THE ROLE OF CYTOCHROME P450 AND THEIR ASSOCIATED ARACHIDONIC ACID METABOLITES IN THE INITIATION AND PROGRESSION OF CARDIAC HYPERTROPHY

By

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Abstract

Heart failure (HF) is the leading cause of mortality and disability in adults worldwide. Cardiac hypertrophy is an independent risk factor and one of the major hallmarks of HF. Research in cardiac hypertrophy is considered as a research into the early events in the development of HF. The expression of cytochrome P450 (CYP) and soluble epoxide hydrolase (sEH) enzymes has been identified in the heart and their levels have been reported to be altered during cardiac hypertrophy and HF. The role of CYP enzymes in cardiac hypertrophy emerge from their ability to metabolize arachidonic acid to the cardioprotective epoxyeicosatrienoic acids (EETs) and the cardiotoxic 20-hydroxyeicosatetraenoic acid (20-HETE) metabolites. Therefore, the objective of the present work was to investigate the role of CYP enzymes, sEH, and CYP-derived arachidonic acid metabolites in the pathogenesis of cardiac hypertrophy. Our results show that cardiac hypertrophy was initiated after 72 hours and 6 hours of isoproterenol treatment in rat and human fetal ventricular cell line, RL-14, respectively. Studies performed at the prehypertrophy phase showed decreases in the expression of CYP epoxygenases and an induction of sEH activity. Consequently, lower EET and higher dihydroxyeicosatrienoic acid (DHETs) levels were observed prior to cardiac enlargement. On the other hand, isoproterenol caused an induction of CYP1A1, CYP1B1, CYP2B1, CYP2B2, CYP4A3 and CYP4F4 expression during the established phase of cardiac hypertrophy, which consequently led to lower levels of EETs and higher levels of 20-HETE. Interestingly, inhibition of sEH by 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS) attenuated the progression of cardiac hypertrophy and fibrosis induced by isoproterenol. Moreover, TUPS significantly inhibited the isoproterenol-

mediated effects on CYP enzymes and their associated metabolites. Furthermore, we showed that fenofibrate significantly induced the cardiac expression of CYP epoxygenases such as CYP2B1, CYP2B2, CYP2C11, and CYP2C23, whereas it decreased the expression of the cardiac ω-hydroxylase CYP4A3. Consequently, fenofibrate significantly increased the formation of cardiac EETs whereas it decreased the cardiac level of 20-HETE. Interestingly, fenofibrate significantly decreased the hypertrophic markers and the increase in heart-to-body weight ratio induced by isoproterenol. Finally, we showed that increasing EET levels by induction of CYP epoxygenases, sEH inhibition, or exogenous administration of EET prevented the initiation of cardiac hypertrophy through a nuclear factor-kB-mediated mechanism. Taken together, these findings reveal a crucial role of CYP, sEH, and CYP-mediated arachidonic acid metabolism in the initiation and progression of cardiac hypertrophy, which may lead to discovery of novel targets for the prevention of HF at an early stage.

Preface

This thesis is an original work done by Mr. Hassan Althurwi. All experimental animal procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Sections 3.1, 3.4, 4.1, and 4.4 of this thesis have been published as Althurwi, H. N., Z. H. Maayah, O. H. Elshenawy and A. O. El-Kadi (2015). "Early Changes in Cytochrome P450s and Their Associated Arachidonic Acid Metabolites Play a Crucial Role in the Initiation of Cardiac Hypertrophy Induced by Isoproterenol." Drug Metabolism and Disposition 43(8): 1254-1266. I was responsible for designing the research, conducting experiments, and analysis as well as the manuscript composition. Z. H. Maayah and O. H. Elshenawy assisted with the analysis and contributed to manuscript edits. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.2 and 4.2 of this thesis have been published as Althurwi, H. N., M. M. Tse, G. Abdelhamid, B. N. Zordoky, B. D. Hammock and A. O. El-Kadi (2013). "Soluble epoxide hydrolase inhibitor, TUPS, protects against isoprenaline-induced cardiac hypertrophy." British Journal of Pharmacology 168(8): 1794-1807. I was responsible for designing the research, conducting experiments, and analysis as well as the manuscript composition. M. M. Tse assisted with experiments conducting. M. M. Tse, G. Abdelhamid, B. N. Zordoky, and B. D. Hammock assisted with the analysis and contributed to manuscript edits. A. O. El-Kadi was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.3 and 4.3 of this thesis have been published as Althurwi, H. N., O. H. Elshenawy and A. O. El-Kadi (2014). "Fenofibrate modulates

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My family, brothers and sisters for being there for me in the time when I needed support.

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List of Abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
ACC/AHA	American College of Cardiology/American Heart Association
ACEI	Angiotensin-converting-enzyme inhibitor
AEPU	1-adamantan-3-(5-(2-(2- ethylethoxy)ethoxy)pentyl) urea
AhR	Aryl hydrocarbon receptor
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
Apo	Apolipoprotein
ATF-6	Activating transcription factor-6
ATP	Adenosine-5'-triphosphate
AUDA	12-(3-adamantan-1-yl-ureido)-dode-canoic acid
BaP	Benzo(a)pyrene
BNP	B-type natriuretic peptide
CaMK	Ca2+/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CHD	Coronary heart disease
COX	Cyclooxygenase
CVDs	Cardiovascular diseases
CYP	Cytochrome P450
DHET	Dihydroxyeicosatrienoic acid
DM	Diabetic mellitus
DMEM	Dulbecco's modified Eagle's medium
EDHF	Endothelium-derived Hyperpolarizing Factor
EET	Epoxyeicosatrienoic acid
ERK	Extracellular signal-regulated kinase
FAO	Fatty acid oxidation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GSK-3b	Glycogen synthase kinase-3b
H/R	Hypoxia and reoxygenation

HW/BW	Heart weight/body weight
HDAC4	Histone deacetylase 4
HETE	Hydroxyeicosatetraenoic acid
HF	Heart Failure
HPETE	Hydroperoxyeicosatetraenoic acid
HTN	Hypertension
I/R	Ischemia/reperfusion
IHD	Ischemic heart disease
IKK	Inhibitor of nuclear factor Kappa B kinase
JNK	c-Jun N-terminal kinase
KATP	ATP-sensitive potassium channel
Kca	Calcium-activated potassium channel
LC-ESI-MS	Liquid Chromatography - Electrospray Ionization - Mass Spectrometry
LOX	Lipooxygenase
LPS	Lipopolysaccharide
LV	Left ventricular
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MEK	MAPK kinase
MEKK	MAPK kinase kinase
MKP-1	MAPK phophatase 1
MMP	Metalloproteinase
MS-PPOH	N-(Methylsulfonyl)- 2-(2-propynyloxy)-benzenehexanamide
mTOR	Mammalian Target of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate tetrasodium
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
NHE	Na+/H+ exchanger
NYHA	New York Heart Association
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin

PGI2	Prostacycline I2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPAR	Peroxisome proliferator activated receptor
ROCK	Rho-associated protein kinase
RTK	Receptor tyrosine kinase
RXR	Retinoic acid X receptor
SD	Sprague Dawley
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
sEH	Soluble epoxide hydrolase
SERCA2a	Sarcoplasmic reticulum Ca2+-ATPase 2a
SHHF	Spontaneously hypertensive heart failure
SHR	Spontaneously hypertensive rat
STAT	Signal transducer and activator of transcription
t-AUCB	Trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxyl]-benzoic acid
TAC	Thoracic aortic constriction
TALH	Thick ascending loop of Henle
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-1	Transforming growth factor-1
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TRPV4	Transient receptor potential Ca2+ channels
TUPS	[1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea]
TxA2	Thromboxane A2
UPREs	Unfolded protein response elements
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
α-MHC	α- Myosin heavy chain
β-MHC	β- Myosin heavy chain

CHAPTER 1. INTRODUCTION

1.1. Heart Failure (HF)

1.1.1. Definition and epidemiology

Although significant progress has been made over the past few decades in heart research, HF is still considered an epidemic public health problem. HF is commonly defined as a clinical syndrome that arises as a consequence of any disorder that impairs the ability of the ventricle to fill with and/or eject blood, rendering the heart unable to pump sufficient blood to meet the metabolic needs of the body (Kaye and Krum 2007). HF affects more than 5 million Americans and 500,000 Canadians with approximately 825,000 and 50,000 new cases every year in the US and Canada, respectively (Ross, Howlett et al. 2006, Yancy, Jessup et al. 2013). Unlike most cardiovascular diseases, the prevalence of HF is projected to increase as the population ages, to affect over 8 million Americans in 2030 (Yancy, Jessup et al. 2013). HF causes a huge economic burden with an estimated expenditure of $50 billion in the US and a total global expenditure of $108 billion annually (Yancy, Jessup et al. 2013, Cook, Cole et al. 2014). Despite the significant advance in understanding this disorder, the prognosis for patients with HF remains poor, with high overall annual mortality. Approximately 50% of patients will die within 5 years of diagnosis (Yancy, Jessup et al. 2013).

1.1.2. Etiology and classification

Numerous cardiac disorders, including those affecting the pericardium, heart valves, and myocardium, can lead eventually to HF (Hunt, Baker et al. 2002). HF can result from any disorder that affects the contracting (systolic dysfunction) and/or relaxing ability (diastolic dysfunction) of the heart. Systolic HF is the classic type

and is characterized by dilated ventricles with a left ventricle ejection fraction (LVEF) of 40% or less; hence it is commonly called HF with reduced ejection fraction (HFrEF) (Yancy, Jessup et al. 2013). On the other hand, HF with preserved ejection fraction (HFpEF) or diastolic HF accounts for about 50% of HF patients and is characterized by a LVEF of 50% or greater with impaired ventricular relaxation and filling (Owan, Hodge et al. 2006). It bears mentioning that systolic and diastolic dysfunction frequently coexist because most cardiovascular diseases can decrease myocardial contractility and cause abnormal ventricular filling (Yancy, Jessup et al. 2013). Common risk factors that cause HF include coronary heart disease (CHD), hypertension (HTN), ventricular hypertrophy, diabetic mellitus (DM), atherosclerotic diseases, thyroid diseases, obesity, cardiotoxins (such as alcohol, chemotherapeutic agents and cocaine), and myocarditis. However, CHD contributes to two-thirds of HFrEF, whereas HTN is the leading cause of HFpEF and accounts for 60%–89% of the cases (Bhuiyan and Maurer 2011).

Due to the progressive nature of HF, the American College of Cardiology and the American Heart Association (ACC/AHA) developed a new staging system that focuses on the risk factors and structural abnormality of the heart. On the contrary, the classification of the New York Heart Association (NYHA) mainly emphasizes the functional limitations (Jessup and Brozena 2003, Yancy, Jessup et al. 2013). Nevertheless, both classifications provide useful information about the development and severity of HF.

1.1.3. Pathophysiology

Over the years, scientists have suggested different paradigms in an attempt to develop a unifying hypothesis that explains the pathophysiology of HF. Nevertheless, no single paradigm was sufficient to explain its complexity as it involves multiple structural, functional, and biological alterations that interact in a complex manner (Jessup and Brozena 2003). Cardiorenal model was among the first suggested paradigms in which excess sodium and water retention were believed to be the problem. The next paradigm was the cardiocirculatory (hemodynamic) model wherein the problem was viewed as inadequate contractility and systemic vasoconstriction. Both models succeeded in rationalizing the use of diuretics, positive inotropic, and vasodilators in the management of disease symptoms, but failed to explain HF progression despite the use of these therapeutic agents (Mann 1999). Consequently, the neurohormonal model was developed to describe the progression of HF. This model recognizes the existence of an initiating event that either damages the heart muscle or disturbs its contractile ability (e.g., MI or long-standing pressure or volume overload), resulting in increased circulating levels of neurohormones and autocrine/paracrine factors. The elevated levels of biologically active molecules such as norepinephrine, angiotensin II, endothelin, aldosterone, and tumor necrosis factor (TNF) are capable of exerting toxic effects on the heart thus, contributing to disease progression (Mann 1999). The latter model rationalizes the successful use of neurohormonal antagonists such as β-blockers and angiotensin-converting-enzyme inhibitors (ACEIs).

In addition to the elevated levels of neurohormones, the left ventricle (LV) undergoes remodeling as a response to the initial insult. LV remodeling is a complex process describing the alteration of heart size, shape, and function as a result of mechanical, neurohormonal, and genetic factors (Braunwald and Bristow 2000). One hallmark of LV remodeling is cardiac hypertrophy which is considered a compensatory response that normalizes wall stress and permits normal cardiovascular function at rest. However, a continued presence of hypertrophy is now recognized as a key component in the pathogenesis of progressive HF and sudden death (Bernardo, Weeks et al. 2010).

1.2. Cardiac Hypertrophy

Cardiac hypertrophy can be defined as an increase in cardiac mass in response to an increase in biomechanical load. At the cellular level, the defining characteristics of hypertrophy include increased cardiomyocyte size, increased protein synthesis, activation of the fetal gene programme, and altered sarcomere organization (Frey and Olson 2003). It is considered as an independent risk factor for many CVDs such as CAD, arrhythmia, HTN and all forms of HF (Bernardo, Weeks et al. 2010). Moreover, cardiac hypertrophy is viewed as an early event in the CVDs continuum where scientists visualize cardiac diseases as a chain of events, which if left untreated will lead eventually to end stage HF and death (Chrysant 2011). Therefore, studying the molecular mechanisms in cardiac hypertrophy can be considered as research into the initial steps of HF (Ritter and Neyses 2003).

1.2.1. Classification of cardiac hypertrophy

1.2.1.1. Physiological vs pathological hypertrophy

Cardiac hypertrophy can be classified into physiological or pathological hypertrophy. Physiological hypertrophy occurs during postnatal development, pregnancy, and in athletes. On the other hand, pathological hypertrophy occurs in response to CVDs such as ischemic heart disease (IHD), cardiomyopathy, or HTN (Bernardo, Weeks et al. 2010). Although physiological and pathological hypertrophy lead to a comparable increase in heart size, there are key structural, functional, metabolic, and biochemical differences between the two conditions. For instance, pathological hypertrophy is correlated with cardiomyocyte loss, fibrosis, and activation of the fetal gene programme, leading to a decrease in heart function and eventually to HF. In contrast, physiological hypertrophy is associated with normal or even enhanced cardiac function in the absence of cardiomyocyte loss and fibrosis (Bernardo, Weeks et al. 2010).

At the metabolic level, fatty acid oxidation (FAO) is decreased in pathological hypertrophy with a shift in substrate utilization and glucose becomes a primary substrate for energy production (Christe and Rodgers 1994). However, glucose utilization also becomes impaired in advanced pathological hypertrophy because insulin resistance increases in failing hearts (Neubauer 2007). In contrast, physiological hypertrophy is characterized by enhanced FAO and glucose utilization, increasing the overall ability of the heart to generate sufficient ATP (Gertz, Wisneski et al. 1988). At the molecular level, an increase in the expression of fetal genes such as β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), B-type natriuretic

peptide (BNP), and α- skeletal actin**,** and a down-regulation of sarcoplasmic reticulum Ca++ ATPase (SERCA) is only reported in pathological hypertrophy (Iemitsu, Miyauchi et al. 2001).

1.2.1.2. Concentric vs eccentric hypertrophy

Cardiac hypertrophy can be further classified, according to shape, as either concentric or eccentric hypertrophy. Concentric hypertrophy is characterized by an increase in myocyte cell width as a result of parallel addition of the sarcomeres. Eventually, concentric hypertrophy can lead to an increase in cardiac mass and wall thickness with relatively unchanged chamber volume (Bernardo, Weeks et al. 2010). In contrast, the length of myocyte is increased in eccentric hypertrophy as a result of series addition of the sarcomeres, leading to an increase in both cardiac mass and chamber volume with normal, decreased, or increased wall thickness (Grossman, Jones et al. 1975, Pluim, Zwinderman et al. 2000, Bernardo, Weeks et al. 2010).

The initiating stimulus of cardiac hypertrophy is the main determinant of its shape. Pressure overload caused by physiological (static exercises such as weight lifting) or pathological stimuli (e.g. HTN or aortic constriction) leads to concentric hypertrophy. On the other hand, volume overload and eccentric hypertrophy develop as a result of physiological stimuli such as pregnancy and dynamic exercises such as swimming or pathological stimuli such as aortic regurgitation (Pluim, Zwinderman et al. 2000, Eghbali, Deva et al. 2005, Bernardo, Weeks et al. 2010). Clinically, pathological eccentric cardiac hypertrophy has been reported to pose greater death risk than concentric cardiac hypertrophy (Berenji, Drazner et al. 2005).

1.2.2. Molecular pathways implicated in mediating cardiac hypertrophy

1.2.2.1. G Protein–Coupled Receptors (GPCRs)

GPCRs represent a group of transmembrane proteins, which play an important role in the regulation and the adaptation of cardiac function to changes in hemodynamic burden (Rockman, Koch et al. 2002). GPCRs are coupled to heterotrimeric GTP-binding proteins (α, β and γ), which disassociate into Gα and Gβγ subunits upon receptor activation. Consequently, several downstream intracellular signaling pathways become activated (Rockman, Koch et al. 2002). With regard to the Gα subunit, G proteins are subdivided into three main subfamilies of Gs, Gi, and Gq/G_{11}, each with distinct downstream signaling molecules and physiological responses (Salazar, Chen et al. 2007).

1.2.2.1.1. Gq/G11 signaling

The most important myocardial Gq/G_{11} coupled receptors include Ang II, ET1, and α-adrenergic receptors. Activation of these receptors has been shown to be sufficient to induce cardiac hypertrophy whereas Ang II-receptor blockers and ACE inhibitors were shown to inhibit cardiac remodeling in patients with cardiomyopathy (Yusuf, Sleight et al. 2000, Frey and Olson 2003). Moreover, genetic overexpression of these receptors and/or their downstream effectors were shown to induce cardiac growth and depressed heart contractility in rodents (D'Angelo, Sakata et al. 1997, Paradis, Dali-Youcef et al. 2000). Upon agonist stimulation, these receptors activate different downstream signaling proteins such as phospholipase C (PLC), mitogen-activated protein kinases (MAPKs), protein kinase A (PKA), and protein kinase C

(PKC) which all have been implicated in the development of pathological cardiac hypertrophy (Bernardo, Weeks et al. 2010).

1.2.2.1.2. Gs/Gi signaling

The β_1-receptor, the most abundant adrenergic receptor in the heart, is coupled to the Gs subunit. Upon agonist stimulation, these receptors activate adenylate cyclase (AC), which results in the production of cAMP (Frey and Olson 2003). Although transgenic overexpression of the β_1-receptor or PKA, the principal target of AC, initially improves cardiac function and responsiveness to isoproterenol, it eventually results in cardiac hypertrophy and fibrosis as seen in animal models (Bisognano, Weinberger et al. 2000, Antos, Frey et al. 2001). Moreover, mice deficient in β-adrenergic receptors exhibit an attenuated hypertrophic and fibrotic response to pressure overload (Kiriazis, Wang et al. 2008). In humans, elevated levels of catecholamines (e.g noradrenaline) along with β_1-receptor downregulation and desensitization have been reported in HF patients (Bristow, Ginsburg et al. 1982).

The β2-receptors are coupled to the Gi subunit, in addition to the Gs subunit, which in turn inhibits AC and opposes Gs-dependent signaling pathways. The β2-receptors have been shown to mediate cardioprotection due to the activation of the phosphoinositide 3-kinase (PI3K) signaling pathway (Chesley, Lundberg et al. 2000).

1.2.2.1.3. Small G proteins

The small G proteins family (also known as small GTPases) consist of Ras, Rho, Ran, Rab, and ADP ribosylation factors (ARFs). They regulate several cellular

processes such as cell growth, cell division, cell survival, and cellular motility (Frey and Olson 2003). Small GTPases were activated in myocytes subjected to GPCR agonists or mechanical stress (Bernardo, Weeks et al. 2010). Cardiac specific overexpression of Ras or Rab in mice results in cardiac hypertrophy, whereas inhibition of Rho Kinase (ROCK), a downstream target of Rho, revealed that GTPases are implicated in pathological cardiac hypertrophy rather than physiological cardiac growth (Balakumar and Singh 2006, Bernardo, Weeks et al. 2010). Small GTPases also have several downstream targets such as raf, MAPKs, and calcineurin/nuclear factor of activated T-cells (NFAT) which all have been shown to be implicated in the development of cardiac hypertrophy (Frey and Olson 2003).

1.2.2.2. Mitogen activated protein kinases (MAPKs) pathway

MAPKs are important regulators of multiple cellular functions through their ability to phosphorylate several transcription factors. MAPKs are divided into three main subfamilies: Extracellular signal receptor-Regulated Kinase (ERK), c-jun NH2-terminal Kinase (JNK) and p38 MAPK (Zhang, Elimban et al. 2003). Several studies have suggested the important role of MAPK in the hypertrophic response (Bueno, De Windt et al. 2000, Zhang, Elimban et al. 2003, Bernardo, Weeks et al. 2010, Wang, Liu et al. 2014). Indeed, inhibition of MAPK pathways, through overexpression of MAPK phosphatase 1 (MKP-1), was shown to attenuate the hypertrophic response *in vivo* and *in vitro* (Bueno, De Windt et al. 2001). Moreover, all MAPK subfamilies were reported to be activated in hypertrophic response to Gq coupled receptor activators, pressure overload, and in failing hearts (Bernardo, Weeks et al. 2010).

Although the activation of the ERK1/2 pathway in hypertrophied hearts has been reported, its potential role in stimulating cardiac growth is still controversial. For instance, overexpression of MEK1 or MEK5, upstream activators of ERK1/2 but not JNK or p38, results in the development of cardiac hypertrophy (Bueno, De Windt et al. 2000, Nicol, Frey et al. 2001). However, mice deficient of ERK1/2 failed to prevent the development of cardiac hypertrophy in response to both pressure and volume overloads (Nicol, Frey et al. 2001). In contrast to ERK1/2, JNK and p38 MAPKs are activated by stress stimuli, in addition to their activation by GPCR and anabolic stimuli. Thus, they are often categorized as stress-responsive MAPKs (Frey and Olson 2003). The JNK and p38 signaling pathways have also been shown to be important regulators of cardiac growth. Mice deficient for MEKK1, which activates MEK7, an upstream activator of JNK, displayed an attenuated hypertrophic response to Gs overexpression (Minamino, Yujiri et al. 2002). Similar to JNK, p38 along with its activators, MEK3 and MEK6, have also been shown to be sufficient to induce cardiac hypertrophy and to activate different transcription factors implicated in cardiac hypertrophy such as myocyte enhancer factor-2 (MEF2) and NFAT (Ueyama, Kawashima et al. 1999, Frey and Olson 2003).

1.2.2.3. Calcium signaling

Calcium plays a vital role in heart contractility and growth. Two calcium-dependant signaling pathways have been suggested as important regulators in pathological hypertrophy: the calcineurin/NFAT and calcium/calmodulin-dependent protein kinase (CaMK) pathways (Bernardo, Weeks et al. 2010).

1.2.2.3.1. Calcineurin/NFAT

Calcineurin, also known as protein phosphatase 2B (PP2B), is a serine/ threonine protein phosphatase that is expressed in human, mouse, and rat hearts (Wilkins and Molkentin 2002). Calcineurin is an important signaling protein in the development of pathological hypertrophy. In humans, calcineurin activity was elevated in hypertrophied and failing hearts. In addition, its activity was also increased in animals subjected to either GPCR agonists or biomechanical stress (Shimoyama, Hayashi et al. 1999, Haq, Choukroun et al. 2001, Saito, Fukuzawa et al. 2003). Moreover, overexpression of calcineurin in the heart resulted in cardiac hypertrophy which rapidly progresses to HF and sudden death in mice. On the other hand, inhibition of calcineurin prevents the development of cardiac hypertrophy in this model (Molkentin, Lu et al. 1998).

The regulatory effect of calcineurin in cardiac hypertrophy is believed to be mediated by the dephosphorylation of NFAT transcription factors. Dephosphorylated or “activated” NFAT translocates to the nucleus and associates with other transcription factors such as MEF2 and GATA4, important regulators of cardiac gene expression (Rockman, Koch et al. 2002). In this regard, NFAT luciferase reporter activity was reported to be increased in pressure overload pathological hypertrophy (Wilkins, Dai et al. 2004). In addition, cardiac-specific NFAT translocation to the nucleus in a transgenic mice model displayed cardiac hypertrophy and HF (Molkentin, Lu et al. 1998).

1.2.2.3.2. Calcium/calmodulin-dependent protein kinases (CaMKs)

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a protein kinase involved in cardiac hypertrophy and HF through its ability to phosphorylate class II histone deacetylase 4 (HDAC4). Phosphorylated HDAC4, in turn, dissociates from the MEF2 transcription factor, rendering it free for nuclear translocation, thus promoting pathological hypertrophy (Bernardo, Weeks et al. 2010). Increased levels of CaMKII have been reported in failing hearts of human and animal (Hoch, Meyer et al. 1999, Bernardo, Weeks et al. 2010). Moreover, transgenic mice with overexpressed forms of CaMKII (nuclear or cytosolic) developed cardiac hypertrophy and HF, whereas genetic inhibition of CaMKII ablated cardiac hypertrophy induced by both pressure overload and GPCR stimulation (Zhang, Johnson et al. 2002, Zhang, Maier et al. 2003, Zhang, Khoo et al. 2005).

1.2.2.4 Phosphoinositide 3-kinase (PI3K) signaling

PI3K is a family of lipid kinases that phosphorylate phosphatidylinositol and release its derivatives from the plasma membrane which in turn mediate a diverse group of cellular functions such as survival, proliferation, cell growth and differentiation (Cantley 2002). PI3Ks are classified into three major classes: class I, II, and III. Of interest, class 1 PI3Ks is further classified into two subclasses; IA which consist of p110 catalytic subunits (α, β or δ) and IB (p110γ) (Vanhaesebroeck, Leevers et al. 1997). p110α and p110γ are the major isoforms that are expressed in heart, and are coupled to receptor tyrosine kinases (RTKs) and GPCRs, respectively (Bernardo, Weeks et al. 2010). Interestingly, mice expressing a cardiac-specific dominant negative (dn) form of (p110α) displayed cardiac hypertrophy in response to pathological stimuli but not to physiological stimuli, suggesting that p110α is a

critical regulator in physiological cardiac growth (McMullen, Shioi et al. 2003). In contrast, p110γ knockout mice subjected to chronic activation of β-ARs exhibited attenuated hypertrophic and fibrotic response, which suggests that p110γ plays an important role in the development of pathological hypertrophy (Oudit, Crackower et al. 2003).

Transgenic mice overexpressing Akt, one of the main targets of PI3K activation, have been reported to develop cardiac hypertrophy (Shioi, McMullen et al. 2002). PI3K/Akt activation is believed to induce cardiac hypertrophy through main downstream targets of Akt, glycogen synthase kinase-3 (GSK-3) and the mammalian target of rapamycin (mTOR). Akt phosphorylates and inhibits the activity of GSK-3 which has been shown to possess an antihypertrophic effect. mTOR, on the other hand, is an important regulator of protein synthesis and cell size through its actions on the 4E binding protein 1 (4EBP-1) and S6 kinase 1 (S6K1) (Frey and Olson 2003, Aoyagi and Matsui 2011).

1.2.2.5 Nuclear factor kappa-B (NF-κB)

NF-κB is a protein complex of homo or heterodimers of p50, p52, p65 (RelA), RelB, and c-rel (Kumar, Takada et al. 2004). Dimeric combinations of NF-κB exert different effects on cell function (Pereira and Oakley 2008). The NF-κB signaling pathway can be activated by various stimuli including cytokines, oxidative stress, viruses, and chemical agents. Upon activation, NF-κB translocates into the nucleus and regulates the expression of many genes involved in the immune response, inflammation, cell growth, and survival (Guijarro and Egido 2001). Several studies have demonstrated the critical role of NF-κB in cardiac hypertrophy and HF

(Zelarayan, Renger et al. 2009, Gordon, Shaw et al. 2011, Gaspar-Pereira, Fullard et al. 2012, Maier, Schips et al. 2012). For instance, the NF-κB signaling pathway was found to be activated in failing, but not in normal, hearts (Frantz, Fraccarollo et al. 2003). Moreover, transgenic mice overexpressing cardiomyocyte-specific IKK2 (leading to NF-κB activation) developed hypertrophied hearts which eventually led to HF (Maier, Schips et al. 2012). In addition, NF-κB has been shown to be implicated in the hypertrophic response of myotrophin, TNF-α, isoproterenol and Ang II (Gupta, Purcell et al. 2002, Higuchi, Otsu et al. 2002, Freund, Schmidt-Ullrich et al. 2005). Recently, inhibition of the NF-κB signaling pathway was reported to be involved in the cardioprotective effect of baicalein against Ang II-induced cardiac hypertrophy in mice (Wang, Song et al. 2015).

1.2.2.6 Miscellaneous signaling pathways

Other signaling pathways have also been shown to be implicated in cardiac hypertrophy (fig 1.1). For instance, Na^+/H^+ exchanger (NHE) activation has been detected in several models of cardiac hypertrophy and HF, whereas its inhibition attenuated isoproterenol-induced cardiac hypertrophy (Cingolani and Camilion de Hurtado 2002, Ennis, Escudero et al. 2003). Moreover, overexpression of the transcription factor signal transducer and activator of transcription 3 (STAT3) was found to be sufficient to induce cardiomyocyte growth both *in vitro* and *in vivo* (Kunisada, Negoro et al. 2000). In addition, cardiac hypertrophy induced by GPCR agonists was found to be dependent on NADPH oxidase activation (Hingtgen, Tian et al. 2006). Expression of matrix metalloproteinase (MMP) was upregulated in cardiac remodeling whereas their inhibition was reported to protect against cardiac hypertrophy, suggesting the involvement of this family of enzymes in the pathogenesis of cardiac hypertrophy (Frey and Olson 2003).

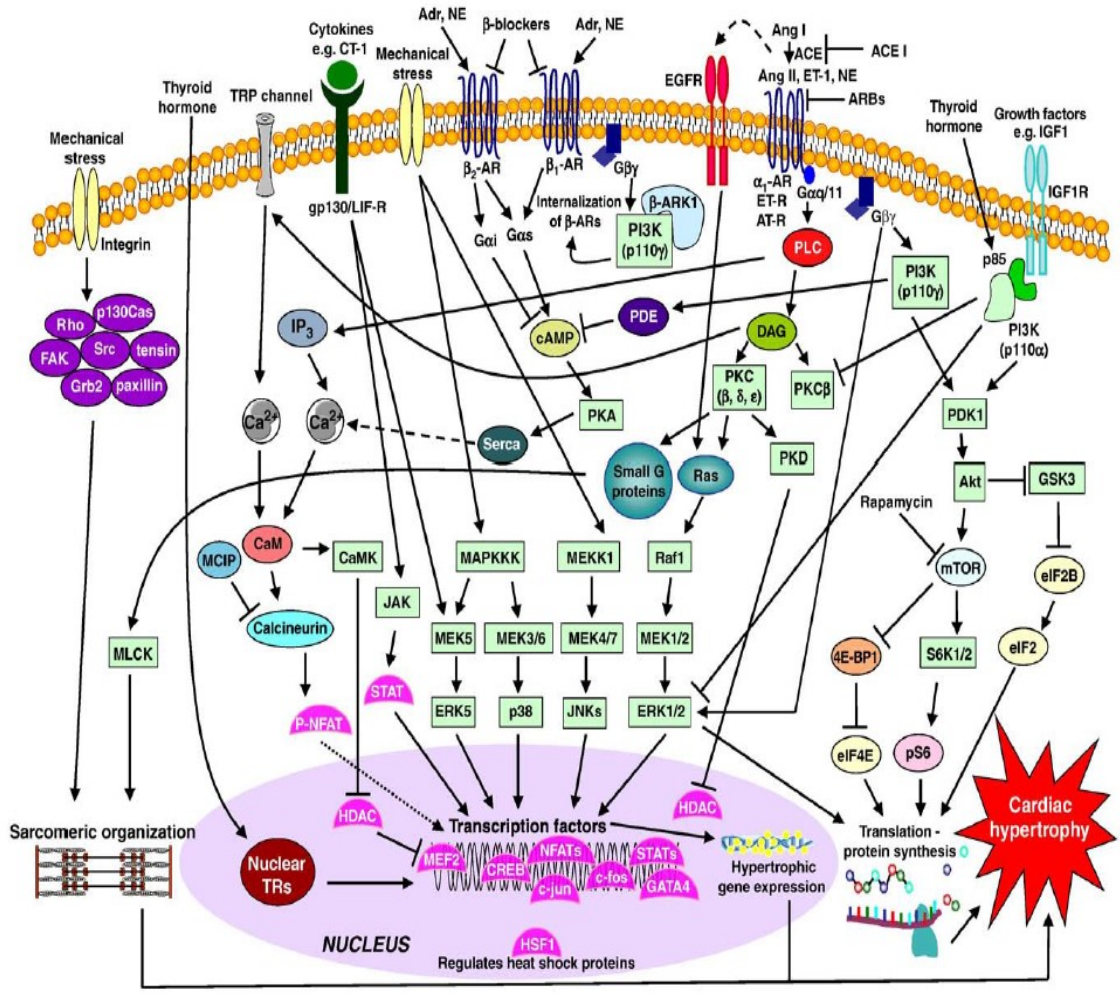

Figure 1.1. Schematic diagram of the possible intracellular signaling pathways involved in cardiac hypertrophy. Numerous signaling pathways have been implicated in the development of cardiac hypertrophy showing a complex and extensive crosstalk between them. However, signaling pathways such as G protein-coupled receptors (GPCRs), mitogen-activated protein kinases (MAPKs), calcineurin–nuclear factor of activated T cells (NFAT), NF-κB and phosphatidylinositol 3-kinase (PI3K)–Akt are increasingly recognized as major signaling pathways that play a pivotal role in the development of cardiac hypertrophy. Reprinted from "*Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies*", Bernardo, B. C., K. L. Weeks, L. Pretorius and J. R. McMullen (2010), Pharmacol Ther 128(1): 191-227, with permission from Elsevier.

1.3. Cytochrome P450 (CYP)

CYP is a superfamily of cysteinato-heme enzymes which play an essential role in the metabolism, biotransformation, and detoxification of endogenous compounds such as steroids, vitamins and fatty acids as well as exogenous compounds (xenobiotics) such as drugs, environmental chemicals, pollutants, and natural plant products (Nelson, Kamataki et al. 1993) . CYP enzymes are expressed mainly in the liver and to a lesser extent in other tissues, including the heart, kidney, and lung (Zordoky and El-Kadi 2008).

1.3.1. Expression of CYP in the heart

Many CYP enzymes have been found to be expressed in explanted human hearts and in the left ventricle of Sprague Dawley (SD) rats as well as other species (Table 1.1) (Thum and Borlak 2000, Thum and Borlak 2002, Michaud, Frappier et al. 2010). At the cellular level, the expression of CYP has been reported in cultured cardiomyocytes and in the cardiac derived H9c2, HL-1 cells, and recently in human fetal ventricular cardiomyocytes, RL-14 cell line (Thum and Borlak 2000, Zordoky and El-Kadi 2007, Elshenawy, Anwar-Mohamed et al. 2013, Maayah, Elshenawy et al. 2015).

1.3.2. Classification and nomenclature of CYP

CYPs are classified, according to the homology in their amino acid sequence, into families and sub-families and comprise of more than 6000 enzymes (Guengerich 2003). Family members have more than 40% homology in their amino acid sequence

whereas enzymes with more than 55% homology in their amino acid sequence are categorized in the same subfamily.

Arabic numbers followed by letters are used to designate members of the same family and subfamilies, respectively. An additional Arabic numbers are added to the subfamily to refer to a specific CYP enzyme. Of interest, families of CYP1, CYP2, and CYP3 are involved in hepatic and extrahepatic drug metabolism whereas other families are believed to play a major role in the metabolism of endogenous substances (Danielson 2002, Lewis 2003).

1.3.2.1. CYP1 family

The CYP1 family contains two subfamilies: CYP1A, which mainly includes CYP1A1 and CYP1A2, as well as CYP1B which only comprises CYP1B1 in humans. Unlike CYP1A2, which is expressed predominantly in hepatic tissue, CYP1A1 and CYP1B1 are mainly expressed in extra-hepatic tissues (Danielson 2002). CYP1A1 and CYP1B1 are mainly involved in the metabolism of polycyclic aromatic hydrocarbons (PAHs), whereas CYP1A2 metabolizes many clinically used drugs such as acetaminophen (paracetamol), theophylline, tamoxifen, and warfarin (Shimada, Hayes et al. 1996, Hu, Sorrentino et al. 2007). In addition to its role in PAHs metabolism, CYP1B1 is also involved in the metabolism of steroid hormones, most notably estradiol (Hayes, Spink et al. 1996). The expression of CYP1 family members is regulated primarily by the aryl hydrocarbon receptor (AhR) (Hankinson 2005). CYP1B1 may also be regulated via non AhR-mediated pathways such as cAMP owing to the fact that CYP1BI has been shown to be constitutively expressed in the hearts of AhR-deficient mice (Shimada, Sugie et al. 2003).

Of interest, the expression of CYP1 members has been reported in human and animal hearts (Table 1.1). For instance, CYP1A1 mRNA was detected in both healthy and diseased human hearts (Thum and Borlak 2000, Thum and Borlak 2002). Moreover, CYP1B1 expression has been reported to be the second most abundantly expressed CYP in explanted human hearts (Bieche, Narjoz et al. 2007). Although CYP1A2 is not expressed in the human heart, its expression has been reported in the pig heart (Messina, Chirulli et al. 2008). In rat hearts, the expression of CYP1A1 and CYP1B1 was induced by benzo(a)pyrene (BaP), a component of cigarette smoke (Messina, Chirulli et al. 2008). *In vitro*, the expression of CYP1A1, CYP1A2, and CYP1B1 has been reported in H9c2, HL-1 and RL-14 cells (Zordoky and El-Kadi 2007, Elshenawy, Anwar-Mohamed et al. 2013, Maayah, Elshenawy et al. 2015). AhR is also reported to be highly expressed in the heart (Korashy and El-Kadi 2006).

1.3.2.2. CYP2 family

The CYP2 family is the largest family of CYP and contains several subfamilies such as CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP2J (Lewis 2004). CYP2A subfamily members CYP2A6, CYP2A7, and CYP2A13, are mainly expressed in the human liver, kidney, and lung (Su and Ding 2004). CYP2A6 metabolizes several pharmaceuticals and toxic compounds whereas little is known about CYP2A7 and CYP2A13 (Su and Ding 2004). Members of the CYP2B subfamily are expressed at very low constitutive levels compared to other CYPs and are mainly expressed in the liver, especially in the presence of inducers such as phenobarbital. Although CYP2B6 accounts for less than 1 % of the hepatic content of CYPs in humans, it metabolizes a diverse class of drugs such as anti-tumours

(e.g. cyclophosphamide), antidepressants (e.g bupropion), and monoamine oxidase inhibitors (e.g. selegiline) (Danielson 2002). The CYP2C subfamily is considered to be the second most pharmacologically important subfamily after CYP3A due to its ability to metabolize many clinical drugs such as warfarin, omeprazole, and paclitaxel (Goldstein and de Morais 1994). In addition, CYP2Cs are involved in the metabolism of endogenous substances such as arachidonic acid and retinoic acids (Rifkind, Lee et al. 1995, McSorley and Daly 2000). Accordingly, CYP2Cs are widely expressed in a variety of tissues including liver, kidney, heart, coronary artery, adrenal gland, brain and lung (Goldstein and de Morais 1994). CYP2D6, on the other hand, constitutes only 2% of the total hepatic CYP content, but it metabolizes over 50 clinically used drugs, especially those acting on the cardiovascular and central nervous systems (Danielson 2002). CYP2E1, the only member of the CYP2E subfamily in humans, was originally believed to be responsible only for ethanol metabolism (Lieber and DeCarli 1968). However, further studies have revealed that CYP2E1 is involved in the metabolism of almost 80 xenobiotics as well as endogenous fatty acids (Hakkak, Korourian et al. 1996). CYP2E1 metabolism of protoxicants such as benzene and acetaminophen is associated with an increase in oxidative stress and depletion of cellular glutathione, which ultimately causes damage to cells expressing this enzyme (Lieber 1997). Unlike CYP2E1, CYP2J2 is mainly involved in the metabolism of arachidonic acid in extrahepatic tissues, where it is mainly expressed (Lieber 1997).

Of interest, the expression of several members of the CYP2 family has been identified in the heart of humans and rodents (Table 1.1). For instance, CYP2A6/7,

CYP2B6/7, CYP2C8/9/19, CYP2D6, and CYP2E1 were detected in human hearts and CYP2J2 is considered to be the most highly and constitutively expressed CYP in the heart (Thum and Borlak 2000, Bieche, Narjoz et al. 2007, Delozier, Kissling et al. 2007). While the expression of CYP2E1 was detected in different parts of the heart, CYP2B6/7, CYP2D6, and CYP2C8/9/19 were detected mainly in the right ventricle (Thum and Borlak 2000). In rats, the expression of CYP2A1/2, CYP2B1/2, CYP2C11/23, CYP2D1/5, CYP2E1, and CYP2J3 was reported in the heart (Thum and Borlak 2002, Imaoka, Hashizume et al. 2005). Of note, the expression of several members of CYP2A, CYP2C, CYP2E, CYP2J subfamilies has been reported to be altered in hypertrophied and failing hearts both in humans and rats (Imaoka, Hashizume et al. 2005, Zordoky and El-Kadi 2008).

1.3.2.3. CYP3 family

Members of the CYP3A subfamily are the most abundant CYP enzymes in the hepatic tissue. They are of major importance because they mediate the biotransformation of more than 30% of clinically used drugs such as benzodiazepines, statins, macrolide antibiotics, dihydropyridine calcium channel blockers and antidepressants (Anzenbacher and Anzenbacherova 2001). Their expression has been mainly detected in hepatic and intestinal tissues. Of interest, the CYP3 family members are not detected in the heart of humans or rats (Thum and Borlak 2002). However, some reports have detected CYP3A mRNA and protein in isolated cardiomyocytes and in the pilot whale's heart tissue, respectively (Celander, Moore et al. 2000, Thum and Borlak 2000) .

1.3.2.4. CYP4 family

The mammalian CYP4 family consists of six subfamilies, namely CYP4A, CYP4B, CYP4F, CYP4V, CYP4X and CYP4Z. Among these subfamilies, the CYP4A and CYP4F enzymes are mainly involved in the hydroxylation of endogenous fatty acids whereas CYP4B enzymes are associated with the bioactivation of some xenobiotics and little is known about other members (Okita and Okita 2001, Danielson 2002, Chaudhary, Batchu et al. 2009). CYP4 family members are expressed mainly in the kidney and to a lesser extent in the hepatic tissue (Anwar-mohamed, Zordoky et al. 2010, Theken, Deng et al. 2011).

Of interest, the expression of CYP4A and CYP4F enzymes has been detected in human, canine and rodent heart tissues (Table 1.1) (Thum and Borlak 2000, Nithipatikom, Gross et al. 2004, Zordoky, Aboutabl et al. 2008, Theken, Deng et al. 2011). Moreover, their expression has been identified at *in vitro* level, in freshly isolated rat cardiomyocyte, H9c2 , HL-1, and RL-14 cells (Thum and Borlak 2000, Elshenawy, Anwar-Mohamed et al. 2013, Tse, Aboutabl et al. 2013, Maayah, Elshenawy et al. 2015).

1.3.2.5. Other CYP families

Similar to the CYP4 family, other CYP families are mainly involved in the metabolism of endogenous substances. For instance, CYP5A1 metabolizes prostaglandin H2 (PGH2) to thromboxane A2 (TxA2), which stimulates platelet aggregation and vasoconstriction (Elbekai and El-Kadi 2006). In contrast to CYP5A1, CYP8A1 metabolizes PGH2 to prostaglandin I2 (PGI2), also known as prostacyclin,

which inhibits platelet aggregation and has a vasodilatory effects (Elbekai and El-Kadi 2006). Members of the CYP7, CYP11, CYP19, and CYP21 families were found to play a role in steroid biosynthesis, whereas CYP27B1 and CYP8B play a role in bile acid synthesis and vitamin D metabolism (Inouye and Sakaki 2001, Danielson 2002, Elbekai and El-Kadi 2006). A few members of these CYP families such as CYP11A and CYP11B have been reported to be expressed in heart tissue (Silvestre, Robert et al. 1998, Young, Clyne et al. 2001).

1.3.3. Modulation of CYP during cardiac hypertrophy and HF

The expression of different CYP enzymes has been reported to be altered during the onset and the progression of different CVDs including cardiac hypertrophy and HF, suggesting an important role of these enzymes in cardiovascular health (Elbekai and El-Kadi 2006, Zordoky and El-Kadi 2008). For instance, the expression of CYP1B1, CYP2J2, CYP2F2, and CYP4A10 has been reported to be increased in failing human hearts (Tan, Moravec et al. 2002, Elbekai and El-Kadi 2006). Moreover, the expression of CYP11B1 and CYP11B2 was identified in failing human hearts, but not in normal hearts (Young, Clyne et al. 2001). In addition, CYP2A6/7 and CYP4A11 were found to be upregulated in hypertrophied human hearts (Thum and Borlak 2002). On the other hand, several animal studies have revealed a significant upregulation of CYP1B1, CYP2A1/2, CYP2B1/2, CYP2E1, and CYP2J3 gene expression in spontaneously hypertensive rats (SHRs) (Imaoka, Hashizume et al. 2005).

1.4. Arachidonic acid metabolism

Arachidonic acid (AA) is a nonessential polyunsaturated fatty acid that is released from its phospholipid storage depot by the action of intracellular phospholipases in response to different stimuli (Buczynski, Dumlao et al. 2009). It has long been recognized that AA is metabolized only by cyclooxygenases (COXs) and lipoxygenases (LOXs). This was until 1981 when CYP monooxygenases emerged as a third enzymes involved in AA metabolism (Fig 1.2) (Roman 2002). COX metabolizes AA into prostaglandins G2 (PGG2) and PGH2, whereas LOX converts AA to hydroperoxyeicosatetraenoic acids (HPETEs), which are further converted to hydroxyeicosatetraenoic acids (HETEs), leukotrienes, or lipoxins (Buczynski, Dumlao et al. 2009). Although CYP enzymes play a limited role in the generation of LOX products, they play an important role in the metabolism of PGH2 to biologically active metabolites such as PGI2 and TxA2 by the action of CYP8A1 (prostacyclin synthase) and CYP5A1 (thromboxane synthase), respectively (Roman 2002). AA metabolites have been shown to be important regulators in the maintenance of body functions as well as successful therapeutic targets in the control of many pathological conditions including pain, inflammation, asthma, allergies and CVDs (Elbekai and El-Kadi 2006).

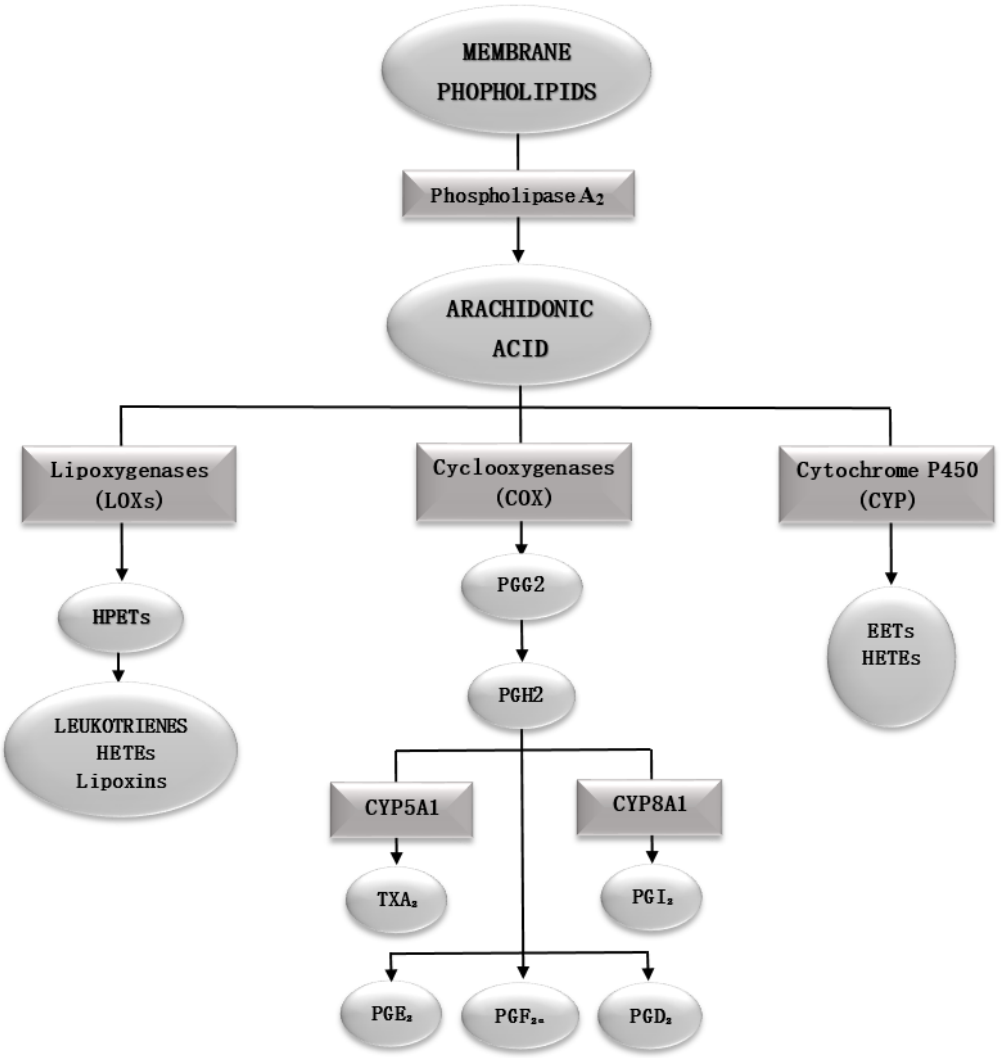

Figure 1.2. Pathways of arachidonic acid metabolism. AA is metabolized by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP). COX metabolizes AA to PGH_2 which is further metabolized by CYP5A1 and CYP8A1 to TxA_2 and PGI_2, respectively. LOX metabolizes AA to HPETEs and subsequently to HETEs, leukotrienes or lipoxins. CYP metabolizes AA to EETs or HETEs.

1.4.1. CYP-mediated arachidonic acid metabolism

In the presence of NADPH and oxygen, free AA can be metabolized by CYP epoxygenases and hydroxylases to epoxyeicosatrienoic acids (EETs) and HETEs, respectively (Fig. 1.3) (Roman 2002).

Four regioisomeric EET metabolites (5,6-, 8,9-, 11,12-, 14,15-EET) are produced by the epoxidation of olefin bonds present on the 5-, 8-, 11-, or 14-positions in AA, by CYP epoxygenases. Enzymes of the CYP subfamilies CYP1A, CYP2B, CYP2C, CYP2E, and CYP2J were reported to produce EETs in various tissues and species (Table 1.1) (Zeldin 2001). However, CYP2Cs and CYP2Js are considered the predominant CYP epoxygenases, especially in cardiovascular tissues (Kroetz and Zeldin 2002, Jenkins, Cedars et al. 2009). EETs can be further hydrolyzed by soluble epoxide hydrolase (sEH) to the corresponding hydrated products 5,6-, 8,9-, 11,12-, 14,15-DHET. In addition, EETs can also undergo β-oxidation or chain elongation to produce 16- or 22-carbon derivatives, respectively (Spector and Norris 2007). Moreover, 5,6-EET and 8,9-EET are further metabolized by COXs to the corresponding 5-hydroxy-PGs and 11-hydroxy-8,9-EET metabolites, respectively (Zhang, Prakash et al. 1992, Carroll, Balazy et al. 1993). The majority of synthesized EETs are incorporated back into membrane phospholipids, thus prolonging their cellular effects (Kaspera and Totah 2009).

On the other hand, regioisomers of HETE can be produced by the hydroxylation activity of different CYPs, such as CYP1A1, CYP1B1, CYP2E1, and CYP4s on AA (Table 1.1) (Elbekai and El-Kadi 2006). The most well characterized of these regioisomers is 20-HETE which can be produced by inserting a hydroxyl

group at the terminal carbon of AA by the action of CYP ω-hydroxylases, mainly CYP4A and CYP4F enzymes (Pat Kunert and Drenjancevic 2011). In humans, several CYP isoforms such as CYP4A11/22 and CYP4F2/3 have been reported to produce 20-HETE whereas CYP4F2 is considered the predominant 20-HETE synthesizing enzyme (Hoopes, Garcia et al. 2015). Similar to EETs, 20-HETE can undergo β-oxidation to 16- or 18- derivatives (Roman 2002), or metabolism by COX to form 20-hydroxy-prostaglandin G2 and H2 (Schwartzman, Falck et al. 1989).

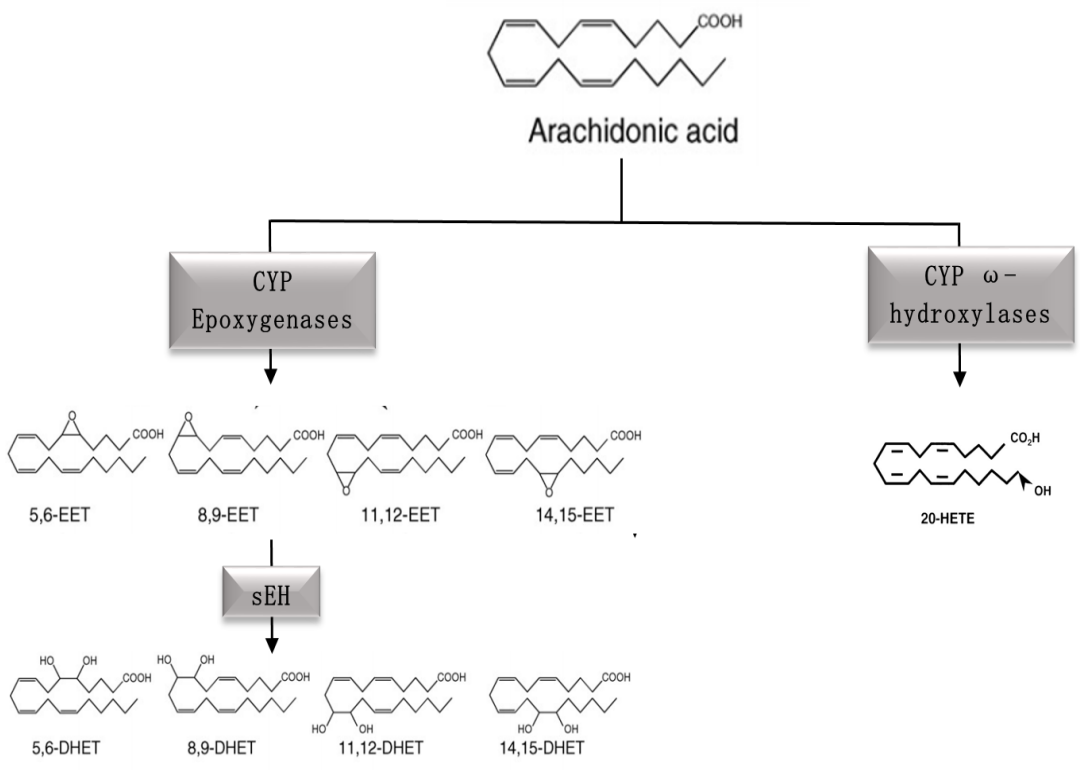

Figure 1.3. Arachidonic acid metabolic pathways by CYPs enzymes. CYP epoxygenases catalyze the epoxidation of AA to form the four regioisomers of EETs (5,6-, 8,9-, 11,12-, 14,15-EET). EETs are further metabolized by sEH to form the corresponding hydrated DHETs (5,6-, 8,9-, 11,12-, 14,15-DHET). On the other hand, CYP ω-hydroxylases catalyze the hydroxylation of AA at the ω-terminal to form 20-HETE.

Table 1.1. The expression of CYP in the heart of humans and rats

Family	Subfamily	Human	Rat	AA metabolites produced	References
CYP1	A	1A1/2	1A1	EETs, HETEs	(Thum and Borlak 2000, Thum and Borlak 2000, Roman 2002, Elbekai and El-Kadi 2006, Maayah, Elshenawy et al. 2015)
	B	1B1	1B1	EETs, HETEs	(Roman 2002, Choudhary, Jansson et al. 2003, Elbekai and El-Kadi 2006, Zordoky, Aboutabl et al. 2008)
CYP2	A	2A6/7	2A1/2	EETs	(Roman 2002, Thum and Borlak 2002)
	B	2B6/7	2B1/2	EETs	(Thum and Borlak 2000, Roman 2002, Thum and Borlak 2002, Elbekai and El-Kadi 2006, Zordoky, Aboutabl et al. 2008)
	C	2C8/9/19	2C11/13/23	EETs	(Thum and Borlak 2000, Roman 2002, Thum and Borlak 2002, Elbekai and El-Kadi 2006, Delozier, Kissling et al. 2007, Zordoky, Aboutabl et al. 2008, Aboutabl, Zordoky et al. 2009)
	D	2D6	----------	EETs	(Roman 2002, Elbekai and El-Kadi 2006, Bieche, Narjoz et al. 2007)
	E	2E1	2E1	EETs, 19-HETE	(Thum and Borlak 2000, Thum and Borlak 2000, Roman 2002, Elbekai and El-

					Kadi 2006, Michaud, Frappier et al. 2010)
	J	2J2	2J3/4	EETs	(Wu, Moomaw et al. 1996, Roman 2002, Elbekai and El-Kadi 2006, Delozier, Kissling et al. 2007)
CYP4	A	4A11	4A1/2/3	19-, 20-HETE	(Roman 2002, Thum and Borlak 2002, Elbekai and El-Kadi 2006, Zordoky, Aboutabl et al. 2008, Aboutabl, Zordoky et al. 2009, Michaud, Frappier et al. 2010)
	B	4B1	----------	----------	(Thum and Borlak 2000, Elbekai and El-Kadi 2006)
	F	4F2/11/12	4F4/5/6	20-HETE	(Cui, Nelson et al. 2000, Roman 2002, Elbekai and El-Kadi 2006, Zordoky, Aboutabl et al. 2008, Aboutabl, Zordoky et al. 2009)

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

1.5.1. Epoxyeicosatrienoic acids (EETs)

The interest in EETs greatly increased when they were defined as Endothelium-Derived Hyperpolarizing Factors (EDHFs) in 1996 (Campbell, Gebremedhin et al. 1996). From then on, EETs were shown to play an important role in modulating different pathophysiological conditions such as inflammation, hypertension, cardiac hypertrophy, ischemic heart diseases and atherosclerosis (Imig 2012).

EETs possess a potent vasodilator effect mediated by their ability to activate the large-conductance calcium-activated potassium channels (K_{ca}) and the transient receptor potential (TRPV4) calcium channels on vascular smooth muscle cells (VSMCs), causing membrane hyperpolarization and vasodilation. (Imig 2012). This vasodilatory response has been observed with all EET regioisomers in numerous organs, highlighting their anti-hypertensive effect. The most extensively studied effect of EETs is their vasodilator effect on the coronary arteries. CYP2Cs are considered the major contributors to EET production in these vessels (Roman 2002). In most of these studies, EETs have a potent vasodilator effect on coronary arteries (Buczynski, Dumlao et al. 2009). However, in renal vasculature, the vasodilator effect of EETs is believed to be regioisomer-dependent because 8.9-,11,12-, and 14,15-EET were able to increase afferent arteriole diameter, whereas 5,6-EET caused vasoconstriction (Imig, Navar et al. 1996). The vasoconstriction effect of 5,6-EET was found to be dependent on COX metabolism (Imig, Navar et al. 1996). In

addition, EETs were reported to be produced in cerebral vascular tissues and astrocytes (Alkayed, Birks et al. 1996, Alkayed, Birks et al. 1997, Munzenmaier and Harder 2000, Imig 2012). Upon astrocyte stimulation by neurotransmitters such as glutamate, EETs are released and act on the VSMC to regulate cerebral blood flow in localized brain regions (Harder, Alkayed et al. 1998). In the pulmonary circulation, the vasodilation caused by EETs is still controversial. In this regard, it has been reported that exogenous administration of EET constricts rabbit pulmonary arteries, and augments pulmonary artery pressure in a murine model of acute hypoxia-induced pulmonary vasoconstriction (Zhu, Bousamra et al. 2000, Keseru, Barbosa-Sicard et al. 2008). However, increasing EET levels was reported to prevent monocrotaline-induced pulmonary hypertension in rats (Revermann, Barbosa-Sicard et al. 2009).

In addition to their vasodilatory effect, increasing evidence suggest that EETs play an important role in vascular homeostasis through their anti-inflammatory (Node, Huo et al. 1999), mitogenic (Roman 2002), antiaggregatory (Fitzpatrick, Ennis et al. 1986), fibrinolytic, and angiogenic actions (Imig 2012). The anti-inflammatory properties of EETs were first identified by Node et al in 1999 (Node, Huo et al. 1999). They demonstrated that EETs were able to decrease cytokine-induced endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) and prevent leukocyte adhesion to vascular walls through a mechanism involving the inhibition of NF-kB (Node, Huo et al. 1999). In addition to their ability to inhibit NF-kB, EETs were also reported to activate the tyrosine kinase and MAPK signaling pathways, which have been implicated in inflammation (Roman 2002). Studies on endothelial and vascular

smooth muscle cells suggest that EETs stimulate angiogenesis through their actions on proliferation and migration (Spector and Norris 2007). All EETs were found to stimulate proliferation of endothelial cells (Fleming 2007). Moreover, EETs were reported to mediate the proliferative effect of vascular endothelial growth factor (VEGF) (Webler, Michaelis et al. 2008). These actions of EETs are believed to be mediated through a complex signal transduction mechanism including PI3K/Akt, MAPK, and cAMP/PKA signaling pathways (Spector and Norris 2007). In addition, EETs display potential antithrombotic and fibrinolytic properties through the induction of tissue-type plasminogen activator (tPA) gene expression, inhibition of platelet aggregation, and inhibition of platelet adhesion to endothelial cells (Fitzpatrick, Ennis et al. 1986, Heizer, McKinney et al. 1991, Zhu, Schieber et al. 1995, Node, Ruan et al. 2001, Krotz, Riexinger et al. 2004).

Several studies have highlighted the cardioprotective properties of EETs in ischemic heart diseases, drug induced-cardiotoxicity, HTN, cardiac hypertrophy and HF (Revermann, Barbosa-Sicard et al. 2009). For instance, exogenous administration of EET or CYP2J2 overexpression was found to decrease the infarct size and protect against ischemia reperfusion injury induced in different animal models (Seubert, Yang et al. 2004, Li, Liu et al. 2009, Zhang, El-Sikhry et al. 2009). Moreover, EET treatment was shown to have protective effects against the cellular hypertrophy induced by Ang II and isoproterenol in primary neonatal cardiomyocytes (Xu, Li et al. 2006) and the H9c2 cell line (Tse, Aboutabl et al. 2013), respectively. Recently, CYP2J2 overexpression or EET treatment was shown to protect against cardiac dysfunction, hypertrophy, and fibrosis in response to Ang II treatment (He,

Zhang et al. 2015). In a similar manner, CYP2J2 overexpression was shown to confer cardioprotection against doxorubicin-induced cardiotoxicity (Zhang, El-Sikhry et al. 2009). Moreover, it has been recently reported that cardiac specific overexpression of CYP2J2 provides cardioprotection against HF induced by Ang II and isoproterenol (Wang, Ni et al. 2014). Additionally, overexpression of CYP2J2 was shown to protect against electrical remodelling induced in response to pathological hypertrophy (Westphal, Spallek et al. 2013). Several signaling pathways have been suggested to be involved in the cardioprotective effect of EETs, such as NF-κB, ATP-sensitive potassium channels, ERK1/2 MAPK pathway, and PKA-dependent signaling pathways (Seubert, Yang et al. 2004, Lu, Ye et al. 2006, Batchu, Law et al. 2009).

EETs are important regulators in cardiac contractility and ischemic injury through their actions on cardiac myocytes and cell membrane channels. In this regard, 5,6-EET and 11,12-EET, but not 8,9-EET and 14,15-EET, were able to increase intracellular calcium and cell shortening in cardiac cells (Moffat, Ward et al. 1993). EETs inhibit cardiac sodium channels, whereas they activate the ATP-sensitive potassium channels (KATP) and L-type calcium channels (Xiao, Huang et al. 1998, Lee, Lu et al. 1999, Lu, Ye et al. 2006).

EETs were also demonstrated to participate in the long-term control of blood pressure through their action on the kidney. In this regard, it has been reported that EETs regulate glomerular filtration by activating the Na^+/H^+ exchanger (Harris, Munger et al. 1990). In addition, EET was shown to inhibit sodium reabsorption in

renal tubules and to mediate pressure-natriuresis (Roman 2002, Dos Santos, Dahly-Vernon et al. 2004).

EETs were shown to have cytoprotective effects through their anti-apoptotic and antioxidant properties (Imig 2012). EET treatment has been reported to protect neonatal rat cardiomyocytes that were exposed to either hypoxia and reoxygenation (H/R) injury or TNF-α treatment through the activation of multiple antiapoptotic targets (Dhanasekaran, Gruenloh et al. 2008, Zhao, Wang et al. 2012). Similarly, overexpression of CYP2J2 was shown to attenuate doxorubicin-induced cardiomyocyte apoptosis in mice (Zhang, El-Sikhry et al. 2009) . Recently, treatment with EETs was shown to abolish the induced endoplasmic stress and apoptotic response in cardiomyocytes mediated by Ang II or isoproterenol (Wang, Ni et al. 2014). The cytoprotective effects of EETs were mediated by their ability to modulate PI3K/Akt, MAPK, and ROCK signaling pathways (Dhanasekaran, Al-Saghir et al. 2006, Ma, Zhang et al. 2010).

1.5.2. 20-Hydroxyeicosatetraenoic acid (20-HETE)

In contrast to EETs, 20-HETE has been reported as a potent vasoconstrictor in coronary, renal, cerebral, pulmonary, mesenteric and skeletal muscle circulation (Imig, Navar et al. 1996, Alonso-Galicia, Falck et al. 1999, Gebremedhin, Lange et al. 2000, Kunert, Roman et al. 2001, Wang, Zhang et al. 2001, Hoopes, Garcia et al. 2015). The vasoconstrictor effect of 20-HETE is believed to be mediated by the blocking of Kca, thus activating voltage-gated calcium channels as a consequence of the increase in intracellular potassium levels (Harder, Gebremedhin et al. 1994, Imig, Zou et al. 1996, Hoopes, Garcia et al. 2015). Other signaling pathways such

as PKC, Rho kinase and MAPK were also reported to mediate the vasoconstrictor effect of 20-HETE (Muthalif, Benter et al. 1998, Obara, Koide et al. 2002, Randriamboavonjy, Busse et al. 2003). Similar to EETs, the effect of 20-HETE on pulmonary circulation was opposite to those seen in the rest of the body. In this regard, 20-HETE was reported to dilate pulmonary arteries in humans and rabbits (Roman 2002). Of note, biologically vasoactive molecules such as Ang II and endothelin-1 were found to increase 20-HETE biosynthesis in VSMCs (Hoopes, Garcia et al. 2015). In fact, 20-HETE was reported to mediate the vasoconstrictor and mitogenic effects of Ang II whereas inhibition of 20-HETE synthesis was shown to attenuate Ang II-induced hypertension in rats (Muthalif, Benter et al. 1998, Alonso-Galicia, Maier et al. 2002, Chabova, Kramer et al. 2007). In addition, higher levels of 20-HETE were observed in animal models of HTN such as SHR and androgen-induced hypertension (Dunn, Renic et al. 2008, Wu, Cheng et al. 2011).

20-HETE was shown to activate NF-κB as well as upregulate the expression of adhesion molecules and proinflammatory cytokines, which promote endothelial activation and inflammation (Ishizuka, Cheng et al. 2008, Hoopes, Garcia et al. 2015).

In addition to its detrimental actions in the pathogenesis of HTN and inflammation, several studies have established a pivotal role of 20-HETE in cardiac hypertrophy and ischemia/reperfusion (I/R) injury. 20-HETE formation was higher in microsomal fractions isolated from hypertrophied hearts than those isolated from control hearts (Zordoky, Aboutabl et al. 2008). Moreover, inhibition of 20-HETE biosynthesis has been shown to protect against benzo(a)pyrene-induced cardiac hypertrophy (Aboutabl, Zordoky et al. 2009). Similarly, 20-HETE levels were

increased in diabetic patients with severe cardiac ischemia. On the other hand, inhibition of 20-HETE synthesis was associated with enhanced cardiac function following I/R injury in diabetic rats (Yousif, Benter et al. 2009). In another study, inhibition of 20-HETE synthesis was shown to attenuate cardiomyocyte apoptosis in rats subjected to I/R injury through a MAPK-mediated mechanism (Lv, Wan et al. 2008). Recently, 20-HETE was shown to promote apoptosis and further mediate the apoptotic response to Ang II treatment in cardiomyocytes (Zhao, Qi et al. 2015).

Nonetheless, several beneficial effects of 20-HETE have been documented. For instance, 20-HETE was shown to exert antihypertensive effects by inhibiting sodium reabsorption in the kidney (Fleming 2005). In this regard, 20-HETE was shown to inhibit the activity of Na^+-K^+-ATPase in the proximal tubules and to block the 70-pS K^+ channel in the apical membrane of the thick ascending loop of Henle (TALH) (Schwartzman, Ferreri et al. 1985, Quigley, Baum et al. 2000, Roman 2002). In addition, 20-HETE was found to inhibit platelet aggregation as well as thromboxane biosynthesis induced by AA in human platelets (Hill, Fitzpatrick et al. 1992).

1.6. Soluble epoxide hydrolase (sEH) as a therapeutic target

The beneficial effects of EETs are limited by their metabolism via sEH to the corresponding less biologically active DHETs. Although some beneficial effects have been reported for DHETs (Oltman, Weintraub et al. 1998), most of the studies demonstrated that DHETs have either no activity or little when compared to EETs (Spector and Norris 2007). Therefore, efforts have been made to increase the

biological level of EETs by either the inhibition of sEH or the induction of CYP epoxygenases.

1.6.1. Soluble epoxide hydrolase (sEH)

Soluble epoxide hydrolase (cytosolic epoxide hydrolase in older literature) is a phase I metabolizing enzyme, which participates in the metabolism of xenobiotics as well as endogenously derived fatty acid epoxides. It is encoded by the EPHX2 gene that is localized on chromosome 8p21-p12 in humans, spans approximately 45 kb, and consists of 18 introns and 19 exons, with the first exon being non-coding (Sandberg and Meijer 1996, Newman, Morisseau et al. 2003). EPHX2 is translated into a 555 amino acid and 62.5 kDa antiparallel homodimer protein. Each monomer is comprised of two distinct structural domains, linked by a proline-rich peptide segment. The ~35-kDa C-terminal domain displays epoxide hydrolase activity by acting on epoxy fatty acids, while the 25-kDa N-terminal domain exhibits phosphatase activity by acting on lipid phosphates (Cronin, Mowbray et al. 2003, Newman, Morisseau et al. 2003, Enayetallah, French et al. 2006). The biological role of epoxide hydrolase activity is well defined; however the action of phosphatase activity is not well understood (Morisseau, Schebb et al. 2012).

1.6.2. Expression of soluble epoxide hydrolase

sEH is widely expressed in tissues such as liver, kidney, intestine, brain, lung, heart, and spleen (Newman, Morisseau et al. 2005). Of interest, sEH was found to be expressed in human heart and blood vessels (Enayetallah, French et al. 2004). In rodents, the sEH gene, protein, and activity were detected in the heart of rats and mice (Johansson, Stark et al. 1995, Monti, Fischer et al. 2008, Zordoky, Aboutabl et

al. 2008). *In vitro*, EPHX2 gene expression was identified in H9c2, RI-14, and rat neonatal cardiomyocytes (Ai, Pang et al. 2009, Tse, Aboutabl et al. 2013, Maayah, Elshenawy et al. 2015).

1.6.3. Regulation of soluble epoxide hydrolase

Although the molecular mechanisms of sEH regulation are not well understood, there are several lines of evidence indicating that sEH is regulated by endogenous chemical mediators and xenobiotics (Tanaka, Kamita et al. 2008). For instance, administration of clofibrate, a peroxisome proliferator activated receptor alpha (PPARα) agonist was found to induce sEH in rodents (Pinot, Grant et al. 1995) despite the absence of PPARα-responsive elements in the upstream region of EPHX2 (Tanaka, Kamita et al. 2008). Likewise, higher activity of sEH was detected in the tissue of male mice compared to female mice despite the absence of androgen-responsive elements in the upstream region of EPHX2 (Pinot, Grant et al. 1995, Tanaka, Kamita et al. 2008). Moreover, the activity of sEH was increased in ovariectomized mice, suggesting a regulatory effect of sex hormones on sEH (Pinot, Grant et al. 1995). Cigarette smoke was also shown to induce the expression of EPHX2 in the heart, lung, and endothelial cells of mice and this effect was suggested to contribute to the hypertensive response of blood vessels to smoking (Maresh, Xu et al. 2005). Similarly, the gene expression of EPHX2 in the Jurkat T cell line was upregulated upon gamma-radiation and this effect was suppressed by NF-κB inhibition, which suggests a transcriptional regulatory effect of NF-κB on sEH (Park, Hwang et al. 2002). Age-dependent changes of EPHX2 gene expression has also been reported in mice, suggesting that age may contribute to sEH regulation (Kaur

and Gill 1985). Interestingly, Ang II was reported to upregulate sEH expression at both the mRNA and protein levels in endothelial cells, and this effect was mediated by c-Jun binding to the AP-1 site of the sEH promoter (Ai, Fu et al. 2007). Moreover, DM has been reported to reduce the expression of sEH by inducing ROS (Ai, Fu et al. 2007). The promoter region of human sEH contains several sites for SP-1 transcription factors (Tanaka, Kamita et al. 2008). Binding of transcriptional factor SP-1 to regulatory elements upstream of EPHX2 gene was shown to be essential for EPHX2 gene expression (Harris and Hammock 2013). Recently, homocysteine treatment was shown to upregulate sEH through a mechanism involving unfolded protein response elements (UPREs) and activating transcription factor-6 (ATF-6) (Zhang, Xie et al. 2012).

1.6.4. Soluble epoxide hydrolase inhibitors

The first inhibitors for sEH were epoxide-containing compounds but they only provide a transient inhibition, which makes them inefficient in cell cultures and *in vivo* (Morisseau and Hammock 2005). Subsequent efforts led to the synthesis of 1,3-disubstituted ureas, carbamates, and amides which are more stable and potent competitive inhibitors of sEH (Morisseau, Goodrow et al. 1999). Earlier compounds such as 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) have limited solubility in water and many organic solvents, which makes them difficult to test pharmacologically (Liu, Tsai et al. 2009). Efforts to improve solubility and decrease metabolic liability without reducing the potency of sEH inhibitors led to formation of many compounds such as 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea (AEPU), trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxyl]-benzoic acid (t-

AUCB), 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxyphenyl)-urea (TUPS), and AR9276 (Chiamvimonvat, Ho et al. 2007, Hwang, Tsai et al. 2007). Of interest, TUPS has displayed a high potency on human sEH and appropriate oral bioavailability and pharmacokinetics in a canine model (Hwang, Tsai et al. 2007).

1.6.5. Soluble epoxide hydrolase in CVDs

Higher activity of sEH has been linked to many cardiovascular diseases in humans and animals, which places sEH inhibitors as promising therapeutic agents to protect against CVDs. The antihypertensive effect of sEH inhibitors has been well documented in several animal models (Imig and Hammock 2009). The antihypertensive effect of sEH inhibitors is believed to be mediated by EETs' ability to decrease vascular resistance and to increase renal sodium excretion (Spector, Fang et al. 2004, Imig and Hammock 2009, Wang, Davis et al. 2013). In addition, inhibition of sEH was shown to play a protective role against organ end damage caused by hypertension (Zhao, Yamamoto et al. 2004). In this regard, sEH inhibition was shown to increase the gene expression of the antiapoptotic Bcl-2 family members and the antioxidant Prdx2 (Simpkins, Rudic et al. 2009).

sEH inhibitors have also been reported to provide anti-inflammatory and lipid lowering effects, which suggests protection against vascular atherosclerosis (Inceoglu, Jinks et al. 2006, EnayetAllah, Luria et al. 2008). In fact, sEH inhibition was reported to reduce the atherosclerotic plaques in apolipoprotein (apo) E-deficient mice (Ulu, Davis et al. 2008). Moreover, inhibition of sEH was found to significantly decrease pro-inflammatory lipid mediators and improve survival in lipopolysaccharide (LPS)-induced systemic inflammation in mice and tobacco

smoke-induced inflammation in SHRs (Schmelzer, Kubala et al. 2005, Smith, Pinkerton et al. 2005, Inceoglu, Jinks et al. 2006). Recently, it has been reported that the cardiac inflammatory response induced by Ang II treatment was attenuated by the inhibition of sEH (Li, Li et al. 2014). Interestingly, COX-2 expression was reported to be down regulated by sEH inhibition, which synergized the effect of non-steroidal anti-inflammatory drugs (NSAIDs). Therefore this combination (NSAIDs + sEH inhibitor) was suggested to produce a beneficial anti-inflammatory effect as well as reducing the dose needed of NSAIDs and subsequently their side effects (Schmelzer, Kubala et al. 2005, Schmelzer, Inceoglu et al. 2006).

Several lines of evidence suggested sEH as an important regulator in the myocardial response to I/R injury. In this regard, sEH gene deletion and/or sEH inhibition in mice subjected to myocardial I/R injury resulted in smaller infarct size with improved functional recovery in comparison to control mice (Seubert, Sinal et al. 2006, Motoki, Merkel et al. 2008, Shrestha, Krishnamurthy et al. 2014). Moreover, inhibition of sEH significantly reduced the infarct size after myocardial ischemia-reperfusion injury in canine hearts (Gross, Gauthier et al. 2008). Furthermore, inhibition of sEH in mice showed an improved cardiac function and prevented the progression of cardiac remodeling post myocardial infarction (Li, Liu et al. 2009).

There is mounting evidence suggesting that the inhibition of sEH provides cardioprotective effects against cardiac hypertrophy and HF (Harris and Hammock 2013, Wang, Davis et al. 2013). For instance, sEH inhibition was reported to prevent and reverse cardiac hypertrophy induced by chronic pressure overload (Xu, Li et al. 2006). Moreover, inhibition of sEH has been shown to protect against cardiac

hypertrophy in stroke-prone spontaneous hypertensive, angiotensin-infused, and BaP-treated rats (Li, Carroll et al. 2008, Ai, Pang et al. 2009, Aboutabl, Zordoky et al. 2011). Additionally, sEH inhibition was shown to prevent the development of cardiac arrhythmias that occurs in response to cardiac hypertrophy (Xu, Li et al. 2006). Interestingly, sEH has been identified as a susceptibility factor for HF in a rat model of human disease (Monti, Fischer et al. 2008). In addition, it has been demonstrated that sEH deletion or sEH inhibition conferred cardioprotection and enhanced ventricular ejection fraction in rats with pressure overload-induced HF (Monti, Fischer et al. 2008). Recently, inhibition of sEH has been reported to confer cardioprotection against an established model of chronic heart failure (Merabet, Bellien et al. 2012).

1.7. Fenofibrate in CVDs

Fenofibrate is a third-generation fibric acid derivative that has been used in the treatment of dyslipidemia since 1975. The lipid-modifying properties of fenofibrate are mediated by the activation of nuclear transcription factor PPARα. PPARα forms heterodimers with the retinoic acid X receptors (RXR). Upon activation, this complex translocates to the nucleus and binds to DNA-specific sequences called peroxisome proliferator–response elements (PPRE). This results in the upregulation of different genes that encode lipid-metabolizing enzymes. PPARα is expressed mainly in tissues with high metabolic activity such as the liver, skeletal muscle, heart, and kidney (Kersten, Desvergne et al. 2000). Interestingly, fenofibrate was reported to confer cardioprotective effects, independent of its beneficial actions on lipid homeostasis (Balakumar, Rohilla et al. 2011). For instance, fenofibrate prevented the development of hypertension in stroke-prone spontaneously hypertensive, Ang II-infused, and Dahl salt-sensitive rats (Shatara, Quest et al. 2000, Diep, Benkirane et al. 2004, Vera, Taylor et al. 2005). In addition, fenofibrate attenuated the development of cardiac hypertrophy and fibrosis in pressure-overloaded aortic banded, Ang II- and aldosterone-treated animals (Ogata, Miyauchi et al. 2002, Lebrasseur, Duhaney et al. 2007). The antihypertrophic effect of fenofibrate was also reported in cultured neonatal cardiomyocytes treated with endothelin-1 (Irukayama-Tomobe, Miyauchi et al. 2004). Fenofibrate was shown to enhance ejection fraction and protect against the progression of HF in high salt diet-fed Dahl salt-sensitive rats (Ichihara, Obata et al. 2006). Moreover, fenofibrate exhibits anti-inflammatory effects by increasing the expression of interleukin-10, an anti-inflammatory cytokine, and

protects against myocarditis (Maruyama, Kato et al. 2002). Similarly, fenofibrate has been reported to exhibits anti-apoptotic effects and decrease apoptosis and necrosis in aldosterone-treated cardiomyocytes (De Silva, Wilson et al. 2009). Furthermore, fenofibrate reduced myocardial infarct size and protect against (I/R) myocardial injury in mice (Tabernero, Schoonjans et al. 2002). The cardioprotection of fenofibrate was also reported in mice treated with doxorubicin (Ichihara, Yamada et al. 2007). The use of PPARα knockdown mice revealed the importance of PPARα in the cardioprotection of fenofibrate (Balakumar, Rohilla et al. 2011). Nevertheless, other signaling pathways such as MAPK, GSK-3, NFATc4, and NF-κB were also reported to be implicated in the cardioprotection of fenofibrate (Li, Zheng et al. 2007, De Silva, Wilson et al. 2009, Zou, Le et al. 2013).

1.8. RATIONALE, HYPOTHESES, AND OBJECTIVES

1.8.1. Rationale

Despite the advances that have been made during the past decades in heart research, cardiovascular diseases remain the leading cause of mortality and morbidity worldwide, and account for 40% of all deaths in the world and more than 30% of deaths in Canada alone (O'Connell 2000, Elbekai and El-Kadi 2006). HF affects more than 23 million people worldwide and more than 5 million people in North America (Bui, Horwich et al. 2011). It is estimated that 90% of Canadians have at least one risk factor for HF (P.H.A.C 2009). One of the major risk factors for development of HF is cardiac hypertrophy. Therefore, identifying the molecular mechanisms responsible for the induction of cardiac hypertrophy can be considered as research into the initial steps of HF (Ritter and Neyses 2003).

In our studies we have used an isoproterenol-induced cardiac hypertrophy model. It is a well-known and established model to develop cardiac hypertrophy and HF without increased blood pressure as a sequence of increased heart load. This model is of clinical importance because it mimics human HF in chronic activation of the sympathetic system which is known to play a pivotal role in the pathophysiology of HF. Thus, β-blockers are considered a standard treatment for those patients (Braunwald and Bristow 2000). However, this model causes stronger and more acute cardiac hypertrophy than what would normally be observed in pathophysiological situations which is considered as a disadvantage of this model.

Several CYP families have been identified in the heart and blood vessels of humans and animals. Increasing evidence has revealed the contribution of CYP to

cardiac health and diseases such as hypertension, coronary heart disease, HF, myocardial infarction, stroke, cardiomyopathy, and arrhythmias (Elbekai and El-Kadi 2006). The role of CYP enzymes in cardiovascular health emerges from their ability to metabolize AA to either the cardioprotective metabolites, 5,6-, 8,9-, 11,12- and 14,15- EETs by the action of CYP epoxygenases, mainly the CYP2C and CYP2J subfamilies, or to the cardiotoxic metabolites, 20-HETE by the action of CYP ω-hydroxylases, namely the CYP4 family (Roman 2002). The expression of several CYP enzymes has been reported to be altered during cardiac hypertrophy and HF. However, it has never been investigated whether these changes are causal or epiphenomenal.

EETs are the major cardioprotective products of AA metabolism catalyzed by CYP enzymes. They are potent vasodilators that function as endothelium-derived hyperpolarizing factors, possess anti-inflammatory effects mediated by the inhibition of NF-κB, and affect endothelial and vascular smooth cell proliferation and angiogenesis (Spector, Fang et al. 2004). Once produced, EETs are either incorporated into membrane phospholipid pools, secreted into the extracellular space, or efficiently hydrolyzed by sEH to biologically less active DHETs, thus reducing their beneficial cardiovascular effects (Spector and Norris 2007). The gene encoding sEH, EPHX2, was found to be significantly induced in different models of cardiac hypertrophy (Zordoky, Aboutabl et al. 2008, Aboutabl, Zordoky et al. 2009, Ai, Pang et al. 2009). On the other hand, inhibition of sEH has been reported to confer cardioprotection against cardiac hypertrophy induced in thoracic aortic constriction (TAC), Ang II, and BaP models (Xu, Li et al. 2006, Ai, Pang et al. 2009,

Aboutabl, Zordoky et al. 2011). However, development of cardiac hypertrophy in these models could be secondary to hemodynamic changes (Fyhrquist, Metsarinne et al. 1995, deAlmeida, van Oort et al. 2010, Gentner and Weber 2011). Therefore, the antihypertrophic effects of sEH inhibition seen in these models might be, at least in part, a result of the antihypertensive activities of EET. Thus, investigation the antihypertrophic effects of sEH inhibition on isoproterenol model of cardiac hypertrophy, along with its effect on the accompanying changes in CYPs and their associated AA metabolites, may enhance our understanding of the cardioprotection of EETs

Fenofibrate is a well-known clinical agent that was found to exert beneficial effects on the heart independent of its lipid-lowering action (Delerive, Fruchart et al. 2001). Of interest, fenofibrate was reported to attenuate cardiac hypertrophy *in vivo* and *in vitro* (Chen, Chen et al. 2007, Zou, Le et al. 2013). Interestingly, fenofibrate was reported to increase renal CYP epoxygenases, thus increasing renal EET bioavailability (Muller, Theuer et al. 2004, Huang, Morisseau et al. 2007). However, the effect of fenofibrate on cardiac CYP enzymes and their associated AA metabolites, and whether this effect protects against isoproterenol-induced cardiac hypertrophy, have never been reported.

1.8.2 Hypotheses

1- CYP and AA-derived metabolites play a crucial role in the initiation of cardiac hypertrophy induced by isoproterenol.

2- Inhibition of sEH will protect against isoproterenol-induced cardiac hypertrophy.

3- Fenofibrate will induce CYP epoxygenase enzymes, increase EET bioavailability in the heart, and protect against isoproterenol-induced cardiac hypertrophy.

1.8.3. Objectives

The specific objectives of the present work are:

1- To examine and compare the expression of CYP and sEH prior to and during cardiac hypertrophy induced by isoproterenol in male SD rats and the RL-14 cell line.

2- To examine and compare the CYP-mediated AA metabolism prior to and during cardiac hypertrophy induced by isoproterenol in male SD rats and the RL-14 cell line.

3- To explore the role of CYP epoxygenases, sEH and their associated AA metabolites in the initiation of cellular hypertrophy in the RL-14 cell lines.

4- To examine the protective effect of sEH inhibition by TUPS on isoproterenol-induced cardiac hypertrophy in male SD rats.

5- To examined the effect of TUPS treatment on CYP enzymes and the formation of CYP-mediated AA metabolites.

6- To investigate the effect of fenofibrate on cardiac expression of CYP epoxygenases, sEH and their associated AA metabolites in male SD rats.

7- To investigate the effect of fenofibrate on isoproterenol-induced cardiac hypertrophy in male SD rats.

CHAPTER 2. MATERIALS AND METHODS

2.1. CHEMICALS and MATERIALS

AA, isoproterenol, fenofibrate, nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH), Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12), and anti-goat IgG secondary antibody were purchased from Sigma-Aldrich Chemical Co (St Louis, MO). 100X Antibiotic–antimycotic (10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of amphotericin B, L-glutamine, fetal bovine serum (FBS) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). High-capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA Technologies Incorporation (San Diego, CA) according to previously published sequences. N-(Methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH) and AA metabolite standards (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 20-HETE, and 14(15)-EET-d11) were obtained from Cayman Chemical (Ann Arbor, MI). 1-(1-Methanesulfonylpiperidin- 4-yl)-3-(4 trifluoromethoxy-phenyl)-urea (TUPS) was synthesized by Dr. Paul Jones (University of California, Davis) as described previously (Tsai, Hwang et al. 2010). Acrylamide, N′N′-bismethylene- acrylamide, β-mercaptoethanol, ammonium persulfate, glycine, bromphenol blue, pure nitrocellulose membrane (0.45 mm) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescent Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Reagents used for liquid

chromatographic-electrospray ionization-mass spectrometry (LC-ESI-MS) were at HPLC-grade. Acetonitrile and water (HPLC grade) were purchased from EM Scientific (Gibbstawn, NJ). Goat anti-rat CYP1A1 polyclonal (sc-9828), rabbit anti-rat CYP1B1 polyclonal (sc-32882), mouse anti-rat CYP2B1/2 monoclonal (clone h7), rabbit anti-human/rat CYP2Js polyclonal (sc-67276), mouse anti-rat CYP4A monoclonal (clone E-6), goat anti–mouse/rat sEH polyclonal, rabbit anti-rat actin polyclonal primary antibodies, and anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti–rat CYP2C11 polyclonal (ab3571), rabbit anti–rat CYP2C23 polyclonal (ab53944), and rabbit anti–human/rats CYP4F polyclonal (ab125399)primary antibodies were purchased from Abcam (Cambridge, United Kingdom). Anti-mouse IgG peroxidase secondary antibody was purchased from R&D Systems (Minneapolis, MN). pCMV-CYP2J2 and TurboFectin 8.0 were purchased from OriGene Technologies, Inc. (Rockville, MD). All other chemicals were purchased from Fisher Scientific Co (Toronto, ON, Canada).

2.2. Animals

All studies involving animals followed the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication no. 85-23, revised 1996). All experimental animal procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Seven-weeks old male Sprague–Dawley (SD) rats weighing 200–250 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were maintained on a 12 h light/dark cycle with food and water available ad libitum.

2.3. Treatments

To determine the changes in CYP and their associated AA metabolites prior to and during cardiac hypertrophy induced by isoproterenol, rats were treated with isoproterenol 5 mg/kg per day [intraperitoneal injection (i.p)], dissolved in sterile 0.9% saline (final volume, 0.25 ml/rat) for time periods: 12, 24, 72, 168, and 240 hours. In the corresponding control group, animals received injections of an equivalent volume of vehicle. Thereafter, animals were euthanized under isoflurane anesthesia at the specified time points, and hearts were excised, rinsed, blotted dry, weighed, and then snap-frozen in liquid nitrogen. The frozen hearts were stored at -80 °C until analyzed.

To test the cardioprotective effect of TUPS on cardiac hypertrophy induced by isoproterenol, rats were injected i.p. with isoproterenol (dissolved in sterile 0.9% saline; final volume = 0.25 mL per rat) at 5 mg/kg/day for 7 days, with or without 0.65 mg/kg/ day TUPS (TUPS was dissolved in PEG400, followed by further dilution in sterile 0.9% saline to 1:3 ratio; final volume = 1 mL per rat). The rats were randomly segregated into four groups. The first group (n = 6) consisted of control rats that received sterile 0.9% saline (i.p.) plus 25% PEG400 (oral gavage). The second group (n = 6) consisted of TUPS-treated rats that received TUPS dissolved in 25% PEG (oral gavage) and sterile 0.9% saline (i.p.). The third group (n = 6) consisted of isoproterenol-treated rats that received isoproterenol dissolved in sterile 0.9% saline (i.p.) plus 25% PEG400 (oral gavage). The fourth group (n = 6) consisted of isoproterenol and TUPS-treated rats that received isoproterenol dissolved in sterile 0.9% saline (i.p.) plus TUPS dissolved in 25%PEG (oral gavage). Thereafter,

animals were sacrificed under isoflurane anaesthesia 24 h after their last injection. Heart, kidney and liver were excised, snap-frozen in liquid nitrogen and stored at -80°C until analyzed.

To investigate whether fenofibrate would induce CYP epoxygenases and increase EET bioavalability in the heart, rats were treated daily for 7 days orally with either 1% methylcellulose (n = 6) or fenofibrate dissolved in 1% methylcellulose (30 mg/kg/day) (n = 6). Thereafter, animals were sacrificed under isoflurane anesthesia 24 hours after the last injection. Heart, kidney, and liver were excised, snap-frozen in liquid nitrogen, and stored at -80 °C until analyzed.

To study the effect of fenofibrate on cardiac hypertrophy induced by isoproterenol, mini-osmotic pumps (Alzet 2001, Alzet corp., Palo Alto, CA) were implanted subcutaneously over the dorsal region of the rats, which were under isoflurane anesthesia, to continuously infuse sterile 0.9% saline or isoproterenol (4.2 mg/kg/day) for 7 days, in the presence or absence of fenofibrate (oral gavage, 30 mg/kg/day). Fenofibrate dosing was started 24 hours before isoproterenol administration and continued concurrently thereafter. Weight-matched controls received the vehicles (1% methylcellulose by oral gavage plus sterile 0.9% saline in mini osmotic pump). Thereafter, animals were sacrificed under isoflurane anesthesia 24 hours after the last injection. Hearts were excised, snap-frozen in liquid nitrogen, and stored at -80 °C until analyzed.

The heart and body weights were recorded for each animal and the heart weight to body weight ratio was calculated.

2.4. Evaluation of heart function and wall thickness by echocardiography.

Randomly selected animals from each group (n=4) were anesthetized (isoflurane), and transthoracic M-mode echocardiography with the Vevo 770 (VisualSonics, Toronto, ON, Canada) small-animal imaging ultrasound system was performed to measure cardiac function and wall thickness. Images were retained and analyzed using VisualSonics software version 3.0.0. Left ventricular (LV) dimensions [left ventricular diameter (LVD), LV posterior wall thickness (PWT), and interventricular septum (IVS)] as well as ejection fraction and fractional shortening were determined using M-mode measurements taken from parasternal long- and short-axis views at the midpapillary level. LV dimensions were recorded in diastole. Measurements were averaged from 3 to 6 cardiac cycles according to the American Society of Echocardiography, digitally transferred online to a computer, and subsequently analyzed by Ms. Donna Beker, who was blinded to the treatment groups.

2.5. Cell culture and treatments

Human fetal ventricular cardiomyocytes, RL-14 cells (American Type Culture Collection, Manassas, VA), were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum, 25 mM L-glutamine, and 1X Antibiotic-Antimycotic mixture. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO2 humidified environment. The cells were seeded in 6- and 12-well cell culture plates in DMEM/F12 culture media for mRNA analysis and immunocytochemistry,

respectively. To detect and compare CYP changes prior to and during cellular hypertrophy, RL-14 cells were grown at a density of 1–1.5 × 10^6 cells per well in a 6-well tissue culture plate. On 60–80% confluence (2–3 days), RL-14 cells were incubated with an appropriate stock solution of isoproterenol to reach final concentrations of 100 μM for 1, 3, 6, 12, 24 hours. For the EET study, RL-14 cells were pretreated with vehicle or 11,12- EET (1 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. For the TUPS study, RL-14 cells pretreated with vehicle or TUPS (1 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. For the fenofibrate study, RL-14 cells pretreated with vehicle, fenofibrate (10 μM), or MS-PPOH (5 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. All treatments were performed in serum-free media and the concentrations used in this study were based on previous studies (Dhanasekaran, Gruenloh et al. 2008, Aboutabl, Zordoky et al. 2011, Tse, Aboutabl et al. 2013, Maayah, Elshenawy et al. 2015).

2.6. Measurement of cell viability

The effects of isoproterenol, TUPS , fenofibrate, 11,12-EET, and MS-PPOH on cell viability were determined by measuring the capacity of the reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals as described previously (Korashy and El-Kadi 2004). Twenty-four hours after incubating the cells with increasing concentrations of these agents in a 96-well plate at 37 °C in 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 nm using a Bio-Tek Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT). The percentage of cell viability was calculated relative to the control wells, which were designated as 100% viable cells.

2.7. Cell Transfection

Cells were transiently transfected with pCMV-CYP2J2 (OriGene Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. Briefly, as the density of the cultured cells reached 50–70%, a complex of TurboFectin 8.0 and pCMV-CYP2J2 plasmid was prepared immediately prior to transfection and added to the culture medium. After 48 hours of transfection, the experimental medium was added to the cells, followed by isoproterenol treatment (100 µM) for 6 h.

2.8. Immunocytochemistry

Treated RL-14 cells grown on sterile coverslips were fixed in 4% formaldehyde at room temperature for 10 minutes, washed three times with ice-cold phosphate-buffered saline, and permeabilized with 0.5% Triton X-100 for 10 minutes. Nonspecific binding of the fixed cells was blocked with phosphate-buffered saline containing 2% bovine serum albumin at 37°C overnight. After washing, cytoskeletal F-actin was stained with Texas red phalloidin (1.5 U/ml) (Molecular Probes®) for 30 minutes. Coverslips were mounted using Prolong Gold Antifade with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes®).

2.9. Measurement of imaged cell surface area

Cells were examined using confocal microscopy. The cell were magnified 200 times and measured by imaging the complete boundary of individual cells using Zeiss Axio Vision software (Carl Zeiss Microscopy GmbH, Jena, Germany). At least 30 cells in each group were counted and regarded as an independent experiment; three independent experiments were performed in each condition.

2.10. RNA extraction and cDNA synthesis

Total RNA from frozen hearts and treated cells was isolated using TRIzol reagent according to the manufacturer's protocol, and quantified by measuring the absorbance at 260 nm. Briefly, 0.6 to 1 mL of TRIzol reagent was added to each six-well cell culture plate to lyse cells or 0.2g of tissue. Cell lysates or tissue homogenates were then collected into 1.5 ml Eppendorf tubes and mixed with 160 µl chloroform followed by centrifugation at 12,000 x g for 15 min at 4 °C. The aqueous upper phase which contains the RNA was then transferred to a fresh tube and 400 µl of isopropyl alcohol was added to each tube to precipitate the RNA by cooling the samples at - 20 °C for at least 4 h. Following centrifugation at 12,000 x g for 10 min at 4 °C, the RNA pellet was washed once with 75% ethanol in ultrapure water (DNase/RNase-Free), and then dissolved in ultrapure water (DNase/RNase-Free). RNA was then quantified by measuring the absorbance at 260 nm. RNA purity was determined by measuring the 260/ 280 ratio.

Thereafter, first-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit 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 10X RT buffer, 0.8 µl 25X dNTP mix (100 mM), 2.0 µl 10X RT random primers, 1.0 µl MultiScribe™ reverse transcriptase, and 3.2 µl 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 seconds, and finally cooled to 4 °C.

2.11. 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). Each 25 µl reaction mix contained 0.1 µl of 10 µM forward primer and 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 (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) and are listed in Table 2.1. No template controls were incorporated onto the same plate 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 seconds, and annealing/extension at 60°C for 1 min. Melting curve (dissociation stage) was performed by the end to ascertain the specificity of the primers and the purity of the final PCR product.

2.12. Real time-PCR data analysis

The real-time PCR data were analyzed using the relative gene expression i.e. (2- ΔΔCT) method as described in Applied Biosystems User Bulletin No.2 and explained further by Livak et al (Livak and Schmittgen 2001). Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by the treatment was studied.

Table 2.1. Primer sequences used for real time-PCR reactions

Gene	Forward primer	Reverse primer
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
β-MHC	AGC TCC TAA GTA ATC TGT TTG CCAA	AAA GGATGAGCCTTTCTTTGCT
PROCOLLAGEN I	TATGCTTGATCTGTATCTGCCACAAT	TCGCCCTCCCGTTTTTG
PROCOLLAGEN III	CAGCTGGCCTTCCTCAGACT	TGCTGTTTTTGCAGTGGTATGTAA
TGF-1	ACCTGCAAGACCATCGACATG	CGAGCCTTAGTTTGGACAGGAT
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1A2	GAATGGCTTCTAGTCCCA	TCATCTTCTCACTAAGGGCT
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2B1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGAT C
CYP2B2	CCATCCCTTGATGATCGTACCA	AATTGGGGCAAGATCTGCAAA
CYP2B6	CCGGGGATATGGTGTGATCTT	CCGAAGTCCCTCATAGTGGTC
CYP2C8	CATTACTGACTTCCGTGCTACAT	CTCCTGCACAAATTCGTTTTCC
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP2C19	GGAAAACGGATTTGTGTGGGA	GGTCCTTTGGGTCAATCAGAGA
CYP2C23	CGTCCAATCACACGGTCAAGT	TTCGGGCTCCTGCTCCTT
CYP2J2	GAGCTTAGAGGAACGCATTCAG	GAAATGAGGGTCAAAAGGCTGT
CYP2J3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
CYP2J4	GCTCGGACCTTCATTCCACA	GATCGTGGCTACCAGAGAGC
CYP4A3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATC
CYP4F2	GAGGGTAGTGCCTGTTTGGAT	CAGGAGGATCTCATGGTGTCTT
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
EPHX2	GATTCTCATCAAGTGGCTGAAGAC	GGACACGCCACTGGCTAAAT
RAT β-actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA
HUMAN β-ACTIN	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA
RAT GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

2.13. Preparation of microsomal and cytosolic fractions

Microsomal and cytosolic fractions were prepared from the heart by differential centrifugation of homogenized tissue. Briefly, approximately 1 g of the tissue was washed in ice-cold potassium chloride (1.15% w/v), cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). The homogenate was centrifuged at 10,000g for 20 minutes, and the resulting supernatant was centrifuged again at 100,000g for 60 minutes to obtain the microsomal pellet and cytosolic supernatant. The final pellet was reconstituted in cold sucrose (0.25 M) and stored at -80°C. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry, Rosebrough et al. 1951).

2.13. Western blot analysis

Western blot analysis was performed according to a previously described method (Gharavi and El-Kadi 2005). Briefly, 10-50 µg of the heart microsomal protein from each treatment group was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with primary antibodies against CYP1A1, CYP1B1, CYP2B1/2, CYP2C11, CYP2C23, CYP2J, CYP4A, CYP4F, sEH and actin for at least 2 h. 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 goat anti-rabbit IgG secondary antibody for (CYP1B1, CYP2C11, CYP2C23, CYP2J, CYP4F, and actin), goat anti-mouse IgG secondary antibody for (CYP2B1/2 and CYP4A), or rabbit anti-goat IgG secondary antibody for (CYP1A1 and sEH) was carried out for 1 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity 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/ii.I).

2.14. Arachidonic acid incubation

Heart microsomes (1 mg protein/ml) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.1 M potassium phosphate buffer pH=7.4) at 37°C in a shaking water bath (50 r.p.m). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. Arachidonic acid was added to a final concentration of 50 μM and incubated for 30 minutes. The reaction was terminated by the addition of 600 μl ice-cold acetonitrile followed by the internal standard. *In vitro*, cells were plated in 100-mm petri dishes and treated with isoproterenol, and thereafter incubated with 50 μM AA for 3 hours. AA metabolites were extracted twice by 1 ml ethyl acetate and dried using speed vacuum (Thermo Fisher Scientific, Ottawa, ON, Canada).

2.15. Analysis of arachidonic acid metabolites

Extracted AA metabolites were analyzed using a liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) (Waters Micromass ZQ 4000 spectrometer; Waters, Milford, MA) method as described previously (Nithipatikom, Grall et al. 2001). The mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The nebulizer gas was obtained from an in house high purity nitrogen source. The temperature of the source was set at 150 °C, and the voltages of the capillary and the cone were 3.51 KV and 25 V, respectively. The samples (10 µl) were separated on a reverse phase C18 column (Kromasil, 250 x 3.2 mm) using linear gradient system of water/acetonitrile with 0.005% acetic acid as mobile phase at flow rate of 0.2 ml/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min, and was held there for 5 min. The concentrations of the eicosanoids in the samples were calculated by comparing the ratios of peak heights to their corresponding standards.

2.16. sEH activity assay

sEH activity was measured using the Morisseau and Hammock method with modifications. 14,15-EET was used as the natural substrate (Morisseau and Hammock 2007). Briefly, the cytosolic fraction was diluted with sodium phosphate buffer (0.076 M, pH 7.4) supplemented with bovine serum albumin (2.5 mg/ml) to 0.4 mg/ml. The assay was initiated by the addition of 14,15-EET (final concentration, 2 mg/ml). The mixture was incubated at 37°C for 10 minutes. The reaction was

terminated by the addition of 600 µl ice-cold acetonitrile followed by the internal standard. *In vitro*, RL-14 cells were plated in 100-mm petri dishes and incubated with 5 µM 14,15-EET for 30 minutes, after being treated with isoproterenol for the indicated time periods. 14,15-EET and its corresponding 14,15-DHET were extracted by ethyl acetate twice, dried using speed vacuum, and analyzed using the LC-ESI-MS method as described previously.

2.17. Preparation of nuclear extract

Nuclear extracts from RL-14 cells were prepared according to a previously described procedure with modifications (Andrews and Faller 1991). Briefly, RL-14 cells were grown on 100-mm petri dishes and were pretreated with 11,12-EET for 2 hours, prior to isoproterenol treatment for 6 hours (100 µM). Thereafter, cells were washed twice with cold phosphate-buffered saline, pelleted, and suspended in cold buffer A (10 mM Hepes-KOH, 1.5 mm MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), pH 7.9, at 4°C. After 15 minutes on ice, the cells were centrifuged at 6500g and the pellets were suspended again in high salt concentration cold buffer C (20 mM Hepes-KOH, pH 7.9; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl2; 0.2 mM EDTA; 0.5 mM dithiothreitol; and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice with vigorous agitation for 60 minutes, followed by centrifugation for 10 minutes at 12,000g at 4°C. The nuclear extracts (supernatant) were stored at -80°C till further use.

2.18. Determination of NF-kB-binding activity

The NF-kB Family EZ-TFA Transcription Factor Assay Chemiluminescent Kit (Millipore, Darmstadt, Germany) was used according to the manufacturer's protocol (Bhattacharya, Sarno et al. 2010). Briefly, nuclear extract was incubated with the NFκB Capture Probe, a double stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NFκB (5'-GGGACTTTCC-3'), in assay buffer. The mixture was then transferred to the streptavidin-coated plate. After washing, NFκB transcription factor subunits (p50 and p65) were detected with specific primary antibodies directed against each individual specific target followed by an HRP-conjugated secondary antibody.

2.19. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M). Comparisons between two groups were analyzed using unpaired, two-tailed t-test. Comparisons among more than two groups were analyzed using Krauskal-Wallis one-way analysis of variance on rank. Statistical analysis was performed using SigmaStat for windows, Systat Software Inc. (San Jose, CA). A result was considered statistically significant where $P < 0.05$.

CHAPTER 3. RESULTS

3.1 Modulation of CYP and their associated AA metabolites prior to and during cardiac hypertrophy induced by isoproterenol *in vivo* and *in vitro*

3.1.1 Assessment of cardiac hypertrophy and fibrosis induced by isoproterenol *in vivo*

Hypertrophy initiation was assessed and confirmed by the following methods: first, echocardiography; second, heart weight/body weight ratio (HW/BW); and third, hypertrophic marker gene expression. Echocardiographic examination of isoproterenol-treated rats showed structural changes in ventricular geometry after 72 hours of isoproterenol treatment. Isoproterenol treatment for 72 hours caused significant increases in interventricular septum (IVS) (0.69 ± 0.02 versus 0.59 ± 0.01 cm/kg), posterior wall thickness (PWT) (0.70 ± 0.02 versus 0.58 ± 0.02 cm/kg), IVS/LVD ratio (0.29 ± 0.01 versus 0.25 ± 0.01) along with significant increases in fractional shortening (FS) (59.0 ± 1.4 vs 50.1 ± 2.0) and ejection fraction (EF) (87.5 ± 1.0 vs 79.7 ± 1.8) as compared to controls (Fig 3.1A and Table 3.1). To further assess the early onset of cardiac hypertrophy, heart-to-body weight (HW/BW) ratio was measured. Our results show that isoproterenol significantly increased the HW/BW ratio at 72, 168, and 240 hours of treatment, by 23%, 21%, and 24%, respectively (Fig 3.1B). Measuring the gene expression of the hypertrophic marker atrial natriuretic peptide (ANP) revealed that isoproterenol caused a significant increase in ANP mRNA at 12, 24, 72, 168, and 240 hours of treatment, by 680%, 690%, 1050%, 660%, and 710%, respectively (Fig 3.2A). Furthermore, the early development of cardiac fibrosis associated with isoproterenol-induced cardiac hypertrophy was assessed by measuring the gene expression of fibrotic markers

procollagen I and procollagen III relative to control. Isoproterenol treatment caused a significant induction of procollagen I, by 250%, 270%, and 320% at 72, 168, and 240 hours of treatment, respectively, and caused a significant induction of procollagen III, by 190%, and 130%, and 150% at 72, 168, and 240 hours of treatment, respectively (Fig. 3.2B).

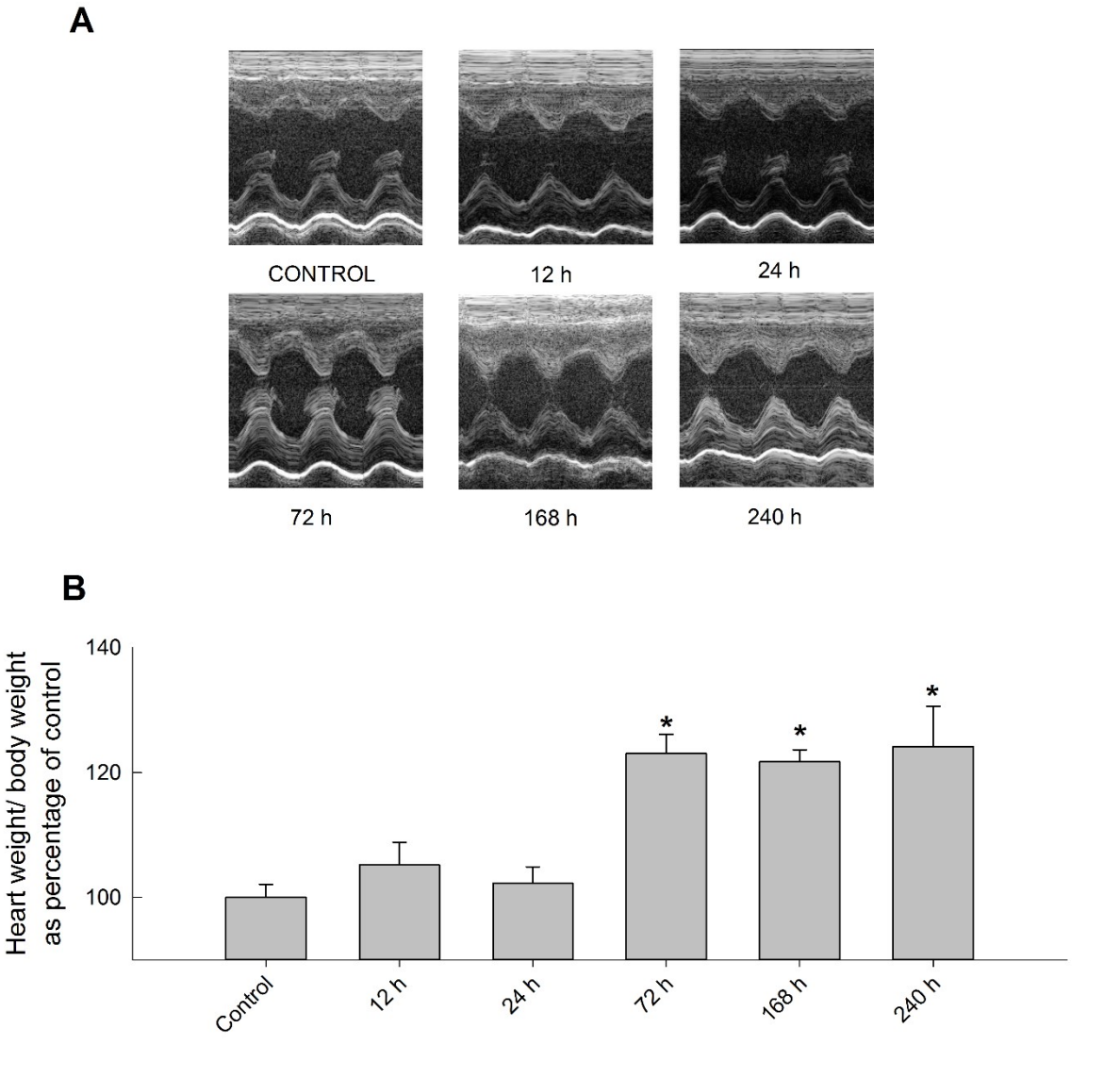

Fig 3.1. Assessment of cardiac hypertrophy induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. (A) Representative short axis M-mode echocardiography images. (B) HW/BW ratio (in milligrams per gram) was determined for each animal after isoproterenol or vehicle treatment. The results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

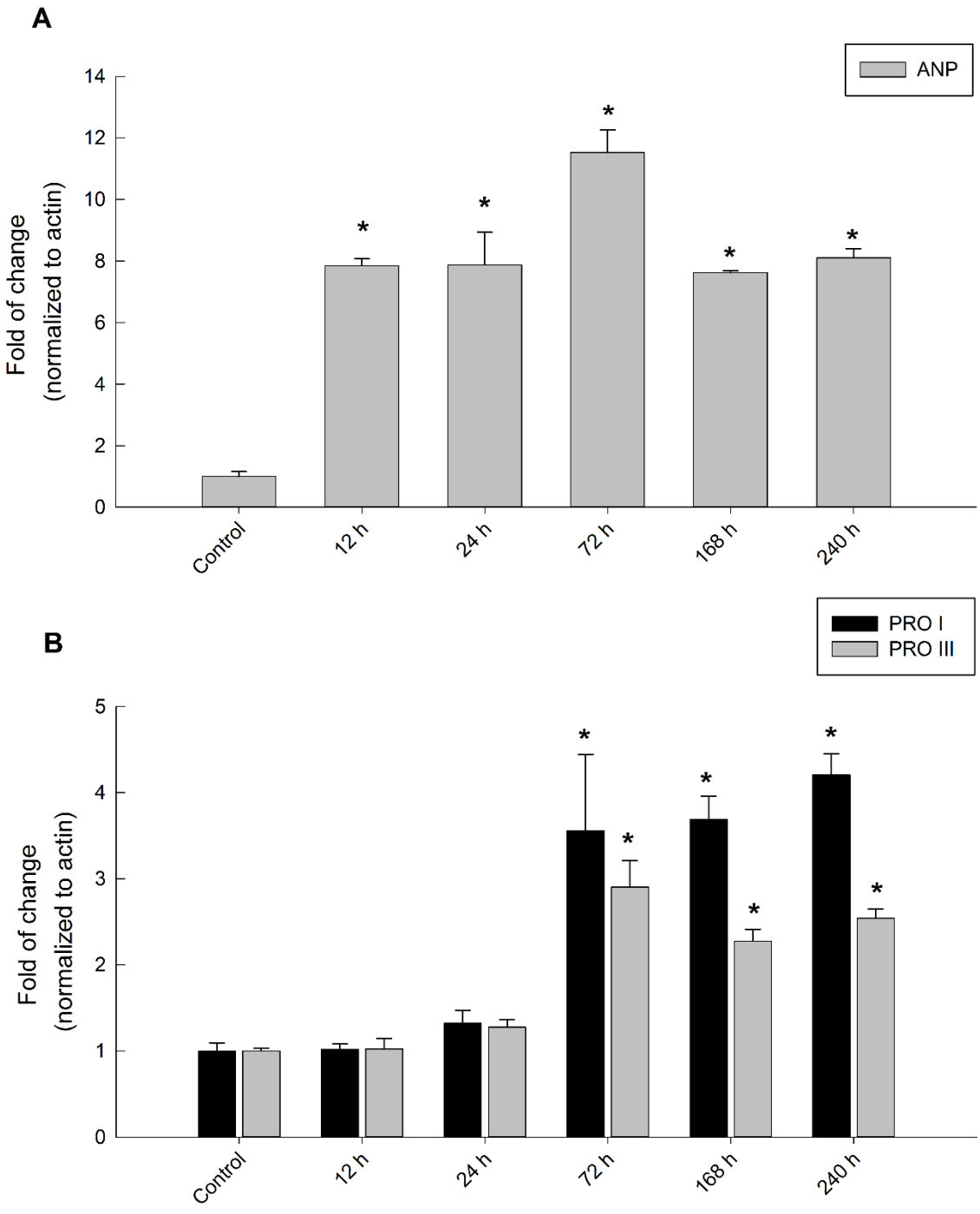

Fig 3.2. Assessment of cardiac hypertrophy and fibrosis induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. Total RNA was isolated from hearts of control and isoproterenol-treated rats; then gene expression of (A) the hypertrophy marker ANP and (B) the fibrotic markers procollagen I (PRO I) and procollagen III (PRO III) was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

Table 3.1. Echocardiographic parameters.

	Control	12 h	24 h	72 h	168 h	240 h
LVD/BW, cm/kg	2.36 ± 0.03	2.36 ± 0.12	2.37 ± 0.08	2.35 ± 0.05	2.20 ± 0.08	2.44 ± 0.10
IVS/BW, cm/kg	0.59 ± 0.01	0.65 ± 0.03	0.64 ± 0.02	0.69 ± 0.02*	0.67 ± 0.02*	0.74 ± 0.04*
PWT/BW, cm/kg	0.58 ± 0.02	0.65 ± 0.05	0.61 ± 0.02	0.70 ± 0.02*	0.68 ± 0.02*	0.75 ± 0.05*
IVS/LVD	0.25 ± 0.01	0.22 ± 0.07	0.27 ± 0.01	0.29 ± 0.01*	0.31 ± 0.01*	0.32 ± 0.01*
EF %	79.7 ± 1.8	81.0 ± 0.5	77.1 ± 2.6	87.5 ± 1.0*	81.9 ± 0.9	84.5 ± 2.0
FS (%)	50.1 ± 2.0	51.2 ± 0.6	47.5 ± 2.4	59.0 ± 1.4*	52.1 ± 1.1	55.3 ± 2.4

Echocardiographic parameters were measured and calculated using a Visualsonics 770 High Resolution Imaging system. Values are expressed as means ± S.E.M. (n = 4). LVD indicates left ventricular diameter; BW, body weight; IVS, interventricular septum; PWT, posterior wall thickness; EF, Ejection fraction; FS, fractional fraction.

3.1.2 Modulation of CYP gene expression prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*

To examine the effect of isoproterenol on CYP gene expression, total RNA was extracted from the hearts of treated rats, and the expression of different CYP genes was measured using reverse transcription followed by real-time PCR. Figure 3.3 demonstrates that, in the prehypertrophy phase (12 and 24 hours), isoproterenol treatment caused a significant decrease in gene expression of the cardiac epoxygenases CYP2C23 and CYP2J3, by 83% and 63% and by 45% and 43% at 12 and 24 hours of treatment, respectively. On the other hand, isoproterenol treatment significantly induced CYP ω-hydroxylase CYP4F4 gene expression, by 78% and 107% at 12 and 24 hours of treatment, respectively (Fig 3.3). However, no significant change was observed in CYP1B1 and CYP2B1 gene expression prior to cardiac hypertrophy.

During the hypertrophy phase (after 72 hours), isoproterenol treatment caused a significant increase in CYP1B1 gene expression at 168 and 240 hours by 196% and 650%, respectively. Moreover, CYP epoxygenase CYP2B1 mRNA was significantly induced at 168 and 240 hours of treatment, by 150% and 185%, respectively, and CYP2J3 mRNA was significantly induced at 240 hours, by 27%. However, the gene expression of CYP2C23 did not change during this phase. On the other hand, CYP ω-hydroxylase CYP4F4 mRNA was significantly increased at all-time points tested during this phase, by 105%, 70%, and 135%, respectively (Fig 3.3).

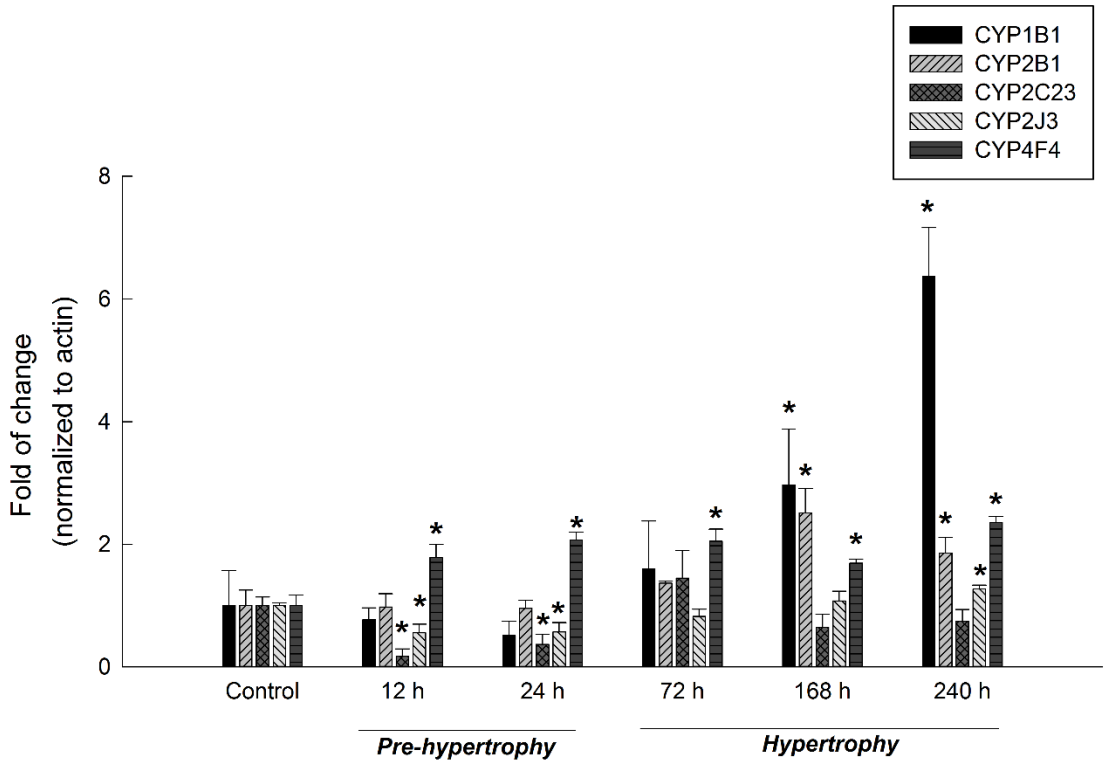

Fig 3.3. Modulation of CYP gene expression prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. Total RNA was isolated from hearts of control and isoproterenol-treated rats; then CYP gene expression was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.3 Modulation of CYP protein expression prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*

To investigate whether changes in CYP gene expression were further translated into proteins, microsomal proteins were prepared from the hearts of control and isoproterenol-treated rats. Thereafter, CYP protein expression was determined by Western blot analysis. Our results show that, in the prehypertrophy phase, isoproterenol treatment significantly decreased protein levels of CYP2C23 at 24 hours, by 25%. In contrast, CYP4F4 protein levels were increased significantly, by 180% and 140%, at 12 and 24 hours of treatment, respectively. However, no significant changes were detected in protein levels of CYP1B1, CYP2B1, and CYP2J3 during this phase (Fig 3.4).

On the other hand, CYP2B1 and CYP4F4 protein levels were significantly induced in the hypertrophy phase, at 168 hours of treatment, by 60% and 50%, respectively. Similarly, isoproterenol significantly induced CYP1B1, CYP2B1 and CYP2J3 at 240 hours, by 275%, 74% and 90%, respectively. However, CYP2C23 protein levels were significantly decreased at 72 and 240 hours, by 30% and 38%, respectively (Fig 3.4).

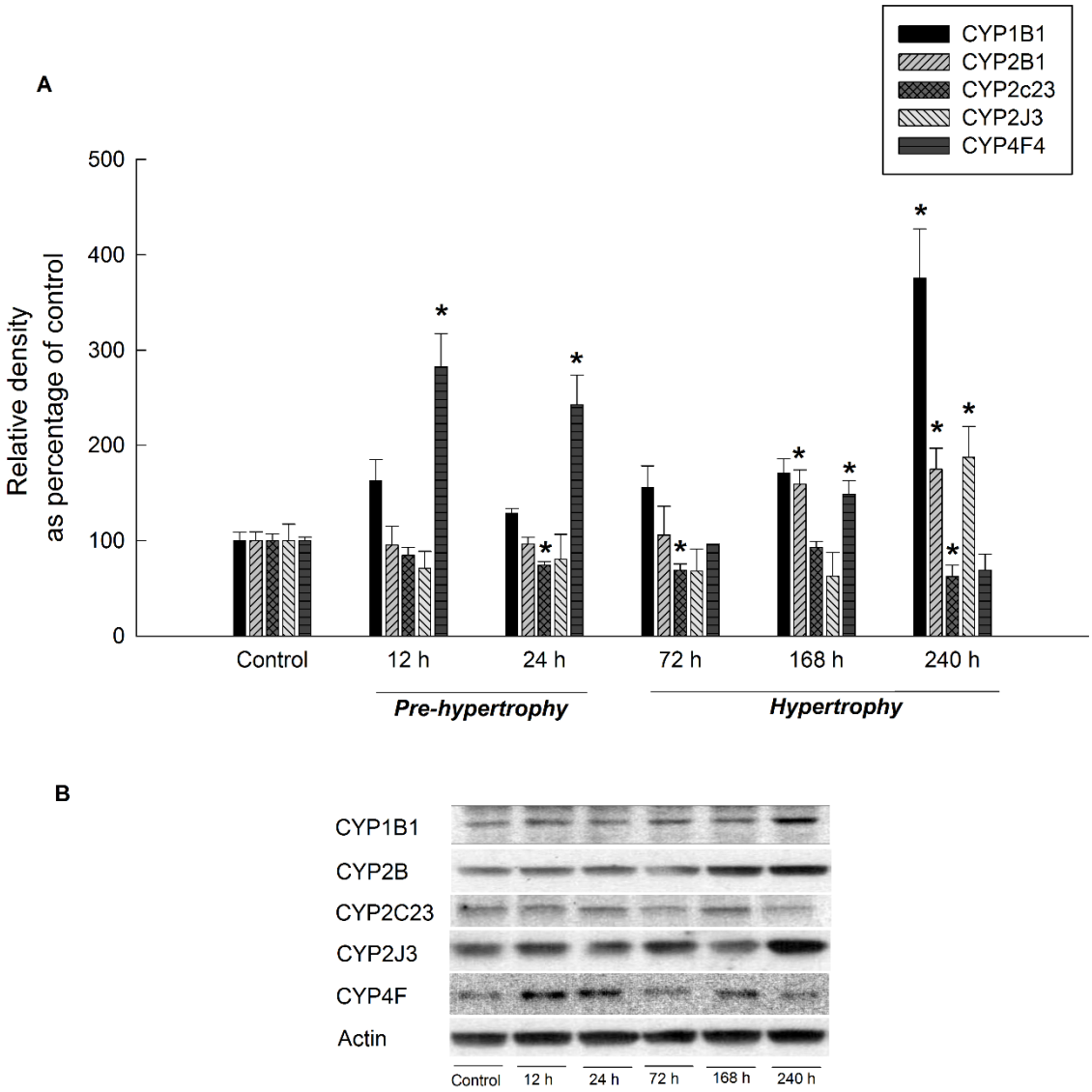

Fig 3.4. Modulation of CYP protein expression prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. Heart microsomal protein was isolated from the hearts of control and isoproterenol-treated rats, and separated on a 10% SDS-PAGE. (A) Relative protein expression of CYP normalized to the expression of β-actin and the results are expressed as percentage of the control (mean ± SEM, n = 4). (B) Representative Western blots are shown. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.4 Modulation of CYP-mediated AA metabolism and sEH activity prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*.

To investigate the effect of isoproterenol treatment on CYP-derived AA metabolites in cardiac hypertrophy, heart microsomes of control and isoproterenol-treated rats were incubated with 50 μM AA for 30 minutes. Thereafter, AA metabolites were determined using LC-ESI-MS. Our results show that, in the prehypertrophy phase, the formation rate of 11,12-EET was significantly decreased, by 40%, in as early as 12 hours. At 24 hours, the formation rates of 5,6-, 8,9-, 11,12-, and 14,15-EET were significantly decreased, by 20%, 30%, 30%, and 20%, respectively (Fig 3.5A). In contrast, formation rates of 8,9-, 11,12-, and 14,15- DHET were significantly increased, by 70%, 20%, and 27% and by 130%, 73%, and 104% at 12 and 24 hours, respectively. 5,6-DHET formation rate was increased only after 24 hours of isoproterenol treatment, by 75% (Fig 3.5B). Similarly, isoproterenol treatment significantly increased the ω-hydroxylase activity (20-HETE formation rate), by 10%, at 24 hours (Fig 3.6A). Moreover, the activity of the sEH, a major determinant of EET levels, was determined, and our results show that isoproterenol treatment caused a significant induction of sEH activity at 12 and 24 hours, by 26% and 35%, respectively (Fig 3.6B).

Studies performed during the hypertrophy phase showed a significant decrease in formation rates of 5,6-, 8,9-, 11,12-, and 14,15-EET, by 16%, 31%, 37%, and 75% and by 20%, 35%, 45%, and 20% at 72 and 168 hours, respectively. However, no significant changes were detected in EET levels at 240 hours in comparison to control hearts (Fig 3.5A). In contrast, formation rate of 14,15-DHET

was significantly increased, by 20%, 42%, and 40% at 72, 168, and 240 hours, respectively. Similarly, isoproterenol significantly increased formation rates of 8,9- and 11,12-DHET, by 45% and 30%, respectively, at 168 hours, whereas 5,6-DHET level was significantly increased at 240 hours, by 78% (Fig 3.5B). On the other hand, the formation rate of 20- HETE was only increased at 168 hours of isoproterenol treatment, by 10% (Fig 3.6A). In addition, sEH activity was increased significantly at all time points tested during this phase, by 25%, 274%, and 47%, respectively (Fig 3.6B).

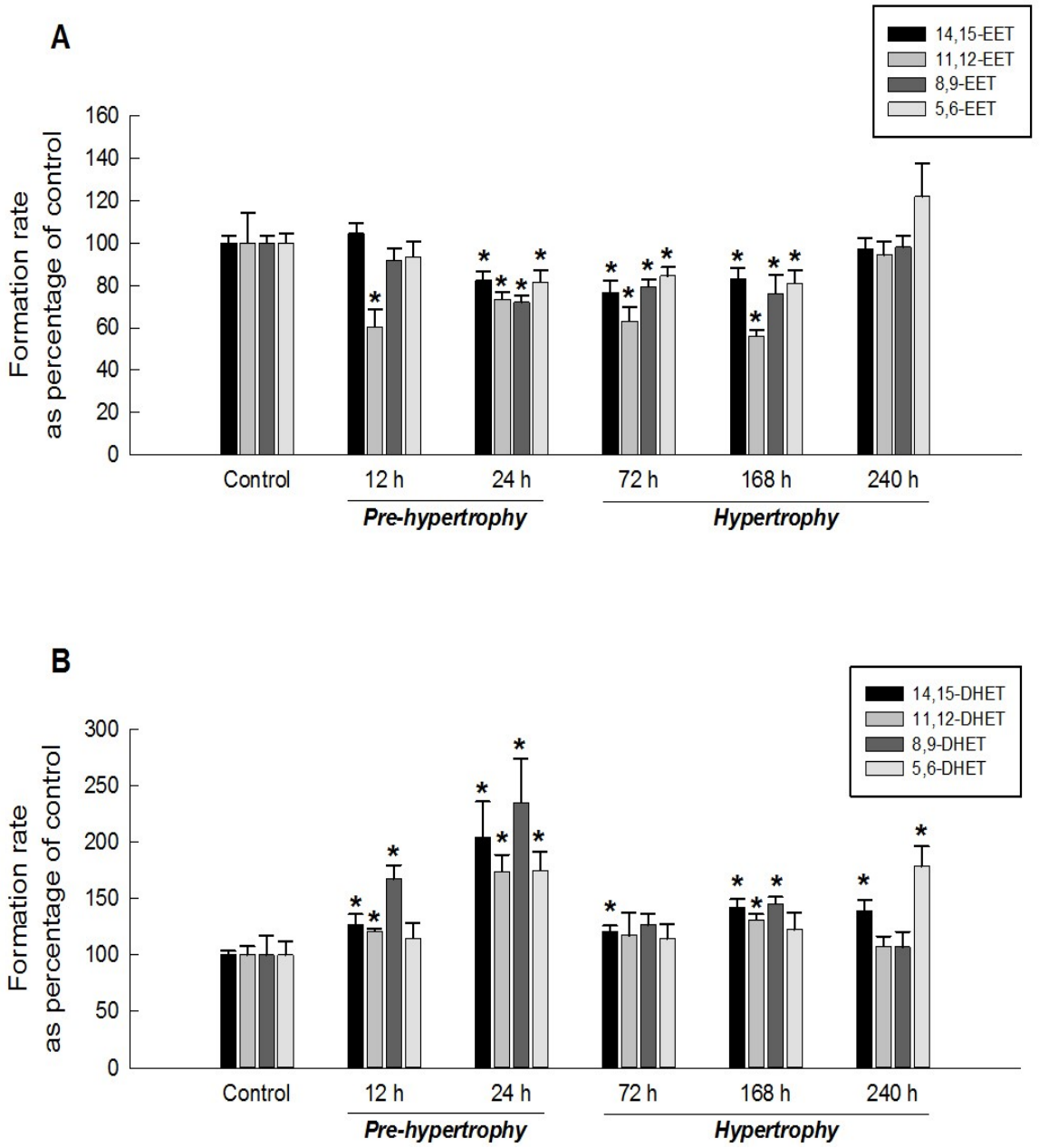

Fig 3.5. Modulation of CYP-mediated AA metabolism prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. Heart microsomes of treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 minutes. The reaction was terminated by the addition of ice-cold acetonitrile. Metabolites were extracted twice by ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) EETs and (B) DHETs. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

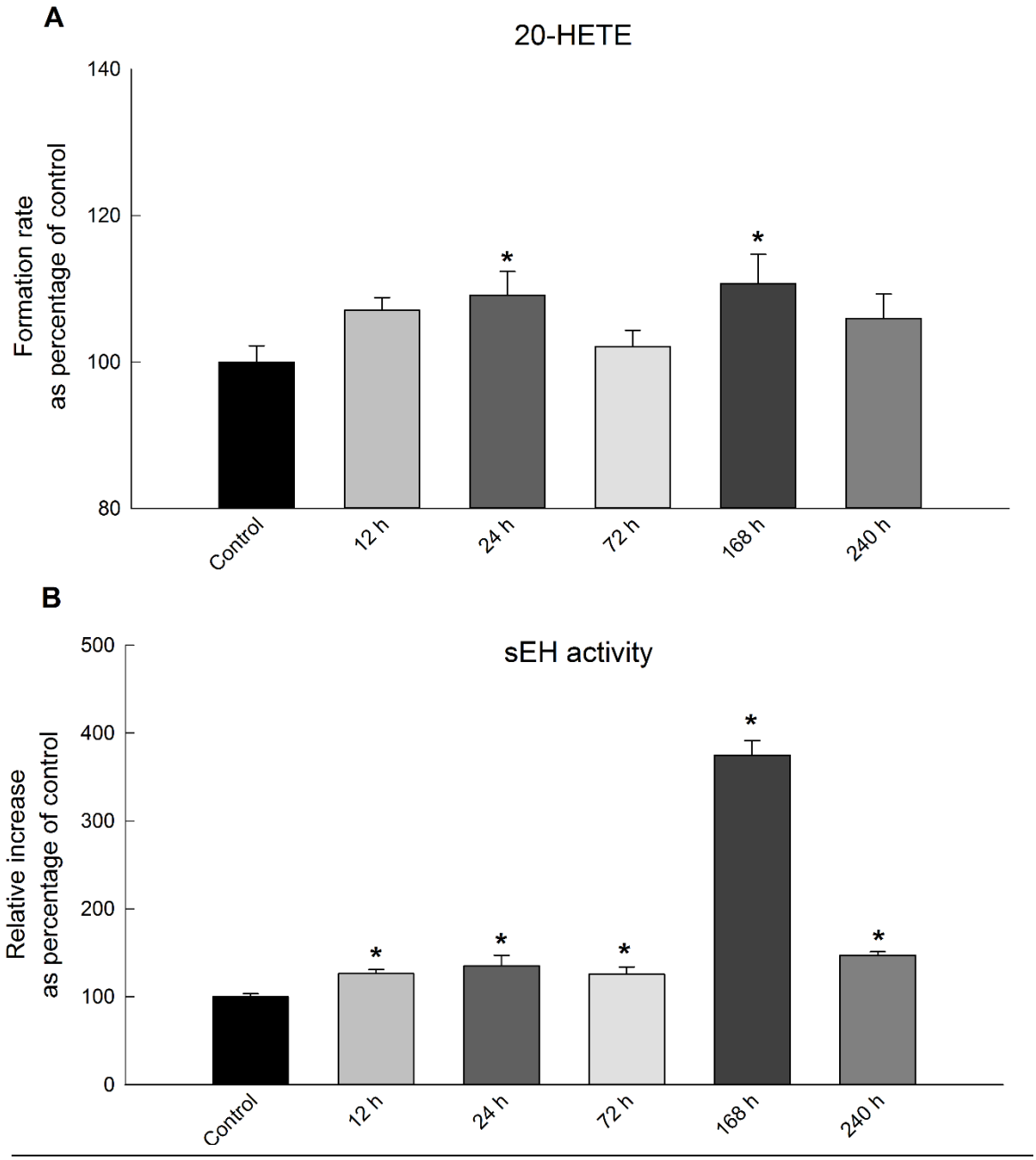

Fig 3.6. Modulation of 20-HETE formation and sEH activity prior to and during cardiac hypertrophy induced by isoproterenol *in vivo*. SD rats received daily injections of vehicle or isoproterenol (5 mg/kg i.p.) for the indicated time periods. Heart microsomes of treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 minutes. The reaction was terminated by the addition of ice-cold acetonitrile. Metabolites were extracted twice by ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) 20-HETE. (B) sEH activity was determined using the sEH assay. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.5 Effect of isoproterenol on human fetal ventricular cardiomyocytes, RL-14, cell viability

RL-14 cells were exposed to different concentrations of isoproterenol for 24 hours. Thereafter, cell viability was determined by MTT assay. MTT assay showed that the maximum nontoxic concentration of isoproterenol was 150 μM Therefore, the observed changes were not due to the decreased cell viability or toxicity (Fig 3.7).

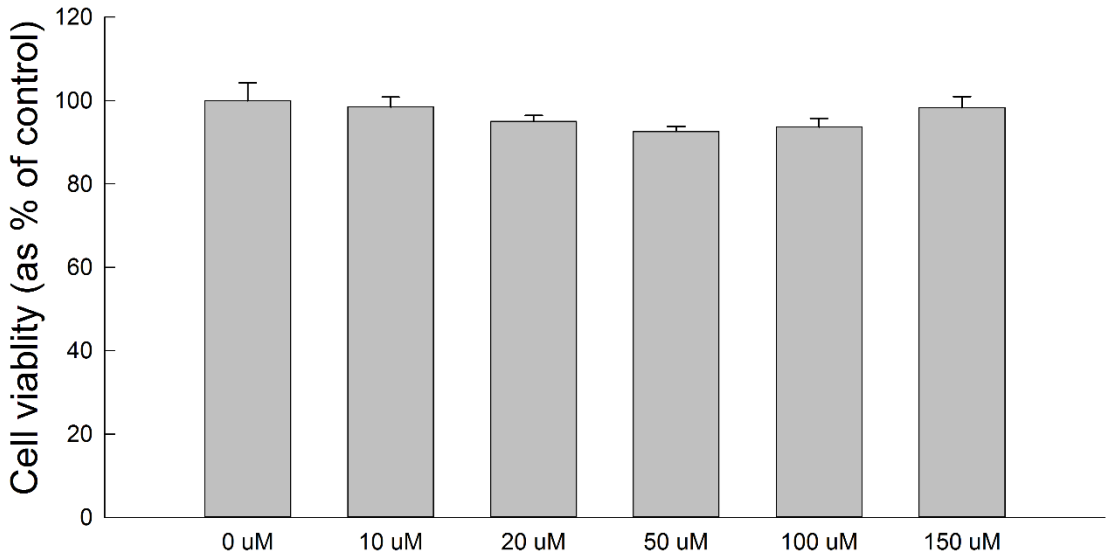

Fig 3.7. Effect of isoproterenol on RL-14 cell viability. RL-14 cells were incubated with increasing concentrations of isoproterenol. Thereafter, the cell viability was measured by the MTT assay and data are presented as a percentage of control (mean ± S.E.M, n=6). Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.6 Assessment of cellular hypertrophy induced by isoproterenol in RL-14 cells.

Assessment of cellular hypertrophy initiation was conducted using gene expression of the cardiac hypertrophy marker ANP and cell size measurements. Isoproterenol treatment significantly increased ANP mRNA at 6, 12, and 24 hours, by 94%, 80%, and 50%, respectively (Fig 3.8). Similarly, isoproterenol treatment showed a significant increase in cell size at 6, 12, and 24 hours, by 82%, 70%, and 180%, respectively, as compared to control cells (Fig 3.9).

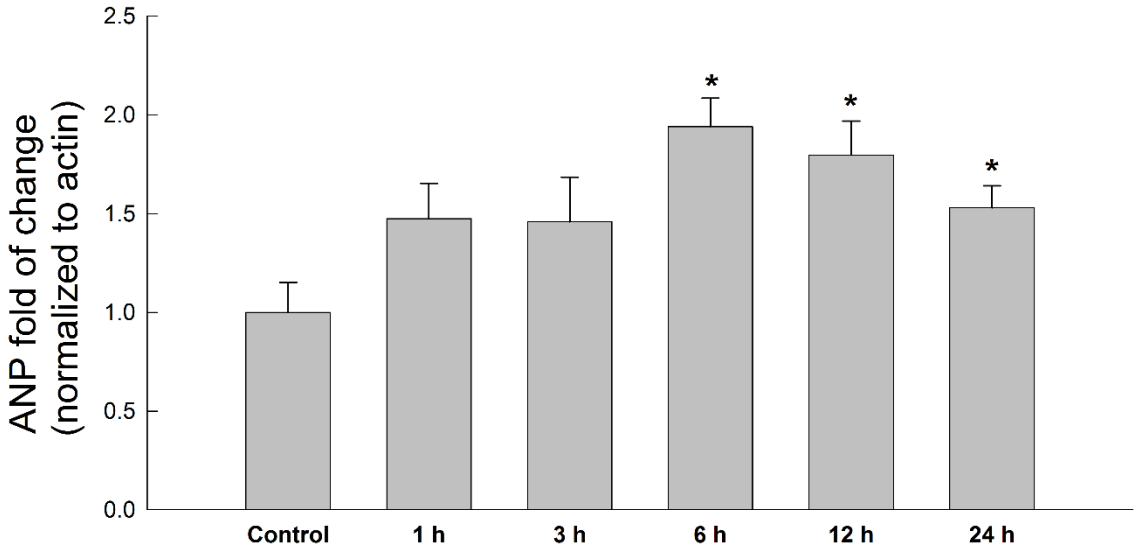

Fig 3.8. Effect of isoproterenol on ANP gene expression in RL-14 cells. Total RNA was isolated from RL-14 cells treated with vehicle or isoproterenol (100 μM) for the indicated time periods. Thereafter, gene expression of the hypertrophic marker ANP was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

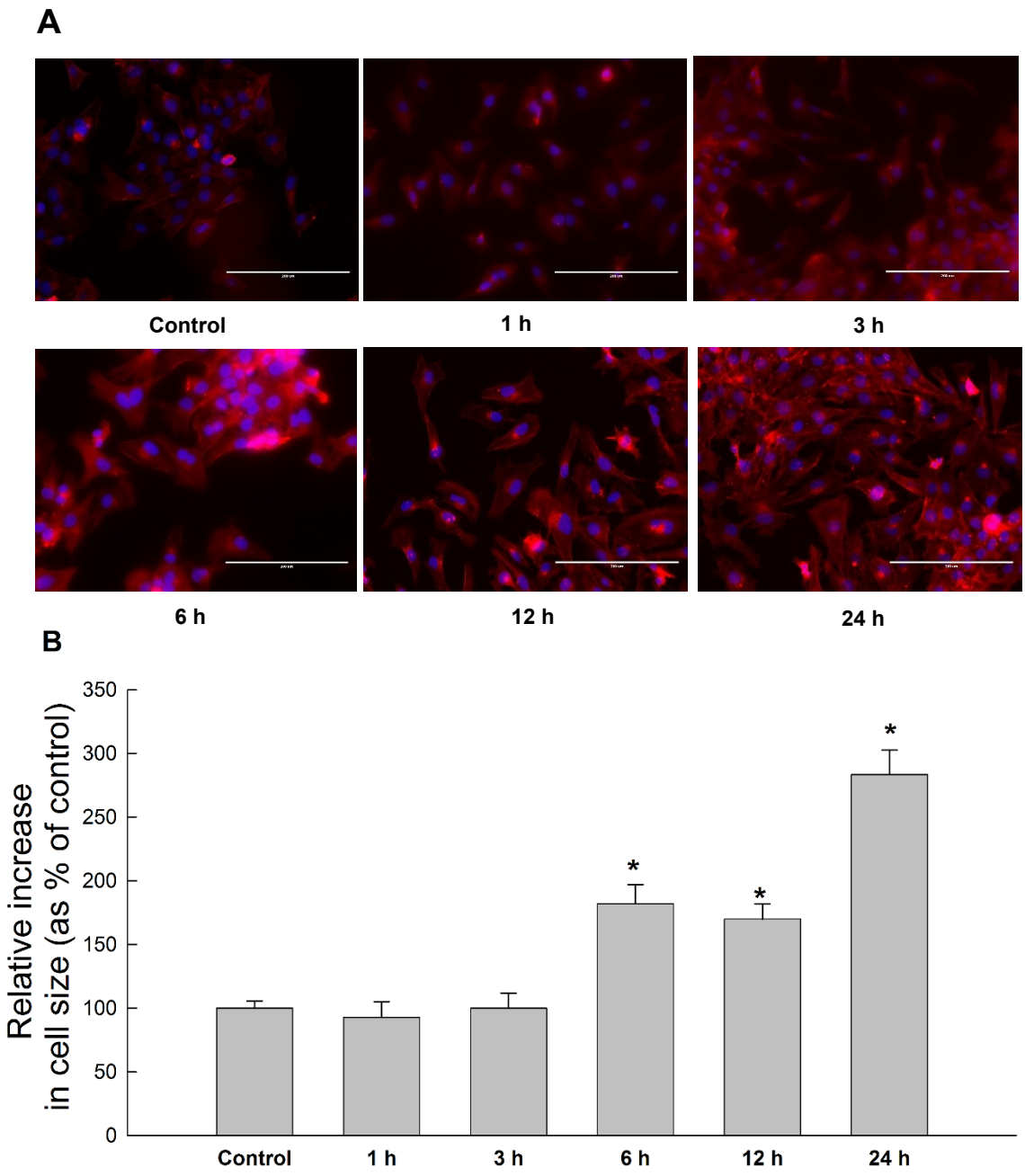

Fig 3.9. Assessment of cellular hypertrophy induced by isoproterenol in RL-14 cells. (A) Representative cell images from RL-14 cells staining with Texas red phalloidin (red) and DAPI (blue) as described in method. (B) Cell size was measured by imaging the complete boundary of individual cells after magnification. Values are the mean ± S.E.M. of 30 cells from different images per treatment. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.7 Modulation of CYP prior to and during cellular hypertrophy induced by isoproterenol *in vitro*.

Treatment of RL-14 cells by isoproterenol during the prehypertrophy phase (1 and 3 hours) caused a significant decrease in CYP2B6 and CYP2C8 gene expression, by 45% and 40% and by 55% and 45%, respectively. In addition, CYP2J2 gene expression was decreased at 3 hours by 24%. However, no significant changes were detected in CYP1A2, CYP2C19, and CYP4F2 gene expression in prehypertrophied cells (Fig 3.10).

Isoproterenol treatment for 6, 12, and 24 hours (hypertrophy phase) caused significant decreases in CYP1A2, CYP2B6, CYP2C8, and CYP2C19 gene expression, by 45%, 60%, 55%, and 42%; by 37%, 73%, 55%, and 40%; and by 64%, 70%, 75%, and 70%, respectively. However, the gene expression of CYP2J2 was only decreased at 6 hours, by 34%, and returned to normal thereafter. In contrast, no significant changes were detected in CYP4F2 mRNA (Fig 3.10).

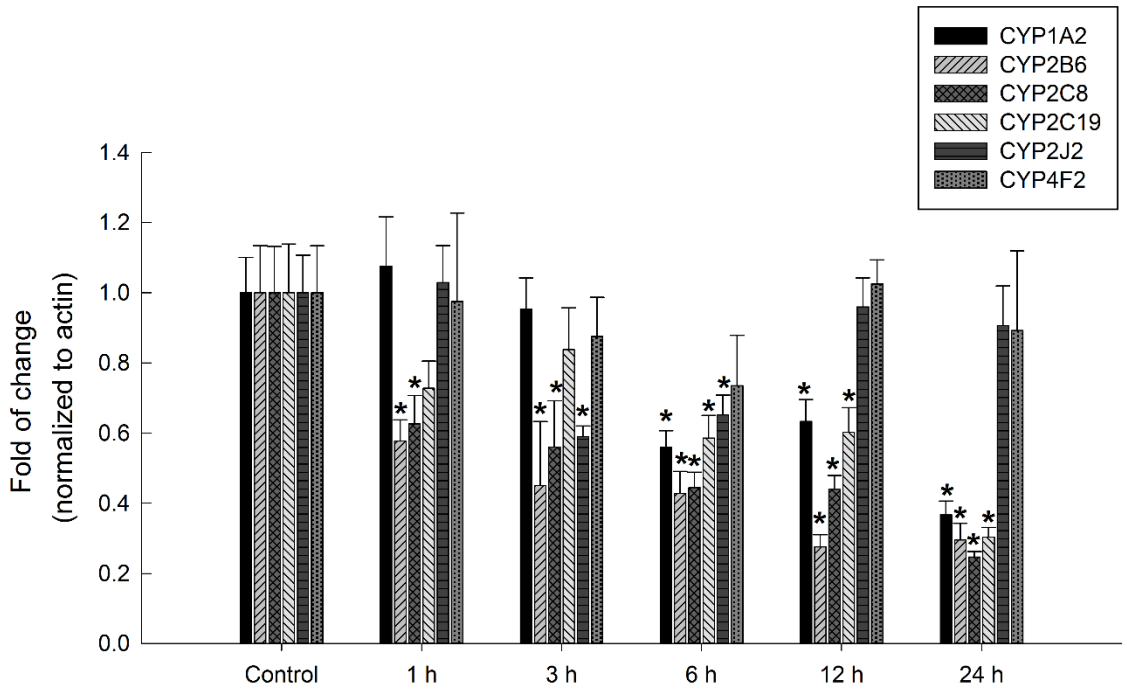

Fig 3.10. Modulation of CYP enzymes prior to and during cellular hypertrophy induced by isoproterenol *in vitro*. Total RNA was isolated from RL-14 cells treated with vehicle or isoproterenol (100 µM) for the indicated time periods. Thereafter, gene expression of CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2J2, and CYP4F2 were determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.1.8 Modulation of CYP-mediated AA metabolism and sEH activity prior to and during cellular hypertrophy induced by isoproterenol *in vitro*.

Treatment of RL-14 cells by isoproterenol during the prehypertrophy phase (1 and 3 hours) caused a significant decrease in 11,12-EET formation rates as early as 1 hour, by 35% and 35%, respectively. In contrast, 11,12-DHET formation rate and sEH activity were significantly increased after 3 hours of isoproterenol treatment, by 60% and 68%, respectively. No significant changes were detected in other measured metabolites during this phase (Fig 3.11 and 3.12).

In the hypertrophy phase, formation rates of 8,9-EET and 14,15- EET were significantly decreased at 6 hours, by 36% and 23%, respectively, and then returned to the control level, whereas 11,12- EET formation rate was significantly decreased at 6, 12, and 24 hours of treatment, by 45%, 40%, and 42%, respectively (Fig 3.11A). In contrast, formation rates of 5,6-, 8,9-, 11,12-, and 14,15-DHET were significantly increased at 6, 12, and 24 hours of treatment, by 50%, 78%, 82%, and 112%; by 65%, 84%, 90%, and 170%; and by 57%, 90%, 63%, and 154%, respectively (Fig 3.11B). Similarly, sEH activity was significantly increased at 6, 12, and 24 hours of treatment, by 99%, 83%, and 79%, respectively (Fig 3.12B). However, no significant changes were detected in 20-HETE formation rate during this phase (Fig 3.12A).

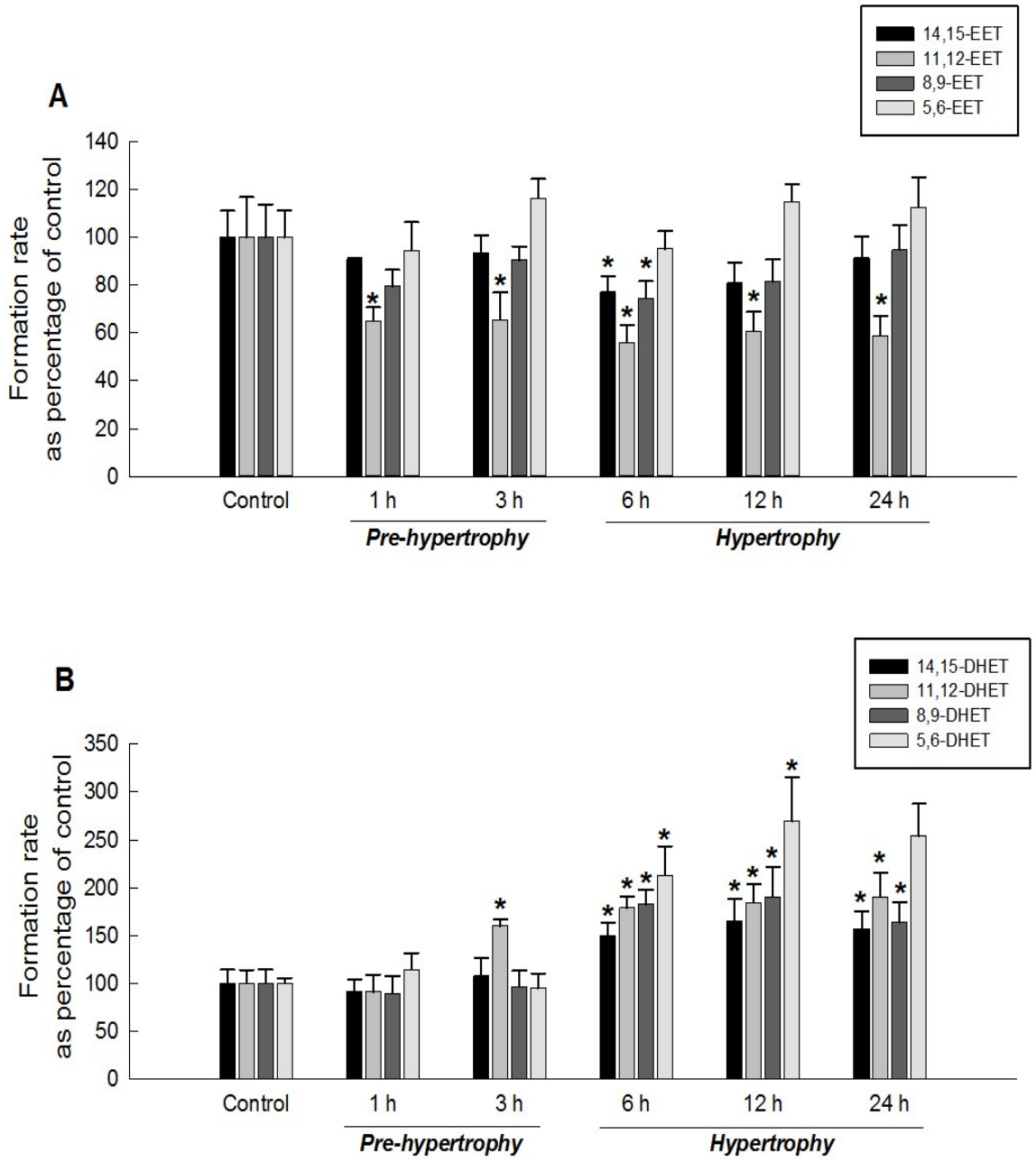

Fig 3.11. Modulation of CYP-mediated AA metabolism prior to and during cellular hypertrophy induced by isoproterenol *in vitro*. RL-14 cells were plated in 100-mm petri dishes and incubated, after having been treated with isoproterenol for the indicated time periods, with 50 μM AA for 3 hours. Metabolites were extracted by ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) EETs and (B) DHETs. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

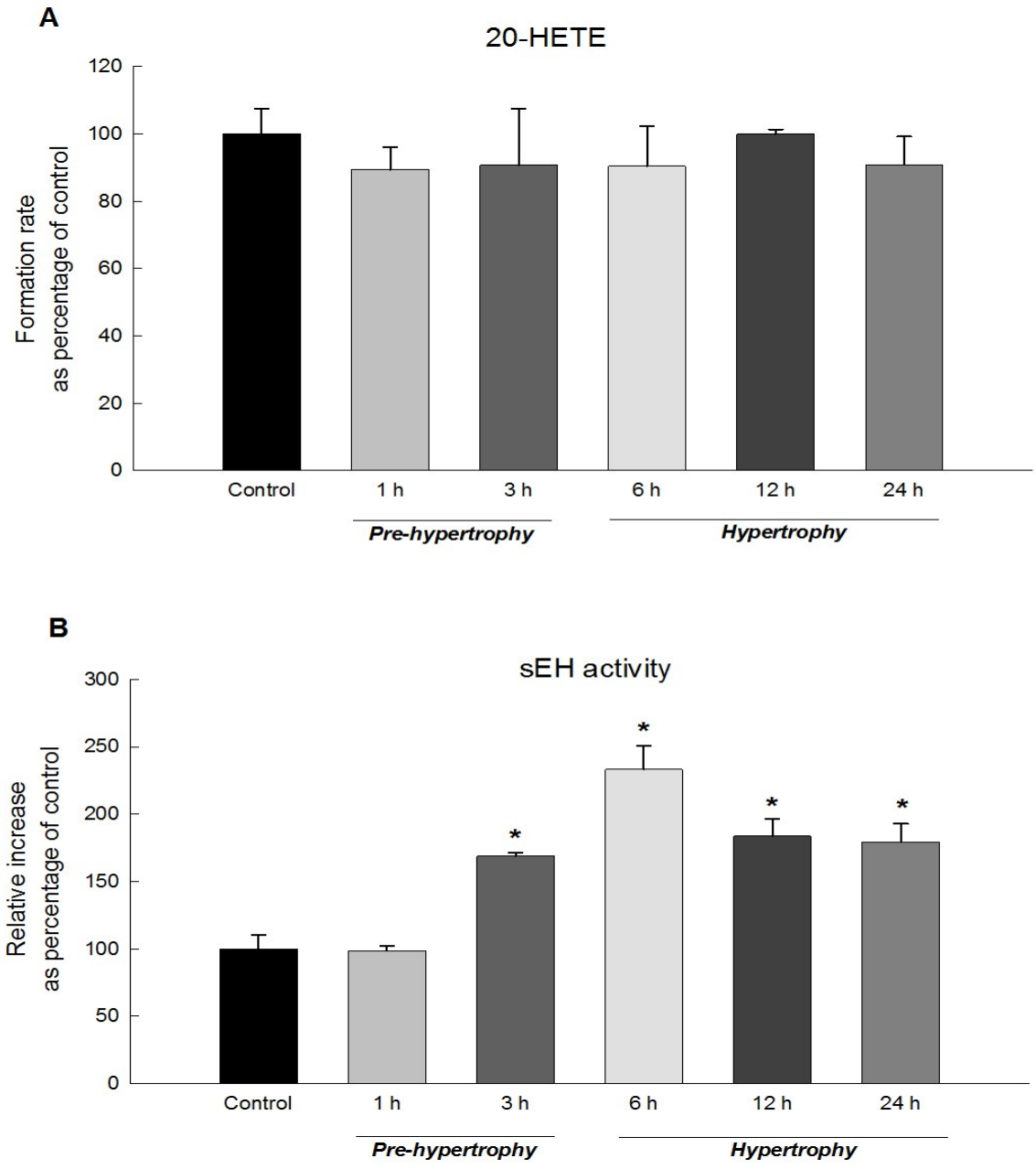

Fig 3.12. Modulation of 20-HETE formation and sEH activity prior to and during cellular hypertrophy induced by isoproterenol *in vitro*. RL-14 cells were plated in 100-mm petri dishes and incubated, after having been treated with isoproterenol for the indicated time periods, with 50 μM AA for 3 hours. Metabolites were extracted by ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) 20-HETE. (B) sEH activity was determined using the sEH assay. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.2 Soluble epoxide hydrolase inhibitor, TUPS, protects against isoproterenol-induced cardiac hypertrophy

3.2.1 Effect of the sEH inhibitor TUPS on the cardiac hypertrophy induced by isoproterenol

To investigate whether the inhibition of sEH confers cardioprotection in isoproterenol-treated rats, we measured the cardiac gene expression of the hypertrophic markers ANP, brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) relative to those in isoproterenol-treated rats. Isoproterenol treatment caused a significant induction in the hypertrophic markers ANP, BNP and β-MHC by 500%, 50% and 265%, respectively (Fig 3.13A). On the other hand, TUPS treatment significantly decreased the isoproterenol-mediated induction of ANP, BNP and β-MHC by 47%, 71%, and 72% respectively (Fig 3.13A). In addition, TUPS treatment alone did not alter the gene expression of ANP, BNP and β-MHC. Moreover, isoproterenol significantly increased the heart weight to body weight ratio by 22%, whereas treatment with TUPS significantly decreased this isoproterenol-mediated increase in the HW/BW ratio by 35%, compared to isoproterenol alone. Furthermore, no significant difference was observed between the control and the TUPS treatment alone (Fig 3.13B).

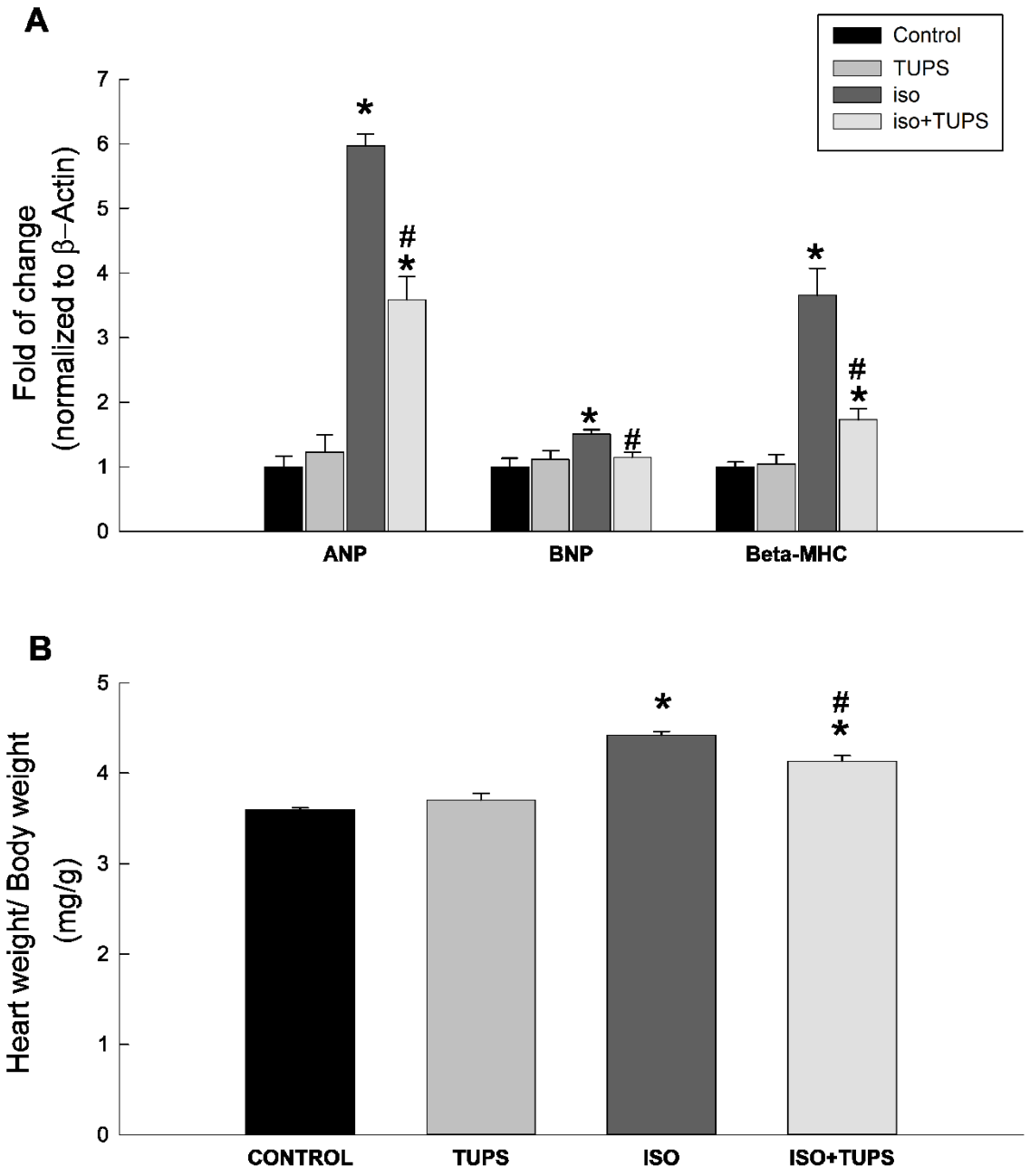

Fig 3.13. Effect of the sEH inhibitor TUPS on the cardiac hypertrophy induced by isoproterenol. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg., i.p.) plus TUPS (0.65 mg/kg, oral) for 7 days; while weight-matched controls received the same volume of 25% PEG400 and saline. (A) The expression of the hypertrophic genes, ANP, BNP and β-MHC were determined in the heart. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). (B) HW/BW ratio (in mg/g) was determined for each animal after the treatment. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.2 Effect of TUPS on fibrotic markers associated with cardiac hypertrophy

To investigate whether the inhibition of sEH has an effect on the fibrosis associated with isoproterenol-induced cardiac hypertrophy, we measured the cardiac gene expression of the fibrotic markers, procollagen I, procollagen III and Transforming growth factor-β1 (TGF-1) relative to isoproterenol-treated rats. Isoproterenol treatment caused a significant induction in the fibrotic markers procollagen I, procollagen III and TGF-1 by 456%, 443% and 123% respectively (Figure 3.14). On the other hand, TUPS treatment significantly decreased the isoproterenol-mediated induction of procollagen I, procollagen III and TGF-1 by 40%, 45% and 40%, respectively, compared to isoproterenol alone (Fig 3.14). Furthermore, no significant difference was observed between the control and the TUPS treatment alone (Fig 3.14).

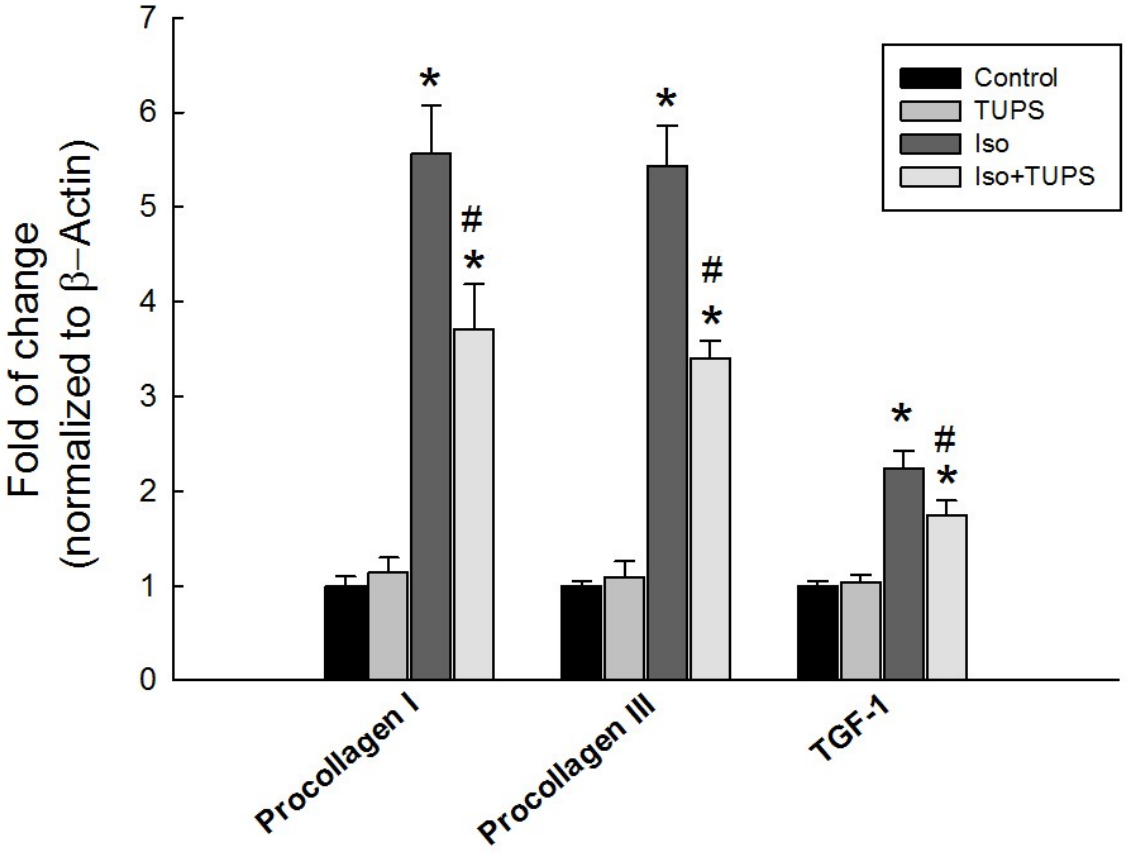

Fig 3.14. Effect of TUPS on the expression of fibrotic markers. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days; while weight-matched controls received the same volume of 25% PEG400 and saline. Total RNA was isolated from hearts of control, TUPS, isoproterenol and isoproterenol+TUPS-treated rats. Gene expressions were determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.3 Effect of TUPS on the changes in CYP gene expression mediated by isoproterenol in heart, kidney, and liver

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

3.2.3.1 Effect of TUPS on the changes in CYP1 family gene expression

Figure 3.15A shows the effect of TUPS treatment on cardiac hypertrophy-induced CYP1A1 gene expression. Isoproterenol treatment caused a significant increase in CYP1A1 gene expression in heart and kidney by about 110% and 94% respectively (Fig 3.15A). On the other hand, TUPS treatment caused significant inhibition of isoproterenol-induced CYP1A1 gene expression by 83% in the heart compared to isoproterenol alone treatment. In the kidney, TUPS alone caused a significant induction of CYP1A1 gene expression by 260% and isoproterenol + TUPS treatment was similar to TUPS alone. In the liver, CYP1A1 mRNA levels were not altered in response to either TUPS or isoproterenol treatment when compared to the control group (Fig 3.15A). Isoproterenol treatment also significantly induced CYP1B1 gene expression in the heart by 320%. In contrast, the CYP1B1 mRNA level was significantly decreased in hearts after treatment with TUPS, by 88% compared to the isoproterenol alone treated group. In the kidney, CYP1B1 mRNA levels were significantly induced in the TUPS- and isoproterenol + TUPS- but not in isoproterenol-treated groups by 151% and 82%, respectively, compared to the

control group. However, no significant changes were observed in the liver (Fig 3.15B).

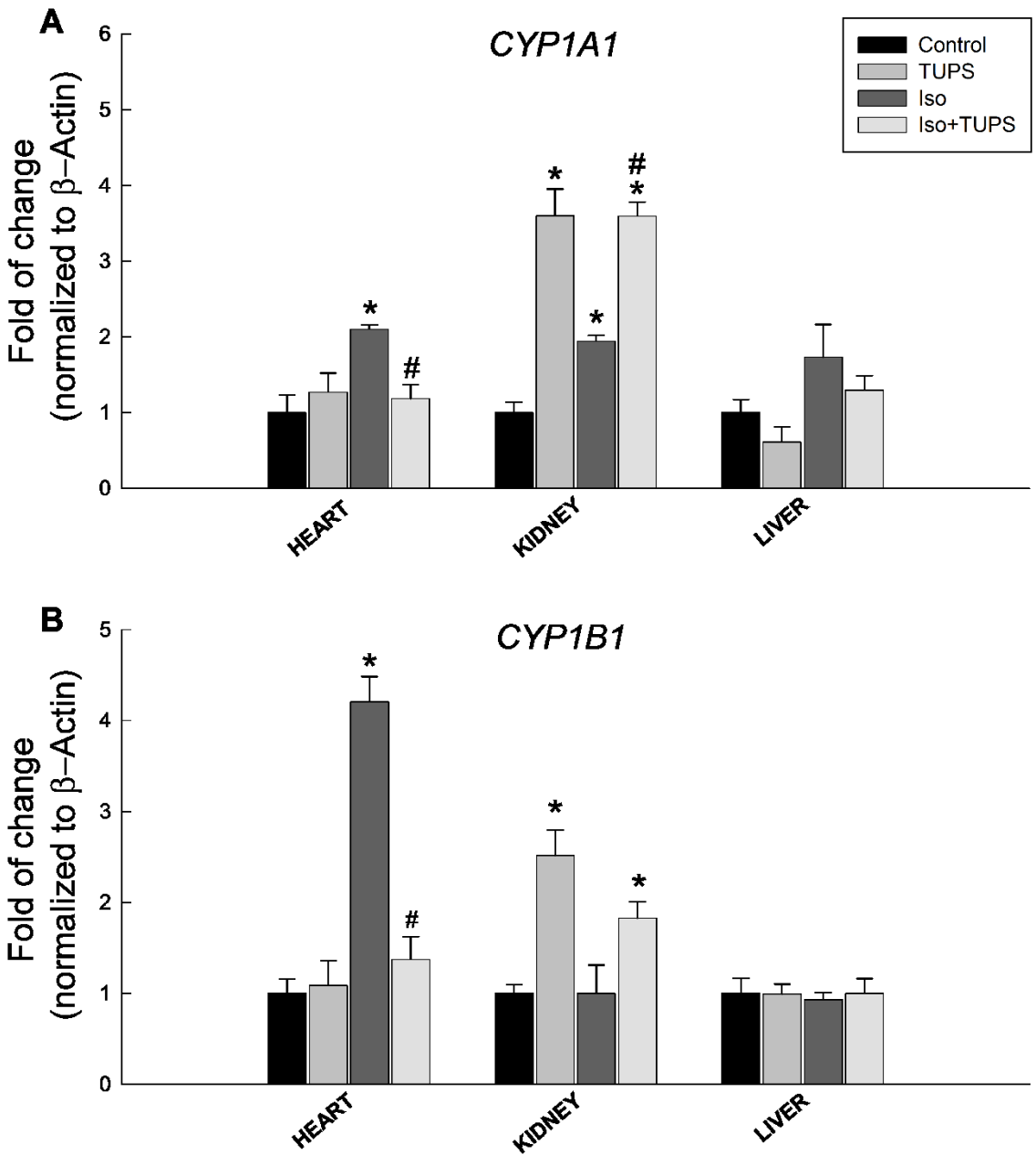

Fig 3.15. Effect of TUPS on the changes in the CYP1 family gene expression mediated by isoproterenol. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days; while weight-matched controls received the same volume of 25% PEG400 and saline. Total RNA was isolated from the heart, kidney and liver of treated rats; and the relative gene expression of (A) CYP1A1 and (B) CYP1B1 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.3.2 Effect of TUPS on the changes in CYP2 family gene expression

With respect to the CYP2 family, isoproterenol treatment caused a significant induction in CYP2B1 and CYP2B2 mRNA levels in the heart by 324% and 407% respectively. In contrast, TUPS treatment significantly inhibited the isoproterenol mediated induction of CYP2B1 and CYP2B2 mRNA by 48% and 64%, respectively, compared to isoproterenol alone. However, no significant changes were observed in either the kidney or the liver (Fig 3.16).

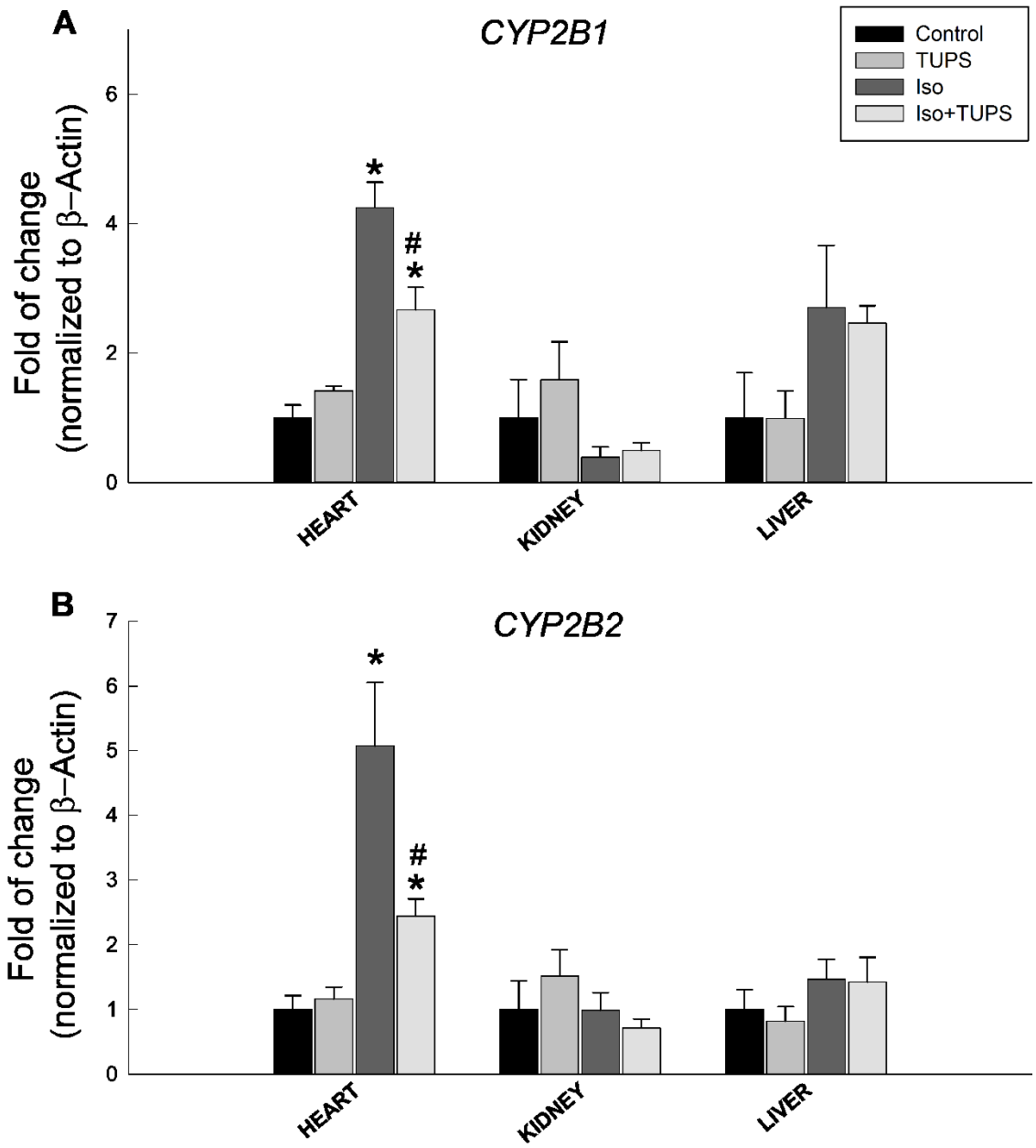

Fig 3.16. Effect of TUPS on the changes in the CYP2 family gene expression mediated by isoproterenol. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Total RNA was isolated from the heart, kidney and liver of treated rats; and the relative gene expression of (A) CYP2B1 and (B) CYP2B2 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.3.3 Effect of TUPS on the changes in CYP4 family gene expression

With regard to the CYP4 family, CYP4A3 gene expression was significantly increased by 1169%, 46% and 103% in the heart, kidney and liver of isoproterenol-treated rats, respectively. On the other hand, the gene expression of CYP4A3 was significantly reduced by 82%, 100% and 89% in the heart, kidney and liver of rats treated with isoproterenol + TUPS, respectively, compared to the isoproterenol alone group (Figure 3.17A). In addition, CYP4F4 gene expression was induced in the heart by 343% but not in the kidney or the liver of isoproterenol-treated rats. Moreover, TUPS treatment significantly reduced the isoproterenol-mediated induction of CYP4F4 mRNA in the heart by 46% (Figure 3.17B).

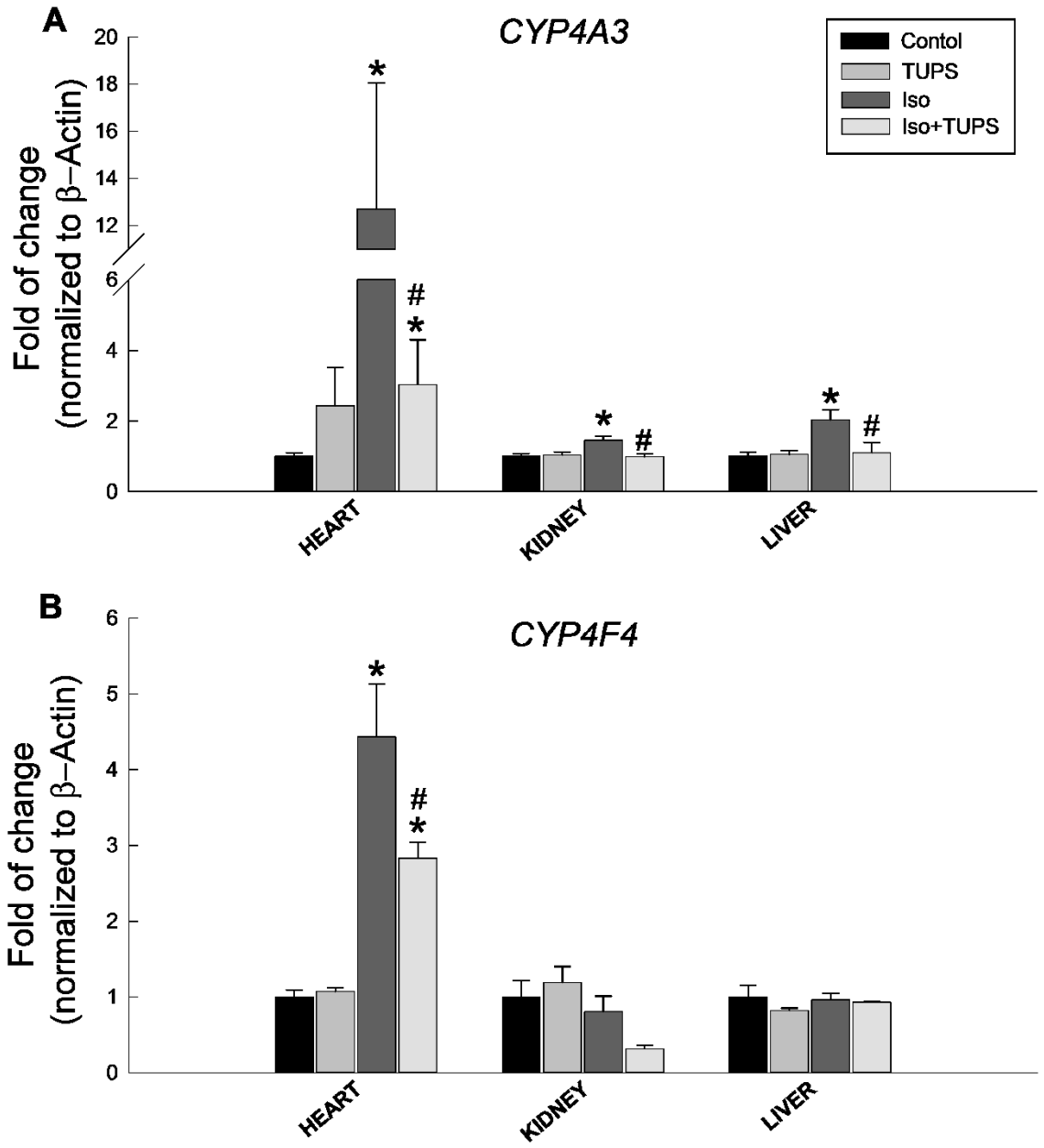

Fig 3.17. Effect of TUPS on the changes in the CYP4 family gene expression mediated by isoproterenol. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Total RNA was isolated from the heart, kidney and liver of treated rats; and the relative gene expression of (A) CYP4A3 and (B) CYP4F4 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.4 Effect of TUPS on CYP protein expression

To investigate whether the changes in CYP gene expression were further translated into protein, microsomal protein was prepared from the hearts of control, isoproterenol- and isoproterenol+TUPS-treated rats. Similar to mRNA levels, isoproterenol treatment significantly increased the protein levels of CYP1A1, CYP1B1, CYP2B1/2 and CYP4A by 480%, 272%, 70% and 96% respectively (Fig 3.18). In contrast, TUPS treatment caused a significant inhibition of isoproterenol-mediated induction of CYP1A1, CYP1B1, CYP2B1/2 and CYP4A protein expression by 76%, 60%, 150% and 160% relative to the isoproterenol alone group, respectively (Fig 3.18).

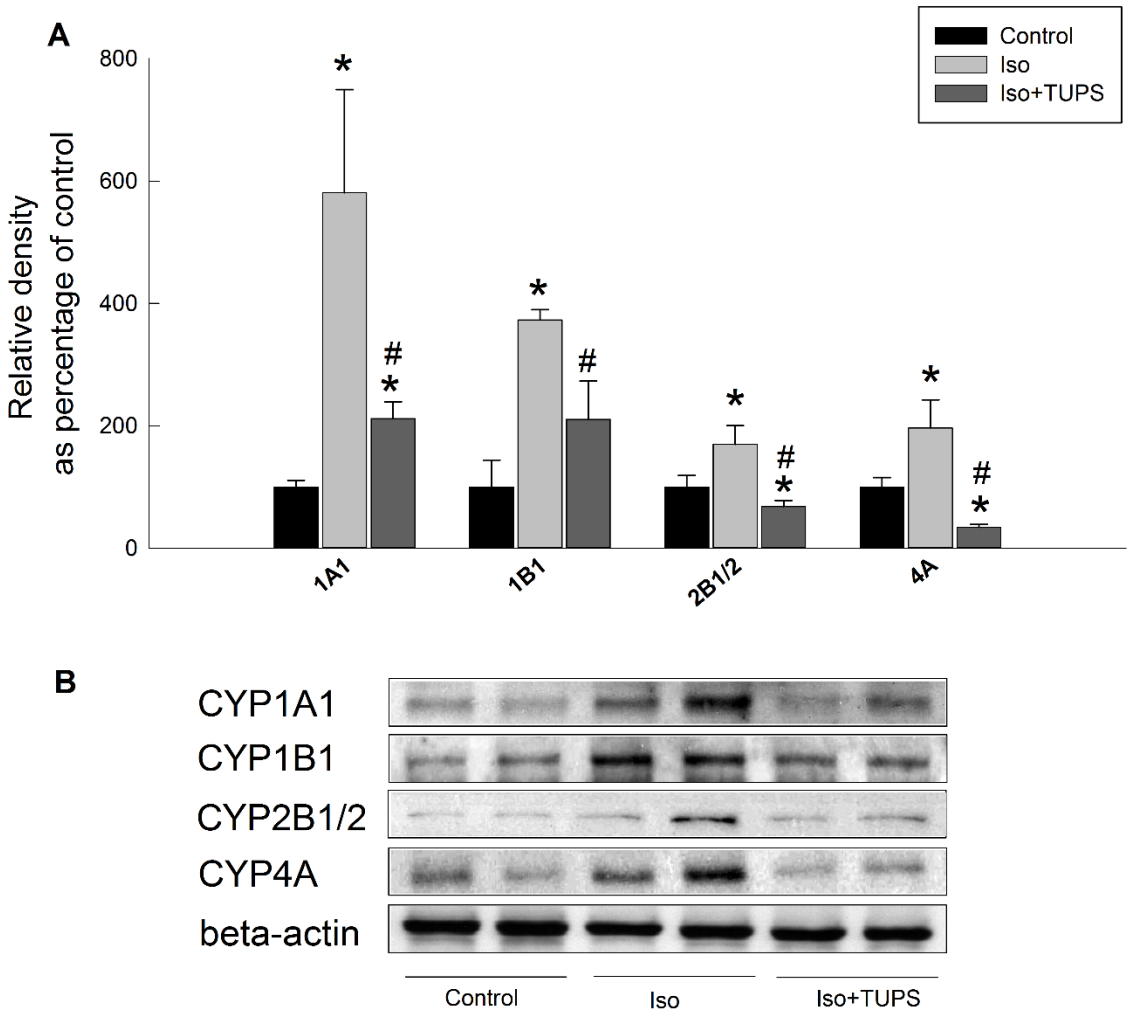

Fig 3.18. Effect of TUPS on the on CYP protein expression. SD rats received daily injections of vehicles, isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Microsomal protein was isolated from the hearts of treated rats, and separated on a 10% SDS-PAGE. (A) Relative protein expression of CYP is normalized to the expression of β-actin and the results are expressed as percentage of the control (mean ± SEM, n = 4). (B) Representative Western blots are shown. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.5 Effect of TUPS on sEH at the gene expression, protein and activity levels

The enzyme sEH is a major determinant of the levels of EETs; therefore, we determined the effect of TUPS treatment on the expression of the EPHX2 gene, which encodes for the sEH enzyme. Isoproterenol treatment did not cause any changes in EPHX2 gene expression in the heart, kidney or liver. Likewise, treatment with TUPS did not alter EPHX2 gene expression in any of the tissues tested (Fig 3.19). To investigate whether changes in EPHX2 gene expression were related to protein levels, we determined sEH protein levels. Isoproterenol treatment significantly increased the protein level of sEH by 1300% relative to the control group. On the other hand, TUPS treatment caused a significant inhibition of isoproterenol-mediated induction of sEH protein expression by 90% (Figure 3.20A). In accordance with this induction in sEH protein expression, isoproterenol treatment caused a significant induction of sEH activity by 86% (Figure 3.20B). On the other hand, TUPS significantly decreased sEH activity by 61% compared to isoproterenol-treated rats (Figure 3.20B).

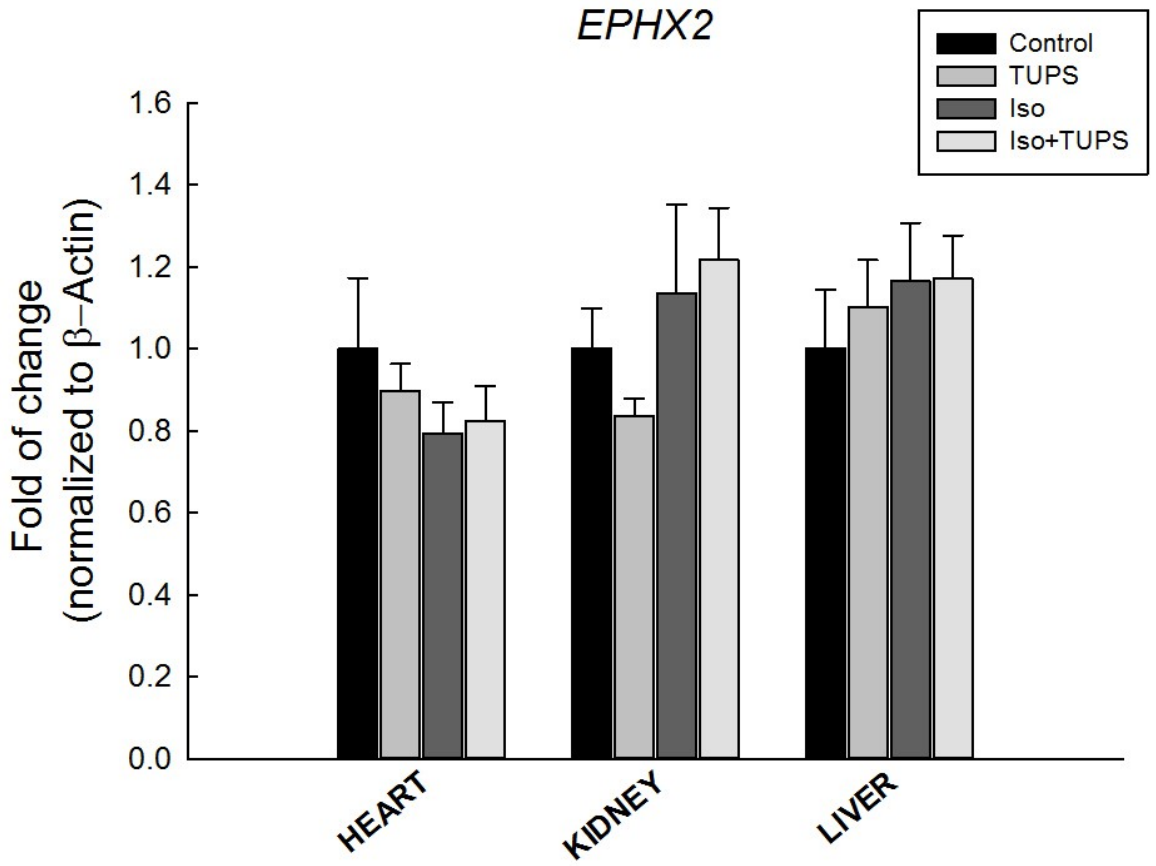

Fig 3.19. Effect of TUPS on EPHX2 gene expression. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Total RNA was isolated from the heart, kidney and liver of treated rats; and the relative gene expression of EPHX2 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

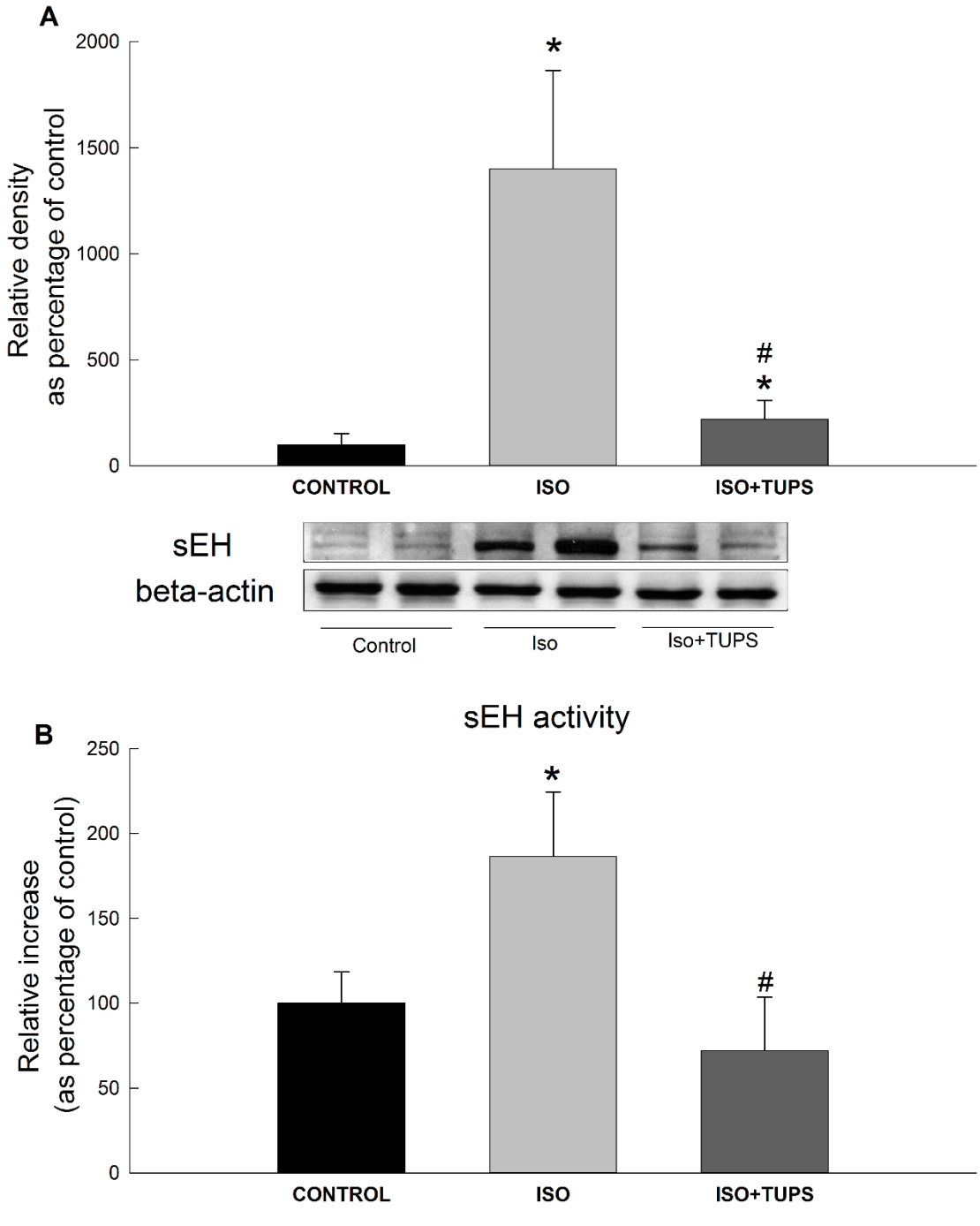

Fig 3.20. Effect of TUPS on sEH protein and activity levels. SD rats received daily injections of vehicles, TUPS (0.65 mg/kg, p.o.), isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. (A) sEH protein level was determined by Western blot analysis. (B) sEH activity was calculated using sEH assay. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.2.6 Effect of TUPS on CYP-mediated AA metabolism

To investigate the effect of TUPS treatment on CYP-derived AA metabolites, heart microsomes of treated rats were incubated with 50 μM AA for 30 min. Thereafter, AA metabolites were determined using LC-ESI-MS. The rates of 5,6-, 8,9-, 11,12- and 14,15-EET formation were significantly lower by 16%, 34%, 50% and 35%, respectively, in hypertrophied heart microsomes compared to control microsomes (Fig 3.21A). In contrast, the formation rates of 8,9- and 14,15-DHET were significantly higher by 120% and 75%, respectively, in hypertrophied heart microsomes compared to control microsomes. However, the formation rates of 5,6- and 11,12-DHET were not significantly altered (Fig 3.21B). TUPS treatment reversed significantly the isoproterenol-induced inhibitory effects on 8,9-, 11,12- and 14,15-EET by 53%, 154% and 151%, respectively, but not that on 5,6-EET levels (Fig 3.21A). Furthermore, TUPS treatment caused a significant reduction in the formation rate of 8,9-, 11,12- and 14,15-DHET by 87%, 70% and 61%, respectively, but not of 5,6-DHET levels (Fig 3.21B).

To determine the effect of TUPS treatment on CYP ω-hydroxylase activity, we measured the formation rate of 20-HETE in microsomes from control, isoproterenol- and isoproterenol+TUPS-treated rats. Isoproterenol treatment significantly increased the 20-HETE formation by 84% in comparison with the control microsomes. On the other hand, TUPS treatment caused a significant reduction in 20-HETE formation rate by 74% compared to the isoproterenol alone group (Fig 3.22).

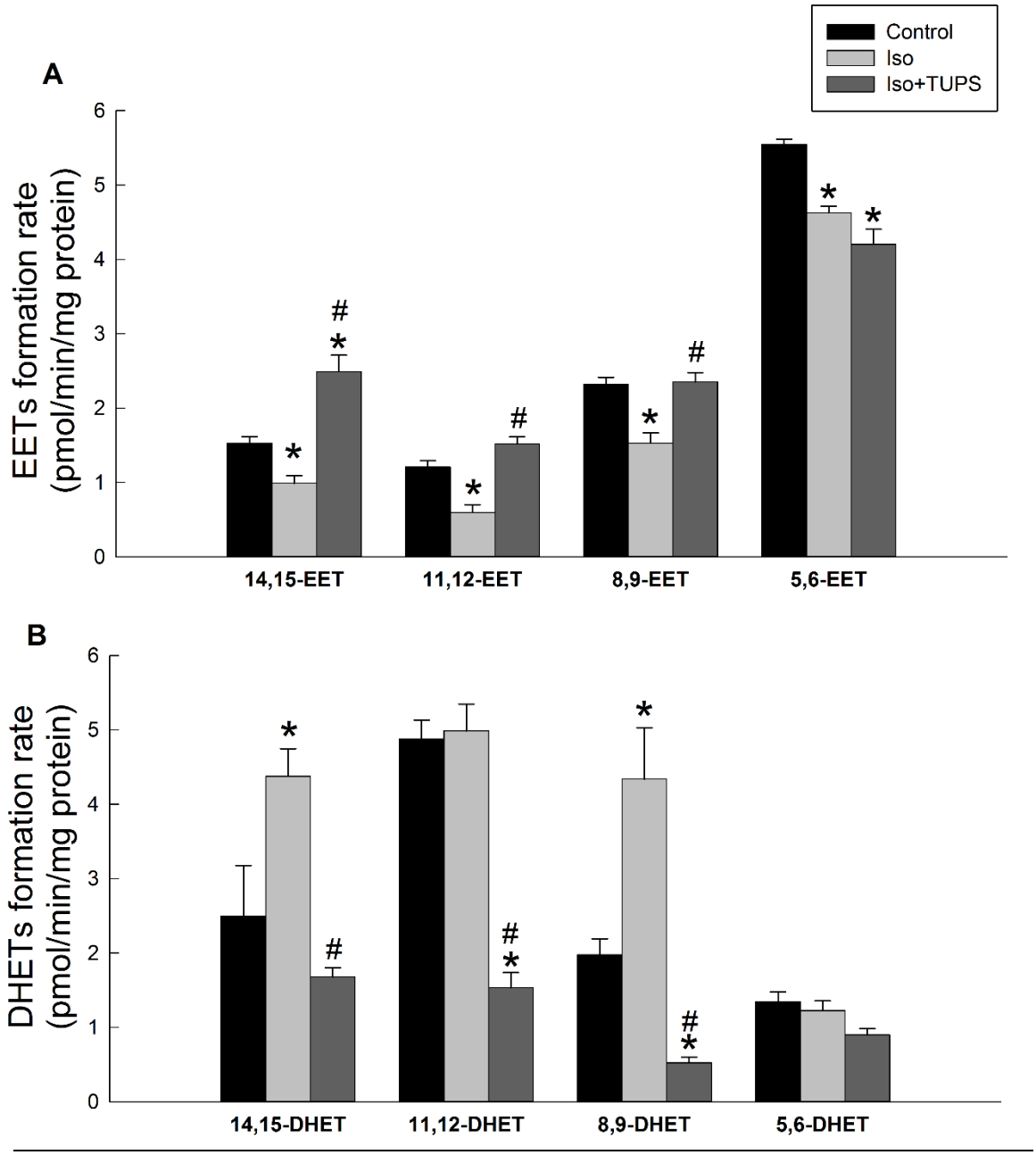

Fig 3.21. Effect of TUPS on CYP-mediated AA metabolism. SD rats received daily injections of vehicles, isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Heart microsomes of treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) EETs and (B) DHETs. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

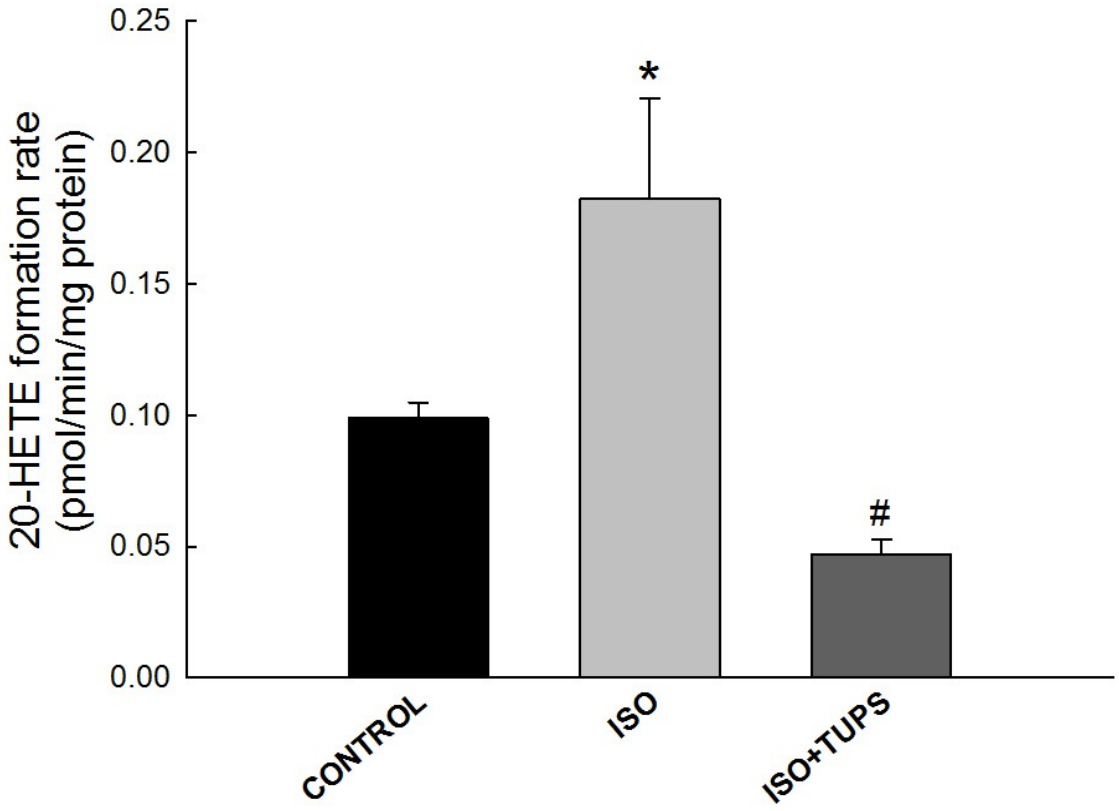

Fig 3.22. Effect of TUPS on 20-HETE formation rate. SD rats received daily injections of vehicles, isoproterenol (5 mg/kg, i.p.) or isoproterenol (5 mg/kg, i.p.) plus TUPS (0.65 mg/kg, p.o.) for 7 days while weight-matched controls received the same volume of 25% PEG400 and saline. Heart microsomes of treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. Metabolites were extracted twice by 1 ml of ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of 20-HETE. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.3 Fenofibrate modulates CYP and AA metabolism in the heart and protects against isoproterenol-induced cardiac hypertrophy

3.3.1 Effect of fenofibrate treatment on CYP gene expression in the heart, kidney, and liver

To examine the effect of fenofibrate on CYP gene expression, total RNA was extracted from the heart, kidney, and liver of control and fenofibrate-treated rats. Thereafter, the expression of different CYP genes was measured using reverse transcription followed by real-time PCR.

Our results demonstrated that fenofibrate treatment causes a significant increase in CYP2B1 and CYP2B2 gene expression in the heart by 100% and 80% respectively. In the liver, fenofibrate treatment significantly induced CYP2B1 and CYP2B2 gene expression by 7200% and 60%, respectively. However, neither CYP2B1 nor CYP2B2 mRNA levels were altered in the kidney (Fig 3.23).

Likewise, fenofibrate treatment caused a significant induction of CYP2C11 and CYP2C23 mRNA levels in the heart by 80% and 230%, respectively compared to control group. However, no significant changes were observed in either kidney or liver (Fig 3.24).

With respect to CYP2J subfamily, a significant induction of CYP2J4 mRNA level was observed in the heart and liver of fenofibrate-treated rats by 50% and 300 %, respectively, whereas CYP2J3 mRNA was significantly induced only in the liver of fenofibrate-treated rats by 100% (Fig 3.25).

Regarding CYP ω-hydroxylases, fenofibrate treatment significantly decreased CYP4A3 gene expression by 170% in the heart of fenofibrate-treated rats,

whereas its expression was significantly increased in the kidney and liver of fenofibrate-treated rats by 100% and 300%, respectively (Fig 3.26A). In contrast, fenofibrate treatment caused a significant induction of CYP4F4 gene expression in the heart by 50%, but not in the kidney or the liver of fenofibrate treated rats (Fig 3.26B).

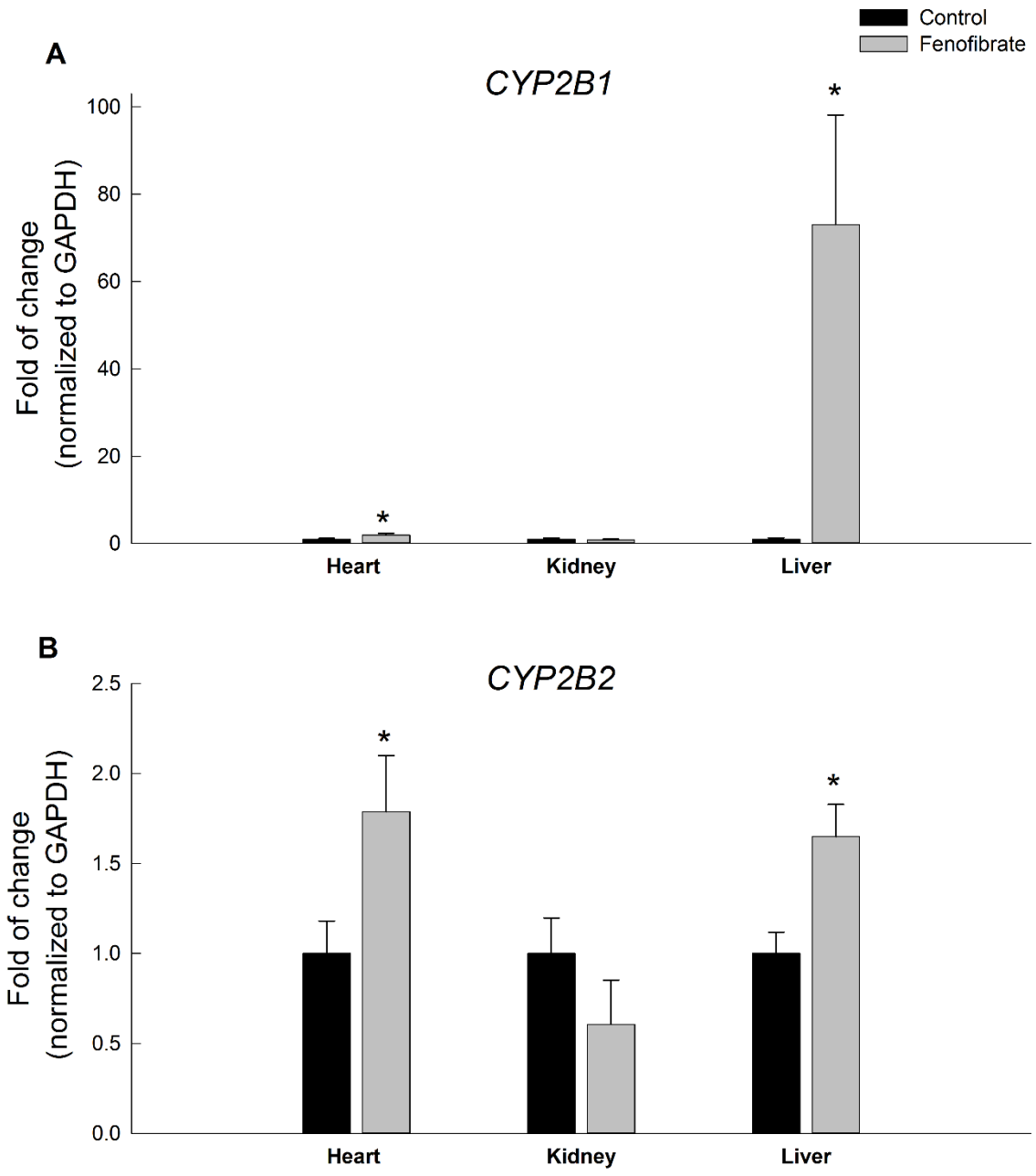

FIG 3.23. Effect of fenofibrate on CYP2B subfamily gene expression in the heart, kidney, and liver. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Total RNA was isolated from the heart, kidney, and liver of control and fenofibrate-treated rats, and the relative gene expression of (A) CYP2B1 and (B) CYP2B2 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

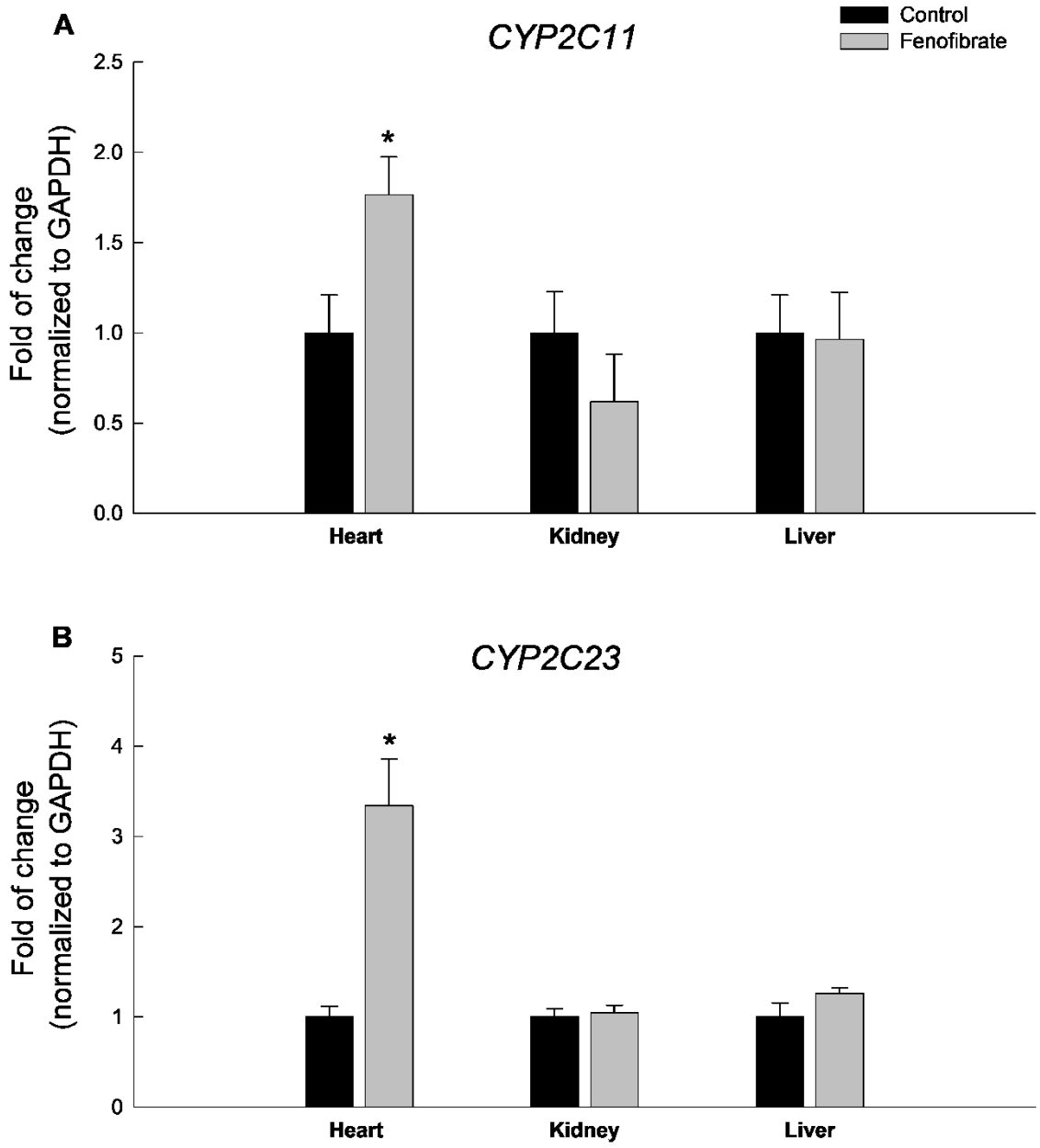

FIG 3.24. Effect of fenofibrate on CYP2C subfamily gene expression in the heart, kidney, and liver. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Total RNA was isolated from the heart, kidney, and liver of control and fenofibrate-treated rats, and the relative gene expression of (A) CYP2C11 and (B) CYP2C23 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

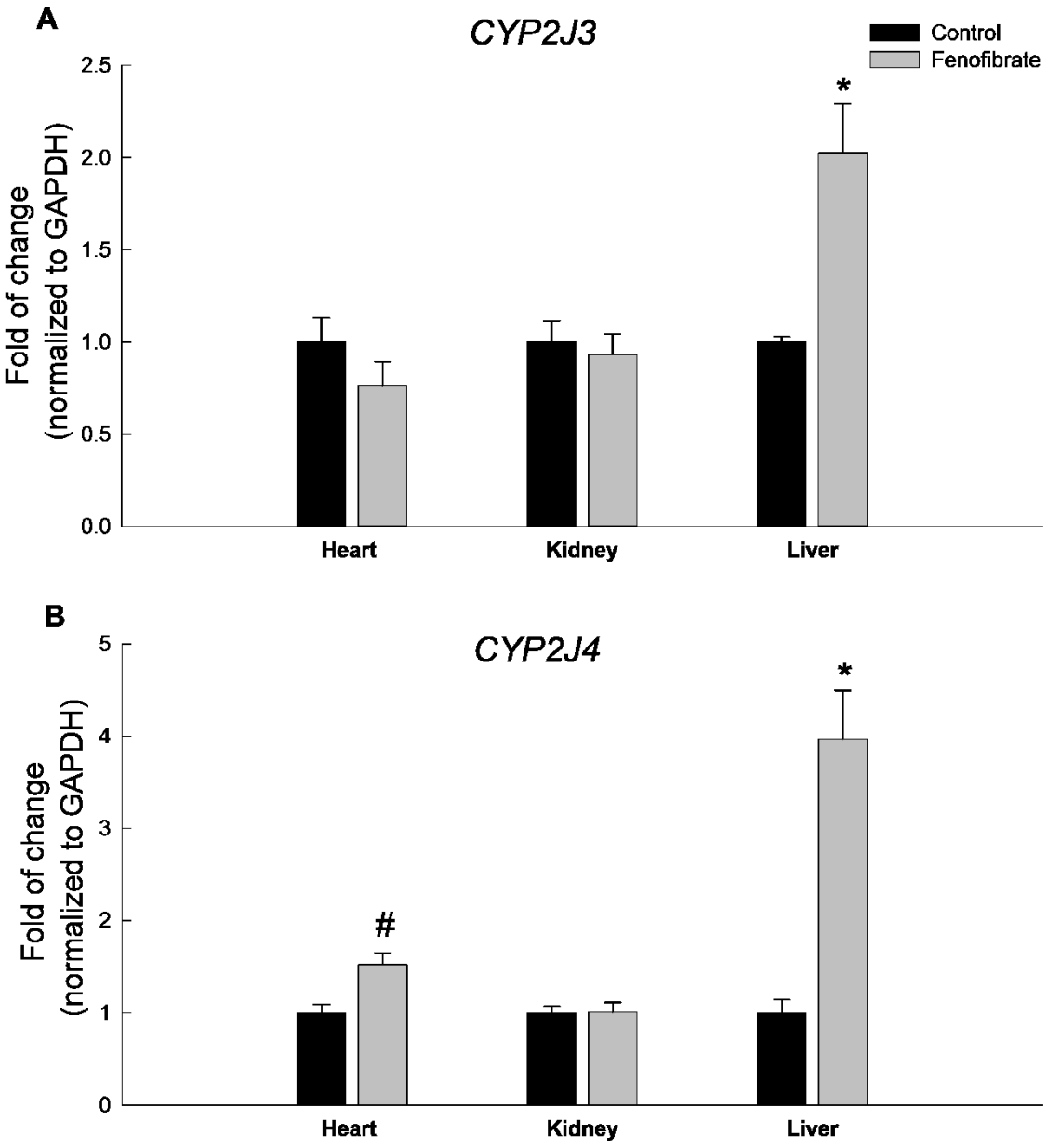

FIG 3.25. Effect of fenofibrate on CYP2J subfamily gene expression in the heart, kidney, and liver. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Total RNA was isolated from the heart, kidney, and liver of control and fenofibrate-treated rats, and the relative gene expression of (A) CYP2J3 and (B) CYP2J4 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

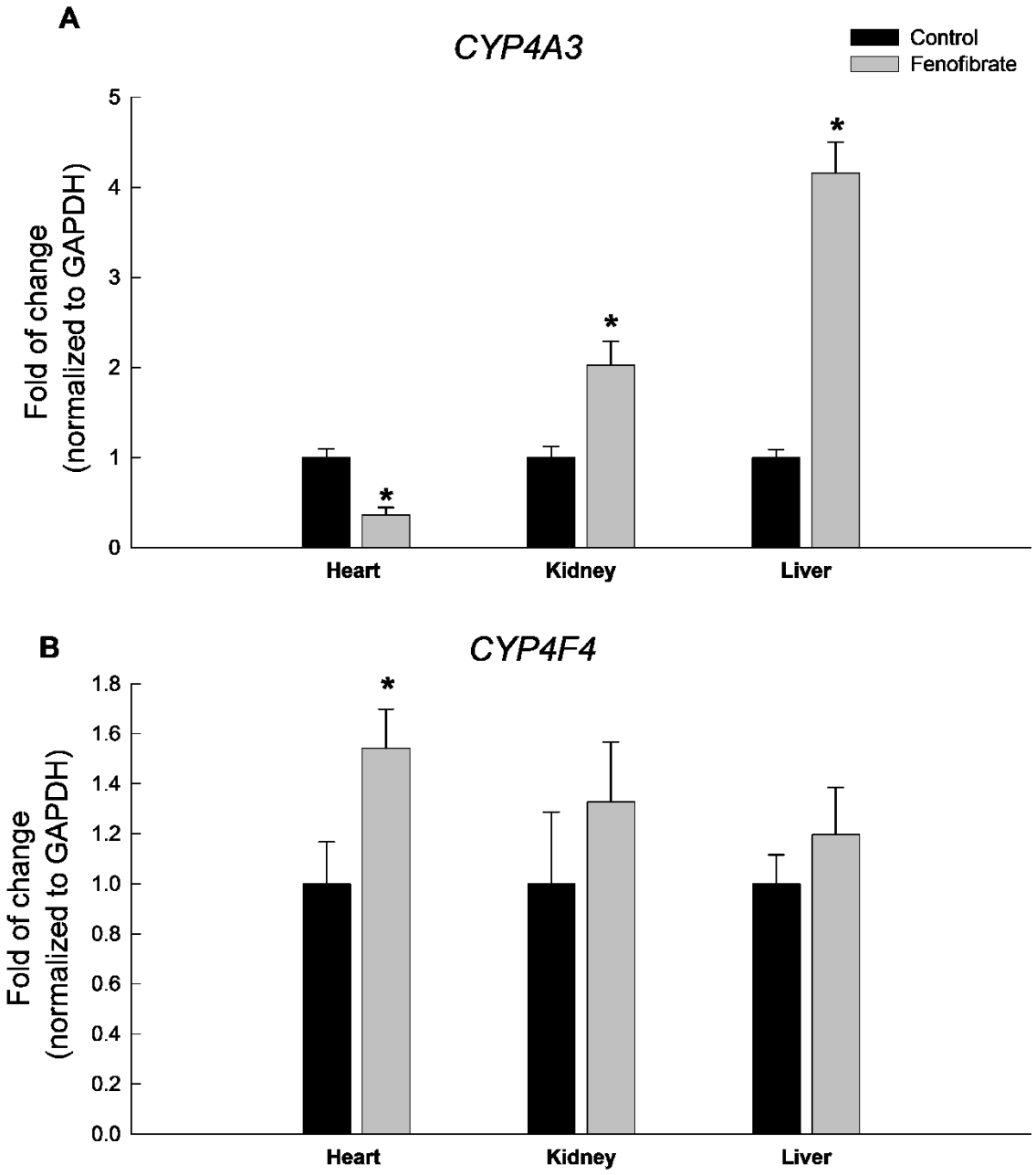

FIG 3.26. Effect of fenofibrate on CYP4 family gene expression in the heart, kidney, and liver. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Total RNA was isolated from the heart, kidney, and liver of control and fenofibrate-treated rats, and the relative gene expression of (A) CYP4A3 and (B) CYP4F4 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

3.3.2 Effect of fenofibrate treatment on CYP protein expression in the heart

To investigate whether the changes in CYP gene expression were further translated into protein, the microsomal protein was prepared from the heart of control and fenofibrate-treated rats. Thereafter, CYP2B1/2, CYP2C11, CYP2C23, and CYP2J protein levels were determined using Western blot analysis.

Similar to the mRNA level, fenofibrate treatment significantly increased the protein level of CYP2B1/2, CYP2C11, CYP2C23, and CYP2J by 190%, 210%, 240%, and 260%, respectively (Fig 3.27). In contrast, fenofibrate treatment caused a significant decrease in CYP4F protein expression by 130%, whereas no significant changes were observed in CYP4A protein expression (Fig 3.27).

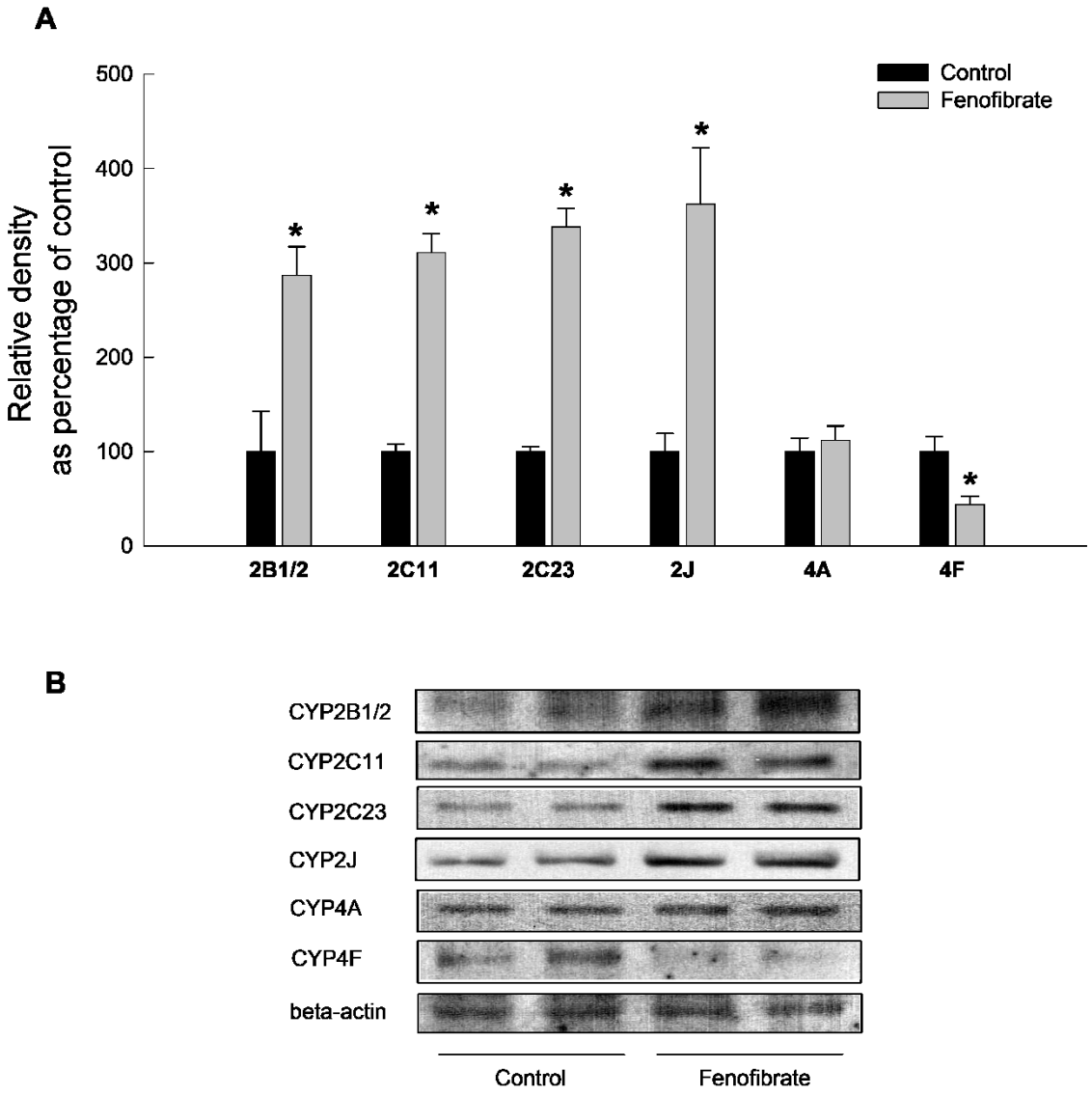

FIG 3.27. Effect of fenofibrate on CYP protein expression in the heart. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Heart microsomal protein was isolated from the hearts of control and fenofibrate-treated rats. Thereafter, microsomal protein was separated on a 10% SDS-PAGE. (A) Relative protein expression of CYP is normalized to the expression of β-actin and the results are expressed as percentage of the control (mean ± SEM, n = 4). (B) Representative Western blots are shown. Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

3.3.3 Effect of fenofibrate treatment on sEH at gene, protein, and activity levels

sEH enzyme is the major determinant of the level of EETs. Therefore, we determined the effect of fenofibrate treatment on the expression of the EPHX2 gene, which encodes for the sEH enzyme. Our results showed that fenofibrate treatment causes a significant induction of EPHX2 gene expression in the kidney and the liver by 140% and 300%, respectively (Fig 3.28). However, fenofibrate did not cause any significant changes in the mRNA, protein, and activity of sEH in the heart (Fig. 3.29).

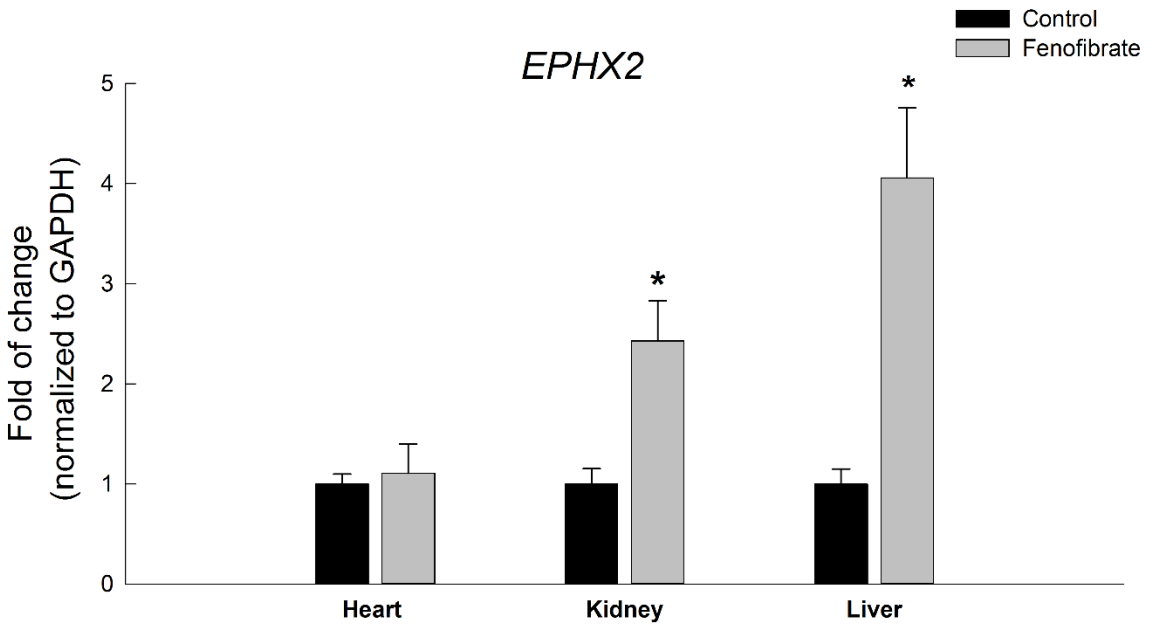

FIG 3.28. Effect of fenofibrate on EPHX2 gene expression in the heart, kidney, and liver. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Total RNA was isolated from the heart, kidney, and liver of control and fenofibrate-treated rats, and the relative gene expression of EPHX2 was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

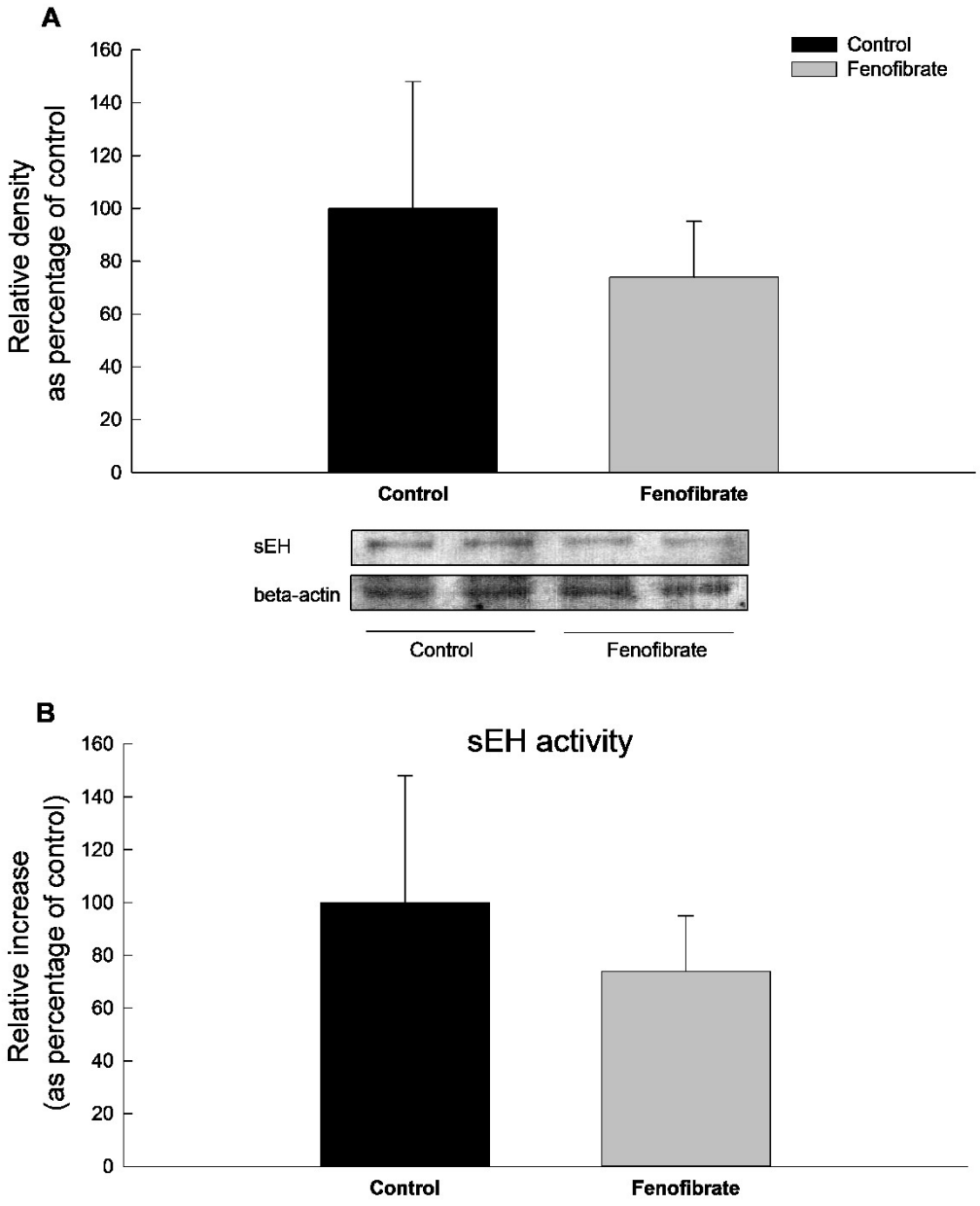

FIG 3.29. Effect of fenofibrate on sEH at protein and activity levels in the heart. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. (A) sEH protein level was determined by Western blot analysis, (B) sEH activity was determined using the sEH assay. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

3.3.4 Effect of fenofibrate treatment on CYP-mediated AA metabolism

To investigate the effect of fenofibrate treatment on CYP-derived AA metabolites, heart microsomes of control and fenofibrate-treated rats were incubated with AA for 30 minutes. Thereafter, AA metabolites were determined using LC-ESI-MS. Our results showed that fenofibrate treatment significantly increases the formation rate of 8,9-, 11,12-, and 14,15-EET by 90%, 90%, and 100%, respectively, in heart microsomes (Fig 3.30A). However, the formation rates of 5,6-EET and DHETs were not significantly altered (Figs 3.30). Moreover, the effect of fenofibrate treatment on the total epoxygenase activity was calculated as the sum of total EETs and DHETs in the control and fenofibrate-treated rats. Our results showed that fenofibrate significantly increases the total epoxygenase activity in the heart microsomes by 110% compared to the control microsomes (Fig 3.31).

To determine the effect of fenofibrate treatment on CYP ω-hydroxylase activity, we measured the formation rate of 20-HETE in heart microsomes from the control and fenofibrate-treated rats. Fenofibrate treatment significantly decreased the 20-HETE formation by 100% in comparison with the control microsomes (Fig 3.32).

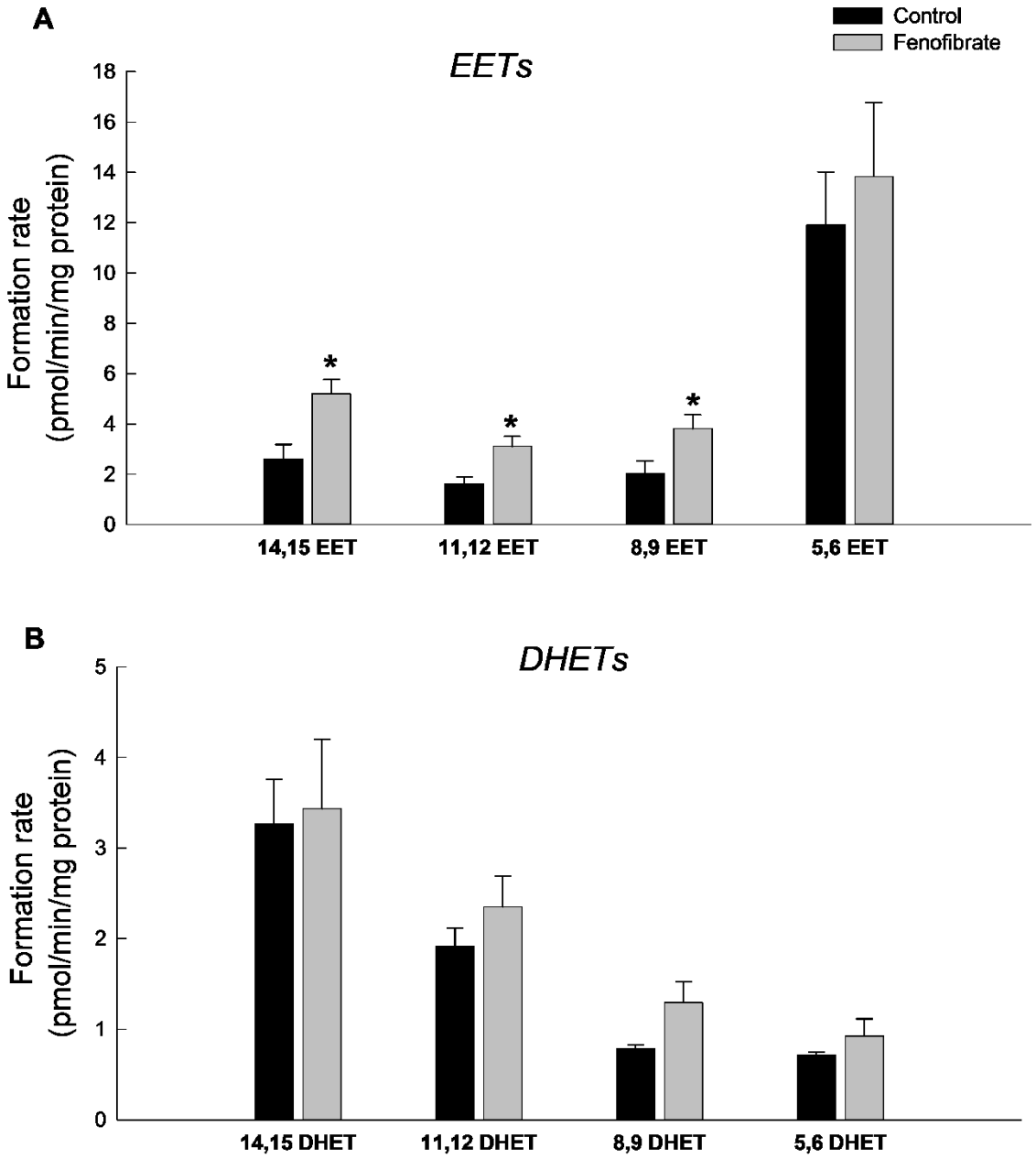

Fig 3.30. Effect of fenofibrate on CYP-mediated AA metabolism. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Heart microsomes of control and fenofibrate-treated rats were incubated with 50 µM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 minutes. The reaction was terminated by the addition of ice-cold acetonitrile. Metabolites were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of (A) EETs and (B) DHETs. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

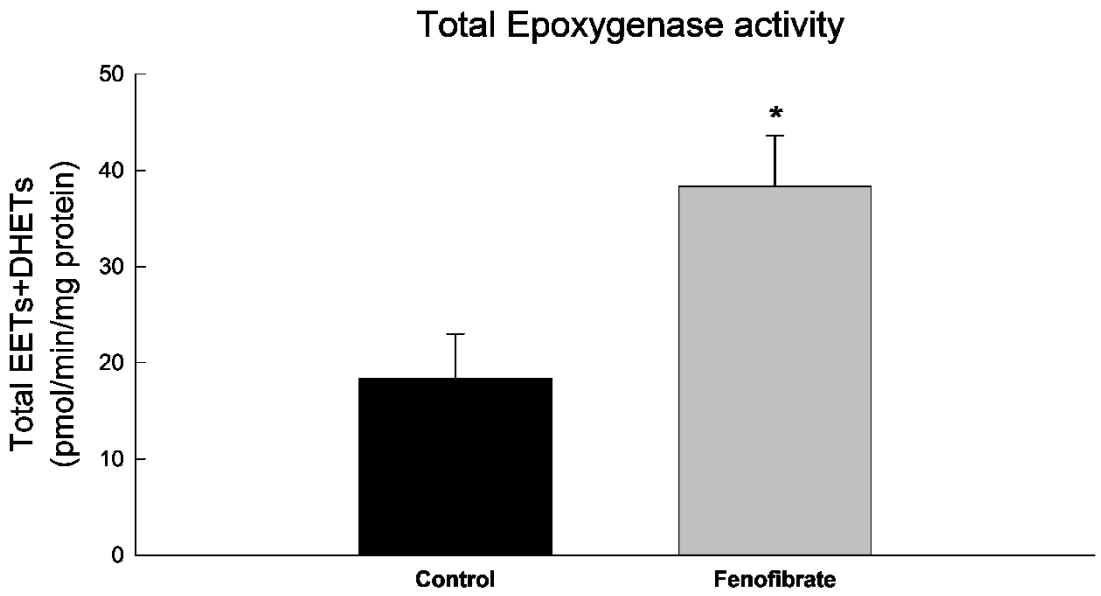

Fig 3.31. Effect of fenofibrate on total epoxygenase activity. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Heart microsomes of control and fenofibrate-treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 minutes. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum. Residues were reconstituted in acetonitrile and injected into an LC-ESI-MS instrument for the determination of total epoxygenase activity. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

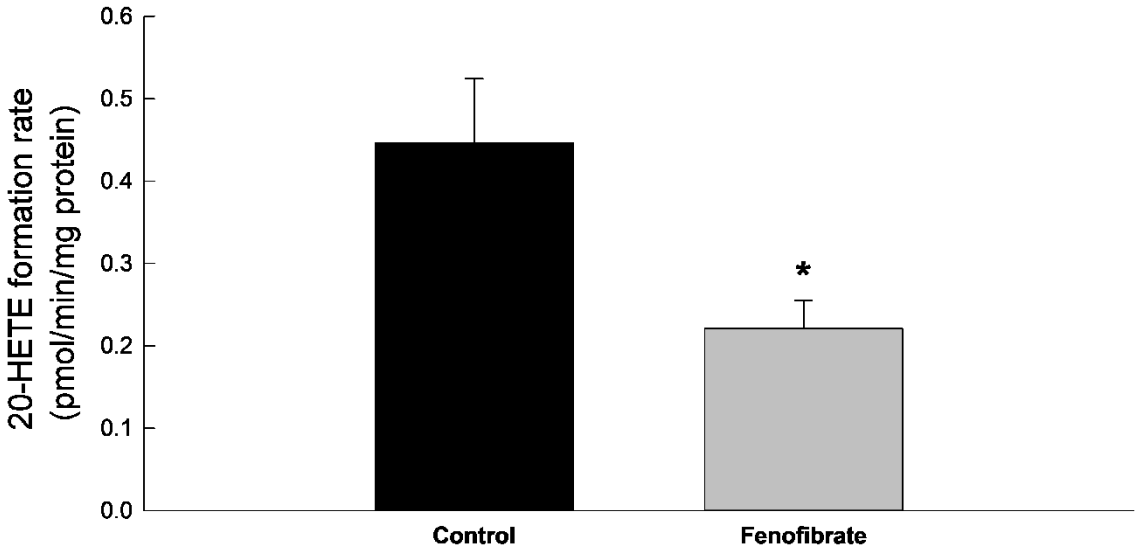

Fig 3.32. Effect of fenofibrate on 20-HETE formation rate. SD rats received daily injections of either 1% methylcellulose or fenofibrate (30 mg/kg, oral), for 7 days. Heart microsomes of control and fenofibrate-treated rats were incubated with 50 μM AA. The reaction was started by the addition of 1 mM NADPH and lasted for 30 minutes. The reaction was terminated by the addition of ice-cold acetonitrile. Metabolites were extracted twice by 1 mL of ethyl acetate and dried using speed vacuum. Reconstituted metabolite was injected into LC-ESI-MS for the determination of 20-HETE. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). Unpaired, two-tailed t-test (* $P < 0.05$ compared to control).

3.3.5 Effect of fenofibrate treatment on the cardiac hypertrophy induced by isoproterenol

To investigate whether the fenofibrate-mediated induction of CYP epoxygenases would confer cardioprotection in isoproterenol-induced cardiac hypertrophy in rats, we measured the gene expression of the hypertrophic markers ANP and BNP. Our results showed that isoproterenol treatment caused significant increases in the gene expression of ANP and BNP by 1900% and 80%, respectively (Fig 3.33). On the other hand, fenofibrate treatment significantly decreased the isoproterenol-mediated induction of ANP and BNP by 60% and 50%, respectively (Fig 3.33). In addition, fenofibrate treatment alone did not alter the gene expression of ANP and BNP. Moreover, isoproterenol significantly increased the HW/BW ratio by 40%, whereas treatment with fenofibrate significantly decreased the isoproterenol-mediated increase in the HW/BW ratio by 10%, compared to isoproterenol alone. Furthermore, no significant difference was observed between control and fenofibrate-treated rats (Fig 3.33).

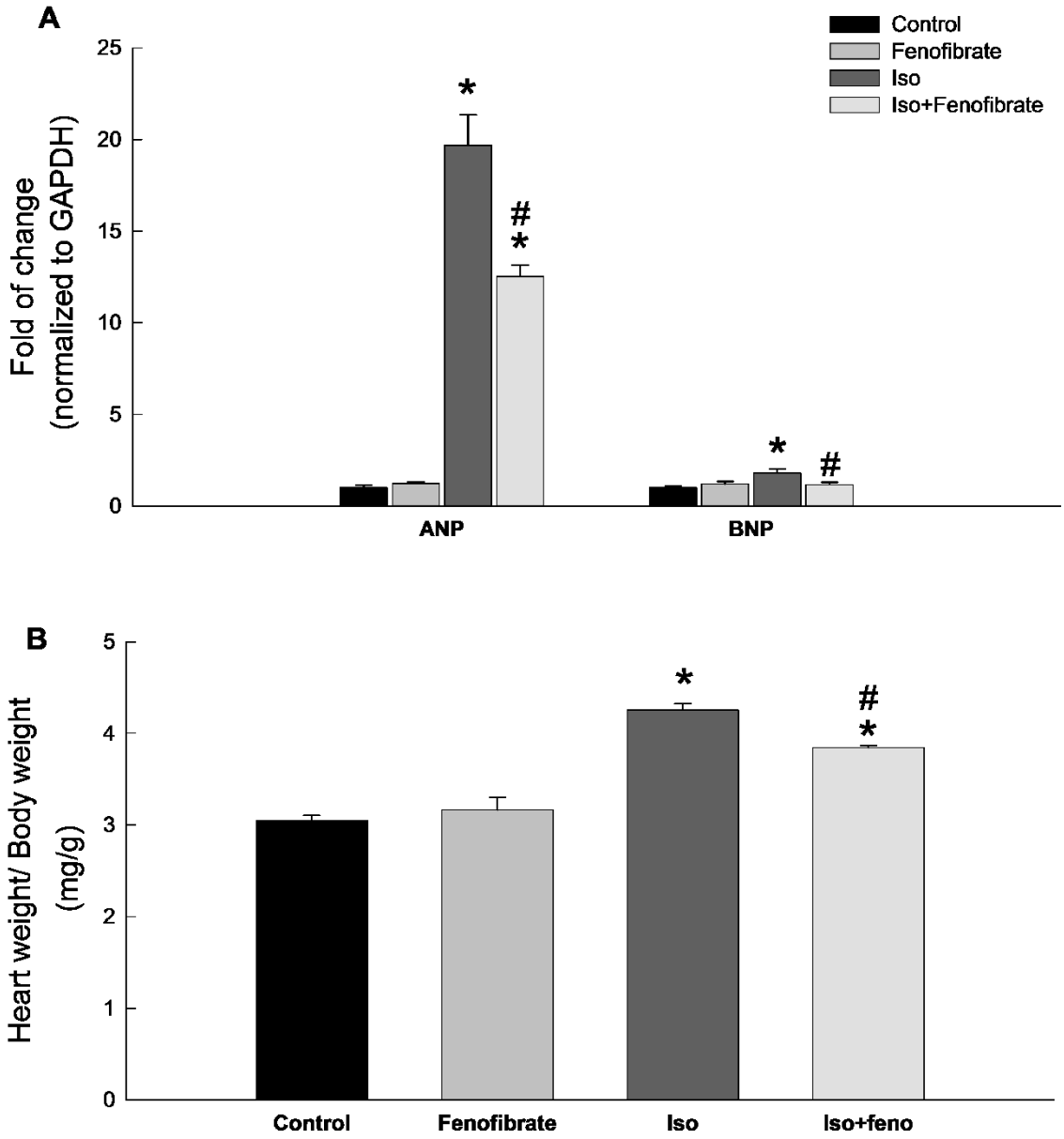

FIG 3.33. Effect of fenofibrate on the cardiac hypertrophy induced by isoproterenol. SD rats received daily injections of vehicles, fenofibrate (30 mg/kg, oral), isoproterenol (mini osmotic pump, 4.2 mg/kg/day), or isoproterenol (mini osmotic pump, 4.2 mg/kg/day) plus fenofibrate (30 mg/kg, oral) for 7 days, whereas weight-matched controls received the same volume of 1% methylcellulose and saline. (A) Total RNA was isolated from the heart of treated rats, and the relative gene expression of ANP and BNP was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± SEM (n = 6). (B) HW/BW ratio was determined for each animal after 7 days of treatment. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated rats).

3.4 Role of CYP epoxygenases and their associated AA metabolites in the initiation of cardiac hypertrophy

3.4.1 Effect of fenofibrate in the initiation of cellular hypertrophy by isoproterenol

To investigate the role of CYP epoxygenases in the initiation of cellular hypertrophy by isoproterenol, RL-14 cells were pretreated with fenofibrate, a CYP epoxygenases inducer, 2 hours prior to isoproterenol treatment in the presence and absence of a selective epoxygenases inhibitor, MS-PPOH.

3.4.1.1 Effect of fenofibrate and MS-PPOH on RL-14 cell viability

RL-14 cells were exposed to different concentrations of fenofibrate and MS-PPOH for 24 hours. In addition, cells were exposed to combination of isoproterenol (100 μM), fenofibrate (10 μM), and MS-PPOH (5 μM). Thereafter, cell viability was determined by the MTT assay. This assay showed that the maximum nontoxic concentration of fenofibrate and MS-PPOH was 10 μM and no toxicity was observed in the combination of these agents with isoproterenol (Figs 34 and 35). Therefore, the observed changes were not due to the decreased cell viability or toxicity.

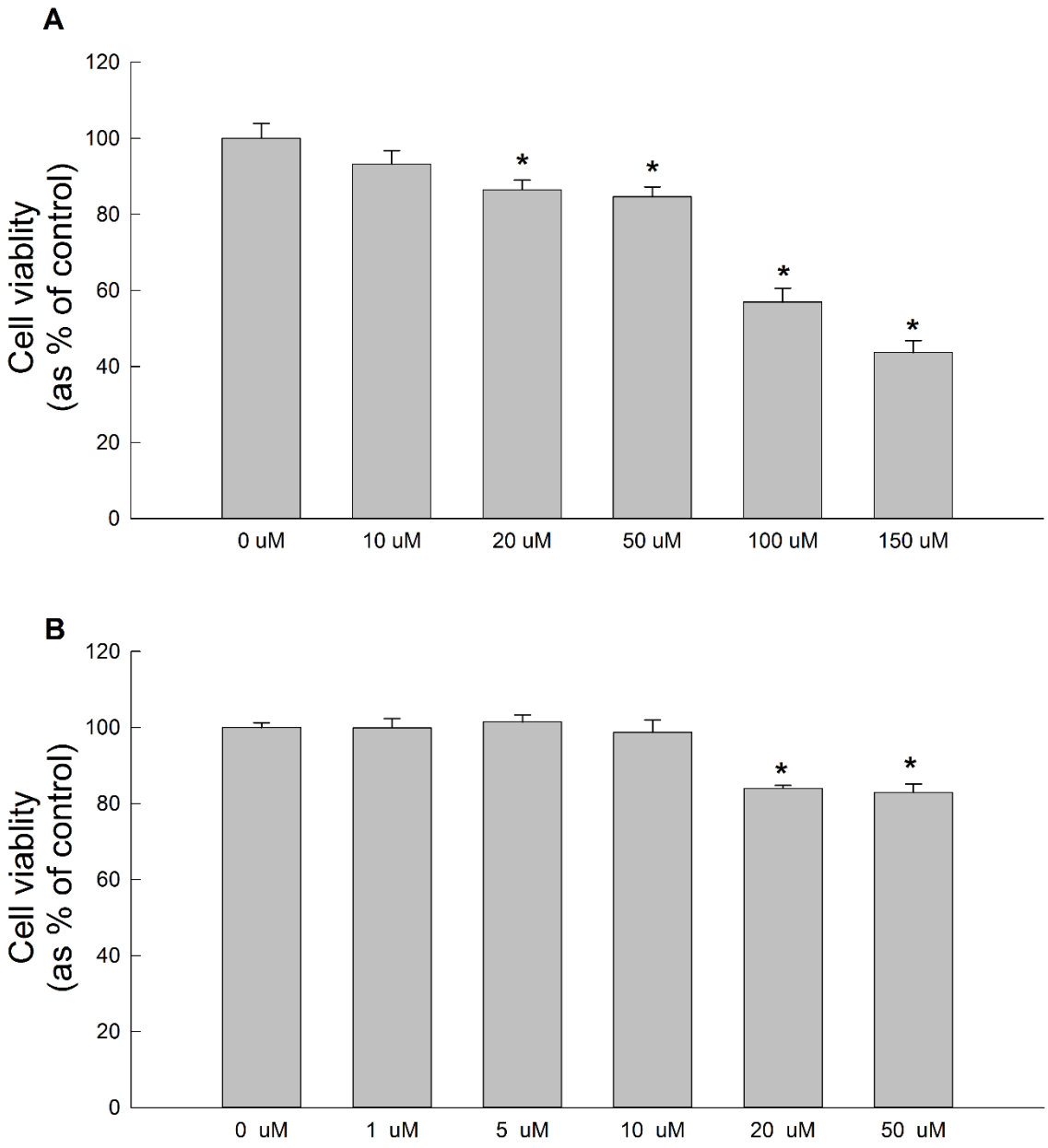

Fig 3.34. Effect of MS-PPOH and fenofibrate on RL-14 cell viability. RL-14 cells were incubated with increasing concentrations of (A) MS-PPOH and (B) fenofibrate. Thereafter, the cell viability was measured by the MTT assay and data are presented as a percentage of control (mean ± S.E.M, n=6). Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

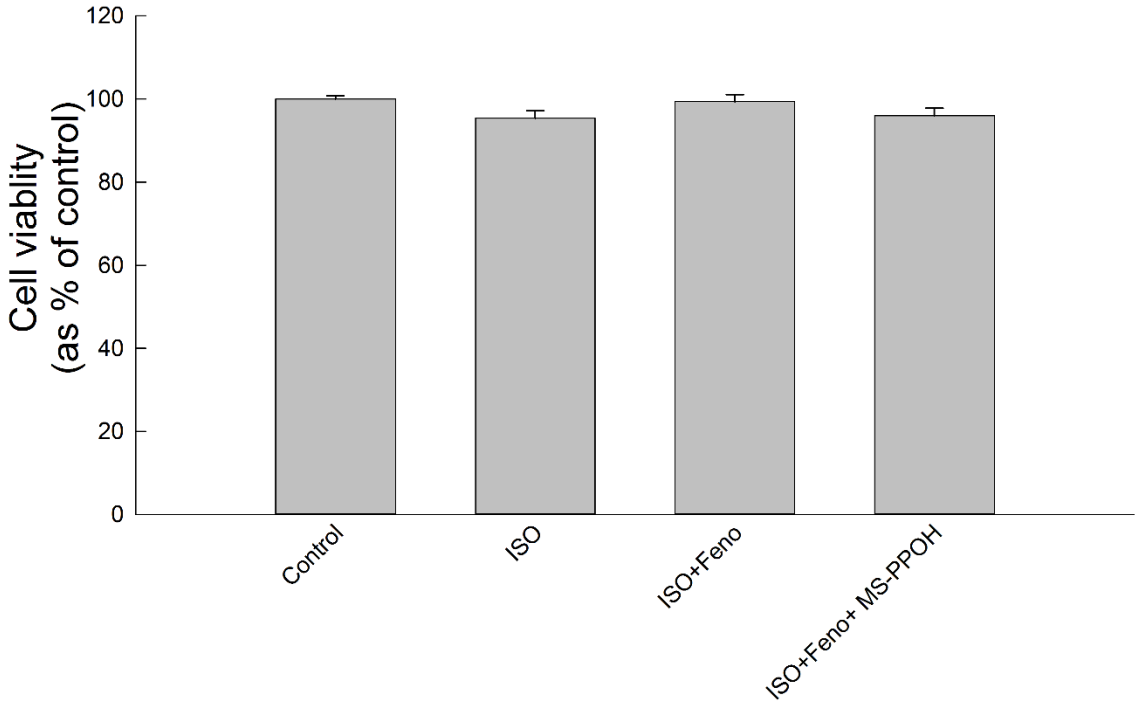

Fig 3.35. Effect of combination treatments of isoproterenol, MS-PPOH, and fenofibrate on RL-14 cell viability. RL-14 cells were incubated with isoproterenol (100 µM), fenofibrate (10 µM), and MS-PPOH (5 µM). Thereafter, the cell viability was measured by the MTT assay and data are presented as a percentage of control (mean ± S.E.M, n=6). Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.4.1.2 Effect of fenofibrate on the initiation of cellular hypertrophy by isoproterenol

Figures 3.36 and 3.37 show that pretreatment of RL-14 cells with fenofibrate prevented the increase in the gene expression of ANP and cell size mediated by isoproterenol. Interestingly, the effect of fenofibrate was abolished by co-treatment with MS-PPOH, a selective epoxygenases inhibitor, suggesting an epoxygenase-dependent mechanism (Fig 3.36).

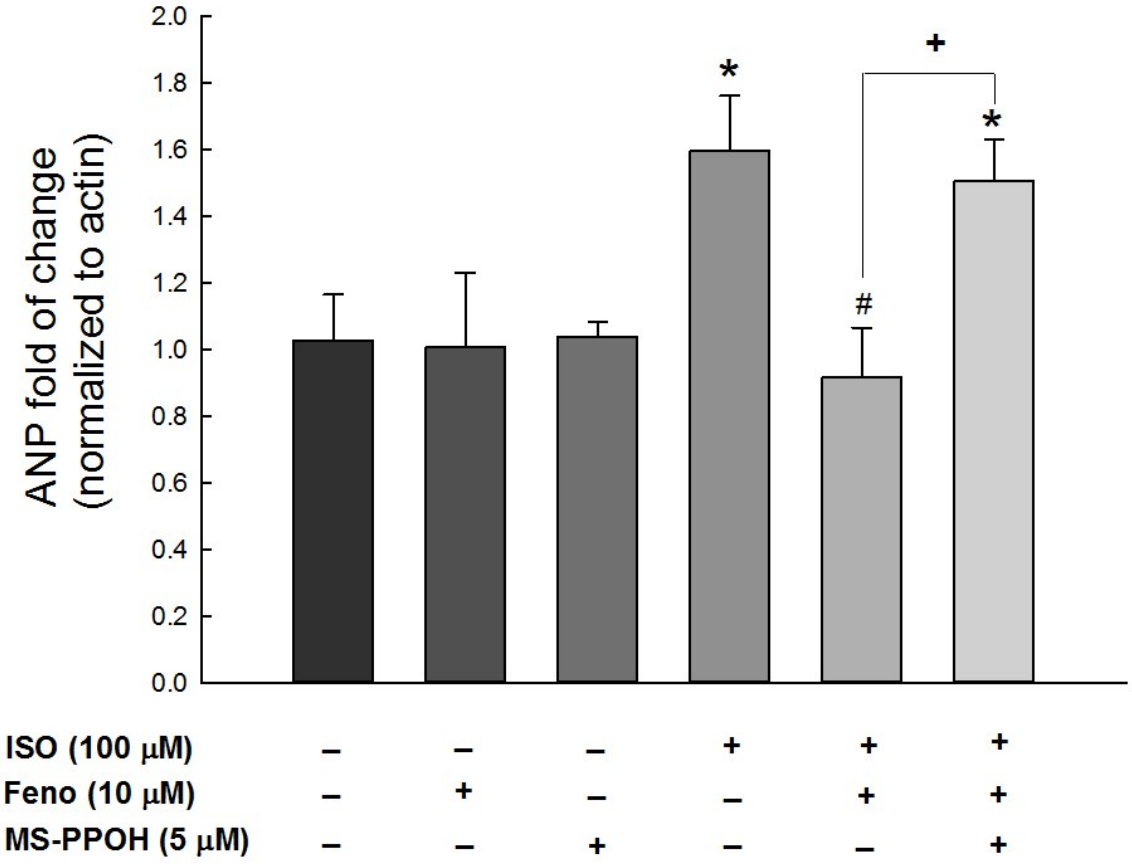

Fig 3.36. Effect of fenofibrate on the initiation of cellular hypertrophy by isoproterenol. Total RNA was isolated from RL-14 cells pretreated with vehicle, fenofibrate (10 μM), or MS-PPOH (5 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. Thereafter, gene expression of ANP was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (*P < 0.05 compared to control; #P < 0.05 compared to isoproterenol; +P < 0.05 compared to isoproterenol + fenofibrate group).

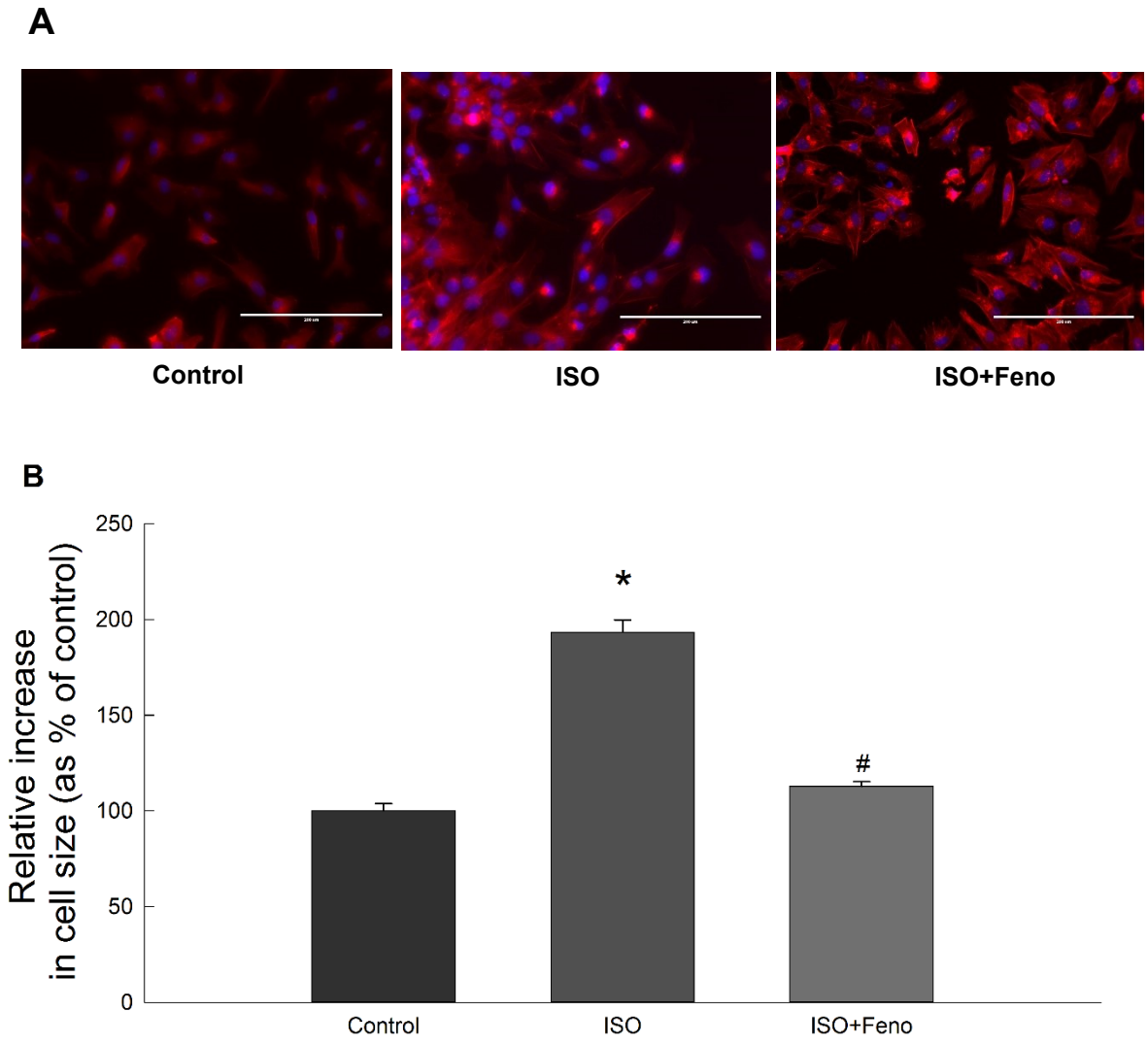

Fig 3.37. Effect of fenofibrate on the initiation of cellular hypertrophy by isoproterenol. RL-14 cells were pretreated with vehicle, fenofibrate (10 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. (A) Representative cell images from RL-14 cells staining with Texas red phalloidin (red) and DAPI (blue) as described in method. (B) Cell size was measured by imaging the complete boundary of individual cells after magnification. Values are the mean ± S.E.M. of at least 30 cells from different images per treatment. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated cells).

3.4.2 Role of CYP2J2 on the initiation of cellular hypertrophy by isoproterenol

To further evaluate the role of CYP epoxygenases in the initiation of cellular hypertrophy, RL-14 cells were transiently transfected with CYP2J2, a predominant CYP epoxygenase in human heart, prior to isoproterenol treatment. Our results showed that pCMVCYP2J2 delivery led to abundant CYP2J2 gene expression (increased by 300% in comparison with control cells). Interestingly, CYP2J2 overexpression abolished the isoproterenol-induced ANP gene expression (Fig 3.38). However, this effect might be due to transfection itself therefore, the effect of mock-transfection should be studied to exclude this possibility.

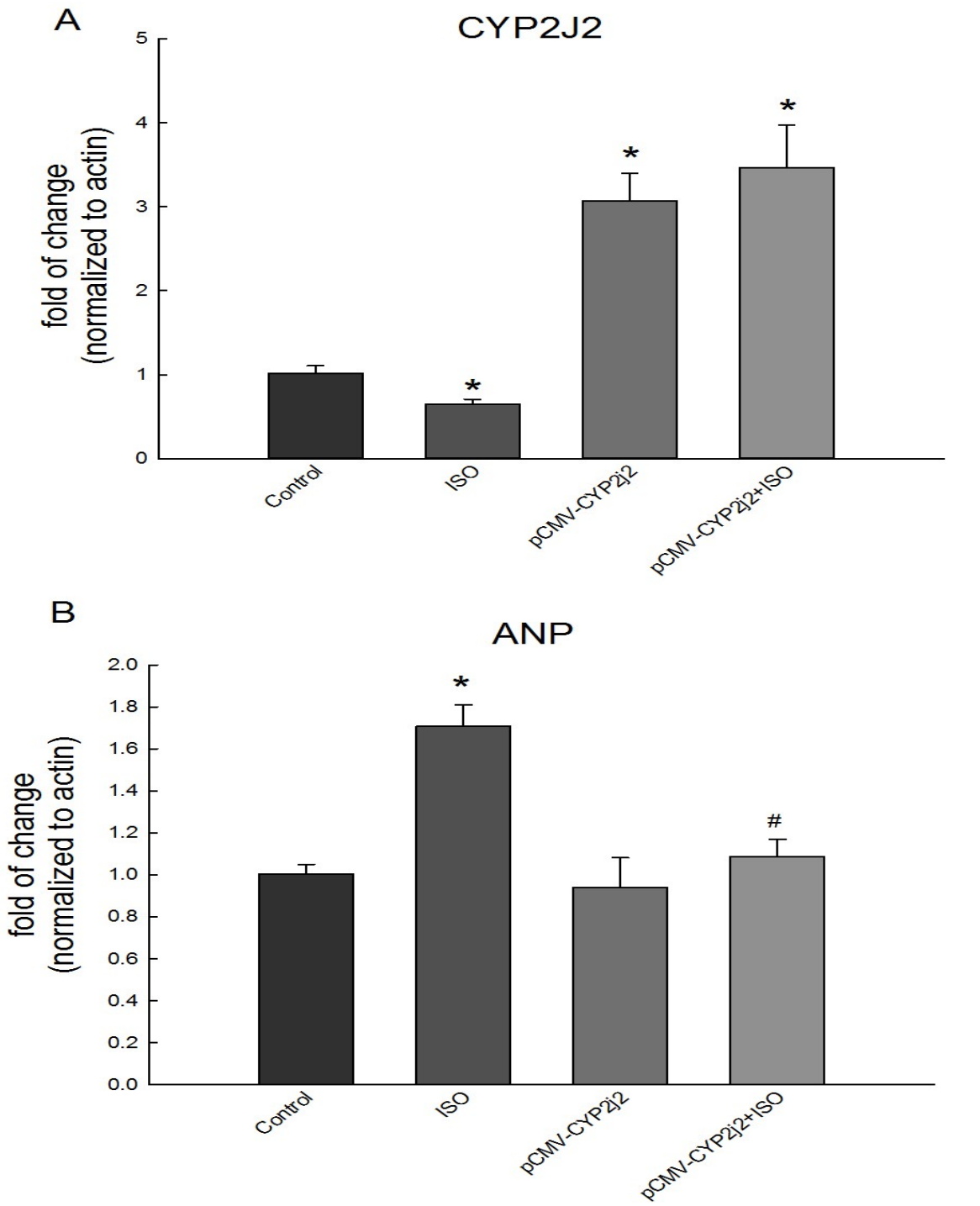

Fig 3.38. Role of CYP2J2 on the initiation of cellular hypertrophy by isoproterenol. Total RNA was isolated from RL-14 cells transfected with pCMV-CYP2J2 for 48 hours prior to isoproterenol treatment (100 μM) for 6 hours. Gene expression of (A) CYP2J2 and (B) ANP was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of four independent experiments ± (n = 4). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated cells).

3.4.3 Role of sEH inhibition and EETs on the initiation of cellular hypertrophy by isoproterenol

3.4.3.1 Effect of TUPS and 11,12-EET on RL-14 cell viability

RL-14 cells were exposed to wide range of concentrations of TUPS and 11,12-EET for 24 hours. In addition, cells were exposed to combination of isoproterenol (100 μM), TUPS (1 μM), and 11,12-EET (1 μM). Thereafter, cell viability was determined by the MTT assay. This assay showed that the maximum nontoxic concentrations of TUPS and 11,12-EET were 20 μM and 10 μM, respectively and no toxicity was observed in the combination of these agents with isoproterenol. Therefore, the observed changes were not due to the decreased cell viability or toxicity (Figs 3.39 and 3.40)

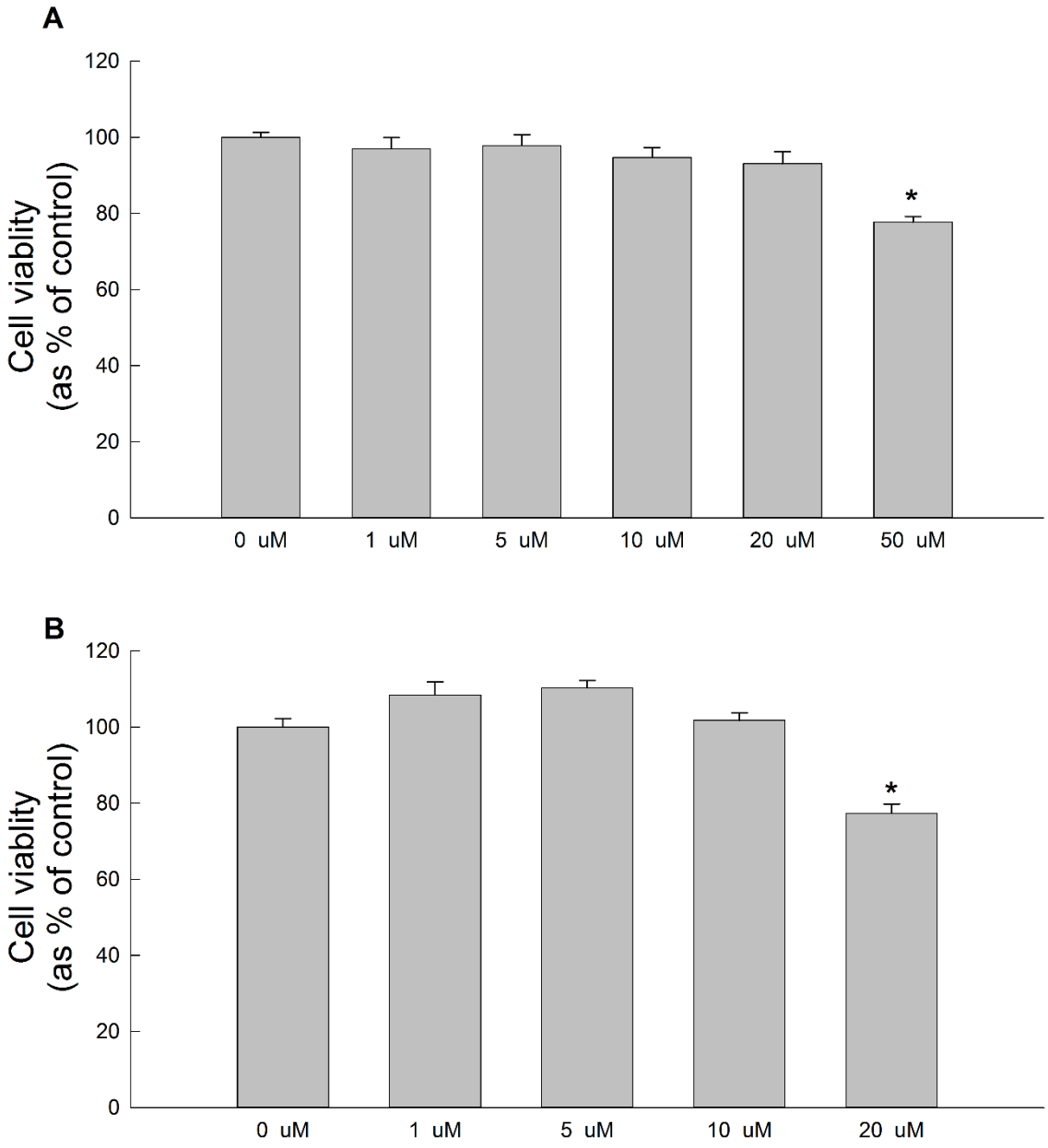

Fig 3.39. Effect of TUPS and 11,12-EET on RL-14 cell viability. RL-14 cells were incubated with increasing concentrations of (A) TUPS and (B) 11,12-EET.Thereafter, the cell viability was measured by the MTT assay and data are presented as a percentage of control (mean ± S.E.M, n=6). Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

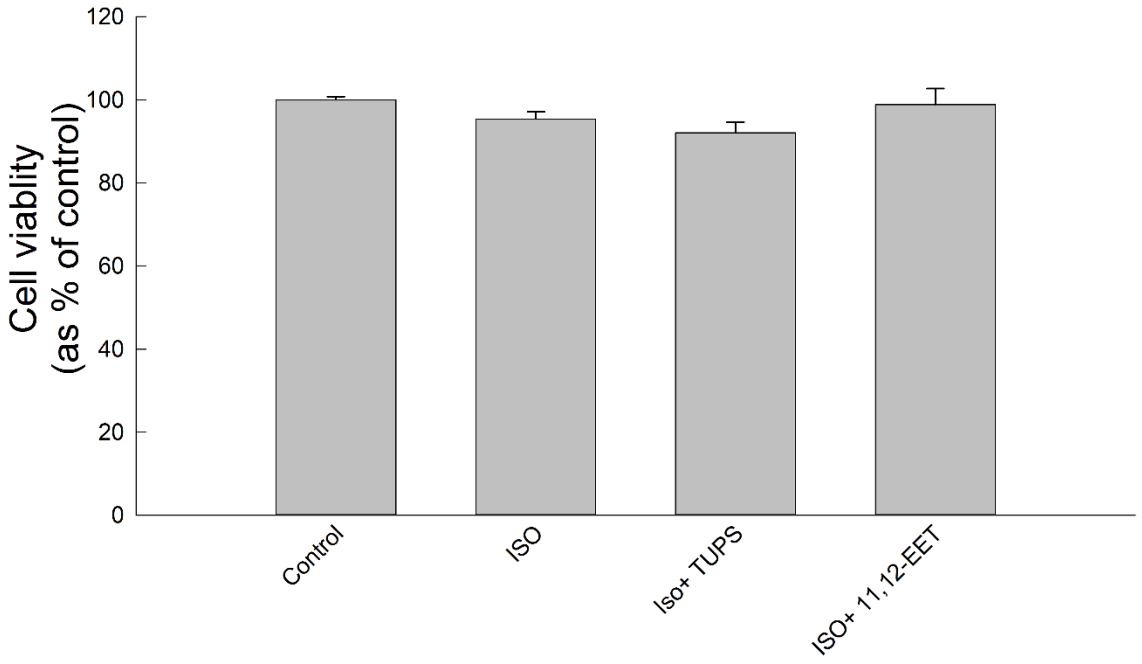

Fig 3.40. Effect of combination treatments of isoproterenol, TUPS, and 11,12-EET on RL-14 cell viability. RL-14 cells were incubated with isoproterenol (100 μM), TUPS (1 μM), and 11,12-EET (1 μM). Thereafter, the cell viability was measured by the MTT assay and data are presented as a percentage of control (mean ± S.E.M, n=6). Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control).

3.4.3.2 Effect of TUPS and 11,12-EET on the initiation of cellular hypertrophy by isoproterenol

To investigate the role of EETs in the initiation of cellular hypertrophy, RL-14 cells were pretreated with either the soluble epoxide inhibitor TUPS or 11,12-EET 2 hours prior to isoproterenol treatment. Figures 3.41 and 3.42 show that pretreatment with either TUPS or 11,12-EET prevented isoproterenol-mediated increase in cell size and ANP gene expression (Fig 3.41 and 3.42).

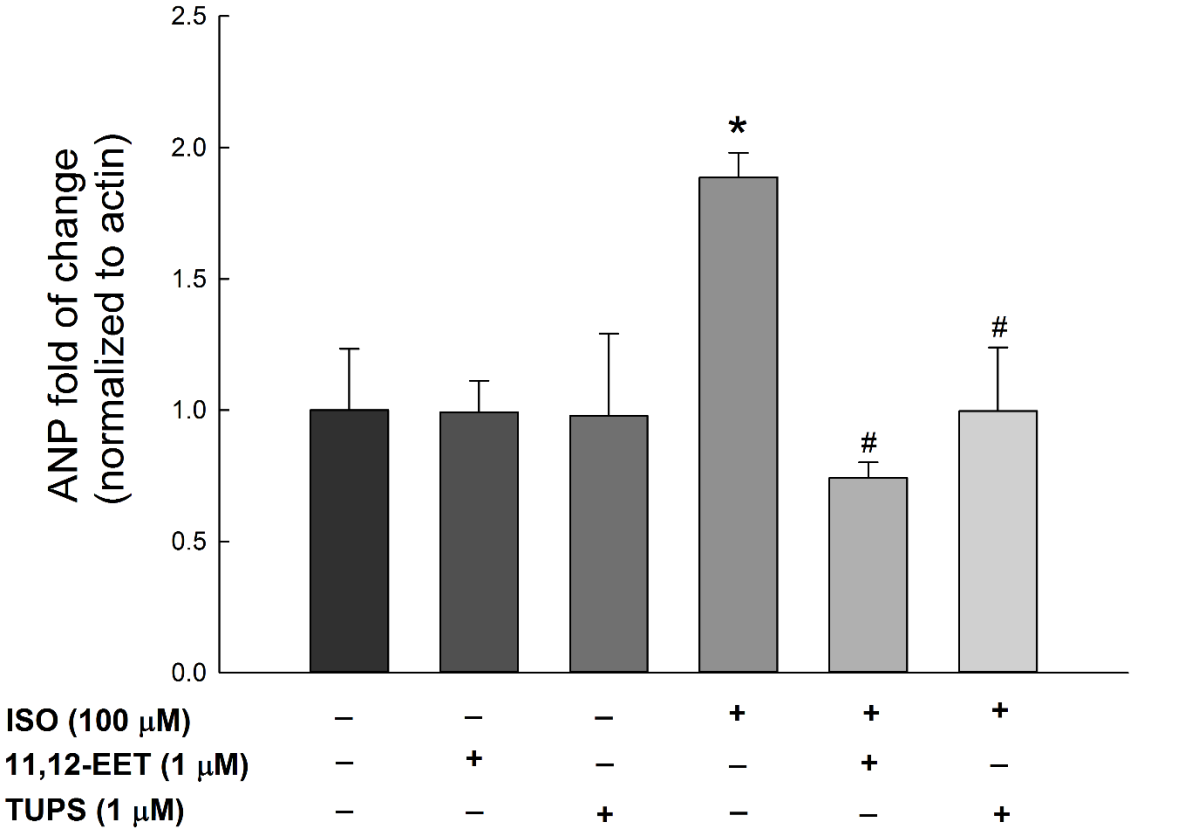

Fig 3.41. Effect of TUPS and 11,12-EET on the initiation of cellular hypertrophy by isoproterenol. Total RNA was isolated from RL-14 cells pretreated with vehicle, TUPS (1 μM), or 11,12-EET (1 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. Thereafter, gene expression of ANP was determined by real-time PCR. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated cells).

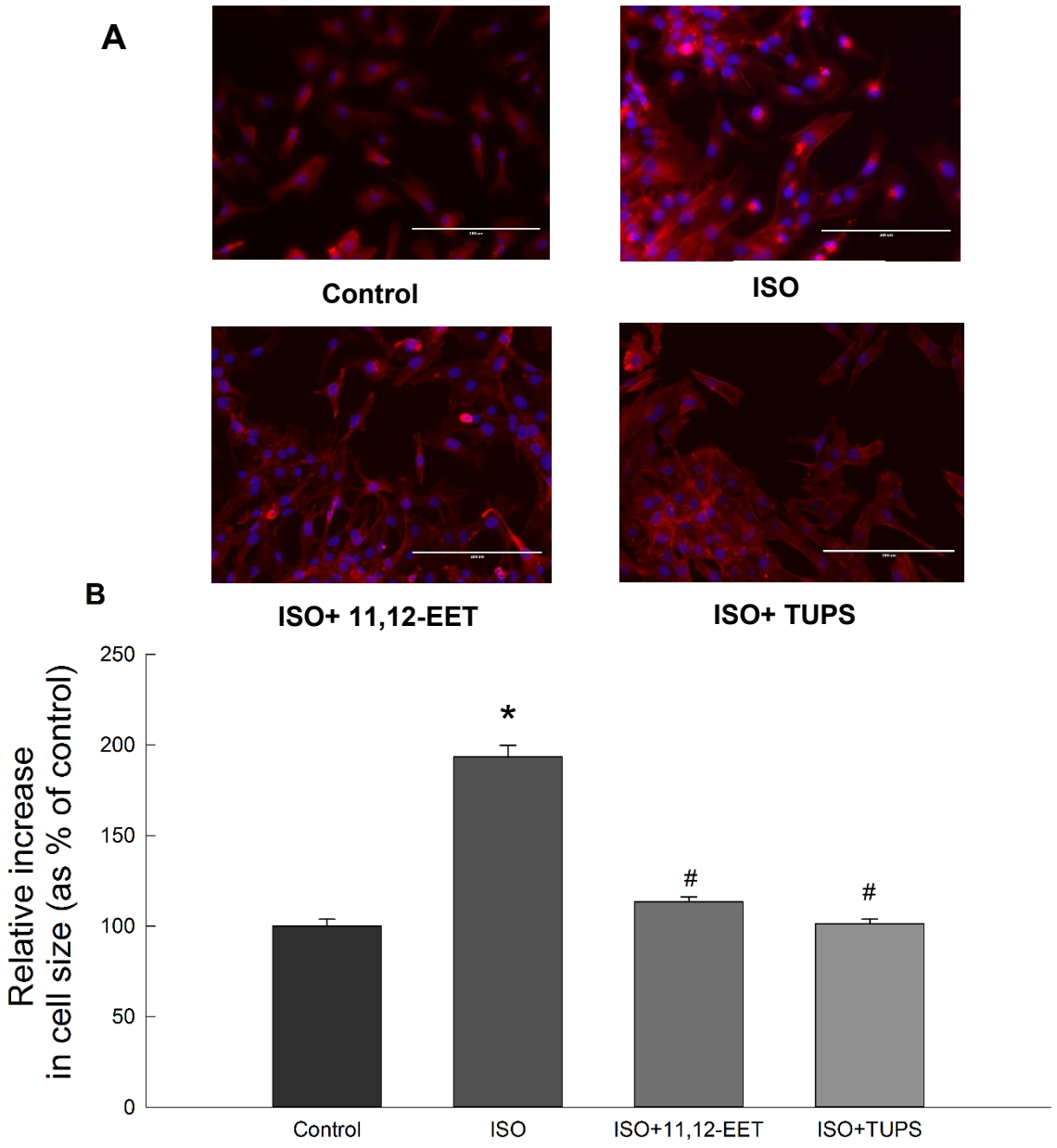

Fig 3.42. Effect of TUPS and 11,12-EET on the initiation of cellular hypertrophy by isoproterenol. RL-14 cells were pretreated with vehicle, TUPS (1 μM), or 11,12-EET (1 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 6 hours. (A) Representative cell images from RL-14 cells staining with Texas red phalloidin (red) and DAPI (blue) as described in method. (B) Cell size was measured by imaging the complete boundary of individual cells after magnification. Values are the mean ± S.E.M. of at least 30 cells from different images per treatment. Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated cells).

3.4.4 Effect of 11,12-EET on isoproterenol-induced NF-kB-binding activity

To investigate the effect of EET on NF-kB activation by isoproterenol, we tested the capacity of 11,12-EET to modulate the binding activity of prototypical NF-κB proteins p65 and p50 using a commercially available kit. Our results showed that isoproterenol significantly increases p50 DNA-binding activity by 30%. However, no significant changes were detected in p65 DNA-binding activity. Although 11,12-EET alone did not alter the binding activity of NF-κB, it significantly abrogated the isoproterenol-mediated induction of p50 DNA-binding activity (Fig 3.43).

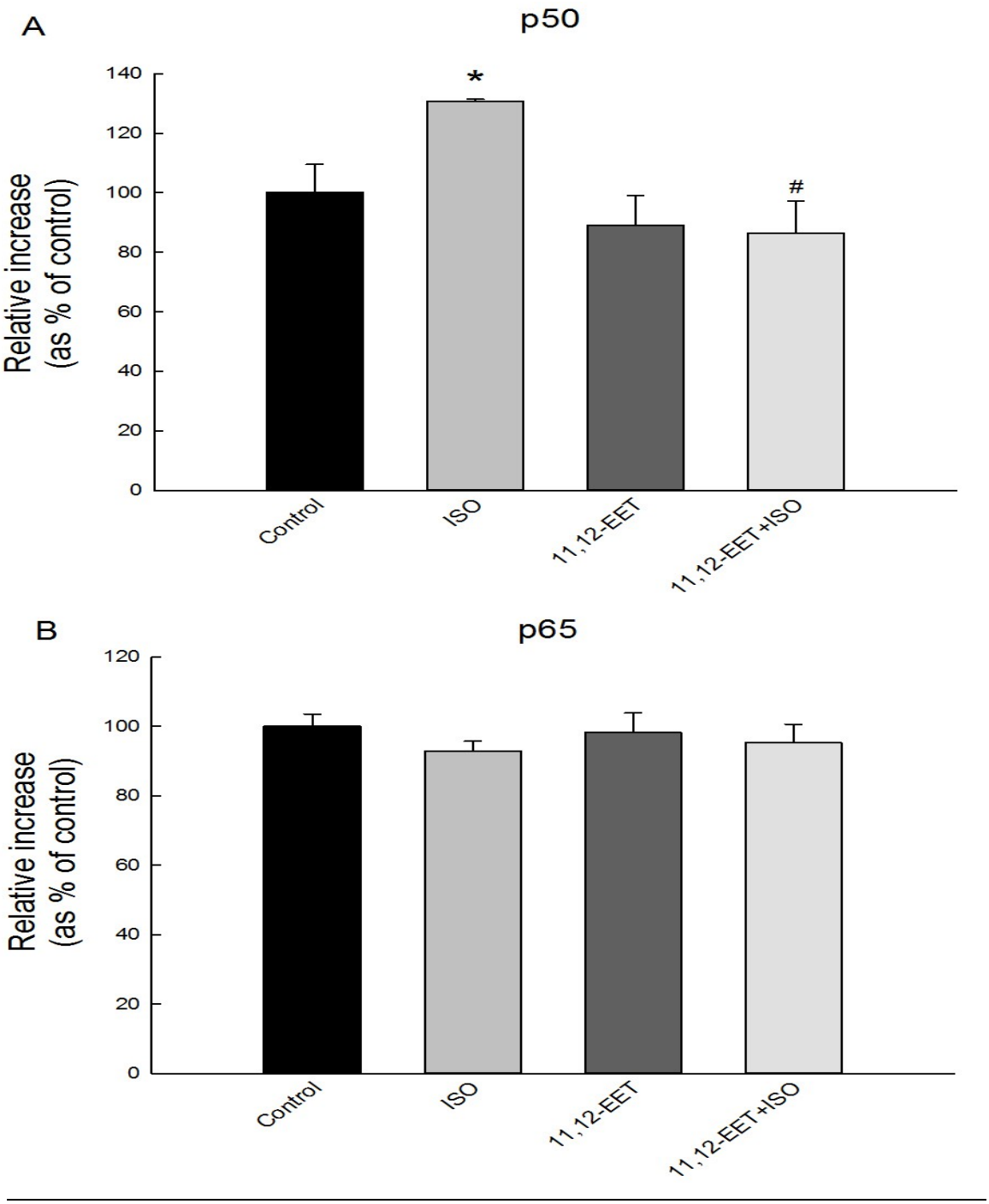

Fig 3.43. Effect of 11,12-EET on isoproterenol - induced NF-kB-binding activity. RL-14 cells were pretreated with vehicle or 11,12-EET (1 μM) for 2 hours prior to isoproterenol treatment (100 μM) for 2 hours. Thereafter, nuclear proteins were prepared and NF-κB activity was determined using a commercially available kit. Duplicate reactions were performed for each experiment, and the results are presented as the means of six independent experiments ± S.E.M. (n = 6). Kruskal-Wallis test and Dunn's post hoc test (* $P < 0.05$ compared to control, # $P < 0.05$ compared to isoproterenol-treated cells).

CHAPTER 4. DISCUSSION

4.1 Modulation of CYP and their associated AA metabolites prior to and during cardiac hypertrophy induced by isoproterenol *in vivo* and *in vitro*.

Identifying the underlying molecular events responsible for the initiation of cardiac hypertrophy has been of great interest for developing new strategies to prevent HF at an early stage. AA metabolism by CYP enzymes to biologically active eicosanoids has been recognized increasingly as an integral mediator in the pathogenesis of cardiac hypertrophy (Ai, Pang et al. 2009, Jenkins, Cedars et al. 2009). However, it is unknown whether these enzymes and metabolites play a role in the initiation of cardiac hypertrophy. Thus, it is important to investigate the changes in CYP and their associated AA metabolites prior to and during the development of cardiac hypertrophy. Therefore, we hypothesized that CYP and their associated AA metabolites play a crucial role in initiation of cardiac hypertrophy.

Echocardiographic examinations and HW/BW ratio measurements confirmed that concentric LV hypertrophy was initiated after 72 hours of isoproterenol treatment, manifested by an increase in LV wall thickness, IVS/LVD ratio at end diastole, and preserved or enhanced cardiac function (Fig 3.1). Upregulation of ANP prior to cardiac enlargement supports the suggestion that ANP can serve as an early and sensitive marker of cardiac stress but not essentially as a hypertrophic marker, especially on an organ level (Fig 3.2) (Cox and Marsh 2014). In the current study, 12 and 24 hours were chosen to determine whether CYP changes precede hypertrophy in rats and may therefore signal its development. These time points were chosen based on the most sensitive estimates of cardiac hypertrophy, echocardiographic measurements and HW/BW ratio.

Much evidence suggests the involvement of CYP epoxygenases in CVDs (Elbekai and El-Kadi 2006). For instance, overexpression of CYP2C and CYP2J subfamilies and their associated cardioprotective EET metabolites increased endothelial cell proliferation and angiogenesis, improved postischemic recovery of the left ventricle, and protected against hypoxia-reoxygenation injury and cardiotoxicity (Yang, Graham et al. 2001, Michaelis, Fisslthaler et al. 2003, Seubert, Yang et al. 2004, Fleming 2007, Zhang, El-Sikhry et al. 2009, Imig 2012). In the current study, we demonstrated that the expression of CYP epoxygenases, specifically CYP2C23 and CYP2J3, was decreased prior to cardiac enlargement, suggesting a signal role of these enzymes in the initiation of cardiac hypertrophy. Although the expression of CYP2C23 continued to be decreased during the hypertrophy phase, other epoxygenases such as CYP2B1 and CYP2J3 were induced late in this phase, suggesting a compensatory response to cardiac hypertrophy (Fig 3.3 and Fig 3.4). In line with the current findings, it has been reported that the expression of CYP2B1 and/or CYP2J3 was increased in SHR, pressure overload–induced cardiac hypertrophy, and isoproterenol-induced cardiac hypertrophy (Thum and Borlak 2002, Zordoky, Aboutabl et al. 2008, El-Sherbeni and El-Kadi 2014).

CYP2B1, CYP2C23, and CYP2J were all reported to produce EETs, and CYP2C and CYP2J subfamilies appear to be the primary CYP epoxygenases, in rat and human heart, respectively (Roman 2002, Imig 2012, El-Sherbeni, Aboutabl et al. 2013). EET levels are critically regulated by the sEH enzyme, as it catalyzes their degradation to DHETs, thus eliminating their biologic activity. Therefore, it was

necessary to investigate the effect of isoproterenol treatment on sEH activity. Our results showed that the activity of sEH was induced as early as 12 hours in prehypertrophied hearts and continued thereafter during the development of cardiac hypertrophy (Fig 3.6). In agreement with our results, higher activity of sEH was reported in spontaneously hypertensive heart failure (SHHF) rats and the BaP model of cardiac hypertrophy (Monti, Fischer et al. 2008, Aboutabl, Zordoky et al. 2011), whereas inhibition of sEH was shown to be effective not only in attenuating cardiac hypertrophy, but also in reversing and/or preventing its development (Xu, Li et al. 2006, Aboutabl, Zordoky et al. 2011).

In the current study, the level of EETs and DHETs in prehypertrophied and hypertrophied hearts could be attributed to different factors including the expression of CYP epoxygenases (synthesis), higher sEH activity (degradation), and/or their incorporation into cell membranes. Thus, further investigation is required to elucidate the contribution of each of these factors in determining their levels.

EETs were shown to protect against various CVDs such as hypertension, endothelial dysfunction, cardiac remodeling, ischemic heart diseases, cardiac hypertrophy and HF (Imig 2012). In addition, EETs were shown to inhibit NF-κB, which is a downstream target of several signaling pathways implicated in cardiac abnormalities, such as angiotensin-II, α-adrenergic stimulation, and the PI3K/Akt, Ras, p38, MEKK1/4-MAPKs, PKC, and gp130 pathways (Harris, Li et al. 2008, Gordon, Shaw et al. 2011).

With regard to CYP ω-hydroxylases, our results demonstrated that the expression of ω-hydroxylase CYP4F4 was significantly induced prior to and during cardiac enlargement (Fig 3.3). The induction of CYP4F4 was associated with a high formation rate of 20-HETE (Fig 3.6). In agreement with our results, the expression of the CYP4F subfamily was found to be upregulated in failing and hypertrophied hearts, and was associated with higher synthesis of 20-HETE in rats (Elbekai and El-Kadi 2006).

In contrast to EETs, 20-HETE, a ω-hydroxylase metabolite, has been shown to exert detrimental cardiovascular effects (Roman 2002). For instance, increasing 20-HETE formation was accompanied by an increase in systolic blood pressure, plasma levels of Ang II, and angiotensin-converting enzyme. On the other hand, inhibition of 20-HETE formation by HET0016, significantly attenuated these changes (Elshenawy, Anwar-Mohamed et al. 2013). Likewise, inhibition of 20-HETE formation prevented the development of hypertension in SHRs, reduced myocardial infarct size, and significantly reversed BaP-induced cardiac hypertrophy (Gross, Nithipatikom et al. 2004, Aboutabl, Zordoky et al. 2009) . Furthermore, 20-HETE was found to be involved in the secretion of endothelin-1– stimulated natriuretic peptide from the heart and increased cellular hypertrophy in H9C2 cells (Lee, Landon et al. 2004, Tse, Aboutabl et al. 2013). Taken together, these findings place EETs and 20-HETE as promising therapeutic targets in the treatment of CVDs.

Animal models are useful tools to study CVDs, but may not be suitable for shedding light on molecular mechanisms involved in human diseases. Therefore, we further investigated the early changes of CYP in the human ventricular

cardiomyocyte RL-14 cell line. RL-14 cells were shown to express CYP at comparable levels to those expressed in adult human primary cardiomyocyte (HMCa) cells and fetal human primary cardiomyocyte (HMC) cells, thus validating their use as a reliable model for studying CYP and their metabolic activity in the heart (Maayah, Elshenawy et al. 2015). Initially, the *in vitro* concentrations of tested compounds used in the current study were chosen based on previous studies after determining the ability of a wide range of concentrations to modulate the expression of CYP and hypertrophic genes without significantly affecting RL-14 cells' viability (Dhanasekaran, Gruenloh et al. 2008, Aboutabl, Zordoky et al. 2011, Tse, Aboutabl et al. 2013, Maayah, Elshenawy et al. 2015).

In RL-14, measurement of cell size and gene expression of the hypertrophic marker ANP confirmed the initiation of cellular growth after 6 hours of isoproterenol treatment (Fig 3.8 and 3.9). Studies performed prior to cellular enlargement (1 and 3 hours) showed that only 11,12-EET and its corresponding 11,12-DHET metabolites were significantly altered during this phase (Fig 3.11). In a manner similar to what was observed *in vivo*, the early decrease in CYP epoxygenases suggests the lower formation rate of 11,12-EET in as little as 1 hour and further suggests CYP stereoselectivity (Fig 3.10). In addition, induction of sEH suggests the higher formation of the corresponding 11,12-DHET during this phase (Fig 3.12).

In contrast to the *in vivo* results, the expression of the ω-hydroxylase CYP4F2 and the level of 20-HETE were not changed in RL-14 cells (Fig 3.10 and 3.12). This discrepancy could be attributed to several factors. First, biological differences between the two models because in vivo studies were conducted using an adult rat

model whereas in vitro data was obtained from human fetal derived cell line. Second, it has been reported that the expression of ω-hydroxylases, in comparison with other CYPs, is low in RL-14 cells (Maayah, Elshenawy et al. 2015). Third, the hemodynamic effects and the complexity of the *in vivo* model in comparison with the *in vitro* model may well explain these conflicting results. In addition to these factors, ω-hydroxylases were reported to be induced in the kidney and liver of isoproterenol-treated rats, which may contribute to higher levels of cardiac 20-HETE (Althurwi, Tse et al. 2013).

In conclusion, we demonstrated for the first time the changes in CYP and their AA metabolites prior to cardiac growth, both *in vivo* and *in vitro*. Demonstrating these changes prior to cardiac growth is necessary (but not sufficient) to establish a signaling role. Taking into account that cardiac hypertrophy can lead ultimately to HF, identification of unique regulators in the initiation of cardiac hypertrophy will provide a better understanding of the mechanisms by which HF develops. Consequently, this will lead to the development of new and innovative pharmacotherapies in the management of HF.

4.2 Soluble epoxide hydrolase inhibitor TUPS protects against isoproterenol-induced cardiac hypertrophy

sEH activity was increased during the established phase of cardiac enlargement, suggesting a role of this enzyme during the progression of cardiac hypertrophy. Therefore, it is important to examine whether the inhibition of sEH will protect against isoproterenol-induced cardiac hypertrophy. The cardioprotective effect of sEH inhibitors appears to be due to their ability to inhibit the degradation of epoxy fatty acids and hence enhance the cardioprotective effect of EETs. In this context, several sEH inhibitors have been synthesized, among which the newly discovered, 1,3-disubstituted ureas, carbamates and amides are considered cutting-edge sEH inhibitors. They are potent and stable transition-state inhibitors of sEH that act through inhibition of the carboxy-terminal domain that possesses the epoxide hydrolase activity of the sEH enzyme (Morisseau and Hammock 2005). Among the different sEH pharmacophores, the urea pharmacophore seems to produce the most potent, competitive and tight-binding inhibitors of sEH (Morisseau, Goodrow et al. 1999). The sEH inhibitor of choice in this study is TUPS, which comprises a highly potent urea pharmacophore (Chiamvimonvat, Ho et al. 2007).

In the current study, we investigated the cardioprotective effect of TUPS treatment on isoproterenol-induced cardiac hypertrophy. Our results demonstrated that TUPS significantly decreased the isoproterenol-mediated induction of the hypertrophic markers ANP, BNP and β-MHC and the increase in the HW/BW ratio (Fig 3.13). In agreement with our results, it has been previously demonstrated that TUPS decreases the left ventricular hypertrophy, HW/BW ratio and the hypertrophic

markers in Ang II–induced cardiac hypertrophy and BaP-induced cardiac hypertrophy, reflecting its cardioprotective effect (Ai, Pang et al. 2009, Aboutabl, Zordoky et al. 2011). Furthermore, TUPS has been reported recently to significantly decrease the isoproterenol-mediated induction of ANP and BNP mRNA level in H9c2 cells, which further confirms the direct effect of both isoproterenol and TUPS at the cardiomyocyte level (Althurwi, Tse et al. 2013).

In addition to its effect on cardiac hypertrophy, TUPS treatment significantly decreased the isoproterenol-mediated increase in fibrotic markers procollagen I, procollagen III and TGF-1, which interestingly seems to be another protective effect of TUPS that extends beyond cardiac hypertrophy (Fig 3.14). Therefore, our study provides a new model in which TUPS is shown to protect against cardiac hypertrophy and fibrosis. However, further studied are needed to confirm this finding using more reliable estimates of cardiac fibrosis such as Masson's Trichrome staining. Nevertheless, it has been recently reported that EET treatment attenuates cardiac fibrosis induced by Ang II infusion in mice and to decrease the level of the fibrotic markers tissue inhibitor of metalloproteinases 1 (TIMP1) and TGF-1 in neonatal cardiomyocytes treated with Ang II (He, Zhang et al. 2015).

It has been shown previously that the induction of cardiac hypertrophy in isoproterenol-treated rats causes significant changes in the expression of several CYP and sEH genes, which is mostly specific to the heart. The overall balance of these changes has led to a higher production of the cardiotoxic metabolite, 20-HETE and a lower production of cardioprotective metabolites, EETs, in the hypertrophied hearts (Zordoky, Aboutabl et al. 2008).

20-HETE is known to be involved in many cardiovascular diseases. Interestingly, it has been demonstrated that the CYP ω-hydroxylase inhibitor, HET0016, partially reverses the BaP-induced cardiac hypertrophy through inhibition of 20-HETE formation, suggesting the involvement of CYP ω-hydroxylases and 20-HETE in the development of cardiac hypertrophy (Aboutabl, Zordoky et al. 2009). In addition 20-HETE has been shown to activate NF-κB and increase the production of inflammatory cytokines in human endothelial cells (Ishizuka, Cheng et al. 2008). Recently, 20-HETE has been shown to induce apoptosis in neonatal rat cardiomyocytes which further confirms its detrimental role in the development of cardiac hypertrophy (Bao, Wang et al. 2011) .

Due to the importance of CYP in the pathogenesis of cardiac hypertrophy, in the current study we investigated the effect of sEH inhibition on the expression of different CYP genes involved in isoproterenol-induced cardiac hypertrophy. Our results demonstrated that isoproterenol significantly induces the gene expression of CYP1A1 in the heart and kidney but not in the liver, whereas CYP1B1 gene expression was induced only in the hypertrophied heart (Fig 3.15). In agreement with our results, these changes have been demonstrated previously in the hearts of isoproterenol-treated rats and SHRs (Thum and Borlak 2002, Zordoky, Aboutabl et al. 2008). CYP1A1 has been shown to be involved in ω-terminal HETE synthesis, whereas CYP1B1 can metabolize AA to both mid-chain HETEs and EETs (Choudhary, Jansson et al. 2004). Moreover, it has been recently demonstrated that CYP1B1 contributes to Ang II–induced hypertension and cardiac hypertrophy (Jennings, Sahan-Firat et al. 2010). In the present study, treatment with TUPS

significantly reduced the isoproterenol-induced CYP ω-hydroxylase enzymes CYP1A1 and CYP1B1 in the heart tissue. However, treatment with TUPS alone caused significant induction of CYP1A1 and CYP1B1 in the kidney and further potentiated the isoproterenol-mediated induction of CYP1A1 (Fig 3.15). In contrast to this finding, it has been shown that TUPS alone does not induce any significant changes in the gene expression of CYP1A1 or CYP1B1 in the kidney (Aboutabl, Zordoky et al. 2011). In our study, TUPS group received 25% PEG400 and saline, whereas in the other study, TUPS group received corn oil and dimethyl sulfoxide. Corn oil contains linoleic acid which can interact with sEH inhibitors leading to change in oxylipin profiles due to high polyunsaturated fatty acids. Therefore, the observed discrepancy between the two studies could be an effect of this interaction.

Previously, it has been reported that treatment with isoproterenol did not cause any significant changes in the cardiac expression of CYP2B1 or CYP2B2 (Zordoky, Aboutabl et al. 2008). However, in the present study, isoproterenol significantly induced the gene expression of both CYP2B1 and CYP2B2 in the heart tissue but not in the kidney or liver (Fig 3.16). This discrepancy might be attributed to differences in the age and weight of animals used (younger rats were used in this study) as it has been reported that the effect of isoproterenol differs in older rats than in younger ones, so the degree of cardiac hypertrophy achieved in this study is not the same as previously published (Kunos, Robertson et al. 1978). Moreover, CYP gene expression has been found to vary with age at both the transcriptional and post-translational level, which provides another possible reason for this discrepancy (Wauthier, Verbeeck et al. 2007). In line with our current findings, it has been

demonstrated that the gene expression of CYP2B1 and CYP2B2 are increased in SHR as compared to normotensive SD rats (Thum and Borlak 2002). Interestingly, treatment with TUPS significantly reduced the isoproterenol-mediated induction of both CYP2B1 and CYP2B2.

With regard to the CYP4 family, our results demonstrated that CYP4A3 gene expression was significantly induced in the heart, liver and kidney of isoproterenol-treated rats as compared to the control. In addition, isoproterenol caused a significant induction of CYP4F4 in the heart alone. Interestingly, treatment with TUPS reduced the increased expression of the CYP4A3 gene in all the tissues examined. Moreover, TUPS significantly reduced the isoproterenol-mediated induction of CYP4F4 gene expression in the heart (Fig 3.17). The premise of this observation emerges from the fact that the CYP4A and 4F subfamilies are important enzymes involved in AA metabolism to 20-HETE (Wang, Stec et al. 1996, Roman 2002).

To investigate whether the alterations in CYP gene expression are further translated into protein, we determined the protein expression of CYP1A1, CYP1B1, CYP2B1/2 and CYP4A. Our results show that the protein levels of CYP1A1, CYP1B1, CYP2B1/2 and CYP4A were significantly increased in the heart of isoproterenol-treated rats; whereas TUPS treatment significantly decreased the isoproterenol-mediated induction of protein expressions of these enzymes (Fig 3.18).

The sEH enzyme is a crucial determinant of EET levels because it catalyses the conversion of EETs to DHETs, thus abolishing their biological activity (Imig, Zhao et al. 2002). Therefore, any change in AA metabolism caused by modification of CYP

can be augmented or opposed by an altered level of sEH. Moreover, the gene encoding sEH was found to be a vulnerability factor for HF in SHHF rats (Monti, Fischer et al. 2008). Therefore, it is essential to investigate the effect of cardiac hypertrophy on EPHX2 expression. Although isoproterenol treatment did not induce any changes in EPHX2 gene expression in heart tissue, it caused a significant induction of EPHX2 in H9c2 cells, which confirms that isoproterenol causes an induction of EPHX2 gene expression at the level of cardiomyocytes (Tse, Aboutabl et al. 2013). The fact that this effect was absent in the present study might be due to factors such as mRNA decay and post-transcriptional regulation which needs further investigation. Nevertheless, isoproterenol caused a significant induction of sEH at both the protein and activity level in the heart (Fig 3.20). Our results accord with those obtained previously where it was shown that sEH activity is higher in SHHF and Ang II treated-rats (Monti, Fischer et al. 2008, Ai, Pang et al. 2009).

To investigate the effect of TUPS treatment on CYP gene expression and AA metabolism, we incubated heart microsomes with AA *in vitro*. We confirmed our previous findings as we showed that the formations of 5,6-, 8,9-, 11,12- and 14,15-EET were significantly decreased in microsomes of hypertrophied hearts in comparison with those from controls (Fig 3.21). The decrease in the formation of EETs was accompanied by a significant increase in 8,9- and 14,15-DHET formation, whereas 5,6- and 11,12-DHET were not significantly altered (Fig 3.21). The decreased formation of EETs during isoproterenol-induced cardiac hypertrophy may be attributed to a higher activity of sEH. The significantly higher formation of 8,9- and 14,15- DHET is consistent with the higher expression of sEH (Fig 3.22). On the other

hand, treatment with TUPS abolished the isoproterenol-mediated reduction in 8,9- and 11,12-EETs and further significantly increased the formation of 14,15-EET as compared to the control group. However, TUPS treatment did not affect the isoproterenol reduction of 5,6-EETs. Moreover, TUPS treatment significantly reduced the increase in 8,9- and 14,15-DHET formation caused by isoproterenol and further reduced the formation of 11,12-DHET significantly in comparison with the control microsomes. In the present study we demonstrated that 20-HETE formation is significantly higher in hypertrophied hearts (Fig 3.22). The increased formation of 20-HETE is suggestive of its role in cardiac hypertrophy. 20-HETE formation is mainly catalysed by CYP ω-hydroxylases (Kroetz and Xu 2005). The CYP ω-hydroxylases involved in the formation of HETEs are CYP1A, CYP1B1, CYP4A and CYP4F (Wang, Stec et al. 1996, Wang, Guan et al. 1999, Roman 2002, Choudhary, Jansson et al. 2004, Elbekai and El-Kadi 2006). Therefore, the increase in 20-HETE formation in the present work could be attributed to the increased expression of CYP1A1, CYP1B1, CYP4A3 and CYP4F4. Interestingly, in heart microsomes treated with TUPS, the isoproterenol mediated increase in 20-HETE formation was abolished. TUPS is not CYP hydroxylase inhibitor so the changes in 20-HETE formation might be due to the reversal effect of sEH inhibition on the cardiac hypertrophy, thus normalizing the level of 20-HETE.

In conclusion, we demonstrated for the first time that treatment with the sEH inhibitor, TUPS, significantly attenuated the progression of established cardiac hypertrophy induced by isoproterenol treatment. In addition, the effects of TUPS on isoproterenol-induced CYP enzymes showed some degree of cardioselectivity,

which is thought to be secondary to its cardioprotective effect. From accumulating evidence that sEH has a role in the pathogenesis of cardiac hypertrophy, sEH inhibition will provide a new therapeutic tool to protect against cardiac hypertrophy. However, more studies are needed to explore the mechanisms by which inhibition of sEH protects against cardiac hypertrophy.

4.3 Fenofibrate modulates CYP and AA metabolism in the heart and protects against isoproterenol-induced cardiac hypertrophy

It is well established that the CYP epoxygenase enzymes and their associated AA metabolites are altered during CVDs, suggesting their roles in cardiovascular health (Elbekai and El-Kadi 2006, Imig 2012). For instance, CYP2B1 and CYP2B2 gene expression were increased in SHRs, whereas CYP2C11 expression was decreased in the hypertrophied hearts (Thum and Borlak 2002, Zordoky and El-Kadi 2008).

It has been reported that fenofibrate induces the expression of renal CYP epoxygenases (Huang, Morisseau et al. 2007). Therefore, we investigated whether fenofibrate would induce CYP epoxygenase enzymes and increase bioavailability of EETs in the heart. Our results demonstrated that fenofibrate significantly induced CYP epoxygenases CYP2B1, CYP2B2, CYP2C11, CYP2C23, and CYP2J4 in the heart at both mRNA and protein levels (Fig 3.27). CYP2B1, CYP2B2, CYP2C11, CYP2C23, and CYP2J were all reported to produce EETs where CYP2C and CYP2J subfamilies seem to be the primary CYP epoxygenases in rat and human heart, respectively (Roman 2002, El-Sherbeni, Aboutabl et al. 2013).

As discussed above, any change in AA metabolism because of the alteration on CYP enzymes can be further influenced by sEH levels. Accordingly, it was necessary to investigate the effect of fenofibrate treatment on sEH expression. Surprisingly, we found that fenofibrate treatment did not cause any changes in sEH mRNA, protein or activity levels in the heart tissue, whereas it caused significant induction of sEH gene expression in the kidney and the liver (Fig 3.28). In line with

our results, it has been previously reported that fenofibrate treatment significantly increases sEH in the liver and the kidney (Schladt, Hartmann et al. 1987). Thus, these results suggest that the effect of fenofibrate on sEH expression is tissue-specific.

In agreement with the CYP epoxygenase mRNA and protein levels, fenofibrate treatment significantly increased the formation rates of 8,9-, 11,12-, and 14,15-EET (Fig 3.30). Moreover, the total epoxygenase activity was significantly increased in fenofibrate-treated heart microsomes compared to the control microsomes (Fig 3.31). In accordance with sEH mRNA, protein and activity levels, formation of DHETs was not significantly altered.

Similar to CYP epoxygenases, the evidence for the involvement of CYP ω-hydroxylases, mainly CYP4A and CYP4F, in CVDs is well established (Roman 2002). In this regard, the expression of CYP4A and CYP4F subfamilies was found to be upregulated in failing and hypertrophied hearts and was associated with higher synthesis of 20-HETE in SD rats (Elbekai and El-Kadi 2006, Aboutabl, Zordoky et al. 2011). In this study, we demonstrated that fenofibrate treatment decreased CYP4A3 gene expression whereas it significantly induced the gene expression of CYP4F4 in the heart (Fig 3.26). However, western blots revealed that the protein level of CYP4A was not changed, whereas the protein expression of CYP4F was significantly decreased in fenofibrate-treated rats (Fig 3.27). The difference observed in CYP expression pattern could be attributed to several factors: first, it has been reported that the transcriptional regulation of CYP4F gene expression differs substantially from the CYP4A subfamily. For instance, a PPARα ligand like clofibrate, which is

known to induce CYP4A expression, either downregulates or has no effect on CYP4F subfamily (Kawashima, Kusunose et al. 1997, Cui, Kawashima et al. 2001). Second, there are several processes between transcription and translation that could be responsible for such a discrepancy and include but are not limited to, RNA and protein stability, and post-transcriptional and post-translational modifications in addition to miRNA. Finally, it is known the transcriptional regulation is a complex process, and usually includes more than one nuclear receptor in the regulation of different CYP isoenzymes in different organs (Palut, Kostka et al. 2002). Hence, further studies need to be conducted to understand such a discrepancy which is beyond the scope of this study. Nevertheless, microsomal incubation with AA revealed that fenofibrate treatment significantly decreased 20-HETE formation in the heart (Fig 3.32). Thus, despite the disparity between CYP ω-hydroxylases mRNA and protein levels, the decrease in 20-HETE formation in fenofibrate-treated rats is suggestive of the lower activity of ω-hydroxylases and consistent with CYP4A3 and CYP4F gene and protein expression, respectively.

To assess whether the cardiac-specific changes seen in fenofibrate treatment would confer cardioprotection, we tested the cardioprotective effect of fenofibrate in the isoproterenol model of cardiac hypertrophy. In this regard, fenofibrate administration significantly decreased the isoproterenol-mediated induction of the hypertrophy markers ANP, BNP, and the increase in the HW/BW ratio (Fig 3.33). In agreement with our results, it has been previously demonstrated that fenofibrate decreases the left ventricular hypertrophy, HW/BW ratio, and the hypertrophy

markers in aortic banding model and aldosterone-induced hypertension model (Irukayama-Tomobe, Miyauchi et al. 2004, Lebrasseur, Duhaney et al. 2007).

Growing evidence has suggested the cardioprotective role of PPARα in the cardiac hypertrophy and HF. For instance, cardiac enlargement and dysfunction in response to TAC were more pronounced in PPARα knockout mice as compared to wild-type mice (Smeets, Teunissen et al. 2008). In addition, fenofibrate treatment attenuates the hypertrophic response and cardiac dysfunction in wild-type mice subjected to chronic pressure overload however, it fails to provide such cardioprotection in PPARα knockout mice subjected to the same pressure overload (Duhaney, Cui et al. 2007). Interestingly, EET has been found to activate PPARα which maybe another mechanism by which EET confer cardioprotection (Wray and Bishop-Bailey 2008). However, further studies are necessary to explore this mechanism in fenofibrate and EETs cardioprotection against cardiac hypertrophy.

In conclusion, we have demonstrated for the first time that treatment with fenofibrate significantly induces the cardiac CYP epoxygenase enzymes and their associated EET metabolites. On the other hand, fenofibrate decreased the cardiac ω-hydroxylase and their associated 20-HETE metabolite. Furthermore, fenofibrate partially protected against isoproterenol-induced cardiac hypertrophy. Taking into account the accumulating evidence for the role of EETs and 20-HETE in the pathogenesis of cardiac diseases, manipulating the expression of epoxygenase and hydroxylase enzymes by fenofibrate will reveal novel points of intervention to be exploited in the development of new therapies for the treatment and prevention of

cardiac diseases. However, more studies are needed to explore the mechanisms by which fenofibrate confers cardioprotection.

4.4 Role of CYP epoxygenases and their associated AA metabolites in the initiation of cardiac hypertrophy

As discussed above, we demonstrated significant changes in CYP epoxygenases and their metabolites prior to cardiac growth, both in vivo and in vitro (Table 4.1). Demonstrating these changes prior to cardiac hypertrophy is necessary (but not sufficient) to establish a signaling role. Therefore, to assess the crucial role of CYP epoxygenases and EETs in the initiation of cellular hypertrophy, different approaches were used. First, RL-14 cells were pretreated with an epoxygenase inducer, fenofibrate, in the presence and absence of a selective epoxygenases inhibitor, MS-PPOH. Fenofibrate has been reported to increase CYP epoxygenases both *in vivo* and *in vitro* (Althurwi, Elshenawy et al. 2014, Maayah, Elshenawy et al. 2015). Second was the genetic approach of overexpressing CYP2J2, a predominant CYP epoxygenase in human heart, in RL-14 cells. Third, RL-14 cells were pretreated with the sEH inhibitor TUPS or 11,12-EET prior to isoproterenol treatment.

Our results showed that increasing EET levels by epoxygenase induction, sEH inhibition, or exogenous administration of EET prevented the initiation of isoproterenol-induced cellular hypertrophy (Figs 3.36, 3.37, 3.38, 3.41, and 3.42). In addition, our result indicated that EETs may be involved, at least in part, in the cardioprotective effect of fenofibrate (Fig 3.36). In line with our results, previous studies have reported that pretreatment with EETs and/or an sEH inhibitor protects against cellular hypertrophy in rat neonatal cardiomyocytes and the H9c2 rat cell line (Ai, Pang et al. 2009, Tse, Aboutabl et al. 2013). Taken together, these findings

confirm the potential role of CYP epoxygenases and EETs in the initiation of cellular hypertrophy.

It has been proposed that EETs exert their effects either by binding to a cellular receptor linked to an intracellular signal pathway, or by direct interaction with ion channels, signal molecules, or transcription factors that produce their response (Spector and Norris 2007). In this regard, the cardioprotection of EET was reported to be mediated by various intracellular targets. For instance, EET activates the pro-survival PI3Kα and its downstream targets in neonatal rat cardiomyocytes (Bodiga, Zhang et al. 2009). As discussed above, PI3Kα is considered as a critical regulator in the development of physiological cardiac hypertrophy. In addition, EET increased the level of antioxidant enzymes, decreased the production of ROS, inhibited ER stress and restored Ca^{++} homeostasis (Wang, Ni et al. 2014). Interestingly, oxidative stress and calcium dysregulation have been shown to mediate isoproterenol cardiac dysfunction. Furthermore, EET was reported to activate STAT-3 which has been implicated in the physiological cardiac growth (Cheranov, Karpurapu et al. 2008). Of particular interest, the NF-kB pathway was shown to be implicated as an early and required player in the initiation of cardiac hypertrophy (Purcell, Tang et al. 2001). Therefore, we tested the capacity of EET to inhibit NF-kB activation by isoproterenol. Our results showed that isoproterenol significantly activated p50 translocation and binding to DNA, whereas 11,12-EET abrogated this activation, highlighting the involvement of NF-kB as a signaling pathway in the protective effect of EETs (Fig 3.43). In line with our results, increased NF-kB-binding activity was observed in isoproterenol-treated rats (Davel, Fukuda et al. 2008). On the other hand, sEH

inhibition was previously found to prevent the activation of NF-kB induced by angiotensin-II, both *in vivo* and *in vitro* (Ai, Pang et al. 2009).

In conclusion, our results provide the first evidence that early changes in CYP epoxygenases and their associated AA metabolites prior to cardiac enlargement play a key role in the initiation of cardiac hypertrophy.

Table 4.1 Summary of CYP modulations

Model	CYP		Pre-hypertrophy phase	Hypertrophy phase
			12 and 24 h	72, 168, and 240 h
RAT	Epoxygenases	CYP2B1	↔	↑
		CYP2C23	↓	↓
		CYP2J3	↓	↑
	ω-hydoxylases	CYP4F4	↑	↑
			1 and 3 h	6,12, and 24 h
RL-14 cell line	Epoxygenases	CYP1A2	↔	↓
		CYP2B6	↓	↓
		CYP2C8	↓	↓
		CYP2C19	↔	↓
		CYP2J2	↓	↓ -----> ↔
	ω-hydoxylases	CYP4F2	↓	↓

↓ indicates decrease in the expression; ↔, no change; ↑, increase in the expression; ↓ -----> ↔, decrease in the expression then returned back to normal levels.

4.5 Summary and General Conclusion

HF is the leading cause of mortality and disability in adults. Significant advances have been made in the therapy of HF in recent decades, leading to the discovery of different classes of therapeutic agents. These agents showed considerable therapeutic success in stabilizing the symptoms of a failing heart but failed to cure HF. Accordingly, there is an urgent need for better therapy options for many patients with HF (Kaye and Krum 2007).

HF is a complex pathophysiological syndrome and research is still needed to understand its pathogenesis and predisposing factors. Cardiac hypertrophy is an independent risk factor and one of the major hallmarks of HF. Therefore, understanding the molecular basis of cardiac hypertrophy is considered as research into the initial steps of HF (Ritter and Neyses 2003).

Increasing evidence has revealed the contribution of CYP and their metabolites in cardiac hypertrophy (Elbekai and El-Kadi 2006, Alsaad, Zordoky et al. 2013). Therefore, this work has been focused on investigating the role of CYP and CYP-mediated AA metabolism in cardiac hypertrophy induced by isoproterenol.

In the present work, we have demonstrated that *in vivo* and *in vitro* hypertrophy was initiated after 72 hours and 6 hours of isoproterenol treatment, respectively. Studies performed at the prehypertrophy phase showed a significant decrease in CYP epoxygenases along with a significant induction of sEH activity. Consequently, lower EET and higher dihydroxyeicosatrienoic acid levels were observed during this phase. However, significant increases in CYP ω-hydroxylase along with its

associated metabolite, 20-HETE, were detected only *in vivo*. The decrease in the cardioprotective EETs and the increase in cardiotoxic 20-HETE may be involved, at least in part, in the development and/or progression of cardiac hypertrophy and subsequently HF.

To assess the involvement of sEH in cardiac hypertrophy progression, we investigated whether inhibition of sEH by TUPS protects against isoproterenol-induced cardiac hypertrophy. Furthermore, we examined the effect of TUPS treatment on CYP enzymes and the formation of CYP-mediated AA lipid mediators. Inhibition of sEH by TUPS significantly decreased the isoproterenol-mediated increases in the hypertrophic, fibrotic markers as well as the HW/BW ratio. Moreover, TUPS significantly inhibited isoproterenol-mediated induction of CYP1A1, CYP1B1, CYP2B1, CYP2B2, CYP4A3 and CYP4F4 gene expression. In addition, TUPS abolished isoproterenol-mediated changes in AA metabolites. Therefore, inhibition of sEH can be considered a potential therapeutic target in the progression of cardiac hypertrophy and consequently HF.

To assess the cardioprotective effect of fenofibrate and whether this effect involves EET, we examined the effect of fenofibrate treatment on cardiac hypertrophy induced by isoproterenol. Furthermore, we studied the effect of fenofibrate treatment on CYP epoxygenases and EETs. Our results showed that fenofibrate significantly induced the cardiac expression of CYP epoxygenases, such as CYP2B1, CYP2B2, CYP2C11, and CYP2C23, whereas it decreased the cardiac expression of the ω-hydroxylase CYP4A3. Moreover, fenofibrate significantly increased the formation of EETs, whereas it decreased the formation of 20-HETE in

the heart. Furthermore, fenofibrate significantly decreased the hypertrophic markers and the increase in HW/BW ratio induced by isoproterenol. This study suggests that the cardioprotective effect of fenofibrate could be mediated by the cardiac-specific changes in CYP and their associated AA metabolites.

In an attempt to dissect the cause-effect relationship between CYP epoxygenases and cardiac hypertrophy, we examined the role of CYP epoxygenases and EETs in the initiation of cellular hypertrophy using different approaches. Our results demonstrated that increasing EET levels by CYP epoxygenase induction, sEH inhibition, or exogenous administration of EET prevented the initiation of cardiac hypertrophy by isoproterenol. Taken together, these findings reveal a signal role of CYP epoxygenases and EETs in the development of cardiac hypertrophy, which could uncover novel targets for prevention of HF at early stages.

A number of signaling pathways hold promise as potential targets for treatment of cardiac hypertrophy. Although the cardioprotection by EET was reported to be mediated by several signaling pathways such as MAPK, PI3K, and KATP channels, NF-κB is the only signaling pathway that has been preliminarily investigated to explain the anti-hypertrophic effect of EET. Therefore, understanding the exact role of NF-κB along with other signaling pathways such as calcineurin/NFAT, GPCRs, MAPK, MMP and NHE may provide insights into the mechanisms by which EET protects against cardiac hypertrophy.

4.6 Future Research Directions

The results of the present work have highlighted the cardioprotection of EETs and the role of CYP, sEH enzymes, and CYP-mediated arachidonic acid metabolism in the initiation and/or progression of cardiac hypertrophy. However, further studies need to be conducted in order to translate this research into clinical practice.

1- To determine the combined effect of sEH inhibitors and epoxygenase inducers on cardiac hypertrophy *in vitro* and *in vivo*.

1- To identify the mechanisms by which cardiac hypertrophy causes these alterations in the CYPs and sEH expression.

2- To identify and understand the molecular regulations of sEH.

3- To determine which regiospecific EETs confer cardioprotection against cardiac hypertrophy *in vitro* and *in vivo*.

4- To identify the molecular mechanisms by which EETs confer cardioprotection.

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