C). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. Total RNA was isolated and the expression of CYP2C11, CYP2E1 and CYP2J3 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

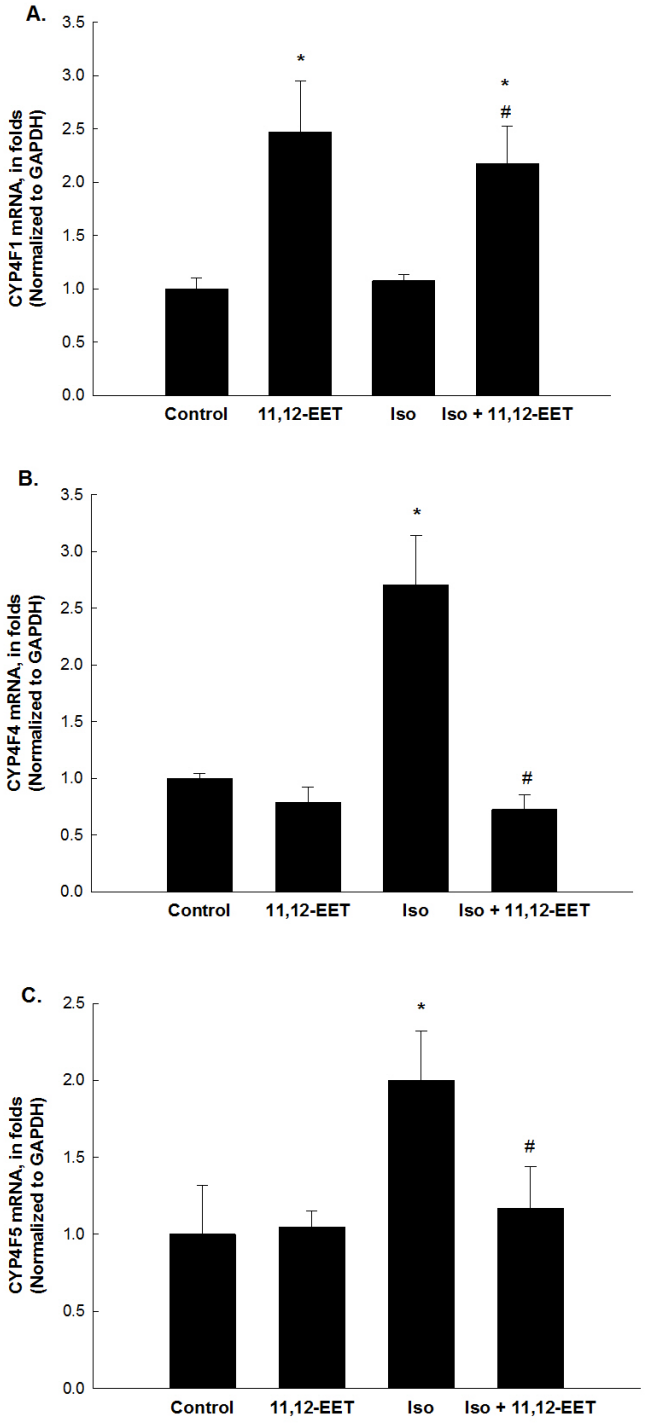

Figure 3.19 Effect of 11,12-EET on the expression of CYP4F1 (A), CYP4F4 (B) and CYP4F5 (C). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. Total RNA was isolated and the expression of CYP4F1, CYP4F4 and CYP4F5 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

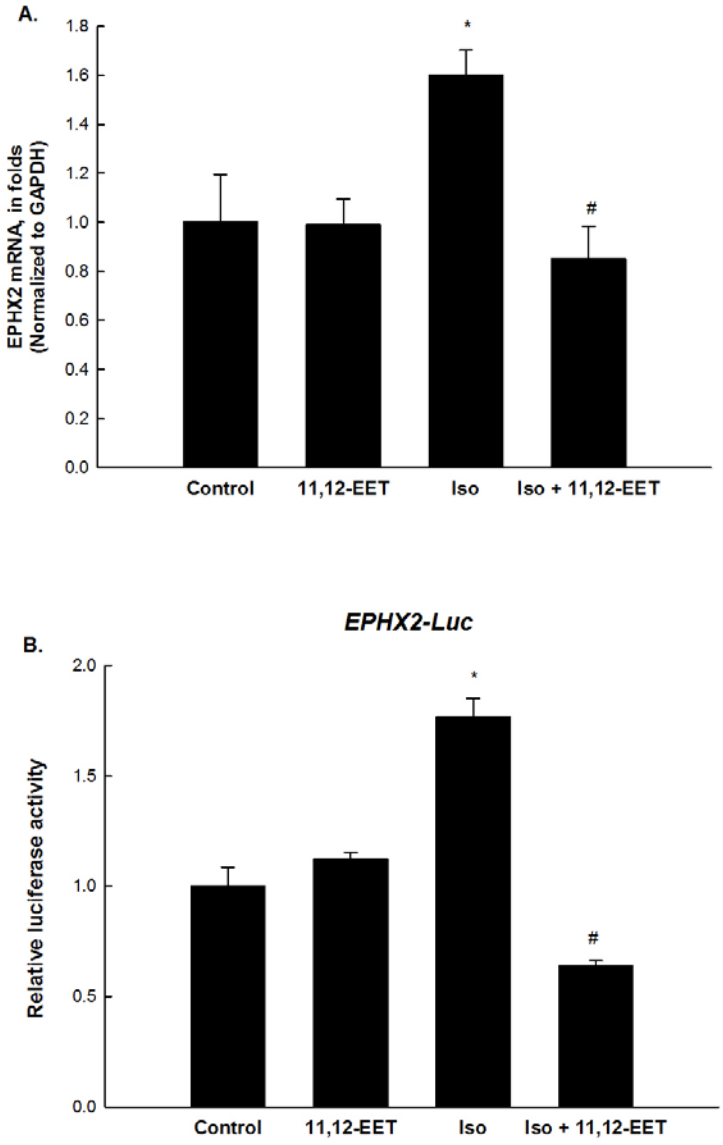

Figure 3.20 Effect of 11,12-EET on the expression of *EPHX2* (A) and the luciferase transcriptional activity of *EPHX2* (B). (A) H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. Total RNA was isolated and the expression of the expression of *EPHX2* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=5-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol. (B) H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 11,12-EET for 24 h. H9c2 cells were lysed and luciferase activities were determined using a Spectra Fluor Plus microplate reader. Fold of induction was calculated as a relative luciferase activity (firefly luciferase activity divided by the control renilla luciferase activity). Data are presented in fold of control (mean $\pm$ S.E.M, n=3/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

3.2.4 Effect of 14,15-EET on the changes of hypertrophic markers in isoproterenol-mediated hypertrophy

To examine the protective effect of 14,15-EET against isoproterenol-induced hypertrophy, H9c2 cells were pretreated with 1 μM of 14,15-EET for 2 h and then 100 μM of isoproterenol, or isoproterenol alone for an additional 24 h. Thereafter, the mRNA expression of the hypertrophic markers ANP, BNP and β-MHC were measured using real-time PCR. Isoproterenol alone caused a significant induction of ANP and BNP. Pre-treatment of 14,15-EET significantly decreased the isoproterenol-mediated induction of ANP and BNP by about 110% and 130%, respectively (Fig. 3.21A-B). However, isoproterenol did not affect the expression of β-MHC, and treatment of 14,15-EET also did not alter the expression of β-MHC (Fig. 3.21C).

3.2.5 Effect of 14,15-EET on the changes of expression of CYPs in isoproterenol-mediated hypertrophy

To further investigate the protective effect of 14,15-EET on isoproterenol-mediated alterations of CYPs, the mRNA expression was assessed using real-time PCR. Consistent with our earlier findings, isoproterenol alone significantly induced the mRNA expression of CYP1A1 and CYP1B1. Treatment of 14,15-EET significantly reduced the isoproterenol-mediated induction of CYP1A1 by 63% (Fig. 3.22A). However, pre-treatment with 14,15-EET did not decrease the isoproterenol-mediated induction of CYP1B1 (Fig. 3.22B).

Similarly, the expression CYP2J3 was significantly induced by the treatment with isoproterenol. Pre-treatment with 14,15-EET caused a significant reduction of CYP2J3 by 84% (Fig. 3.23C). Among the CYPs we have measured, the expression of CYP2C11, CYP2E1 and CYP4F1 were modulated by the treatment of 14,15-EET, but not by the treatment of isoproterenol. Fig. 3.23A shows a significant inhibition in the mRNA levels of CYP2C11 in the 14,15-EET alone and isoproterenol plus 14,15-EET groups to about 0.6-fold of control. In contrast, 14,15-EET caused a significant induction of CYP2E1 in the 14,15-EET

alone and isoproterenol plus 14,15-EET groups to about 1.7 and 3.5-fold, respectively (Fig. 3.23B). Furthermore, treatment of 14,15-EET significantly increased the expression of CYP4F1 in the 14,15-EET alone and isoproterenol plus 14,15-EET groups to about 2.4 and 1.6-fold, respectively (Fig. 3.24A). On the other hand, isoproterenol significantly increased the expression of CYP4F4 and CYP4F5 as we mentioned earlier. Treatment of 14,15-EET significantly attenuated the isoproterenol-mediated induction of CYP4F4 and CYP4F5 by 127% and 126%, respectively (Fig. 3.24B-C).

3.2.6 Effect of 14,15-EET on the changes of *EPHX2* and the luciferase transcriptional activity of *EPHX2* in isoproterenol-mediated hypertrophy

Regarding the expression of *EPHX2*, our results demonstrated a similar induction caused by isoproterenol as mentioned earlier. Pre-treatment with 14,15-EET significantly inhibited the isoproterenol-mediated induction of *EPHX2* by 105% (Fig. 3.25A). Furthermore, we examined the effect of isoproterenol on the transcriptional activity of *EPHX2*; a transient expression assay based on a reporter firefly luciferase gene was employed. Figure 3.25B shows that the treatment with 14,15-EET caused a significant reduction in the isoproterenol-induced luciferase activity by 134% (Fig. 3.25B).

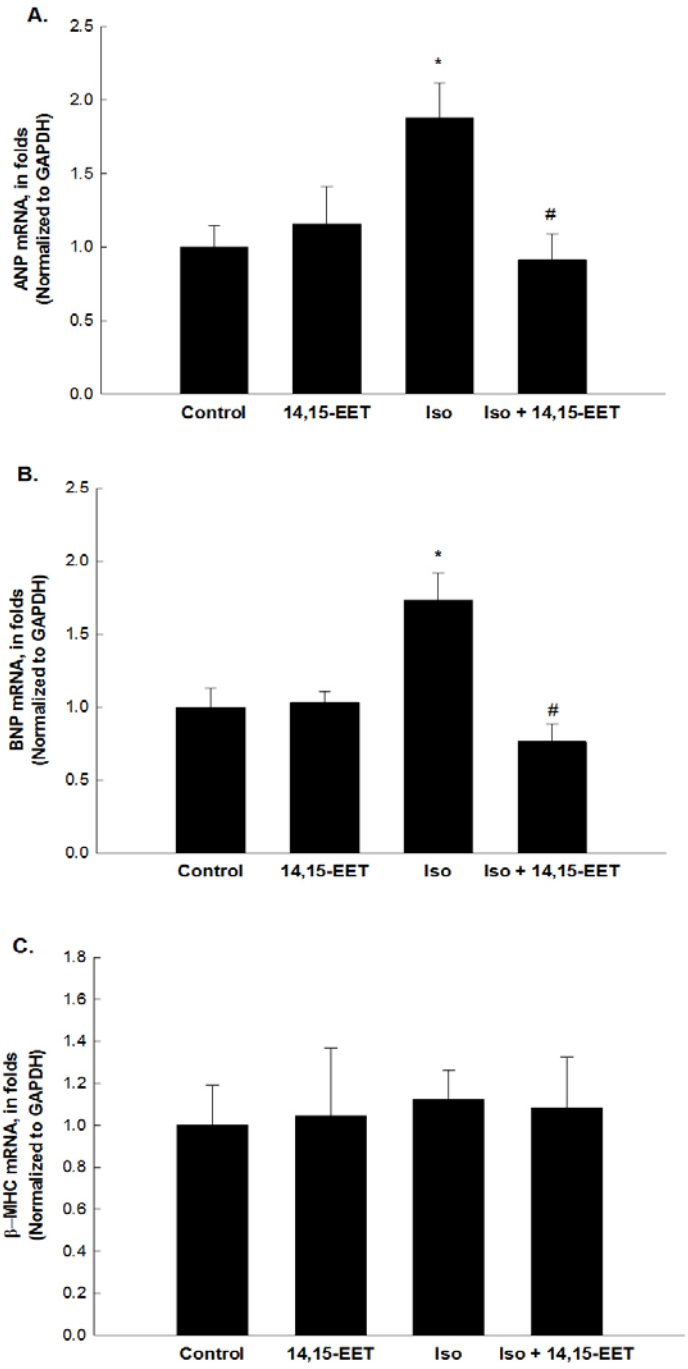

Figure 3.21 Effect of 14,15-EET on the expression of hypertrophic markers, ANP (A), BNP (B) and β-MHC (C). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 14,15-EET for 24 h. Total RNA was isolated and the expression of the hypertrophic markers ANP, BNP and β-MHC were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

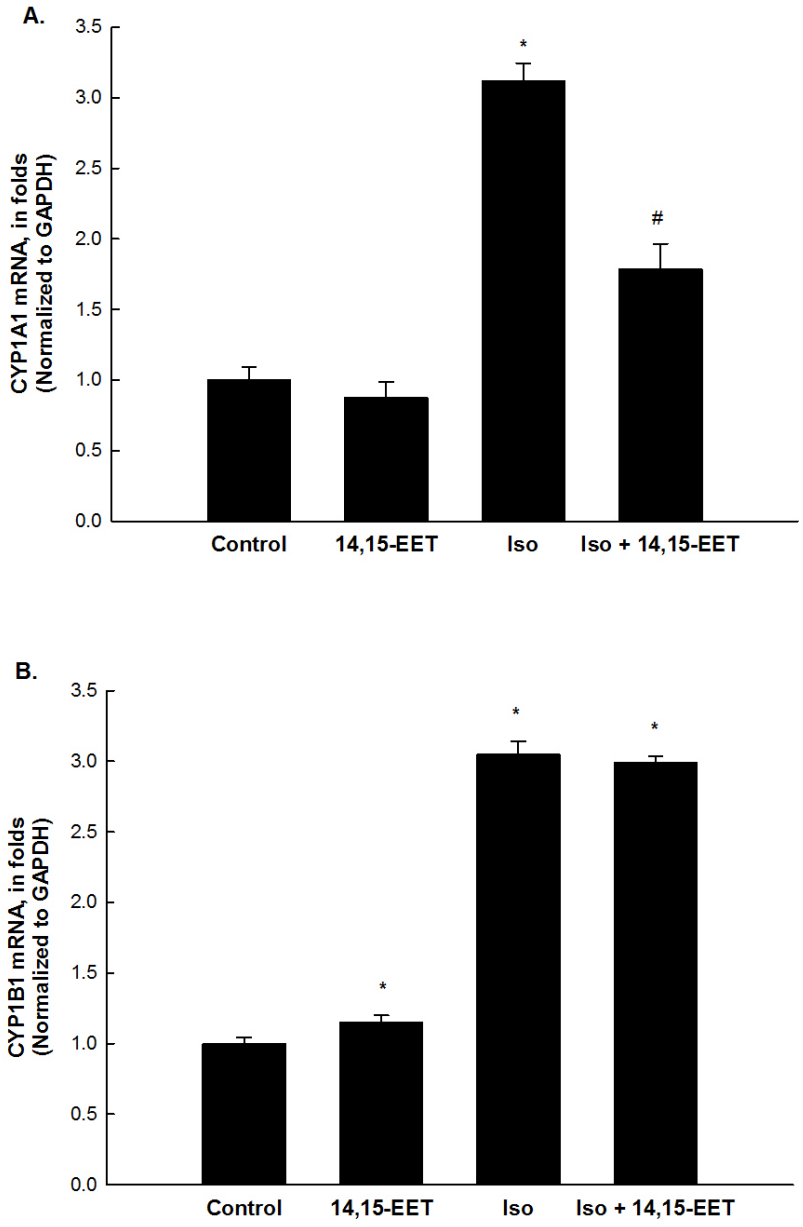

Figure 3.22 Effect of 14,15-EET on the expression of CYP1A1 (A) and CYP1B1 (B). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 14,15-EET for 24 h. Total RNA was isolated and the expression of CYP1A1 and CYP1B1 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

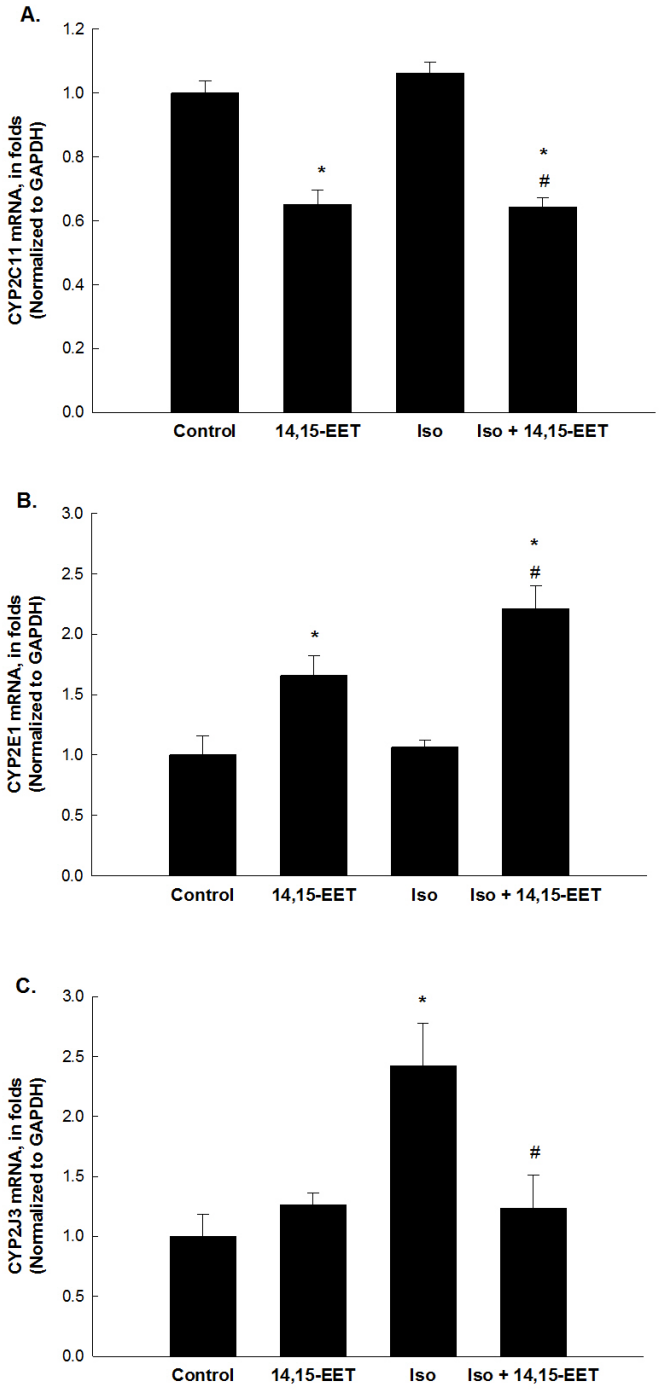

Figure 3.23 Effect of 14,15-EET on the expression of CYP2C11 (A), CYP2E1 (B) and CYP2J3 (C). H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 14,15-EET for 24 h. Total RNA was isolated and the expression of CYP2C11, CYP2E1 and CYP2J3 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

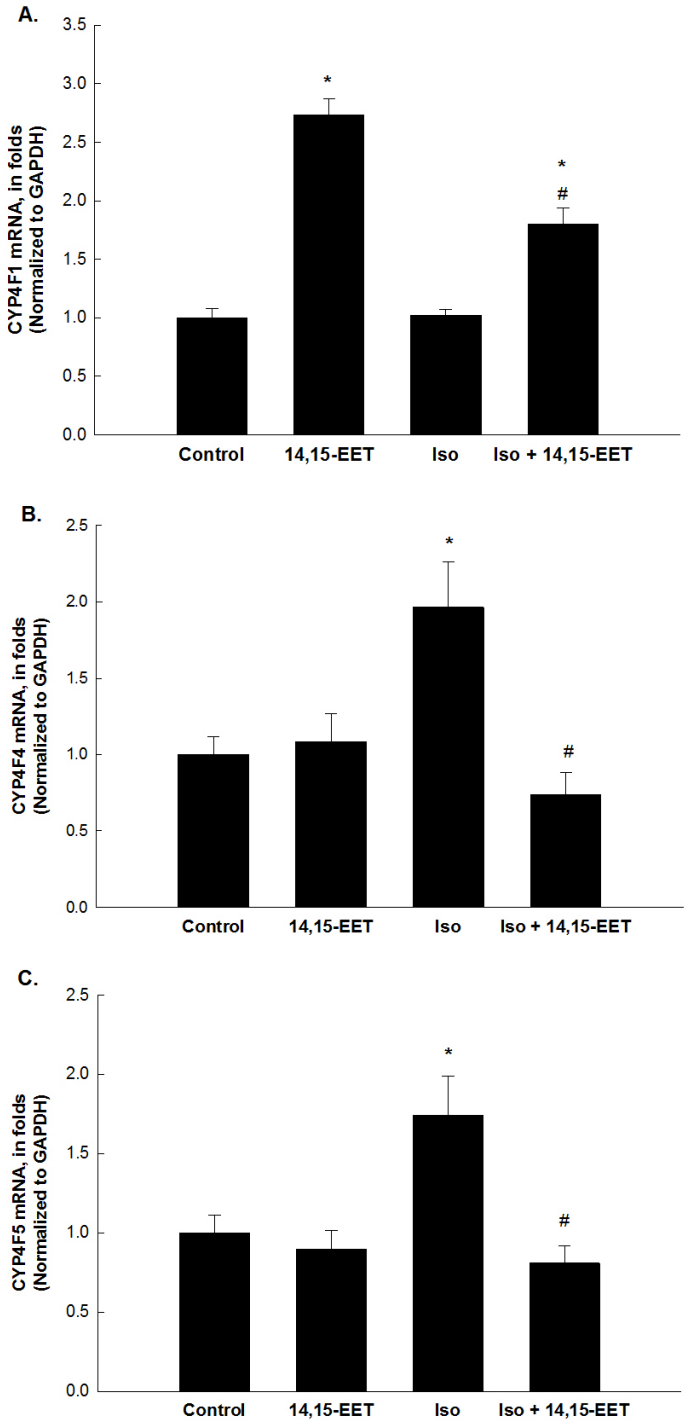

Figure 3.24 Effect of 14,15-EET on the expression of CYP4F1 (A), CYP4F4 (B) and CYP4F5 (C). H9c2 cells were incubated with 100 µM isoproterenol in the presence or absence of 1 µM 14,15-EET for 24 h. Total RNA was isolated and the expression of CYP4F1, CYP4F4 and CYP4F5 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

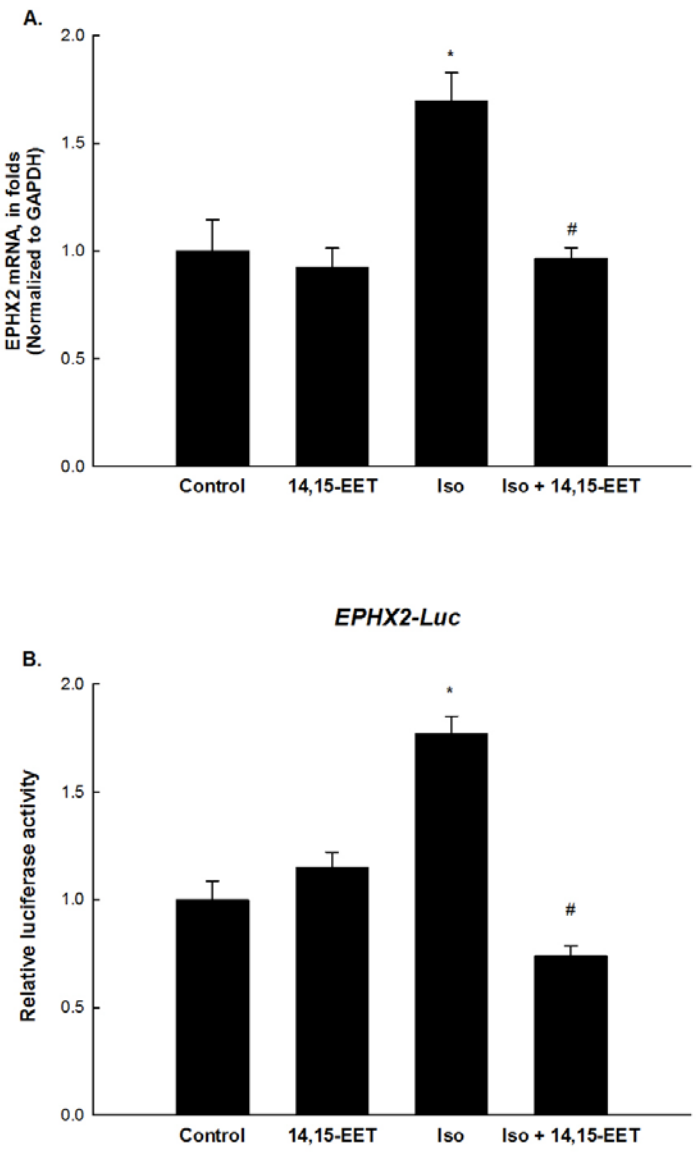

Figure 3.25 Effect of 14,15-EET on the expression of *EPHX2* (A) and the luciferase transcriptional activity of *EPHX2* (B). (A) H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 14,15-EET for 24 h. Total RNA was isolated and the expression of the expression of *EPHX2* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol. (B) H9c2 cells were incubated with 100 μM isoproterenol in the presence or absence of 1 μM 14,15-EET for 24 h. H9c2 cells were lysed and luciferase activities were determined using a Spectra Fluor Plus microplate reader. Fold of induction was calculated as a relative luciferase activity (firefly luciferase activity divided by the control renilla luciferase activity). Data are presented in fold of control (mean ± S.E.M, n=3/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

3.3 Inhibition of sEH by a sEH inhibitor, TUPS protects against isoproterenol-induced cellular hypertrophy in H9c2 cells

3.3.1 Effect of TUPS on cell viability

To determine the cytotoxic effect of TUPS, H9c2 cells were incubated with increasing concentrations of TUPS (0.1, 1, 5, 10 μM) for 24 h. Thereafter, cell viability was evaluated by the MTT assay. The MTT assay showed that the TUPS concentrations ranging from 0.1-10 μM did not significantly affect cell viability as compared to the control (Fig. 3.26). Therefore, the observed changes in the gene expression are not due to the decreased cell viability or toxicity.

3.3.2 Effect of TUPS on the changes of hypertrophic markers in isoproterenol-mediated hypertrophy

To investigate whether the inhibition of sEH by TUPS confers cardioprotection in isoproterenol-mediated cellular hypertrophy, H9c2 cells were pretreated with 10 μM of TUPS for 2 h and then 100 μM of isoproterenol, or isoproterenol alone for an additional 24 h. We measured the expression of hypertrophic markers ANP, BNP and β-MHC using real-time PCR. Isoproterenol caused a significant induction of ANP and BNP as we observed earlier. TUPS treatment significantly decreased the isoproterenol-mediated induction of ANP and BNP by 75% and 130%, respectively (Fig. 3.27A-B). Consistent with our earlier findings, isoproterenol did not cause any significant changes in the expression of β-MHC. In addition, pre-treatment with TUPS did not alter the expression of β-MHC (Fig. 3.27C).

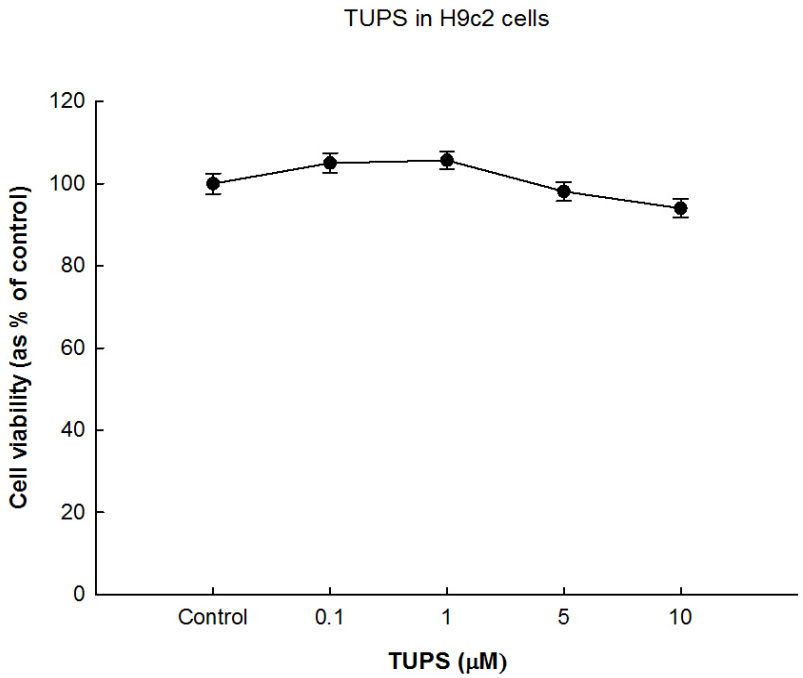

Figure 3.26 Effect of TUPS on cell viability. H9c2 cells were incubated with increasing concentrations of TUPS (0.1, 1, 5 and 10 μM) for 24 h. The cell viability was measured by the MTT assay as described in materials and methods. Data are presented as a percentage of control (mean ± S.E.M, n=8). * $p < 0.05$ compared to control.

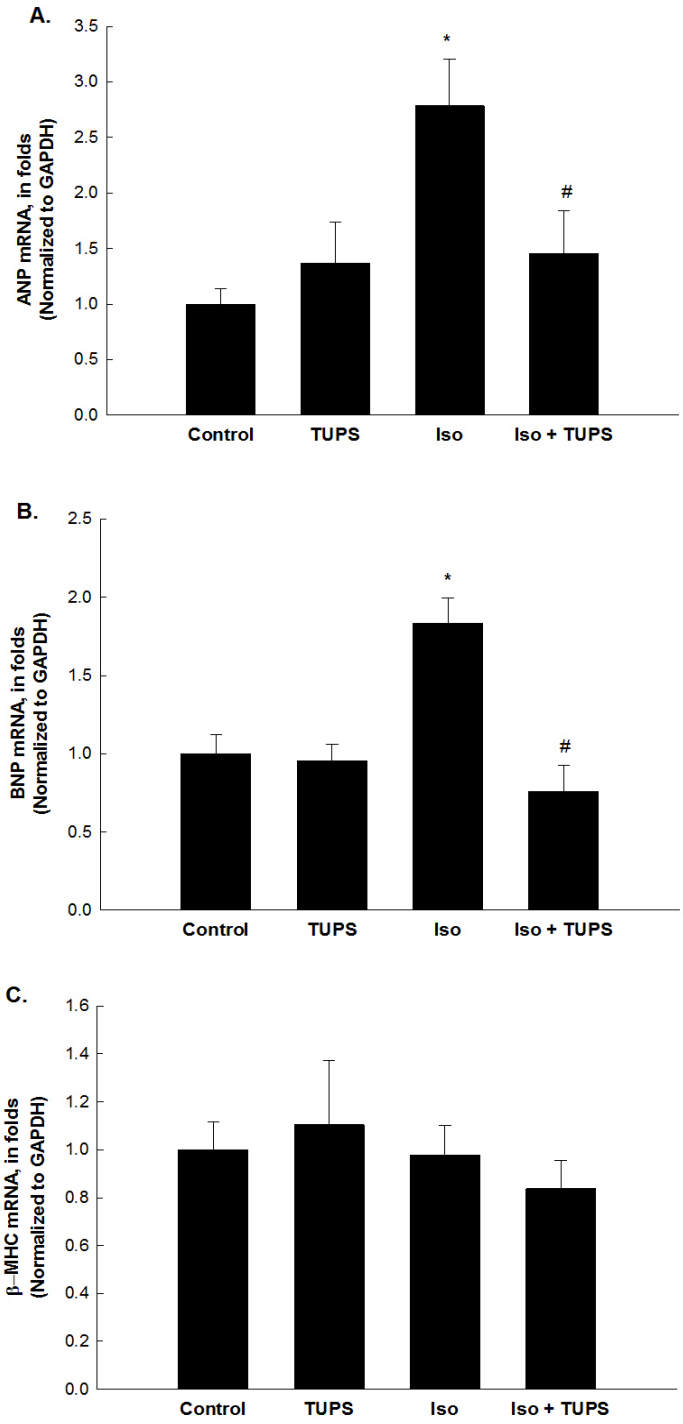

Figure 3.27 Effect of TUPS on the expression of hypertrophic markers, ANP (A), BNP (B) and β-MHC (C). H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. Total RNA was isolated and the expression of the hypertrophic markers, ANP, BNP and β-MHC were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

3.3.3 Effect of TUPS on the changes of CYPs expression in isoproterenol-mediated hypertrophy

To examine the protective effect of TUPS on isoproterenol-mediated alterations of CYPs, the mRNA expression was evaluated using real-time PCR. Figure 3.28A shows the effect of TUPS treatment on isoproterenol-mediated induction of CYP1A1 expression. Our results demonstrated that isoproterenol caused a significant increase in the CYP1A1 mRNA expression. Treatment of TUPS caused a significant inhibition of the isoproterenol-induced CYP1A1 expression by 52% (Fig. 3.28A). On the other hand, isoproterenol induced the CYP1B1 expression as we demonstrated earlier. However, treatment of TUPS caused no significant changes in the isoproterenol-mediated induction of CYP1B1. Furthermore, TUPS alone significantly increased the expression of CYP1B1 to about 1.7-fold of control (Fig. 3.28B).

Consistently, isoproterenol induced the expression of CYP2J3. Pre-treatment of TUPS significantly attenuated the isoproterenol-induced effect of CYP2J3 by about 115% (Fig. 3.29C). As we observed earlier, isoproterenol did not alter the expression of CYP2E1. Moreover, treatment of TUPS did not cause a significant change in the expression of CYP2E1 (Fig. 3.29B). Interestingly, the expression of CYP2C11 and CYP4F1 was modulated by the treatment of TUPS, but not by the treatment of isoproterenol. Fig. 3.29A shows a significant inhibition in the mRNA level of CYP2C11 in the TUPS alone and isoproterenol plus TUPS groups to about 0.4-fold of control. Contrarily, treatment of TUPS significantly increased CYP4F1 in the TUPS alone and isoproterenol plus TUPS groups to about 2.5 and 2.2-fold, respectively (Fig. 3.30A). On the other hand, isoproterenol significantly increased the expression of CYP4F4 and CYP4F5 as we observed earlier. Treatment of TUPS significantly attenuated the isoproterenol-mediated induction of CYP4F4 and CYP4F5 by 137% and 97%, respectively (Fig. 3.30B-C).

3.3.4 Effect of TUPS on the changes of *EPHX2* expression and the luciferase transcriptional activity of *EPHX2* in isoproterenol-mediated hypertrophy

As for the expression of *EPHX2*, we showed that isoproterenol caused a similar induction of *EPHX2* as mentioned earlier. Pre-treatment of TUPS significantly inhibited the isoproterenol-mediated induction of *EPHX2* by 105% (Fig. 3.31A). Moreover, we examined the effect of isoproterenol on the transcriptional activity of *EPHX2*, a transient expression assay based on a reporter firefly luciferase gene was adopted in this study. Our findings demonstrated that treatment of TUPS significantly inhibited the isoproterenol-induced luciferase activity by approximately 150% (Fig. 3.31B).

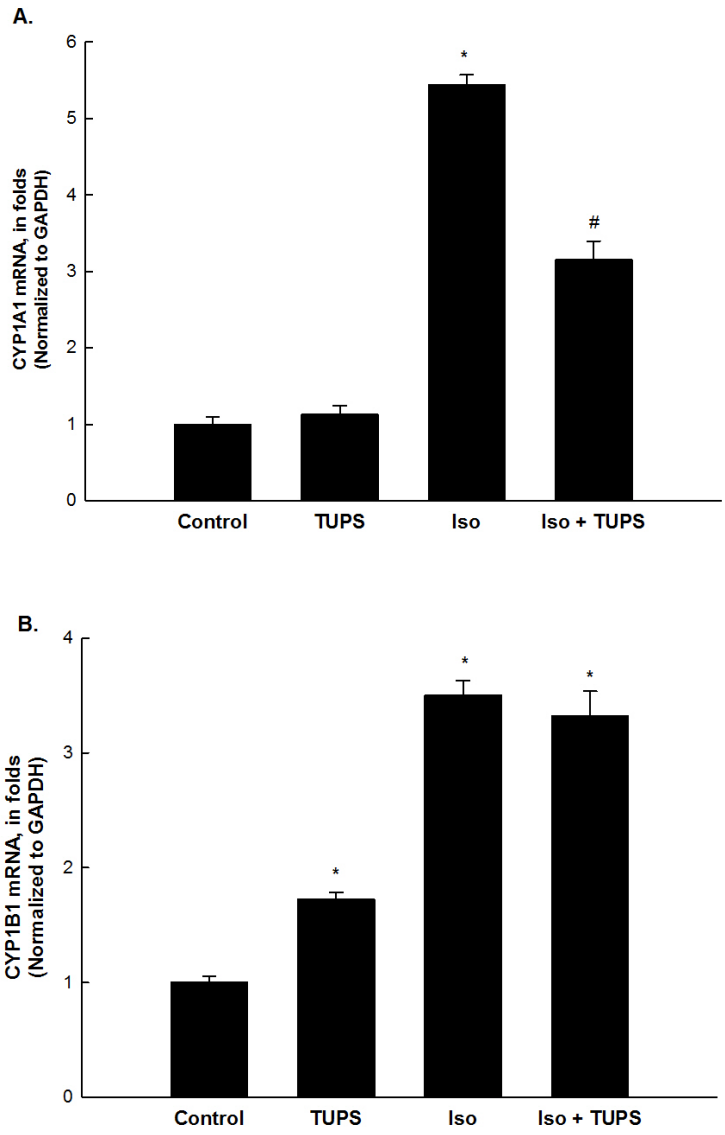

Figure 3.28 Effect of TUPS on the expression of CYP1A1 (A) and CYP1B1 (B). H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. Total RNA was isolated and the expression of CYP1A1 and CYP1B1 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=5-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

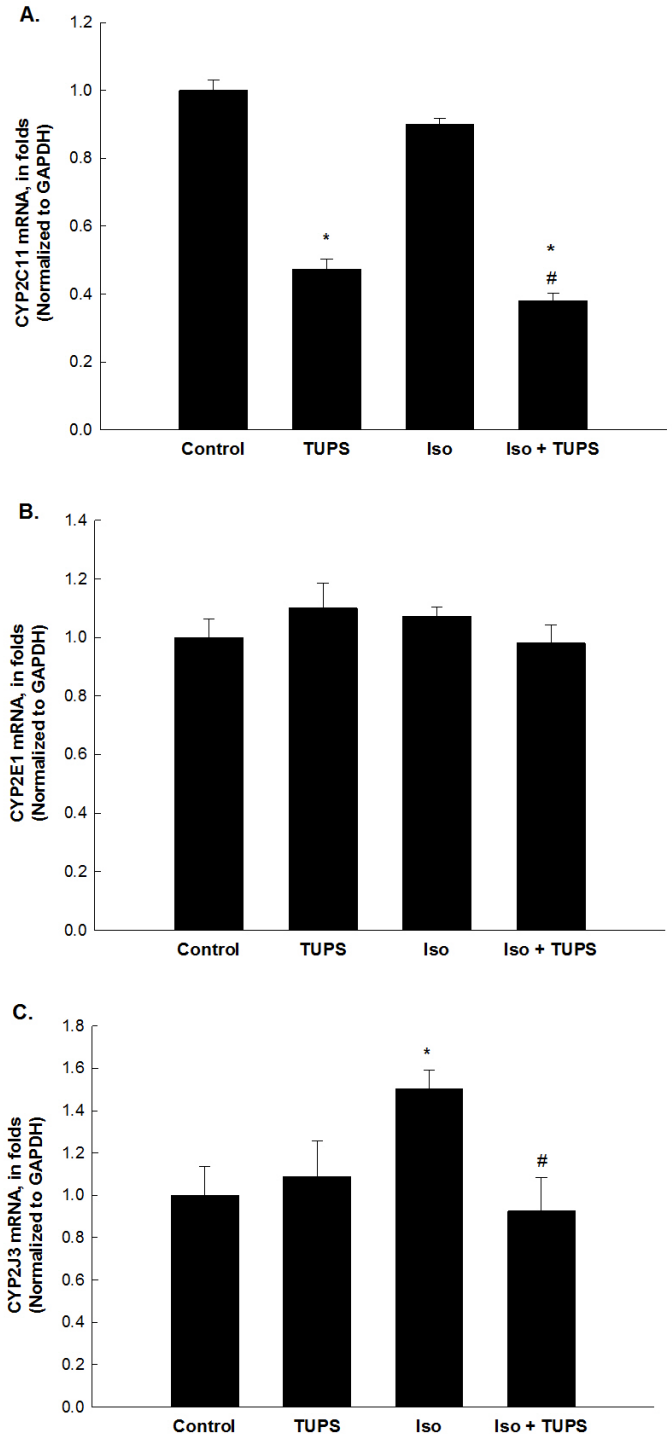

Figure 3.29 Effect of TUPS on the expression of CYP2C11 (A), CYP2E1 (B) and CYP2J3 (C). H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. Total RNA was isolated and the expression of CYP2C11, CYP2E1 and CYP2J3 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

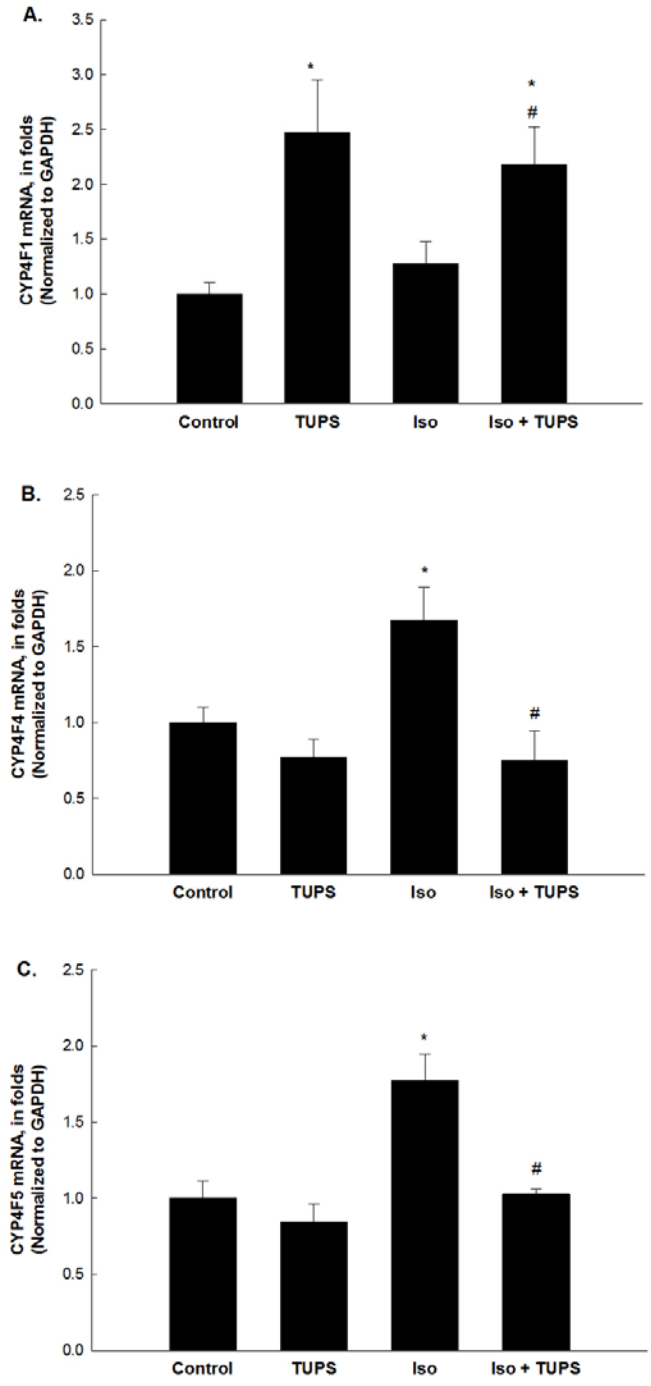

Figure 3.30 Effect of TUPS on the expression of CYP4F1 (A), CYP4F4 (B) and CYP4F5 (C). H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. Total RNA was isolated and the expression of CYP4F1, CYP4F4 and CYP4F5 were determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean $\pm$ S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

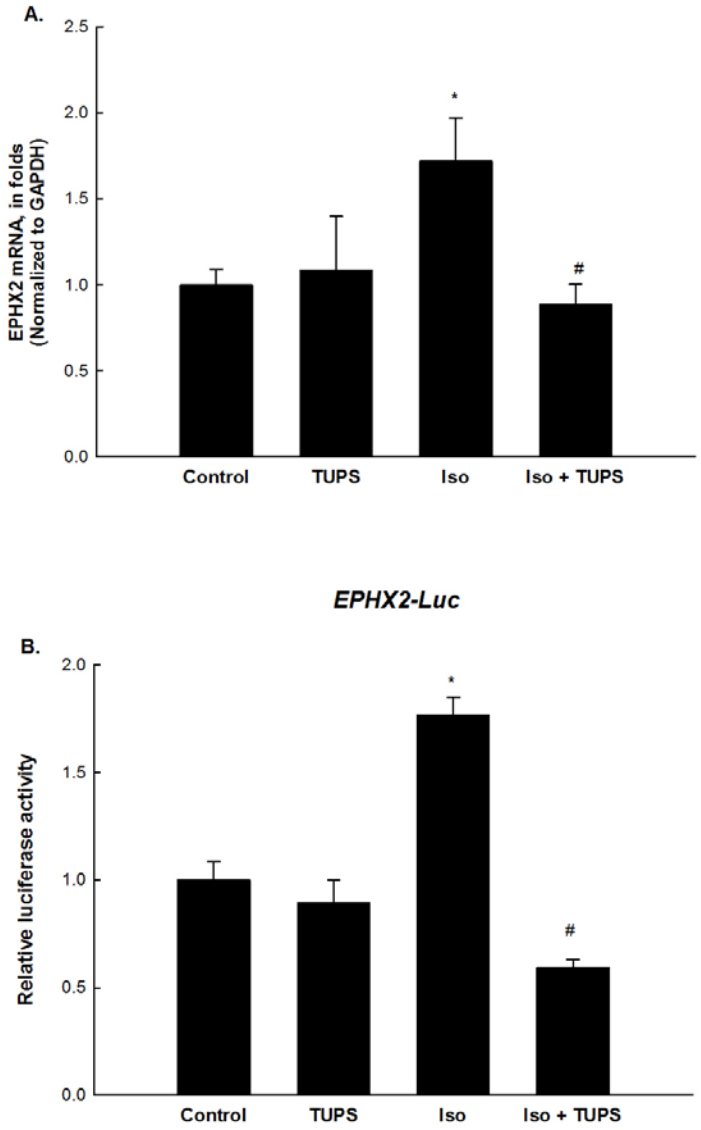

Figure 3.31 Effect of TUPS on the expression of *EPHX2* (A) and the luciferase transcriptional activity of *EPHX2* (B). (A) H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. Total RNA was isolated and the expression of the expression of *EPHX2* was determined by real-time PCR. Fold of induction was calculated as target gene expression (normalized to GAPDH) divided by the control values (the control value was set as 1). Data are presented in fold of control (mean ± S.E.M, n=4-6/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol. (B) H9c2 cells were pretreated with 10 μM TUPS for 2 h and then 100 μM isoproterenol, or isoproterenol alone for an additional 24 h. H9c2 cells were lysed and luciferase activities were determined using a Spectra Fluor Plus microplate reader. Fold of induction was calculated as a relative luciferase activity (firefly luciferase activity divided by the control renilla luciferase activity). Data are presented in fold of control (mean ± S.E.M, n=3/group). * $p < 0.05$ compared to control. # $p < 0.05$ compared to isoproterenol.

4. Discussion

4.1 Isoproterenol induces cellular hypertrophy and modulates the gene expression of cytochromes P450 and *EPHX2* in rat cardiomyoblast H9c2 cells, but not in rat hepatoma H4IIE cells.

In the present study, we investigated the effect of isoproterenol on rat cardiomyoblast H9c2 cells and rat hepatoma H4IIE cells and its modulation on the expression of *CYP* genes. Our results have demonstrated that isoproterenol caused cellular hypertrophy in H9c2 cells at both 24 and 48 h, as manifested by a significant induction of the hypertrophic markers ANP and BNP. Previously, isoproterenol has been shown to induce cellular hypertrophy as indicated by an induction of hypertrophic markers, an increase in cardiomyocyte size and an enhance protein systhesis in H9c2 cells and rats (Clarke and Ward 1983; Chen, Bu et al. 2012). In agreement with our results, the induction of ANP and BNP has been found in several *in vivo* models of cardiac hypertrophy (Shimoike, Iwai et al. 1997; Magga, Vuolteenaho et al. 1998; Masson, Arosio et al. 1998; Zordoky, Aboutabl et al. 2008; Ai, Pang et al. 2009). Similarly, numerous *in vitro* models of cellular hypertrophy caused by vasopressin, insulin-like growth factor II, angiotensin II, lipopolysaccharides, doxorubicin, hydrogen peroxide, TCDD, and βNF showed an induction of ANP and BNP in H9c2 cells (Van der Bent, Church et al. 1994; Chu, Tzang et al. 2008; Huang, He et al. 2008; Liu, Cheng et al. 2008; Zordoky and El-Kadi 2008; Saeedi, Saran et al. 2009; Zordoky and El-Kadi 2010; Oyama, Takahashi et al. 2011; Watkins, Borthwick et al. 2011). Furthermore, this is consistent with previous findings from our lab and others that the expression of ANP and BNP was significantly increased in rat hearts of isoproterenol-induced cardiac hypertrophy (Sucharov, Mariner et al. 2006; Tshori, Gilon et al. 2006; Lin, Wang et al. 2008; Zordoky, Aboutabl et al. 2008; Tan, Li et al. 2011).

In an attempt to understand the role of CYPs in the development of cardiac hypertrophy, we measured the mRNA expression of different *CYP* genes caused by isoproterenol-induced hypertrophy in H9c2 cells. We demonstrated that treatment of isoproterenol significantly increased the mRNA expression of

CYP1A1 and CYP1B1 at both 24 and 48 h time points. The induction of CYP1A1 and CYP1B1 is consistent with a previous report from our lab illustrating the increased mRNA expression of CYP1A1 and CYP1B1 in rat hearts following isoproterenol treatment (Zordoky, Aboutabl et al. 2008). Furthermore, the expression of CYP1A1 and CYP1B1 was significantly increased in left ventricular tissues of SHRs as compared to normotensive rats (Thum and Borlak 2002). CYP1A1 has been shown to be involved in synthesis of ω-terminal HETEs (Schwarz, Kisselev et al. 2005; Lucas, Goulitquer et al. 2010), while CYP1B1 can metabolize arachidonic acid to both mid-chain HETEs and EETs (Choudhary, Jansson et al. 2004).

With regard to the CYP2 family, no significant changes were observed for the expression of CYP2B1, CYP2C11 or CYP2E1 at 24 or 48 h in the isoproterenol-treated H9c2 cells. Previous studies reported that CYP2B1 mRNA expression was not altered during isoproterenol-induced cardiac hypertrophy in a rat model (Zordoky, Aboutabl et al. 2008). Also, the expression of CYP2B1 was not significantly changed in the rat hearts in AhR ligands; 3-MC and BaP-induced hypertrophy (Aboutabl, Zordoky et al. 2009). Similarly, the expression of CYP2B1 was not altered in various *in vitro* models of cellular hypertrophy, including doxorubicin and the AhR ligands; TCDD and βNF-induced cellular hypertrophy in H9c2 cells (Zordoky and El-Kadi 2008; Zordoky and El-Kadi 2010). Additionally, the expression of CYP2C11 in our current study is consistent with several studies from our lab. We previously showed that CYP2C11 expression was not altered by 3-MC and BaP treatments in rat hearts, and during TCDD and βNF-induced cellular hypertrophy in H9c2 cells (Aboutabl, Zordoky et al. 2009; Zordoky and El-Kadi 2010). The importance of these enzymes emerges from their ability to metabolize arachidonic acid to various EETs (Kroetz and Zeldin 2002). CYP2B1 epoxygenase produces 14,15-EET predominantly, whereas CYP2C11 metabolizes arachidonic acid into 11,12- and 14,15-EETs (Laethem, Halpert et al. 1994; Ng, Huang et al. 2007). As for CYP2E1, there have been some discrepancies regarding its expression in different models of cardiac hypertrophy. In several models of cardiac hypertrophy, significant inductions of

CYP2E1 expression were observed by treatment with AhR ligands and doxorubicin in rats and H9c2 cells (Zordoky and El-Kadi 2008; Aboutabl, Zordoky et al. 2009; Zordoky and El-Kadi 2010). Contrarily, another study showed that isoproterenol treatment caused a significant reduction of CYP2E1 expression in the hypertrophied hearts of rats (Zordoky, Aboutabl et al. 2008). However, our results from the current study demonstrated that CYP2E1 expression was not significantly altered. These discrepancies are likely due to different inducers of experimental cardiac hypertrophy and the use of animal or cell line models.

Our results demonstrated that isoproterenol significantly increased the mRNA expression of CYP2J3 during isoproterenol-mediated cellular hypertrophy in H9c2 cells. In agreement with our findings, a previous study showed that the mRNA expression of CYP2J3 was significantly increased by 4-fold in the left ventricular tissues of SHRs (Thum and Borlak 2002). Furthermore, other studies have demonstrated a significant induction of CYP2J3 in TCDD, βNF and doxorubicin-treated H9c2 cells (Zordoky and El-Kadi 2008; Zordoky and El-Kadi 2010). However, several papers have shown that the mRNA expression of CYP2J3 was not significantly altered in 3-MC, BaP and isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008; Aboutabl, Zordoky et al. 2009). These discrepancies may be explained by the use of animal or cell line models, different types of experimental stimuli, and the period of treatment in these studies. Further studies will be needed to better define the factors that regulate the expression of CYP2J3. In this context, it is important to mention that CYP2J3 is one of the major enzymes involved in the formation of EETs in extrahepatic tissues. It is the major epoxygenase that is highly expressed in the heart (Wu, Moomaw et al. 1996; Wu, Chen et al. 1997; Scarborough, Ma et al. 1999). CYP2J3 mainly produces 11,12- and 14,15-EETs with a ratio of 40% and 60%, respectively (Kaspera and Totah 2009).

CYP4A and CYP4F subfamilies are considered the most important CYP ω-hydroxylases which are involved in the metabolism of arachidonic acid to 20-HETE (Kroetz and Xu 2005; Rifkind 2006). In the current study, the expression

of CYP4A1, CYP4A2 and CYP4A3 were not detected in the H9c2 cells. Moreover, there was no significant change in the expression of CYP4F1 in the isoproterenol-treated H9c2 cells. Consistent with previous findings, CYP4F1 expression was not altered in AhR agonists; TCDD and βNF-induced models of cellular hypertrophy in H9c2 cells (Zordoky and El-Kadi 2010), or 3-MC and BaP-induced models of cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2009). On the other hand, we demonstrated that treatment with isoproterenol significantly increased the mRNA expression of CYP4F4 at both 24 and 48 h in H9c2 cells. Our results are consistent with several previous studies showing that the expression of CYP4F4 was significantly induced in TCDD and βNF-induced cellular hypertrophy in H9c2 cells (Zordoky and El-Kadi 2010), as well as in 3-MC, BaP and isoproterenol-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2009; Althurwi, Tse et al. in press). Furthermore, our findings also showed a significant induction of CYP4F5 in isoproterenol-treated H9c2 cells at both time points. Similar to a previous study, the expression of CYP4F5 was significantly increased in hearts of 3-MC and BaP-treated rats (Aboutabl, Zordoky et al. 2009). However, the expression of CYP4F5 was not altered in two other hypertrophy studies, including TCDD and βNF-treated H9c2 cells (Zordoky and El-Kadi 2010), and isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008). Further studies will be needed to clarify these discrepancies.

The sEH enzyme is an important factor in determining the levels of EETs, as it catalyses the conversion of EETs to DHETs, thereby diminishing their biological effects (Imig, Zhao et al. 2002). A previous study has identified *EPHX2*, the gene encodes the sEH enzyme, as a susceptibility factor for heart failure in SHHF rats using linkage analyses with genome-wide expression profiling (Monti, Fischer et al. 2008). Therefore, we investigated the effect of cardiac hypertrophy on the expression of sEH. In the current study, the transcript and protein levels of sEH were found to be increased in isoproterenol-induced cellular hypertrophy in H9c2 cells. Consistent with our results, a study has shown an increase in the transcript, protein, and enzymatic activity levels of sEH in

SHHF rats (Monti, Fischer et al. 2008). Another study has also indicated that the expression of sEH was induced in spontaneous hypertensive rats (Ai, Fu et al. 2007). Furthermore, an *in vitro* study showed an increase in the protein expression of sEH in Ang II-induced cellular hypertrophy in rat neonatal cardiomyocytes and H9c2 cells (Monti, Fischer et al. 2008; Pang, Li et al. 2011). Correspondingly, the expression of sEH was detected to be increased in the rat myocardium of Ang II-induced cardiac hypertrophy (Ai, Pang et al. 2009). More recently, the mRNA expression of sEH was shown to be induced in rat hearts of isoproterenol-induced cardiac hypertrophy (Zordoky, Aboutabl et al. 2008). Similarly, the protein level of sEH was significantly increased in isoproterenol-induced cardiac hypertrophy in rats (Althurwi, Tse et al. in press).

To determine whether the hypertrophic effect of isoproterenol and the modulation of CYP expression due to the isoproterenol-mediated cellular hypertrophy were specific to cardiac cells, we investigated whether isoproterenol has a similar effect and modulates the expression of CYPs in rat hepatoma H4IIE cells. In the current study, we showed that the expression of hypertrophic markers ANP and BNP were not expressed in H4IIE cells, but only expressed in H9c2 cells. Similar to a previous study in our lab, the mRNA expression of ANP and BNP was only detected in the heart tissues, and not in liver tissues in isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008). Furthermore, the mRNA expression of CYPs was measured in the isoproterenol-treated H4IIE cells. Our results demonstrated that the expression of CYP1A1, CYP1B1, CYP2B1, CYP2C11 and CYP2E1 was not significantly changed in the H4IIE cells by treatment with isoproterenol. Consistent with a previous study, the mRNA expression of these CYPs were not altered in the liver in isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008). On the other hand, the expression of the major epoxygenase, CYP2J3 was found at very low or undetectable levels in H4IIE cells. As shown in a previous study, even at a detectable level in the liver of rats, isoproterenol did not mediate any significant changes in the mRNA expression of CYP2J3 (Zordoky, Aboutabl et al. 2008). As for the CYP4 family, our study showed that the expression of

CYP4A1, CYP4A2, CYP4A3, CYP4F1, CYP4F4 and CYP4F5 was not significantly altered by the treatment with isoproterenol in H4IIE cells. Similar findings were observed in a previous study. The expression of these CYPs was not significantly changed in the liver of isoproterenol-treated rats (Zordoky, Aboutabl et al. 2008). Altogether, these findings suggest that the hypertrophic effect of isoproterenol is specific to cardiac cells. Furthermore, the modulation of CYP expression was likely due to the isoproterenol-mediated cellular hypertrophy, rather than the effect of isoproterenol itself as we did not observe a similar modulation in the H4IIE cells.

In conclusion, our study showed that isoproterenol induces cellular hypertrophy in H9c2 cells, but not in H4IIE cells. Furthermore, isoproterenol-induced cellular hypertrophy caused significant changes in the expression of several CYPs and *EPHX2*, which is mostly specific to cardiac cells. Therefore, the cardiac CYPs 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* and *EPHX2* gene expression.

4.2 11,12- and 14,15-EETs protect against the isoproterenol-induced cellular hypertrophy through the modulation of gene expression of *CYPs* and *EPHX2* in H9c2 cells.

To investigate the protective effect of 11,12- and 14,15-EETs against isoproterenol-induced cellular hypertrophy in H9c2 cells, we performed real time-PCR on the expression of the hypertrophic markers, CYPs and *EPHX2*. We found that both 11,12- and 14,15-EETs significantly decreased the isoproterenol-mediated induction of ANP and BNP in H9c2 cells. For the first time, the protective effect of EETs was being examined in cellular hypertrophy. Previous studies have only shown that the exogenous administration of EETs exerts a protective effect against ischemia-reperfusion injury and myocardial infarction (Moffat, Ward et al. 1993; Wu, Chen et al. 1997; Nithipatikom, Moore et al. 2006; Motoki, Merkel et al. 2008). Likewise, other studies have only

demonstrated the protective effects of sEH inhibitors against cardiac hypertrophy. Nevertheless, we have shown that an sEH inhibitor decreased the hypertrophic markers ANP and BNP in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011). Similarly, another study showed that inhibition of sEH by TUPS attenuated the hypertrophic markers in Ang II-mediated hypertrophy in rat hearts and neonatal rat cardiomyocytes (Ai, Pang et al. 2009). Two other sEH inhibitors, AEPU and AUDA were also reported to decrease the hypertrophic markers in thoracic aortic constriction murine model (Xu, Li et al. 2006). Our study is the first to show that exogenous administrations of 11,12- and 14,15-EETs attenuated the expression of ANP and BNP in isoproterenol-mediated cellular hypertrophy in H9c2 cells. Increasing levels of EETs by adding exogenous EETs or by inhibiting sEH, the enzyme responsible for the degradation of EETs, both showed a protective effect against cellular hypertrophy and decreased the expression of hypertrophic markers.

Due to the importance of CYPs in the pathogenesis of cardiac hypertrophy, we examined the effect of 11,12- and 14,15-EETs on the expression of different CYPs that are mediated by isoproterenol-induced cellular hypertrophy. Our results demonstrated that 11,12- and 14,15-EETs significantly decreased the isoproterenol-mediated induction of the CYP ω-hydroxylase enzyme CYP1A1 in H9c2 cells. However, 11,12- and 14,15-EETs were unable to decrease the other ω-hydroxylase enzyme, CYP1B1 in our study. Most studies have indicated that CYP1A1 is regulated by the aryl hydrocarbon receptor (AhR) signaling pathway (Fujii-Kuriyama, Imataka et al. 1992; Abel and Haarmann-Stemmann 2010). On the other hand, both rat and mouse CYP1B1 is regulated by the AhR and cyclic AMP (cAMP)-mediated pathways. Transcription factor-1 motifs have been found to associate with cAMP-dependent transcriptional activation of genes in the 5' upstream regulatory sequences of the mouse CYP1B1 gene (Brake and Jefcoate 1995; Zhang, Savas et al. 1998). Rat CYP1B1 has been found to be inducible by AhR agonists and by adrenocorticotropic hormone (ACTH) (Brake and Jefcoate 1995). ACTH treatment of cultured cells of rat adrenal cortex resulted in an increase in CYP1B1 expression. Interestingly, the

response towards ACTH was greater than the corresponding response elicited by treatment with dioxin. Other steroid hormones, such as benzanthracene and estradiol also increased the expression of CYP1B1 in rat mammary stromal fibroblasts (Christou, Savas et al. 1995). Many studies have indicated the inducible expression of CYP1B1 may also involve non-AhR-mediated mechanisms, thus providing further evidence for the involvement of multiple cellular pathways in the regulation of CYP1B1 (Murray, Melvin et al. 2001). Therefore, the unresponsive effect of CYP1B1 in our study may be due the regulation of this gene by other pathways.

Nonetheless, treatment with 11,12- and 14,15-EETs also significantly reduced the isoproterenol-mediated induction of CYP2J3. Interestingly, treatment with 11,12- and 14,15-EETs alone caused a significant reduction in the expression of CYP2C11 whereas, a significant induction was observed in the expression of CYP2E1. Consistent with a previous study, increasing EET concentrations by the inhibition of sEH resulted in an induction of CYP2E1 in the heart tissues of rats (Aboutabl, Zordoky et al. 2011). Likewise, the expression of CYP4F1 was also significantly increased by the treatment of 11,12- and 14,15-EETs alone in H9c2 cells. The modulation of CYP2C11, CYP2E1 and CYP4F1 by 11,12- and 14,15-EETs could be attributed to the involvement of EETs in regulating CYP-dependent pathways during cardiac hypertrophy. With regard to the other CYP4 members, our study showed that the treatment of 11,12- and 14,15-EETs significantly inhibited isoproterenol-mediated induction of CYP4F4 in H9c2 cells. Our results are consistent with several studies showing that the expression of CYP4F4 was significantly attenuated by the inhibition of sEH with TUPS in BaP and isoproterenol-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011; Althurwi, Tse et al. in press). Similarly, the expression of CYP4F5 was also significantly decreased by treatment with 11,12- and 14,15-EETs in isoproterenol-induced cellular hypertrophy. In agreement with our results, a previous study showed that TUPS reduced the expression of CYP4F5 in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011).

In addition, our findings indicated that 11,12- and 14,15-EETs significantly attenuated the mRNA expression of *EPHX2* in isoproterenol-induced cellular hypertrophy. To confirm the effect of 11,12- and 14,15-EETs on the transcriptional activity of *EPHX2*, we employed the luciferase gene reporter assay under the control of *EPHX2* promoter. We found that isoproterenol activated the *EPHX2* promoter, and the treatment with 11,12- and 14,15-EETs decreased the isoproterenol-mediated induction of *EPHX2*-luciferase activity. Consistently, other studies have shown that inhibition of sEH by TUPS also decreased the mRNA expression of sEH in BaP and isoproterenol-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011; Althurwi, Tse et al. in press). Altogether, these findings suggest that EETs exert a protective effect against cellular hypertrophy by down regulating the gene expression of *EPHX2*. EETs play an important role in the inhibition of NF-κB, which is a downstream target of several signaling pathways implicated in cardiac hypertrophy, including pathway involving α-adrenergic stimulation, angiotensin II, PI3K/Akt, p38, ras, MEKK1/4, PKC and gp130 (Zechner, Craig et al. 1998; Rouet-Benzineb, Gontero et al. 2000; Craig, Wagner et al. 2001; Force, Kuida et al. 2004; Fischer and Hilfiker-Kleiner 2007). In addition to NF-κB, EETs have several other downstream targets that may explain their cardioprotective effects (Harris, Li et al. 2008). EETs modulate several signal transduction pathways such as those involving p42/p44 MAPK, cAMP-PKA, IKK-IKB, and ATP-sensitive potassium channels, suggesting that EETs act by binding to membrane receptors to initiate signaling cascades (Seubert, Yang et al. 2004; Lu, Ye et al. 2006; Batchu, Law et al. 2009).

In conclusion, we demonstrated for the first time that treatment with 11,12- and 14,15-EETs significantly attenuated isoproterenol-induced cellular hypertrophy in H9c2 cells. In addition, 11,12- and 14,15-EETs also inhibited the isoproterenol-mediated induction of CYPs and *EPHX2*. Altogether, our results suggest the role of CYPs in the development of cardiac hypertrophy and the protective effects of 11,12- and 14,15-EETs against cellular hypertrophy may be through the involvement of CYPs. Further investigation is needed to confirm our findings and to examine the mechanisms by which CYPs are implicated in cardiac

hypertrophy and the pathways by which EETs mediate to protect against cardiac hypertrophy.

4.3 Inhibition of sEH by a sEH inhibitor, TUPS, protects against isoproterenol-induced cellular hypertrophy through the modulation of gene expression of *CYPs* and *EPHX2* in H9c2 cells.

The cardioprotective effects of sEH inhibitors emerge from their ability to inhibit the degradation of EETs and other epoxy fatty acids, thereby prolonging the cardioprotective effect of EETs. Among the different sEH inhibitors that have been synthesized, the urea pharmacophore is the most potent, competitive and tight binding inhibitor of sEH (Morisseau, Goodrow et al. 1999). In the current study, we employed TUPS as the choice of sEH inhibitor, which comprises a highly potent urea pharmacophore (Chiamvimonvat, Ho et al. 2007). We investigated the cardioprotective effect of the inhibition of sEH by TUPS in isoproterenol-induced cellular hypertrophy. Our results demonstrated that TUPS significantly decreased the isoproterenol-mediated induction of hypertrophic markers ANP and BNP in H9c2 cells. In agreement with our results, it has been demonstrated previously that TUPS decreased the hypertrophic markers, ANP and BNP in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011). Similarly, TUPS attenuated the hypertrophic markers and left ventricular hypertrophy in Ang II-induced hypertrophy in rat hearts and neonatal rat cardiomyocytes (Ai, Pang et al. 2009). Recently, our lab also demonstrated that TUPS decreased the hypertrophic markers of isoproterenol-induced cardiac hypertrophy in rats (Althurwi, Tse et al. in press). Furthermore, two other sEH inhibitors, AEPU and AUDA, were also reported to decrease the hypertrophic markers and prevent cardiac hypertrophy in a thoracic aortic constriction murine model (Xu, Li et al. 2006). Therefore, these findings suggest that sEH inhibitors exert a cardioprotective effect against cardiac hypertrophy and decrease the expression of hypertrophic markers.

Due to the importance of CYPs in the pathogenesis of cardiac hypertrophy, we investigated the effect of sEH inhibition on the expression of

different *CYP* genes involved in isoproterenol-induced cellular hypertrophy. Our results demonstrated that TUPS significantly decreased the isoproterenol-mediated induction of CYP1A1 in H9c2 cells. In agreement with our results, another study showed that TUPS significantly attenuated the expression of CYP1A1 in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011). On the other hand, TUPS did not decrease the expression of CYP1B1, which is consistent with the findings we observed earlier with 11,12- and 14,15-EETs. Although a previous study demonstrated that TUPS decreased the expression of CYP1B1 in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011), we did not observe this reduction in our current study. This discrepancy is likely due to the different models between animals and cell lines.

With regard to the CYP2 family, treatment with TUPS significantly decreased the isoproterenol-mediated induction of CYP2J3. Interestingly, treatment with TUPS alone caused a significant reduction in the expression of CYP2C11. Consistent with the findings we observed earlier by the treatment of 11,12- and 14,15-EETs, which alone also decreased the expression of CYP2C11. On the other hand, the expression of CYP4F1 was significantly increased by the treatment of TUPS, which is consistent with the results obtained from the treatment of 11,12- and 14,15-EETs. The modulation of CYP2C11 and CYP4F1 by TUPS could be attributed to the increasing levels of EETs through the inhibition of sEH, and possibly through the involvement of EETs in regulating the CYP-dependent pathways during cardiac hypertrophy. On the other hand, our study showed that the treatment of TUPS significantly inhibited the isoproterenol-mediated induction of CYP4F4 in H9c2 cells. Consistent with our results, several studies showed that TUPS significantly attenuated the expression of CYP4F4 in BaP and isoproterenol-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011; Althurwi, Tse et al. in press). Furthermore, our study also showed that treatment of TUPS significantly decreased the expression of CYP4F5 in isoproterenol-induced cellular hypertrophy in H9c2 cells. In agreement with our results, a previous study showed that TUPS reduced the expression of CYP4F5 in BaP-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011).

The expression of *EPHX2* was found to be induced in spontaneously hypertensive rats with heart failure (Monti, Fischer et al. 2008). Moreover, an increased level of sEH was detected in the rat myocardium of Ang II-induced cardiac hypertrophy (Ai, Pang et al. 2009). Previous studies from our lab have also demonstrated that the expression of sEH was significantly induced in BaP-mediated cardiac hypertrophy (Aboutabl, Zordoky et al. 2009) and isoproterenol-induced cardiac hypertrophy in rats (Zordoky, Aboutabl et al. 2008). In our current study, we demonstrated that TUPS significantly attenuated the mRNA expression of sEH in isoproterenol-induced cellular hypertrophy. To confirm the effect of TUPS on the transcriptional activity of *EPHX2*, we adopted the luciferase gene reporter assay under the control of the *EPHX2* promoter. We found that isoproterenol increased the *EPHX2*-luciferase activity, and the treatment of TUPS significantly attenuated the isoproterenol-mediated induction of *EPHX2*-luciferase expression. Consistently, previous studies have shown that inhibition of sEH by TUPS decreased the mRNA expression of sEH in BaP and isoproterenol-induced cardiac hypertrophy in rats (Aboutabl, Zordoky et al. 2011; Althurwi, Tse et al. in press). Altogether, these findings suggest that TUPS exerts a protective effect against cellular hypertrophy by down regulating the gene expression of *EPHX2*. Inhibition of sEH by TUPS increases the endogenous levels of EETs; the EETs play a critical role in the inhibition of NF-κB activation, which is a downstream target of several signaling pathways implicated in cardiac hypertrophy (Zechner, Craig et al. 1998; Rouet-Benzineb, Gontero et al. 2000; Craig, Wagner et al. 2001; Force, Kuida et al. 2004; Fischer and Hilfiker-Kleiner 2007). In addition to NF-κB, EETs also target several other downstream signaling pathways that may explain their cardioprotective effects (Harris, Li et al. 2008). EETs activate the p42/p44 MAPK pathway, ATP-sensitive potassium channels, as well as the PKA-dependent signaling pathway (Seubert, Yang et al. 2004; Lu, Ye et al. 2006; Batchu, Law et al. 2009).

In conclusion, we demonstrated that the treatment with TUPS significantly attenuated isoproterenol-induced cellular hypertrophy in H9c2 cells. In addition, TUPS also inhibited the isoproterenol-mediated induction of CYPs and *EPHX2*

gene expression. Taking into account the accumulating evidence of sEH involved in the development of cardiac hypertrophy, sEH inhibitors may emerge as a new therapeutic tool for the treatment of cardiac hypertrophy and heart failure. However, more studies are needed to further demonstrate the protective effect of sEH inhibitors against cardiac hypertrophy and to examine the mechanisms by which CYPs are implicated in cardiac hypertrophy.

4.4 General conclusion

Cardiac hypertrophy remains as a major pathological event, in which prolonged hypertrophy can lead to heart failure and sudden death. Therefore, research into the molecular basis of cardiac hypertrophy can elucidate possible treatment approaches to prevent hypertrophy and subsequently heart failure at an early stage. Hence, our work has been focused on investigating the protective effect of EETs, and the role of CYP enzymes and sEH in cardiac hypertrophy.

In the current study, we have demonstrated that treatment with isoproterenol increased the expression of the hypertrophic markers ANP and BNP in H9c2 cells. Isoproterenol-induced cellular hypertrophy also caused a significant induction of the *CYP1A1*, *CYP1B1*, *CYP2J3*, *CYP4F4*, *CYP4F5* and *EPHX2* gene expression. Of these CYP enzymes, CYP1A1, CYP1B1, CYP4F4 and CYP4F5 are the major CYP ω-hydroxylases, which were significantly increased by isoproterenol-mediated hypertrophy in our study. As the main CYP epoxygenase in cardiac cells, the mRNA expression of CYP2J3 was also significantly induced in isoproterenol-induced cellular hypertrophy. Furthermore, the expression of sEH was increased at both the mRNA and protein levels. Altogether, these findings suggest a modulation of CYP expression, which leads to a possible alteration in the balance of CYP-mediated arachidonic acid metabolites, EETs and HETEs during cardiac hypertrophy.

In an attempt to examine the protective effect of EETs against cardiac hypertrophy, we investigated whether administration of 11,12- and 14,15-EETs can prevent hypertrophy by modulating the isoproterenol-mediated induction of CYPs and *EPHX2* expression in cellular hypertrophy. Our results demonstrated

that treatment with 11,12- and 14,15-EETs significantly inhibited the isoproterenol-mediated induction of the hypertrophic markers ANP and BNP in H9c2 cells. Furthermore, 11,12- and 14,15-EETs also significantly attenuated the isoproterenol-mediated induction of *CYP1A1*, *CYP2J3*, *CYP4F4*, *CYP4F5* and *EPHX2* gene expression. Thus, we confirmed the protective effect of EETs against isoproterenol-induced cellular hypertrophy and the involvement of CYPs and sEH in the development of cardiac hypertrophy.

In order to further address the role of sEH in cardiac hypertrophy, we examined whether the inhibition of sEH would confer cardioprotection in isoproterenol-mediated cellular hypertrophy. Our study showed that treatment with TUPS significantly decreased the isoproterenol-mediated induction of ANP and BNP in H9c2 cells. Moreover, TUPS caused a significant reduction in the isoproterenol-mediated induction of *CYP1A1*, *CYP2J3*, *CYP4F4*, *CYP4F5* and *EPHX2* gene expression. These findings indicate a role of the sEH inhibitor TUPS in protection against isoproterenol-mediated cellular hypertrophy and the involvement of CYP enzymes in the development of cardiac hypertrophy.

4.5 Future directions

The results of the present work have highlighted the protective effect of EETs and the role of CYPs and sEH in the pathogenesis of cellular hypertrophy. However, more studies are needed to confirm the findings and further address the underlying molecular basis of cardiac hypertrophy.

1) To investigate whether EETs protect against cardiac hypertrophy *in vivo*.
2) To determine whether CYP epoxygenase inducers confer cardioprotection against cardiac hypertrophy *in vitro* and *in vivo*.
3) To determine the combined effect of sEH inhibitors and epoxygenase inducers on cardiac hypertrophy *in vitro* and *in vivo*.
4) To identify the mechanisms by which cardiac hypertrophy causes these alterations in the CYPs and sEH expression.
5) To investigate whether CYP ω-hydroxylase inhibitors confer cardioprotection against cardiac hypertrophy *in vitro* and *in vivo*.

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