University of Alberta

ROLE OF CYP-MEDIATED ARACHIDONIC ACID METABOLITES IN DEVELOPMENT OF CARDIAC HYPERTROPHY AND CHRONIC DOXORUBICIN-INDUCED CARDIOTOXICITY

By

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THIS WORK IS DEDICATED TO

MY MOTHER

MONIRAH Bint ABDULRAHMAN ALRAJIH

&

MY FATHER

MOHAMMED Bin SAAD ALSAAD

ABSTRACT

Drug-induced cardiotoxicity is classified as a predisposing factor leading to cardiac hypertrophy and heart failure. Of importance, doxorubicin (DOX, adriamycin) is considered as an effective anticancer agent whose major limiting side effect is cardiotoxicity. Of importance, several studies showed that acute DOX cardiotoxicity alters cytochrome P450 (CYP)-mediated arachidonic acid (AA) metabolism. However, the clinical situation involves chronic drug administration. Therefore, we investigated the effect of chronic DOX treatment on expression of cardiac CYP enzymes and CYP-mediated AA metabolism in male Sprague–Dawley (SD) rats. Our results showed that chronic DOX treatment significantly induced gene expression and activity of CYP ω-hyroxylase and soluble epoxide hydrolase (sEH) enzymes. Inhibition of these enzymes significantly prevented DOX-mediated induction of hypertrophic markers in H9c2 cells confirming the role of these enzymes in DOX cardiotoxicity. In conclusion, CYP ω-hyroxylase and sEH enzymes might be considered as novel targets to treat and/or to protect against DOX cardiotoxicity.

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LIST OF ABBREVIATIONS

3-MC	3-methylcholanthrene
Ang ll	Angiotensin II
ANP	Atrial natriuretic peptide
B(a)P	Benzo(a)pyrene
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CVDs	Cardiovascular diseases
CYP	Cytochrome P450
DHET	Dihydroxyeicosatrienoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
EET	Epoxyeicosatrienoic acid
ERK	Extracellular signal-regulated kinase
HET0016	N-hydroxy-N'-(4-butyl-2-methylphenyl) formamidine
HETE	Hydroxyeicosatetraenoic acid
IKB	Inhibitor of nuclear factor Kappa B
IKK	Inhibitor of nuclear factor Kappa B kinase
InsP3	Inositol-3,4,5-triphosphate
IP	Intraperitonial
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LC-ESI-MS	Liquid chromatography-electrospray ionization-mass spectrometry
LOX	Lipooxygenase
MAPK	Mitogen-activated protein kinases

mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate tetrasodium
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
P38	P38 mitogen-activated protein kinases
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
SD	Sprague Dawley
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
sEH	Soluble epoxide hydrolase
SHR	Spontaneously hypertensive rat
TBS	Tris-Buffered Saline
TEMED	N,N,N',N'-tetramethylethylenediarnine
α-MHC	α-myosin heavy chain
β-MHC	β-myosin heavy chain
βNF	β-naphthoflavone

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Heart Failure (HF) is becoming a major health concern that accounts for half a million deaths per year in the United States (Roger, et al., 2012). In 2008, the National Health and Nutrition Examination Survey data showed that HF affects 5,700,000 Americans with age of ≥20 years (Roger, et al., 2012). By 2030, it is also estimated that 3 millions of new cases will be added to the current statistics in the United States (Heidenreich, et al., 2011). In Canada, HF also affects 500,000 Canadians with 50,000 new cases every year (Ross, et al., 2006). Indeed, both the increased incidence and survival of HF patients might be the reasons behind the growing prevalence of HF (Roger, 2010). However, to date, HF prognosis remains poor with an estimated survival rate of 50% after 5 years (Roger, 2010).

As an independent risk factor for HF, cardiac hypertrophy is considered the most indicative predictor of adverse cardiovascular events (Gradman & Alfayoumi, 2006). Furthermore, several studies have investigated the pathogenesis of cardiac hypertrophy to identify the molecular targets that can be exploited to develop new therapies for cardiac hypertrophy and HF. We and other investigators have identified that cytochrome P450 enzymes (CYP) have an important role in the pathogenesis of cardiac hypertrophy through altering the CYP-mediated arachidonic acid (AA) metabolism (Aboutabl, Zordoky, & El-Kadi, 2009; Certikova Chabova, et al., 2010; Zordoky, Aboutabl, & El-Kadi, 2008). These CYP enzymes are involved in the metabolism of AA and generation of cardiotoxic and cardioprotective metabolites (Zordoky & El-Kadi, 2008b). Of importance, 20-hydroxyeicosatetraenoic acid (20-HETE), an eicosanoid produced by CYP ω-hydroxylases, has been shown to have a detrimental effect on several

cardiovascular functions (Bao, et al., 2011; Certikova Chabova, et al., 2010; Yousif, Benter, & Roman, 2009). On the other hand, CYP epoxygenase products, epoxyeicosatrienoic acids (EETs), have been demonstrated to be cardioprotective in several models of cardiovascular diseases (CVDs) (Deng, Theken, & Lee, 2010; Elbekai & El-Kadi, 2006; Roman, 2002). Inhibition of soluble epoxide hydrolase (sEH) enzyme, an enzyme responsible for conversion of EETs to biologically less active metabolites dihydroxyeicosatrienoic acids (DHETs), was also proven to reduce and to reverse cardiac hypertrophy (Xu, et al., 2006).

Of interest, both CYP and sEH enzymes are expressed in the cardiovascular system (Delozier, et al., 2007; Michaud, Frappier, Dumas, & Turgeon, 2010; Nithipatikom, et al., 2001). In addition, alteration in their expression and the subsequent change of 20-HETE and/or EETs levels were observed in pathological conditions such as cardiac hypertrophy and HF (Zordoky & El-Kadi, 2008b). Both expression of these enzymes and their altered expression in cardiac hypertrophy and HF will be reviewed in the following sections.

1.2 CYTOCHROME P450 ENZYMES (CYP)

1.2.1 Overview

CYP is a super family of heme containing enzymes involved in phase I drug metabolism and mediates oxidation of different xenobiotics or endogenous compounds (Walker, Pollenz, & Smith, 1997). It can be classified into different families and subfamilies according to their amino acid sequences. Only four families are involved in drug metabolism i.e. the CYP1, CYP2, CYP3, and to less extent CYP4 families. These

enzymes are expressed mainly in the liver and to different extent in heart, kidney, and lung (Imaoka, Hashizume, & Funae, 2005).

1.2.2 Expression of CYP and sEH in cardiovascular tissues

Several studies have shown that CYP and sEH enzymes are detected in cardiac tissue *in vivo* as well as in cultured cardiomyocytes and in the cardiac derived H9c2 cells (Fulton, Mahboubi, McGiff, & Quilley, 1995; Geetha, Marar, & Devi, 1991; McCallum, Horton, Falkner, & Bend, 1993; Thum & Borlak, 2000a; Yamada, Kaneko, Takeuchi, Oguri, & Yoshimura, 1992; Zordoky & El-Kadi, 2007). The following sections will review the expression of CYP and sEH enzymes in the cardiovascular tissues.

1.2.2.1 CYP1 Family

There are two subfamilies that are detected in rats left ventricles i.e. CYP1A1 and CYP1B1 mRNAs (Thum & Borlak, 2002). CYP1A1 mRNA was detected in the right ventricle and the left atrium of patients with dilated cardiomyopathy and in the left ventricle of healthy subjects (Thum & Borlak, 2000b, 2002). Also, left ventricular tissue of explanted human hearts contains CYP1A1 mRNA (Michaud, et al., 2010). In addition, it has been reported that CYP1A1 is inducible in the heart by treatment with β-naphthoflavone (βNF) and aroclor 1254 (Brauze, Widerak, Cwykiel, Szyfter, & Baer-Dubowska, 2006; Granberg, Brunstrom, & Brandt, 2000; Thum & Borlak, 2000a). Also, *CYP1B1* is considered the second most abundantly expressed *CYP* gene in explanted human hearts (Bieche, et al., 2007). It has been reported at a higher basal level than CYP1A1 in human cardiac fibroblasts (Dubey, et al., 2005). Also, it has been shown that CYP1B1 mRNA is induced in rat hearts using concentrated ambient particles (Ito, et al.,

2008). Also, cigarettes, which contains benzo(a)pyrene (BaP) was reported to induce CYP1A1 and CYP1B1 in rat heart (Aboutabl, et al., 2009). Within the heart, it has been also reported that aryl hydrocarbon receptor (AhR) which is a transcriptional factor that regulates CYP1A1 and CYP1B1, is highly expressed in the heart (Korashy & El-Kadi, 2006). Apart from the heart, both CYP1A and CYP1B subfamilies are also involved in generation of CYP-mediated AA metabolites within the vascular system. CYP1A1 was detected in the pulmonary aorta (Thum & Borlak, 2000b, 2002). Also, CYP1A1 was detected in smooth muscle cells of human coronary artery (Dubey, Jackson, Gillespie, Zacharia, & Imthurn, 2004). CYP1B1 is expressed in vascular smooth muscle cells (Kerzee & Ramos, 2001). In human, CYP1B1 was detected in the veins (Bertrand-Thiebault, et al., 2004). In mice, CYP1B1 protein was detected in the endothelial cells and aortic smooth muscle cells.

1.2.2.2 CYP2 Family

In the cardiovascular system, the CYP2 subfamily is widely expressed. It has been reported that subfamilies such as CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP2J are expressed in human cardiovascular tissues (Delozier, et al., 2007; Thum & Borlak, 2000b). With regard to the isozymes that are responsible for production of EETs, it has been shown that CYP2C8, CYP2C9 and CYP2J2 are responsible for the epoxygenase activity within the cardiac tissue. All theses isoforms are expressed constitutively in the normal human heart where the CYP2J2 is considered the highest expressed enzyme (Bieche, et al., 2007; Delozier, et al., 2007; Thum & Borlak, 2002). Both CYP2C8 and CYP2C9 are expressed in the arteries where the mRNA and protein expression of these isoforms were induced after the ischemic injury (Bolz, et al., 2000;

Mancy, Dijols, Poli, Guengerich, & Mansuy, 1996). In contrast, CYP2J2 were expressed significantly in normal hearts 1,000 times higher than CYP2C8 and CYP2C9 (Delozier, et al., 2007). Regarding the expression of other CYP2 subfamilies within the heart, there are limited data on the cardiac expression of these isoforms. Within the right ventricle and aorta of patients with dilated cardiomyopathy, it has been reported that CYP2B6/7 are expressed (Thum & Borlak, 2000b). Within the normal hearts, it has been reported also that CYP2D6 and CYP2E1 mRNA are expressed as well as within the dilated cardiomyopathy (Bieche, et al., 2007; Thum & Borlak, 2000b). While the CYP2D6 was expressed in the right ventricle, it has been reported that CYP2E1 is expressed in atria, ventricles, and the ventricular septum (Thum & Borlak, 2000b). Moreover, it has been reported that CYP2B6/7, CYP2D6, and CYP2C8-19 are expressed predominantly in the right ventricle (Thum & Borlak, 2000b). Also, it has been shown that CYP2B6 and CYP2C9 mRNAs were detected at low levels in some of the failing human hearts (Michaud, et al., 2010). In rat ventricular tissues, it has been reported that CYP2A1/2, CYP2B1/2, CYP2C23, CYP2E1 and CYP2J3 are expressed (Imaoka, et al., 2005; Thum & Borlak, 2002).

1.2.2.3 CYP3 Family

Several studies have shown that CYP3A4, CYP3A5, CYP3A7, and CYP3A47 mRNA were not detected in human heart tissues (Bieche, et al., 2007; Thum & Borlak, 2000b). Only one study has identified the binding of CYP3A4 antibody, which was detected by immunohistochemistry in heart tissues (Minamiyama, et al., 1999). With exception of CYP3A1 mRNA that was detected in isolated cardiomyocytes (Thum & Borlak, 2000a), CYP3A1/2 isoforms were not found in the cardiovascular tissues. Therefore, the expression of the CYP3 family within the cardiovascular tissues remains to be elucidated.

1.2.2.4 CYP4 Family

Several studies have reported that CYP4A1 was detected in dog heart tissues and freshly isolated rat cardiomyocytes (Nithipatikom, et al., 2004; Thum & Borlak, 2000a). Also, it has been reported that Cyp4a12 mRNA was expressed in mice hearts (Theken, et al., 2011). Within the human and dog heart, other studies has detected the CYP4F (Bylund, Bylund, & Oliw, 2001; Cui, Nelson, & Strobel, 2000; Nithipatikom, et al., 2004). Apart from the heart, CYP ω-hydroxylases are also expressed in endothelium and vascular tissues of human, mice, and rats. In human, CYP4A11, CYP4F2 and CYP4F3 are expressed in different tissues (Christmas, et al., 2001; Lasker, et al., 2000; Powell, Wolf, Jin, & Lasker, 1998). In rats, CYP4A1, CYP4A2, CYP4A3, and CYP4A8, CYP4F1, CYP4F4, CYP4F5 and CYP4F6 are also expressed in different tissues (Gebremedhin, et al., 2000; Hardwick, Song, Huberman, & Gonzalez, 1987; Kimura, Hanioka, Matsunaga, & Gonzalez, 1989).

1.2.2.5 Soluble epoxide hydrolase (sEH) enzyme

Among different tissues, the sEH enzyme is highly expressed in the liver followed by the kidney and then by other tissues where it is localized in cytosol, microsomes, and peroxisomes (Newman, Morisseau, & Hammock, 2005; Oesch, Schladt, Hartmann, Timms, & Worner, 1986; P. Wang, Meijer, & Guengerich, 1982; Z. Yu, et al., 2004). Within the heart, it has been reported that sEH enzyme is expressed in cardiomyocytes (Enayetallah, French, Thibodeau, & Grant, 2004; Motoki, et al., 2008; J. M. Seubert, et al., 2006; Xu, et al., 2006). Also, it has been reported that sEH is expressed in the blood vessels using immunostaining (Enayetallah, et al., 2004). Also, it has been shown that sEH was highly expressed in the human coronary endothelial cells (VanRollins, Kaduce, Knapp, & Spector, 1993) while lower levels of sEH were detected in arteries and vascular smooth muscle cells (Z. Yu, et al., 2004). Recent studies from our lab have detected the mRNA expression of sEH in the heart of SD rats and in the cardiac derived H9c2 cells (Zordoky & El-Kadi, 2008b, 2010). Also, other studies have determined a high level of sEH activity in the heart of SD rats (Oesch, et al., 1986; Schladt, Worner, Setiabudi, & Oesch, 1986).

1.2.3 Alterations in CYP and sEH expression during cardiac hypertrophy and HF

It has been previously reported that during pathological conditions such as cardiac hypertrophy and HF there is a change in expression of CYP and sEH enzymes leading to derailed CYP-mediated AA metabolism (Zordoky & El-Kadi, 2008b). During cardiac hypertrophy and HF, several human and animal studies have reported that CYP ω-hydroxylases are induced. For instance, it has been reported that gene expression of *CYP4A11* induced by 2-3 fold in cardiac hypertrophy in human (Thum & Borlak, 2002). With regard to CYP epoxygenases, the expression of these enzymes was also altered during cardiac hypertrophy and HF. For instance, in the failing hearts, it has been reported that CYP2J2 was upregulated (Tan, et al., 2002). Furthermore, the expression of sEH has been altered in several cardiovascular conditions. It has been reported that *EPHX2*, the gene encoding sEH, is a susceptibility factor for HF in spontaneously hypertensive HF rats using linkage analyses with genome-wide expression profiling (Monti, et al., 2008). Therefore, several studies have confirmed the role of sEH in HF through *EPHX2* knockout mice where the *EPHX2* gene ablation protected from pressure overload-induced HF (Monti, et al., 2008).

1.3 CYP-MEDIATED ARACHIDONIC ACID METABOLISM

1.3.1 CYP-mediated arachidonic acid metabolism

Generally, there are three metabolic pathways that are involved in the metabolism of AA (Panigrahy, Kaipainen, Greene, & Huang, 2010). First, AA can be metabolized by lipooxygenase (LOX) to form hydroperoxyeicosatetraenoic acids and dihydroxyeicosatetraenoic acids, which can be further converted to hydroxyeicosatetraenoic acids, leukotrienes, or lipoxins. Second, AA can be metabolized by cyclooxygenase (COX) into prostaglandin H_2, which can be further converted to prostacyclin or thromboxane A_2. Third, AA can be metabolized into EETs, 19-HETE, and 20-HETE by CYP enzymes.

Of importance, formation of CYP-mediated AA metabolite 20-HETE is mainly mediated through CYP4A and CYP4F subfamilies (Kroetz & Xu, 2005). However, other enzymes may have some contribution to 20-HETE formation such as CYP1A1 and CYP1B1 (Choudhary, Jansson, Stoilov, Sarfarazi, & Schenkman, 2004; Elbekai & El-Kadi, 2006). Those enzymes usually catalyze ω-hydroxylation of xenobiotics (phase I metabolic reactions) and are normally expressed in the heart and/or vascular tissues. Production of 20-HETE usually increases in response to the reduced diameter of blood vessels or induced activity of ω-hydroxylase enzymes during pathological conditions (Miyata & Roman, 2005). Therefore, 20-HETE and the enzymes produce/degrade 20-HETE are considered as therapeutic targets of CVDs. In addition, formation of 19-HETE is mainly mediated through ω-1 hydroxylases i.e. CYP2E1, CYP4A, CYP2C, and CYP2J9 (Laethem, Balazy, Falck, Laethem, & Koop, 1993; Luo, Zeldin, Blaisdell, Hodgson, &

Goldstein, 1998; Poloyac, et al., 2004; Qu, et al., 2001). This metabolite (19-HETE) has been reported to play pivotal roles in regulation of ion transport and vascular tone in the kidney (Escalante, Falck, Yadagiri, Sun, & Laniado-Schwartzman, 1988).

On the other hand, EETs are produced by CYP epoxygenases that converts olefinic bonds at the 5-, 8-, 11-, or 14- positions of AA to epoxide products 5,6-, 8,9-, 11,12-, 14,15-EETs. These EETs are mainly produced by CYP2J and CYP2C, however, other CYP enzymes have reported to produce certain amount of EETs (Zeldin, 2001). Families of CYP1A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2G, CYP2J and CYP4A have been shown to metabolize AA to EETs in various tissues and species (Kroetz & Zeldin, 2002). The CYP2C8/9/19, CYP1A2, CYP2J2 and CYP2B6 enzymes are all expressed in human and found to produce EETs when incubated with AA (Elbekai & El-Kadi, 2006).

1.3.2 Metabolic fate of CYP-mediated arachidonic acid metabolites

After formation of EETs, these metabolites are stored into the cell membrane phospholipids (Roman, 2002; Weintraub, et al., 1997). As a metabolic fate, EETs can be metabolized through a major enzymatic hydrolysis pathway to a less active form DHETs by sEH (Imig, 2000, 2012; Spector & Norris, 2007). In addition, both β-oxidation or chain elongation are considered the predominant metabolic pathways of EETs where the β-oxidation of 11,12- or 14,15- EET yields the 16 carbon epoxy fatty acid (Fang, et al., 2001; Fang, et al., 2002) and the chain elongation yields the 22 carbon epoxy fatty acid. Furthermore, CYP ω-oxidases on 8,9-, 11,12-, 14,15-EET add a hydroxyl group at the EETs's ω-terminal. With regards to COX enzymes, 5,6-, and 8,9-EET can be converted to 5,6-epoxy-PGEl (Carroll, Balazy, Margiotta, Falck, & McGiff, 1993) and 11-hydroxy-

8,9-EET (J. Y. Zhang, Prakash, Yamashita, & Blair, 1992), respectively. All EETs are subjected to glutathione-S-transferase to form glutathione conjugates (Spearman, et al., 1985). Of importance, these EETs have been reported to have different beneficial effects such as anti-apoptotic (J. K. Chen, Capdevila, & Harris, 2001), anti-fibrotic (Levick, Loch, Taylor, & Janicki, 2007), anti-inflammatory (Campbell, 2000), and vasodilating effects (Y. Zhang, et al., 2001b). On the other hand, the formed 20-HETE can be rapidly converted to 16- or 18- derivatives by β-oxidation (Fang, et al., 2001; Roman, 2002). Also, COXs convert 20-HETE to 20-OH-prostaglandin G2/H2 (Schwartzman, Falck, Yadagiri, & Escalante, 1989). Of importance, 20-HETE has been reported to have a vasoconstriction effect on the vasculature (Imig, et al., 1996; Zhu, Zhang, Medhora, & Jacobs, 2002) and other potential effects on the heart.

Furthermore, sEH enzyme is an important enzyme that is highly expressed during cardiac hypertrophy and has been implicated in development of several CVDs (Imig, 2005; Imig, Zhao, Capdevila, Morisseau, & Hammock, 2002; Kaur & Gill, 1985; Monti, et al., 2008; Sinal, et al., 2000). This implication arises due to metabolic conversion of fatty acid epoxides such as EETs to the biologically less active metabolite DHETs, which diminishes the beneficial effects of EETs on the heart functions. **Figure 1.1** summarizes the metabolic pathways of CYP enzymes and formation of CYP-mediated AA metabolites (20-HETE, EETs, and DHETs).

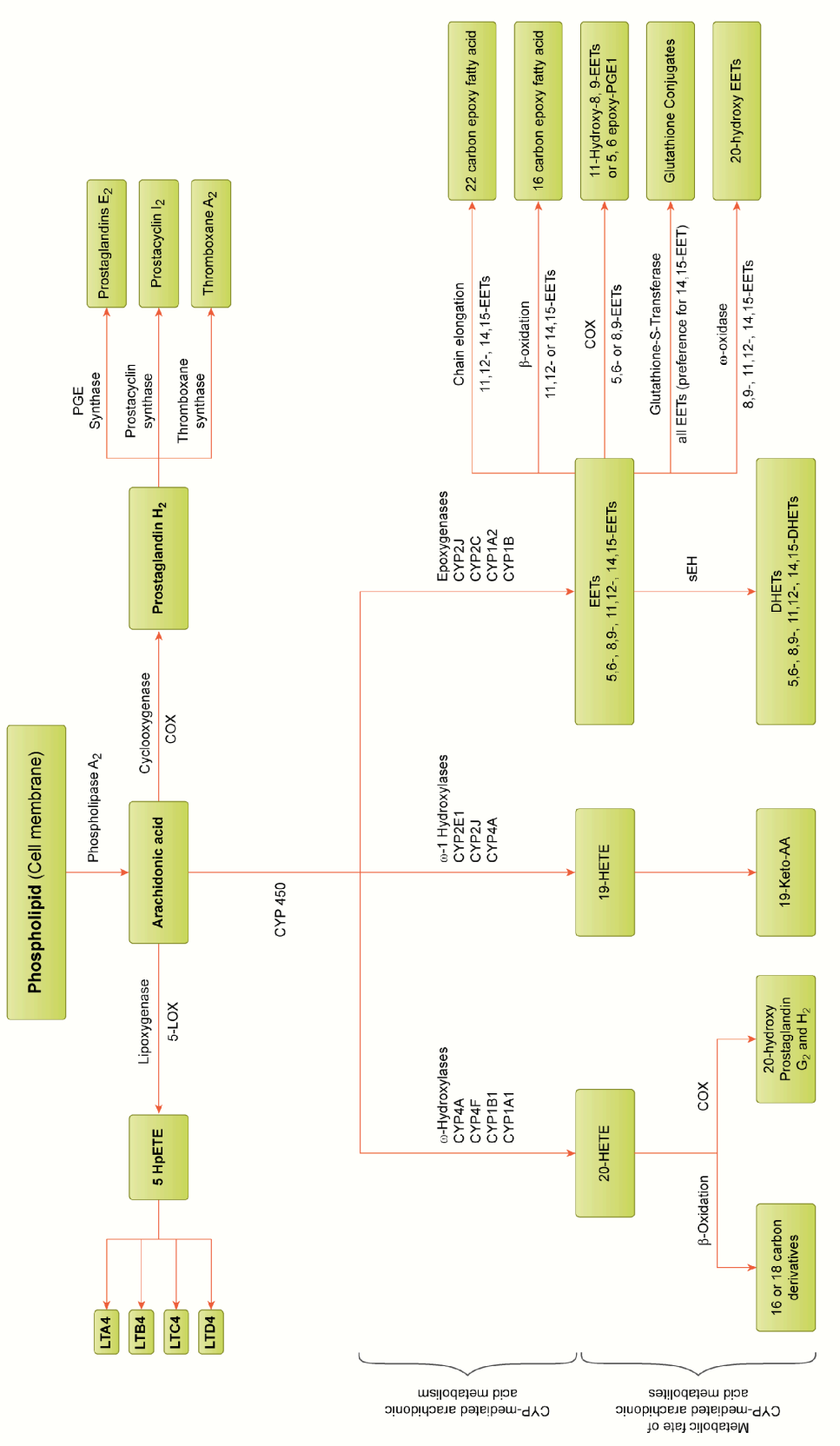

Phospholipid (Cell membrane)
Phospholipase A2
Arachidonic acid
Cyclooxygenase
COX
Prostaglandin H2
PGE Synthase
Prostacyclin synthase
Thromboxane synthase
Prostaglandins E2
Prostacyclin I2
Thromboxane A2
Lipoxygenase
5-LOX
5 HpETE
LTA4
LTB4
LTC4
LTD4
CYP 450
ω-1 Hydroxylases CYP2E1 CYP2J CYP4A
19-HETE
19-Keto-AA
Epoxygenases CYP2J CYP2C CYP1A2 CYP1B
EETs 5,6-, 8,9-, 11,12-, 14,15-EETs
Chain elongation
11,12-, 14,15-EETs
β-oxidation
11,12- or 14,15-EETs
22 carbon epoxy fatty acid
16 carbon epoxy fatty acid
COX
5,6- or 8,9-EETs
11-Hydroxy-8, 9-EETs or 5, 6 epoxy-PGE1
Glutathione-S-Transferase
all EETs (preference for 14,15-EET)
Glutathione Conjugates
ω-oxidase
8,9-, 11,12-, 14,15-EETs
20-hydroxy EETs
sEH
DHETs 5,6-, 8,9-, 11,12-, 14,15-DHETs
ω-Hydroxylases CYP4A CYP4F CYP1B1 CYP1A1
20-HETE
COX
20-hydroxy Prostaglandin G2 and H2
β-Oxidation
16 or 18 carbon derivatives
CYP-mediated arachidonic acid metabolism
Metabolic fate of CYP-mediated arachidonic acid metabolites

Figure 1.1 CYP-mediated arachidonic acid metabolism and metabolic fate of CYP-mediated arachidonic acid metabolites (20-HETE, EETs, 19-HETE, and DHETs). Three metabolic pathways are involved in the metabolism of arachidonic acid (AA). These pathways include: cytochrome P450 (*CYP*), cyclooxygenase (COX), and lipoxygenase (LOX) pathways. CYP pathway generates 20-HETE (20-hydroxyeicosatetraenoic acid), 19-HETE (19-hydroxyeicosatetraenoic acid), and EETs (epoxyeicosatrienoic acids) (CYP enzymes are arranged in an order that reflects their contribution in the metabolism from the highest to the lowest). Thereafter, CYP-mediated AA metabolites are subjected to further metabolism as a metabolic fate affecting the levels of these metabolites. Also, AA can be metabolized by COXs to generate prostaglandin H_2, which can be further metabolized into prostaglamdin E_2, prostacyclin I_2, and thromboxane A_2. On the other hand, AA can be metabolized by 5-LOX to generate HpETE, which can be further metabolized into leukotrienes. HpETE, hydroperoxyeicosatetraenoic acid; *PGE,* prostaglandin; $LT(A,B,C,D)_4$, leukotriene $(A,B,C,D)_4$.

1.4 ROLE OF CYP-MEDIATED ARACHIDONIC ACID METABOLITES IN DEVELOPMENT OF CARDIAC HYPERTROPHY AND HEART FAILURE

1.4.1 CYP-mediated arachidonic acid metabolites and cardiac hypertrophy

Cardiac hypertrophy can be defined as a thickening of the ventricular wall and septum which can be recognized by an increase in cardiomyocytes size, protein synthesis, altered sarcomeric organization, and changes in size/geometry of the heart chambers leading to cardiac remolding (Braunwald & Bristow, 2000; Liang & Gardner, 1999; Rohini, Agrawal, Koyani, & Singh, 2010). At the molecular level, cardiomyocytes usually respond to stimuli by increasing the cardiac output in order to meet the body demand. However, this response may progress to cardiac hypertrophy and HF (Nadal-Ginard, Kajstura, Leri, & Anversa, 2003). Of interest, 20-HETE affects cardiomyocytes by triggering potential intracellular signaling cascades, which are known to cause changes in gene transcription, protein synthesis, and resulting in pathological cardiac hypertrophy. These signaling cascades regulate several cardiac functions during normal and pathological conditions. Furthermore, cardiotoxic effects of 20-HETE may occur as a result of its direct action on cardiomyocytes. Recently, it has been reported that 20-HETE stimulates an L-type Ca^{+2} channel, through a protein kinase C (PKC) dependent mechanism, leading to contractile dysfunction in rat cardiomyoctes (Zeng, et al., 2010). On the other hand, EETs have been shown to inhibit several signaling pathways involved in the development of pathological hypertrophy. However, further studies are required to examine the role of CYP-mediated AA metabolites on the induction of pathological vs. physiological cardiac hypertrophy and/or concentric vs. eccentric cardiac hypertrophy.

Figure 1.2 represents the potential effects of 20-HETE and EETs on the intracellular signaling pathways that may be involved in the pathogenesis of cardiac hypertrophy.

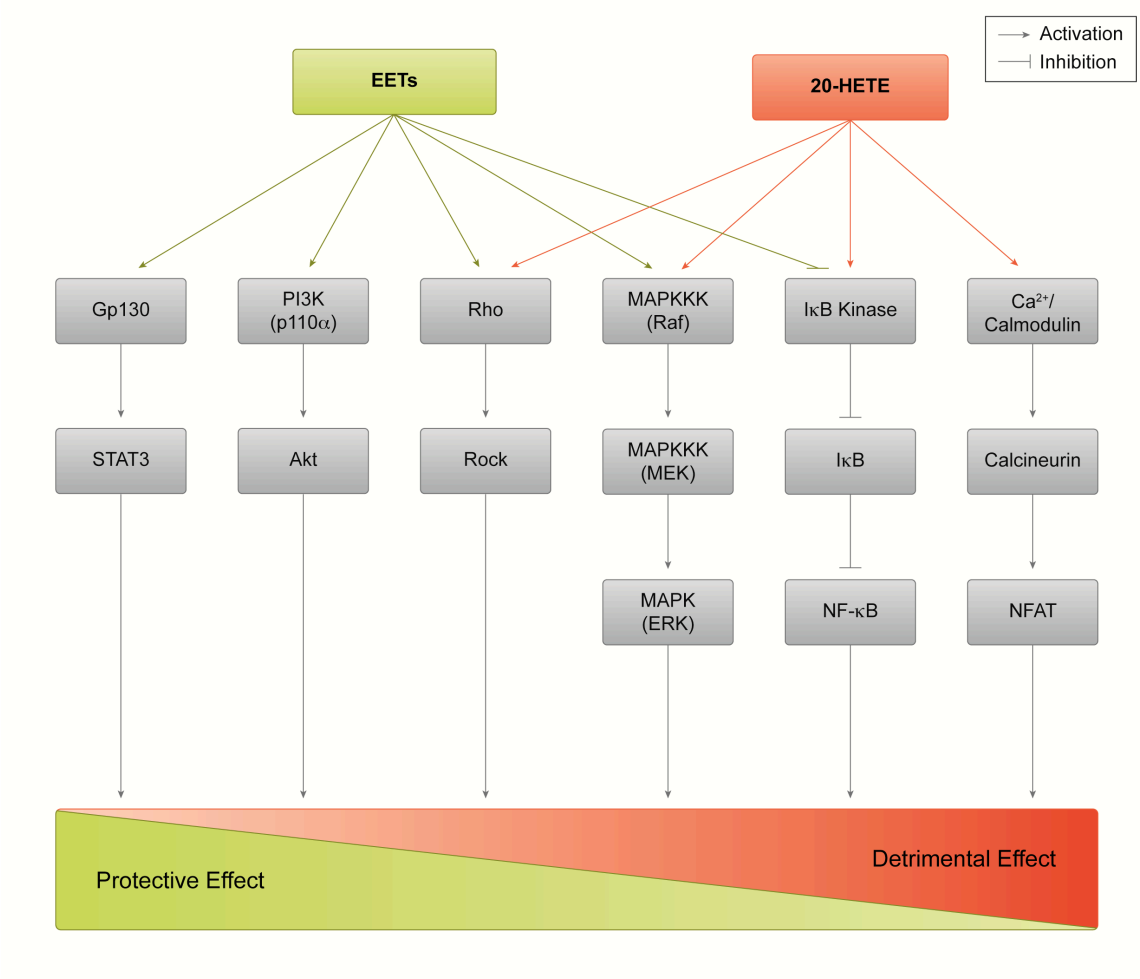


Figure 1.2 Potential effects of 20-HETE and EETs on the intracellular signaling pathways that may be involved in the pathogenesis of cardiac hypertrophy. 20-HETE and EETs mediate their detrimental or protective roles, respectively, through several signaling pathways. These pathways include: NFAT, NF-κB, MAPK, ROCK, PI3K, and STAT-3. With the exception of the NF-κB pathway, the effects of (20-hydroxyeicosatetraenoic acid) and reduced EETs (epoxyeicosatrienoic acids) on these pathways were demonstrated in non-cardiac cells, and their roles in the pathogenesis of cardiac hypertrophy induced by alteration in CYP-mediated AA metabolites need to be confirmed. ECM: extracellular matrix degradation.

1.4.2 Potential intracellular targets of EETs and 20-HETE within the heart

Although 20-HETE and EETs are seldom found in the blood at significant levels, these lipid mediators have been proposed to work as autocrine and paracrine factors that regulate several biological functions in the heart and vasculature. The autocrine response is elicited by lipid mediators that are synthesized from the membrane phospholipids by the action of phospholipase A_2 (Spector, 2009). On the other hand, the paracrine response is elicited by lipid mediators that are taken from the extracellular fluid of adjacent cells. Mechanistically, it is proposed that response to these metabolites can be mediated through activation of membrane receptor linked to intracellular signaling cascades. According to this hypothesis, it has been shown that a cell surface protein was recognized as a stereoselective binding site for 14(R),15(S)-EET where 14,15-EETs down regulate putative receptor through activation of protein kinase A (PKA) and through elevation of cAMP in guinea pig monocytes (P. Y. Wong, Lai, & Falck, 2000; P. Y. Wong, Lai, Shen, Belosludtsev, & Falck, 1997). On the other hand, it is also proposed that these lipid mediators can directly interact with intracellular components leading to the observed response. This hypothesis involves interaction of lipid mediators with fatty acid binding protein, transcription factors, ion channels, and cellular protein (Fang, et al., 2001; Fang, Kaduce, Weintraub, & Spector, 1997; Lee, et al., 1999; Lu, Hoshi, Weintraub, Spector, & Lee, 2001). Of interest, a recent study showed CYP4A/CYP4F 20-HETE system is expressed in endothelial progenitor cells where the 20-HETE can act as an autocrine and paracrine factor (Guo, et al., 2011). Indeed, lipid mediators such as 20-HETE and EETs exist in high concentrations within the tissue due to high lipophilicity, high binding affinities to albumin (Widstrom, Norris, & Spector, 2001), and storage in the membrane

phospholipids limiting the availability of these mediators within the vascular space (VanRollins, Kaduce, Fang, Knapp, & Spector, 1996).

In the following sections, several targets that are affected by 20-HETE and/or EETs will be discussed. First, these metabolites may act directly on the cardiomyocytes by modulating several intracellular signaling cascades considered as potential players in the pathogenesis of cardiac hypertrophy. Second, potential effects of these metabolites on the apoptosis, inflammation, and extracellular matrix degradation will eventually contribute to the transition toward the pathological hypertrophy.

1.4.2.1 Calcineurin/Nuclear factor of activated T-cells

Nuclear factor of activated T-cells (NFAT) is a family of transcription factors that have essential roles in development of cardiac system. It is considered as a calcium (Ca^{+2})-dependent transcription factor, which has a role in non-immune cells such as cardiomyocytes (Hill-Eubanks, Gomez, Stevenson, & Nelson, 2003). The NFAT family can be classified as: NFATc1, c2, c3, and c4. In resting cells, NFAT exists in the cytoplasm. Upon the activation, NFAT is dephosphorylated and translocated to the nucleus where it becomes an active transcription factor (Crabtree & Olson, 2002). The most common pathway to activate NFAT is associated with receptors linked to calcium where calcineurin works as a protein phosphatase. Therefore, calcineurin is directly involved in activation of NFAT and its translocation to nucleus. Consequently, the activated NFAT induces the expression of different mediators and represents a potential target for different inhibitors such as tacrolimus (J. Liu, et al., 1991).

Recent studies have shown that the hypertrophic response of cardiomyocytes is linked to alteration in calcium regulation, which in turn activates calmodulin-activated calcineurin (Wilkins & Molkentin, 2004). Thereafter, calcineurin activates NFAT as a downstream signaling cascade mediating the hypertrophic response through different kinases (Wilkins & Molkentin, 2004). Therefore, the calcium-calcineurin-NFAT cascade is considered as a potent pro-hypertrophic pathway. Both calcineurin-NFAT and MAPK signaling cascades are shown to be dependent on each other where they are involved in organization of hypertrophy response (Molkentin, 2004). Interestingly, it has been demonstrated that 20-HETE induced NFAT3 translocation in pulmonary artery smooth muscle cells (Yaghi & Sims, 2005). Therefore, activation of NFAT3 by 20-HETE could be a possible mechanism that explains the pro-hypertrophic effect of 20-HETE. However, it is still not known if 20-HETE can also induce NFAT translocation in cardiomyocytes. On the other hand, to the best of our knowledge, the effect of EETs on NFAT signaling is not known.

1.4.2.2 Nuclear factor kappa B (NF-κB)

NF-κB is a transcription factor having a pivotal role in regulation of normal cardiac functions and has been linked to development of several cardiac diseases (Valen, Yan, & Hansson, 2001). It can be described as a transcription factor found in different cells in the inactive from, and upon activation, it regulates several genes (Baldwin, 1996; Gutierrez, Kuri, & del Castillo, 2008; Miyamoto & Verma, 1995; Sen & Baltimore, 1986; Van der Heiden, Cuhlmann, Luong le, Zakkar, & Evans, 2010). In almost all cells, NF-κB exists in the cytoplasm in the inactive form (trimeric). Upon activation, NF-κB (dimer) is released by proteases and translocated to the nucleolus to activate transcription

of several genes (E. T. Wong & Tergaonkar, 2009). Over the years, the role of NF-κB signaling cascade in the development of cardiac hypertrophy and HF has been discussed extensively. It has been previously reported that NF-κB activation is sufficient to cause hypertrophy in rat neonatal ventricular cardiomyocytes (Purcell, et al., 2001). Activation of NF-κB was essential to induce hypertrophic markers such as atrial natriuretic factor and to increase size of cardiomyocytes. In addition, many studies showed that NF-κB is able to control the pro-inflammatory cytokines and to induce expression of immune proteins such as tissue necrosis factor alpha (TNFα), which has a depressant effect on the heart leading to HF (Gutierrez, et al., 2008; Meldrum, 1998).

Of interest, 20-HETE has been shown to activate NF-κB in human endothelial cells (Ishizuka, et al., 2008). Mechanistically, 20-HETE can activate NF-κB and its downstream events by stimulating the inhibitor of NF-κB phosphorylation (IκB) in endothelial cells (Ishizuka, et al., 2008). First, 20-HETE initiates the activation of NF-κB phosphorylation (IκB) inhibitor, which keeps NF-κB in the inactive form through protein-protein interactions. Subsequently, the free NF-κB is able to interact with DNA and activates gene transcription (F. Chen, Castranova, Shi, & Demers, 1999; Ghosh, May, & Kopp, 1998). In addition, activation of NF-κB may attributed to generation of reactive oxygen species (ROS) and oxidative stress where NF-κB is known to be a redox-sensitive transcription factor (Kabe, Ando, Hirao, Yoshida, & Handa, 2005). This activation will increase the expression of intracellular cell adhesion molecule. Similar to the NFAT pathway, NF-κB activation may be another mechanism by which 20-HETE can elicit direct hypertrophic effect on cardiomyocytes. However, future research should investigate the effect of 20-HETE on the NF-κB cascade within the cardiac tissue.

On the other hand, several studies have shown that EETs inhibit NF-κB signaling cascade. For instance, EETs have been demonstrated to inhibit NF-κB and I-κB kinase in human endothelial cells (Node, et al., 1999). Similarly, CYP epoxygenase transfection or exogenous addition of EETs has been shown to inhibit NF-κB nuclear translocation and NF-κB DNA binding in mouse aortic endothelial cells (Moshal, et al., 2008). Of interest, sEH inhibitors, which increase the biological levels of EETs, were reported to protect against cardiomyocyte hypertrophy by blocking the NF-κB activation (Xu, et al., 2006). Therefore, the inhibition of NF-κB signaling by EETs is a proven mechanism by which EETs can protect against cardiac hypertrophy.

1.4.2.3 Mitogen-activated protein kinases (MAPKs)

MAPKs can be described as a triple kinase pathway, which includes MAPK kinases kinase (MKKK) and MAPK kinase (MKK) (Raman, Chen, & Cobb, 2007; Rose, Force, & Wang, 2010). At the intracellular level, MAPKs are considered as signaling cascades that activate and control different intracellular functions such as cell proliferation and death. Regulation of these intracellular functions is mediated through phosphorylation of specific amino acids in target proteins (Vatner, et al., 2000). Activation of tyrosine kinase receptor usually leads to activation of Ras (master regulator that activate MAPKs) and Raf (MAPK kinase kinase) (Muslin, 2005). Accordingly, MKK1/2 are activated, which in turn activate ERK1/2. The activated ERK1/2 play pivotal roles in regulation of cellular functions of cardiomyocytes and eventually produce cardiac hypertrophy (Glennon, Sugden, & Poole-Wilson, 1995). On the other hand, other MAPKs such as p38 and JNK are shown to be involved in regulation of cell functions (Raman, et al.,

2007). Indirectly, JNK and p38 are activated through activated GTP-bound ras, which directly activate PI3Kα. The activated PI3Kα activates rac GTPase (Rodriguez-Viciana, et al., 1994; Tsakiridis, Taha, Grinstein, & Klip, 1996), which in turn activates MEKK known as the MKKKs for signaling cascades such as p38 and JNK (Nimnual, Yatsula, & Bar-Sagi, 1998). As an independent activation pathway, other stimuli such as ROS are shown to be involved in activation of p38 and JNK without activation of rac (Urano, et al., 2000).

With regard to cardiovascular disease models, there are four members of MAPKs that are involved in the development of cardiac hypertrophy i.e. ERK1/2, JNK, p38, and ERK5 (Rose, et al., 2010). Accordingly, extensive research has revealed that those MAPKs are involved in modulation of cardiac hypertrophy resulting from the pressure overload and other cardiovascular disease models. While the activation of ras-Raf-MKK-ERK signaling cascade causes cardiac hypertrophy, activation of JNK or p38 does not lead to cardiac hypertrophy (Petrich, et al., 2004). Usually, MAPKs regulate hypertrophic response to pressure overload by ras-Raf-MKK-ERK cascade. As a result, Raf-MKK-ERK1/2 will promote the survival of cardiomyocytes. In addition, activation of Ras/MAPK regulates other cellular signaling molecules involved in cell proliferation. On the other hand, these effects are antagonized by MKKK-MKK4/7-JNK and MKKK-MKK3/6-p38 cascades (Heineke & Molkentin, 2006). Moreover, MAPKs can promote contractile dysfunction leading to ventricular dilatation through p38α (Sugden, 2001). Activation of p38α was reported to cause contractile dysfunction of cardiomyocytes (Y. Wang, et al., 1998; S. Zhang, et al., 2003). However, simultaneous activation of JNK, ERK, and p38 results in pathological cardiac hypertrophy (Heineke & Molkentin, 2006).

Furthermore, MAPKs have been reported to have other roles that include regulation of cardiac remodeling after myocardial infarction, modulation of atherosclerosis, and vascular stenosis. Both p38α and JNK1/2 are involved in pathological cardiac remodeling (See, et al., 2004). Also, JNK and p38α were shown to be involved in regulation of macrophages foam cell formation leading to formation of atherosclerotic lesion (Rahaman, et al., 2006; Ricci, et al., 2004; Silverstein & Febbraio, 2000).

Although few studies are available to describe the precise action of ERK5 in the cardiovascular disease models, ERK5 has been shown to be involved in development of cardiac hypertrophy. A recent study showed that activation of Gq-dependent ERK5 in cardiomycytes has an important physiological role through Gα(q)/PKCζ/ERK5 signaling cascade (Garcia-Hoz, et al., 2012). Within the cardiomyocytes, different stimuli such as Ang II are also reported to activate ERK5 signaling cascade (Ikeda, et al., 2005; Nicol, et al., 2001). This activation is mediated through G protein coupled receptors during the cardiac hypertrophy (Kacimi & Gerdes, 2003). In addition, other study showed that ERK5 has important roles in cardiomyocyte hypertrophy and survival through myocyte enhancer factor (MEF2) of neonatal cardiomyocytes (Z. Zhao, et al., 2010). It has been also shown that ERK5 is activated in Ang II-induced hypertrophy of human aortic smooth muscle cells though activation of MEF-2C (Z. Zhao, et al., 2009) and through AT1/PKC/PKD pathway (Geng, et al., 2009). Also, PKC epsilon-dependent ERK5 phosphorylation was reported to mediate hypertrophic response in cardiomyocytes (Z. Zhao, et al., 2010).

Of interest, a recent study has shown that inhibition of ω-hydroxylases by the selective inhibitor HET0016 was effective to decrease apoptosis through the activated ERK1/2 in ischemic reperfusion heart (Lv, et al., 2008). This effect is mediated through enhanced activity of ERK1/2 but not through JNK or p38α. Furthermore, 20-HETE has been shown to cause a five-fold increase in the protein expression of ERK1 and ERK2 in renal microvessels (Sun, Falck, Harder, & Roman, 1999). It has been demonstrated that 20-HETE activates the Raf/MEK/ERK pathway in renal epithelial cells (Akbulut, et al., 2009). Similarly, EETs have been shown to activate ERK and p38 MAPKs in endothelial cells (Y. Wang, et al., 2005). Moreover, it has been demonstrated that CYP2J2-derived metabolites are cardioprotective after ischemia by a mechanism that involves p42/p44 MAPK activation (J. Seubert, et al., 2004). In summary, CYP-mediated AA metabolites, 20-HETE and EETs are reported to interact with several MAPKs involved in cell survival and/or hypertrophic response. However, further research is urgently needed to demonstrate whether these interactions are detrimental or protective with regard to the pathogenesis of cardiac hypertrophy.

1.4.2.4 Rho-kinases (ROCKs)

Rho-kinases are classified as effectors of small G protein RhoA that regulates cellular functions such as proliferation and apoptosis (Etienne-Manneville & Hall, 2002; Sahai & Marshall, 2002). However, the abnormal activation of this signaling pathway has been reported to be associated with cardiac hypertrophy (Kuwahara, et al., 1999). During cardiac hypertrophy, the downstream zinc finger transcription factor (GATA-4) is linked to the Rho/ROCK signaling cascade (Yanazume, et al., 2002). Furthermore, Rho inhibitor protects against excessive endurance exercise training-induced cardiac

hypertrophy (Ho, Huang, Huang, & Lin, 2012). A recent study showed that RhoA/ROCK might be involved in cardiac hypertrophy induced by experimental hyperthyroidism (Na, et al., 2012). 20-HETE induces contraction of small coronary arteries through the activation of Rho-kinase (Randriamboavonjy, Busse, & Fleming, 2003). Similarly, it has been shown that an 8,9-EET analog protects pulmonary artery smooth muscle cells from apoptosis through the ROCK pathway (J. Ma, et al., 2010). Similar to the MAPK pathway, how the interactions between these eicosanoids and the ROCK pathway can be involved in the pathogenesis of cardiac hypertrophy remains an important question that requires further research.

1.4.2.5 Phosphoinositide 3-kinase (PI3K/Akt)

PI3K has several isoforms that possess distinct biological functions (Aoyagi & Matsui, 2011). Among PI3K isoforms, p110α and p110γ are highly expressed in the heart (Crackower, et al., 2002; Oudit & Penninger, 2009). Cardiac PI3K (p110γ) is induced by GPCRs and it has been shown to be activated in a model of pressure overload of cardiac hypertrophy (Naga Prasad, Esposito, Mao, Koch, & Rockman, 2000). In addition, deficiency of PI3K (p110γ) has been shown to protect against isoproterenol-induced HF (Oudit, et al., 2003). On the other hand, PI3K (p110α) that is regulated through receptor tyrosine kinase has been shown to be essential for the physiological hypertrophy and to protect against dilated and hypertrophic cardiomyopathy (McMullen, et al., 2007; McMullen, et al., 2003). Therefore, it is believed that PI3K (p110α) signaling cascade is important in the physiological exercise-induced hypertrophy, whereas PI3K (p110γ) may be implicated in the pathological hypertrophy (Bernardo, Weeks, Pretorius, & McMullen, 2010). Indeed, a conditional activation of Akt for 2 weeks resulted in the

physiological hypertrophy, whereas a more prolonged Akt activation caused the pathological hypertrophy with a marked decrease in cardiac function (Shiojima, et al., 2005).

Two downstream targets i.e. glycogen synthase kinase-3 (GSK-3) and mammalian target of rapamycin (mTOR) are thought to be involved in mediating the PI3K/Akt's activation of cardiac hypertrophy (Fingar, Salama, Tsou, Harlow, & Blenis, 2002; Frey & Olson, 2003). It has been shown that 20-HETE induced nitric oxide production in pulmonary artery endothelial cells through activation of PI3K/Akt pathway (Bodiga, et al., 2010). Similarly, 20-HETE stimulates migration of vascular smooth muscle cells through pathways that involve PI3K (Stec, Gannon, Beaird, & Drummond, 2007). Moreover, it has been shown that 20-HETE increases Akt phosphorylation, a common downstream target of PI3K activation, in pulmonary artery endothelial cell (Y. Chen, Medhora, Falck, Pritchard, & Jacobs, 2006). Nevertheless, the effects of 20-HETE on PI3K/Akt signaling cascade and specific PI3K isoforms have never been investigated in the heart. On the other hand, several studies have shown that EETs activate the PI3K/Akt signaling cascade. Pretreatment with 8,9-, 11,12-, or 14,15-EET increased the activity of the prosurvival PI3K enzyme and its downstream targets in neonatal rat cardiomyocytes subjected to hypoxia and reoxygenation (Dhanasekaran, et al., 2008). Indeed, the anti-apoptotic properties of EETs have been demonstrated to depend mainly on activating the PI3K (p110α) signaling pathway (Batchu, et al., 2011b). Therefore, EETs are thought to have a cardioprotective effect through activating the PI3K (p110α) signaling pathway. Nevertheless, it is important to investigate whether EETs can induce and/or potentiate the physiological hypertrophy through activating this pathway.

1.4.2.6 Gp130/STAT3 signaling

Several genes involved in hypertrophic and survival signaling pathways are induced through activation of Gp130 activation, which causes the translocation of STAT3 to the nucleus (Yamauchi-Takihara & Kishimoto, 2000). Also, it is well reported that STAT3 overexpression induced cardiomyocyte hypertrophy in transgenic mice (Kunisada, et al., 2000; Kunisada, et al., 1998). However, the pressure overload initiated by aortic band ligation caused a dilated cardiomyopathy accompanied by massive apoptosis using the gp130 deficient mice (Hirota, et al., 1999). Thus, several studies have showed that this signaling pathway is necessary to promote survival of cardiomyocytes (Frey & Olson, 2003). Of interest, it has been reported that 14,15-EET was able to activate STAT-3 in human dermal microvascular endothelial cells (Cheranov, et al., 2008). Therefore, STAT-3 could also be a possible mechanism by which EETs exert their cardioprotective effects. However, there are no reports about the effect of 20-HETE on STAT3 signaling pathway.

1.4.2.7 β-adrenergic receptors/cAMP

Several studies have demonstrated that β-receptors are expressed in cardiomyocytes (Lohse, Engelhardt, & Eschenhagen, 2003). Activation of β_1 leads to activation of adenylate cyclase (Xiang & Kobilka, 2003; R. P. Xiao, et al., 2006). This activation increases cAMP, which in turn activates protein kinase A (PKA) leading to phosphorylation of Ca^{2+} channel. As a result, this activation leads to increased contractility and heart rate (Bers, 2002). However, desensitization of β-adrenergic

receptors alters cAMP signaling through reduction of cAMP-phosphodiesterases leading to cardiac hypertrophy (Abi-Gerges, et al., 2009).

Of interest, it has been shown that EETs activates cAMP/PKA cascades through activation of adenosine 2A receptor leading to vasodilating effect in rat preglomerular microvessels (Carroll, et al., 2006). Also, it has been shown that overexpression of CYP2J2 enhanced the cardiac L-type Ca^{2+} currents through cAMP-PKA-dependent phosphorylation of the L-type Ca^{2+} channel in cardiomyocytes of CYP2J2 transgenic mice (Y. F. Xiao, et al., 2004). On the other hand, a recent study has showed that 20-HETE can induce hyperglycemia in CYP4F2 transgenic mice through cAMP/PKA-PhK-GP (Lai, Wu, Liu, & Zhao, 2012). In short, cAMP is an important second messenger that mediate different hypertrophic or anti-hypertrophic signaling cascades, however, further investigations are required to examine the influence of CYP-mediate AA metabolites on cAMP.

In conclusion, 20-HETE and EETs have been demonstrated to modulate several signaling pathways that are involved in the pathogenesis of cardiac hypertrophy. In general, 20-HETE activates the pathways that result in the pathological hypertrophy e.g. NFAT and NF-κB. On the other hand, EETs activate the pathways that usually result in cardioprotection and/or physiological hypertrophy e.g. PI3K (p110α) and STAT-3. In addition, EETs inhibit NF-κB that is involved in the development of the pathological cardiac hypertrophy. Inhibition of NF-κB by EETs is the only signaling pathway that has been experimentally demonstrated to explain the anti-hypertrophic effect of EETs. The potential role of 20-HETE and EETs in the modulation of other pathways is extrapolated from cells other than cardiomyocytes and/or not experimentally proven to be directly

involved in cardiac hypertrophy. Therefore, a lot of research is still needed to explore how the interactions between CYP-mediated eicosanoids and these signaling pathways within the cardiomyocytes can provide the mechanistic explanation of their documented role in the pathogenesis of cardiac hypertrophy.

1.4.2.8 Apoptotic cascades

HF is associated with an increase in the cardiomyocytes apoptosis to 10- to 100-fold higher than that observed in healthy hearts (Konstantinidis, Whelan, & Kitsis, 2012). In order to demonstrate that increased apoptosis can cause HF, Wencker et al have used transgenic mice that express a conditionally active caspase in the myocardium. Increased cardiomyocytes apoptosis caused a lethal dilated cardiomyopathy and inhibition of cardiomyocytes death prevented HF in these mice (Wencker, et al., 2003). Cardiomoycytes apoptosis is regulated through several signaling pathways that have been reviewed extensively in literature (Dorn, 2009; Foo, Mani, & Kitsis, 2005; MacLellan & Schneider, 1997). In brief, apoptosis is usually activated due to activation of cell surface receptors "death receptors", as an extrinsic signaling cascade, while the changes in mitochondria represent the intrinsic pathway for its activation (Nagata, 1997; Tsujimoto, 1998). The activation of death receptors is mediated through activation of caspase-8 (Nagata, 1997), For instance, upregulation of TNF-α induces apoptosis in cardiomyocytes through induction of Fas receptors and matrix metalloproteases (MMP-2) (Krown, et al., 1996; Shen, O'Brien, & Xu, 2006; Torre-Amione, et al., 1996). Bcl-2 is also shown to be involved in regulation of the intrinsic pathway by causing release of pro-apoptotic factors such as cytochrome c (X. Liu, Kim, Yang, Jemmerson, & Wang, 1996; Narula, et al., 1999; Tsujimoto, 1998). Cytochrome c usually binds to Apaf-1 and

caspase-9 leading to activated form (Li, et al., 1997; H. Zou, Henzel, Liu, Lutschg, & Wang, 1997). Thereafter, caspase-3 pathway is activated leading to damage of DNA and several structural changes associated with pathological cardiac hypertrophy (Akyurek, et al., 2001; Li, et al., 1997; Narula, et al., 1996; Olivetti, et al., 1997). Both the extrinsic and intrinsic pathways have been described in the cardiomyocytes (Chiong, et al., 2011).

Of interest, 20-HETE has been reported to induce the apoptotic pathways in cardiomyocytes through the intrinsic mitochondrial pathways in neonatal rat cardiomyocytes (Bao, et al., 2011). Moreover, inhibition of CYP ω-hydroxylases reduces cardiomyocyte apoptosis in rat myocardial ischemia-reperfusion (Lv, et al., 2008). On the other hand, EETs are known to have several anti-apoptotic properties. It has been shown that pretreatment with 8,9-, 11,12-, or 14,15-EET inhibited apoptosis in neonatal rat cardiomyocytes after exposure to hypoxia and reoxygenation mainly through activating the pro-survival enzyme PI3K (Dhanasekaran, et al., 2008). Similarly, 11,12-EET has been demonstrated to inhibit doxorubicin-induced activation of caspase-3 in cardiac derived H9c2 cells (Y. Zhang, et al., 2009). Recently, EETs have also been shown to inhibit TNF-α-induced apoptosis and cardiac injury both *in vitro* and *in vivo* (G. Zhao, et al., 2012). The anti-apoptotic properties of EETs are also extended to other cell types. An 8,9-EET analog has been shown to protect pulmonary artery smooth muscle cells from apoptosis (J. Ma, et al., 2010). Similarly, CYP epoxygenases overexpression attenuated apoptosis in bovine artery endothelial cells in a PI3K- and MAPK-dependant mechanism (S. Yang, et al., 2007).

Therefore, it is clear that 20-HETE or EETs can play a detrimental or a protective role in the transition toward the pathological hypertrophy, respectively. In addition to apoptotic

cell death, other pathways of non-apoptotic programmed cell death e.g. programmed necrosis and autophagy are also crucial in development of the pathological cardiac hypertrophy (Dorn, 2009; Nishida, Kyoi, Yamaguchi, Sadoshima, & Otsu, 2009). However, there are no reports about the effect of CYP-mediated AA metabolites, 20-HETE and EETs on these pathways.

1.4.2.9 Inflammatory cytokines

In cases of cardiac hypertrophy or HF, it is well documented that there is an elevation in certain inflammatory cytokines, which are considered as maladaptive mechanisms to protect the heart against further detrimental damage (Damas, et al., 2001; Eiken, et al., 2001; Mallat, et al., 2004; Torre-Amione, et al., 1996). This elevation is mainly characterized by the presence of TNFα and interleukin 1b (IL1b) that contribute to the development of HF through cardiac hypertrophy and contractile dysfunction. Also, TNFα is considered as a modest hypertrophic agent *in vitro* working on renin-angiotensin-aldosterone system (Flesch, et al., 2003). For instance, in mice, IL-1 is considered as a potent inducer of cardiac hypertrophy (Nishikawa, et al., 2006; Palmer, Hartogensis, Patten, Fortuin, & Long, 1995). Mechanistically, IL-1 is shown to be able to activate PI3K, which in turn increases protein synthesis in general and atrial natriuretic peptide expression. Furthermore, stimulation of gp130 (a receptor for IL6-cytokines) was showed to induce cardiac hypertrophy in mice through STAT3-dependent signaling pathway (Kunisada, et al., 1998). In H9c2, inflammatory cytokines induces cellular hypertrophy by activating calcium calciurin/nuclear factor of activated T-cells (C. J. Liu, et al., 2008).

Of interest, it has been shown that 20-HETE stimulates the production of inflammatory cytokines in human endothelial cells through activating NF-κB (Ishizuka, et al., 2008). Mechanistically, 20-HETE induces inflammation through activation of NF-κB, which in turn activates immune system and inflammatory cytokines in endothelial cells leading to endothelial dysfunction. Also, 20-HETE demonstrates cross talk with NF-κB and MAPK/ERK signaling pathways to mediate regulation of inflammatory cytokines. In 2008, Ishizuka and his group (Ishizuka, et al., 2008) showed that inhibition of NF-κB activation and ERK1/2 phosphorylation inhibited 20-HETE-mediated intracellular cell adhesion molecule expression. Both effects suggest that MAPK/ERK cascades are essential in the activation of NF-κB. Therefore, the pro-inflammatory properties of 20-HETE may also be involved in 20-HETE-induced cardiac hypertrophy.

On the other hand, EETs have been demonstrated to be key anti-inflammatory mediators (Norwood, Liao, Hammock, & Yang, 2010). The first report of the anti-inflammatory properties of EETs was described by Node et al who demonstrated that both 11,12-EET and CYP2J2 overexpression inhibits inflammation by a mechanism involving NF-κB inhibition (Node, et al., 1999). Similarly, inhibition of the sEH has been shown to inhibit inflammation in several animal models (Fleming, 2007; J. Y. Liu, et al., 2010). Furthermore, overexpression of CYP epoxygenase and disruption of sEH have been shown to attenuate acute vascular inflammatory response in mice (Deng, et al., 2011). Therefore, the anti-inflammatory properties of EETs may also be involved in their protective effect against cardiac hypertrophy and HF.

1.4.2.10 Oxidative stress

Superoxide anion is a product of one electron reduction of O_2, which are produced in large amounts by NADPH oxidase enzyme. It is considered as a free radical leading to cellular toxicity. In cardiomyocytes, 20-HETE induces the production of NADPH-derived superoxide anion, which in turn activates L-type Ca^{+2} channel through PKC dependent mechanism (Anrather, Racchumi, & Iadecola, 2006; Zeng, et al., 2010). This induction will lead to oxidative stress and generation of superoxide anion, which is the mediator of NF-κB activation. In addition, 20-HETE effects on ROS production illustrate the role of these species as a messenger for different effects of 20-HETE, which eventually lead to cardiac hypertrophy and HF (Akki, Zhang, Murdoch, Brewer, & Shah, 2009; Thannickal & Fanburg, 2000). Of interest, there is a positive association between 20-HETE levels and markers of oxidative damage in hypertensive patients (Ward, Puddey, Hodgson, Beilin, & Croft, 2005). Therefore, the increase in oxidative stress may also add to the detrimental effect of 20-HETE in the pathogenesis of cardiac hypertrophy and the subsequent HF. On the other hand, CYP2J2 transfection has been shown to decrease the amount of extracellular superoxide in bovine aortic endothelial cells (B. Yang, et al., 2001). Similarly, 11,12-EET has been shown to attenuate ROS level in carcinoma cells treated with arsenic trioxide (L. Liu, et al., 2011). On contrast to 20-HETE, the potential of EETs to relieve oxidative stress may explain their protective properties against cardiac hypertrophy.

1.4.2.11 Extracellular matrix degradation

Matrix metalloproteases (MMP) is classified as a family of enzymes responsible for remodeling of matrix under several CVDs (Dollery, McEwan, & Henney, 1995; Galis, et al., 1994). Their involvement in development of cardiac hypertrophy requires activation of MMP. Thereafter, there is a conversion of partially active form to the fully active form, which requires stromelysin MMP-3 (Dollery, et al., 1995). Activation of MMP-3 is considered the critical step in the process of MMP activation. Furthermore, MMP-9 has shown to be involved in degradation of myocardial matrix and metalloproteases activation. Of interest, CYP ω-hydroxylases have been shown to upregulate MMP-9 in non-small cell lung cancer (W. Yu, et al., 2011). On the other hand, both CYP2J2 transfection and exogenous EETs have been shown to attenuate MMP-9 through inhibition of NF-κB in mouse aortic endothelial cells (Moshal, et al., 2008).

Therefore, in addition to the effect of 20-HETE and EETs on the aforementioned signaling pathways, their opposing effects on apoptosis, inflammation, oxidative stress, and MMP-9 may also explain the detrimental role of 20-HETE and the protective role of EETs against the development of cardiac hypertrophy and HF.

1.4.3 Indirect effects of CYP-mediated arachidonic acid metabolites on the heart

20-HETE is classified as a pro-hypertensive eicosanoid having a pivotal role in regulation of blood pressure where it is involved in the pathogenesis of hypertension and other CVDs. It is generally accepted that 20-HETE is a potent vasoconstrictor produced in arteries of the heart, brain, and kidney where 20-HETE formation is induced by Ang II and serotonin (Cambj-Sapunar, Yu, Harder, & Roman, 2003; Imig, et al., 1996; Y. H. Ma, et al., 1993; Miyata & Roman, 2005; A. Oyekan, Balazy, & McGiff, 1997; A. O. Oyekan & McGiff, 1998; A. P. Zou, et al., 1996). Of interest, 50% reduction in vasoconstriction induced by these agents has been linked to reduced formation of 20-HETE (Alonso-Galicia, Maier, Greene, Cowley, & Roman, 2002; Carroll, et al., 1996; Croft, McGiff, Sanchez-Mendoza, & Carroll, 2000; Hercule & Oyekan, 2000; Imig, et al., 2000; A. Oyekan, et al., 1997).

Mechanistically, several studies have showed that elevated 20-HETE levels in vascular tissues is considered one of the leading causes of endothelial dysfunction and subsequent pathological complications, which in turn increase the systemic vascular resistance and after-load leading to cardiac hypertrophy and HF (Cheng, et al., 2010; Ishizuka, et al., 2008; J. S. Wang, et al., 2006). Furthermore, several lines of evidence have linked the overproduction 20-HETE with the increased vasoconstrictions and after-load. For instance, endothelial 20-HETE induces renal injury and hypertension in SD rats, which occurs due to induced levels of angiotensin converting enzyme and Ang II leading to activated renin-angiotensin system (Inoue, et al., 2009; Sodhi, et al., 2010). Also, 20-HETE contributes to the hypertension in postmenopausal hypertension in female spontaneously hypertensive rats where cerebral 20-HETE is elevated and renal levels of

20-HETE are reduced (Ando, et al., 1990). The vasoconstriction and endothelial dysfunction induced by 20-HETE is initiated by activation of signaling cascades such as MAPKs, Rho-kinase, inflammatory cytokines, and others that are essentially involved in regulation of vascular homeostasis (Ishizuka, et al., 2008; Muthalif, et al., 1998; Muthalif, et al., 2000; Muthalif, et al., 2001; Nowicki, et al., 1997; Obara, Koide, & Nakayama, 2002; Randriamboavonjy, et al., 2003; Sun, et al., 1999). Furthermore, 19-HETE has been shown to be involved in the enhancement of renal cortical Na^+/K^+ ATPase (Escalante, et al., 1988) and proximal tubule transporters (Quigley, Baum, Reddy, Griener, & Falck, 2000). In rats, it has been reported that 19-HETE has a vasodilating effect on renal arcuate arteries (Y. H. Ma, et al., 1993). However, there is no information about the effect of 19-HETE on the heart and further research is required.

On the other hand, EETs have a vasodilator effect where EETs activate the calcium-activated potassium channels (K_{Ca}) (Pomposiello, Carroll, Falck, & McGiff, 2001; Pratt, Li, Hillard, Kurian, & Campbell, 2001; Y. Zhang, et al., 2001a). Furthermore, EETs were reported to work as an endothelium-derived hyperpolarizing factor through vasodilating and hyperpolarizing effects on vascular smooth muscle cells (Gebremedhin, Harder, Pratt, & Campbell, 1998; Hecker, Bara, Bauersachs, & Busse, 1994; Medhora, Narayanan, Harder, & Maier, 2001). With regard to the effect of EETs on the kidney, EETs also mediate natriuresis that may, indirectly, protect against the development of cardiac hypertrophy by lowering the blood pressure and after-load (Dos Santos, Dahly-Vernon, Hoagland, & Roman, 2004). **Table 1.1** summarizes the potential mechanisms for the pro-hypertrophic effect of 20-HETE and the anti-hypertrophic effect of EETs.

Table 1.1 Potential mechanisms for the pro-hypertrophic effect of 20-HETE and the anti-hypertrophic effect of EETs.

Mechanism (Signaling Pathway)	**Effect of 20-HETE**	**Effect of EETs**
NFAT	Activation	Not known
NF-κB	Activation	Inhibition
MAPKs	Activation	Activation
ROCK	Activation	Activation
PI3K/Akt	Activation	Activation
STAT-3	Not Known	Activation
Apoptosis	Pro-apoptotic	Anti-apoptotic
Inflammatory cytokines	Pro-inflammatory	Anti-inflammatory
Oxidative stress	Increase	Decrease
MMP-9	Activation	Inhibition
Indirect effect (via vasculature)	Vasoconstriction	Vasodilatation
Indirect effect (via kidney)	Natriuretic	Natriuretic

Abbreviations: ED, Endothelial dysfunction; MMP, Matrix metalloproteinases; MAPKs, Mitogen activated protein kinases; NF-κB, Nuclear factor kappa B; ROS, Reactive oxygen species; NFAT, Nuclear factor of activated T-cells; PI3K, phosphatidyinositol 3 kinase; ROCK, Rho-kinases.

1.5 POTENTIAL THERAPEUTIC TARGETS WITHIN THE CYP-MEDIATED ARACHIDONIC ACID METABOLISM

A plethora of studies have identified the role of CYP-mediated AA metabolites in the pathogenesis of several CVDs. Therefore, several researchers have been investigating how to therapeutically target this pathway in order to prevent and/or treat these diseases. There are several approaches to target the CYP-mediated AA metabolism pathway. First, ω-hydroxylase inhibitors have been used to inhibit the production of the cardiotoxic metabolite, 20-HETE. Second, sEH inhibitors (sEHIs) have been developed to prevent the degradation of the cardioprotective metabolites, EETs, and thus increasing their levels. Finally, epoxygenase inducers have been investigated to induce the epoxygenase enzymes responsible for the synthesis of the EETs. In the following sections, we will highlight the use of these approaches to prevent and/or treat cardiac hypertrophy. **Figure 1.3** summarizes the potential therapeutic targets within the CYP-mediated AA metabolism and how they can prevent and/or treat cardiac hypertrophy and HF.

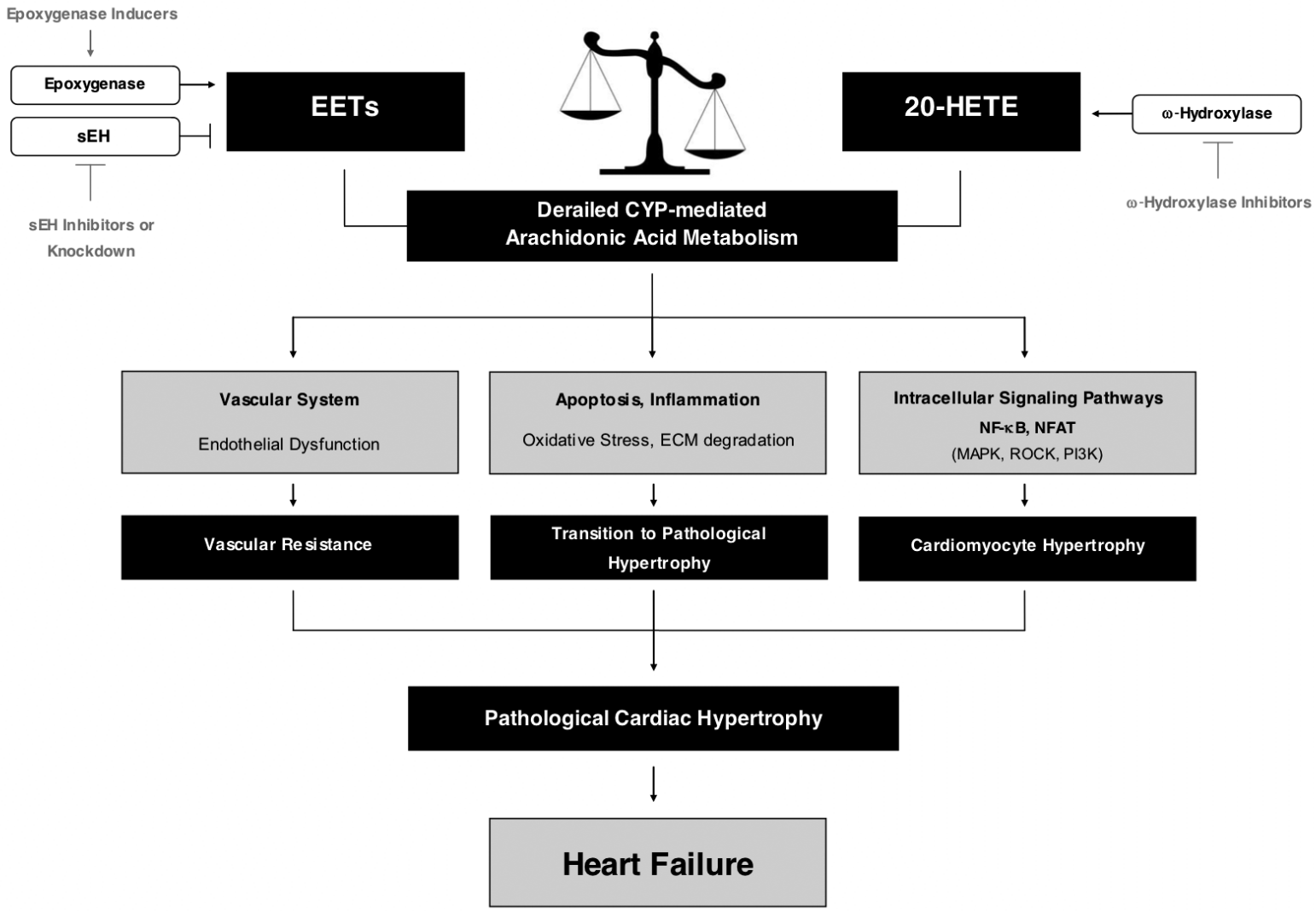

Epoxygenase Inducers
Epoxygenase
sEH
sEH Inhibitors or Knockdown
EETs
20-HETE
ω-Hydroxylase
ω-Hydroxylase Inhibitors
Derailed CYP-mediated Arachidonic Acid Metabolism
Vascular System
Endothelial Dysfunction
Apoptosis, Inflammation
Oxidative Stress, ECM degradation
Intracellular Signaling Pathways
NF-κB, NFAT
(MAPK, ROCK, PI3K)
Vascular Resistance
Transition to Pathological Hypertrophy
Cardiomyocyte Hypertrophy
Pathological Cardiac Hypertrophy
Heart Failure

Figure 1.3 Potential therapeutic targets within the CYP-mediated AA metabolism and how they can prevent and/or treat cardiac hypertrophy and HF. Alteration in the expression of CYP and sEH enzymes occurs during several cardiovascular diseases. This alteration usually results in elevated level of 20-HETE (20-hydroxyeicosatetraenoic acid) and/or reduced EETs (epoxyeicosatrienoic acids) levels. Accordingly, the altered levels of these metabolites mediate different effects through intracellular signaling cascades within the cardiovascular system leading to endothelial dysfunction and cardiomyocytes hypertrophy. Furthermore, transition to pathological hypertrophy is augmented by apoptosis, inflammation, oxidative stress, and extracellular matrix degradation. Inhibitors of CYP ω-hydroxylases, inhibitors of soluble epoxide hydrolase (sEHIs), and the inducers of CYP epoxygenase are considered as therapeutic approaches in treatment and/or prevention of cardiac hypertrophy. ED, endothelial dysfunction; ECM: extracellular matrix degradation.

1.5.1 Protective role of sEH inhibitors (sEHIs)

Two possible approaches were studied to minimize or to stop degradation of EETs and to reduce consequent detrimental effects associated with reduced level of EETs on the heart and the vasculature. These approaches include silencing of sEH enzyme or inhibiting its activity. A recent study showed that silencing of sEH was effective to reduce apoptosis induced by doxorubicin and to increase the expression of Bcl-2 using interfering RNA in the cultured rat cardiomyocytes (Du, Lv, He, & Ma, 2011). Also, knockdown of sEH reversed the arsenic-induced hypertrophic markers in H9c2 cells (Anwar-Mohamed, et al., 2012a). Furthermore, targeting the sEH enzyme activity with specific sEHIs is also considered as a feasible approach to reduce the metabolic conversion of EETs. This approach has been shown to be effective against cardiac hypertrophy in the murine model of thoracic aortic constriction resulting in a complete resolution of cardiac hypertrophy (Harris, Li, Chiamvimonvat, & Hammock, 2008). Also, it has been shown that sEHIs reduced cardiac hypertrophy through the elevation of EETs level, which inhibits NF-κB (Xu, et al., 2006). Moreover, these sEHIs are effective against other CVDs using cardiomyocytes or cell lines (Pang, et al., 2011) and animal models (Sinal, et al., 2000). Recently, it has been demonstrated that sEHI (AR9281) is a potent and selective inhibitor with adequate pharmacodynamic and pharmacokinetic properties in healthy human subjects (D. Chen, et al., 2012). Therefore, the cardiovascular effects of these sEHIs have been extensively studied and linked to reduced hypertension (Chiamvimonvat, Ho, Tsai, & Hammock, 2007; Imig, et al., 2005), post-myocardial infarction damage (Chaudhary, Abukhashim, Hwang, Hammock, & Seubert, 2010), post-cerebral ischemia damage (Dorrance, et al., 2005), vascular smooth

muscle cells proliferation (Davis, et al., 2006; Davis, et al., 2002), vasoconstrictions (Fang, et al., 2001; Fang, et al., 1998; Larsen, et al., 2006), and inflammation (Y. Liu, et al., 2005; Schmelzer, et al., 2005; Smith, et al., 2005). In addition, data from our lab demonstrated that inhibition of sEH protected against BaP-induced cardiac hypertrophy and corrected the BaP-induced derailment in CYP-mediated AA metabolism (Aboutabl, Zordoky, Hammock, & El-Kadi, 2011).

Although sEHIs were shown to be effective against several CVDs (Marino, 2009), there are some possible limitations that might hinder the clinical use of these inhibitors. These limitations include, pulmonary hypertension (Keseru, et al., 2008; Pokreisz, et al., 2006) and angiogenesis promotion in cancer patients (Pozzi, et al., 2005). A recent study showed that lipid mediators such as EETs have a role in cancer (Panigrahy, Greene, Pozzi, Wang, & Zeldin, 2011). Accordingly, active research is now directed to investigate the role of EETs' inhibitors as a novel approach to reduce tumor growth and metastasis (Jiang, et al., 2005; Nithipatikom, et al., 2010). In agreement with these findings, another recent study demonstrated that sEHIs promote the growth of primary tumor and metastasis through elevation of EETs levels in different mouse models (Jiang, et al., 2005; Panigrahy, et al., 2012). These studies raised the concern about the use of these drugs in human (D. Wang & Dubois, 2012). Thus, the use of sEHIs that are proven to be effective in treatment and/or prevention of CVDs must be carefully discussed before transferring sEHIs to the clinical practice.

1.5.2 Protective role of epoxygenase inducers or EETs stabilizers

In addition to stabilization of produced EETs by sEHIs, another effective approach to regulate the balance between EETs and 20-HETE is to increase their syntheses. Studies showed that activation of PPAR-α in obese rats improves renal endothelial functions through induction of epoxygenase CYP2C11 and CYP2C23 using fenofibrate in Zucker rats (X. Zhao, et al., 2006). Furthermore, fenofibrate was shown to be effective, in a synergetic manner with sEHIs, such as 12-(3-adamantane-1-yl-ureido) dodecanoic acid (AUDA), to reduce hypertension and renal damage by minimizing sodium retention and vascular constriction in obese rats (Huang, et al., 2007). A dual acting agent that has both EET-mimetic and sEH inhibitory effects such as UA-8 (13-(3-propylureido)tridec-8-enoic acid) was effective against ischaemia reperfusion injury where the cardioprotection is attributed to reduced mitochondrial dysfunction through class-I PI3K signaling (Batchu, et al., 2011a). Taken together, this approach may have a great potential in prevention and treatment of CVDs; however, further research is required.

1.5.3 Protective role of ω-hydroxylase inhibitors

Since the alteration in the expression of CYP ω-hydroxylase enzymes are associated with overproduction of 20-HETE in CVDs, ω-hydroxylase inhibitors have been extensively studied to investigate the role of 20-HETE in the biological system under different disease conditions. For instance, these inhibitors are effective in reducing the infarct size after ischemic reperfusion (Kroetz & Xu, 2005; Miyata, et al., 2005; Omura, et al., 2006; Renic, et al., 2009).

Of interest, inhibition of ω-hydroxylases by the selective inhibitor HET0016 has shown to be effective to protect against BaP-induced cardiac hypertrophy in rats (Aboutabl, et al., 2009). Also, HET0016 alleviates hypertension and oxidative stress in rat treated with 5-α-dihydrotestosterone (Singh, et al., 2007). A newer selective inhibitor, TS-011 [N-(3-chloro-4-morpholin-4-yl) phenyl-N'-hydroxyimido formamide] reverses the vasospasm resulting from subarachnoid hemorrhage and reduces the infarct size in stroke ischemic models (Miyata, et al., 2005). Furthermore, simultaneous inhibition of 20-HETE formations and metabolism of EETs reduces the development of hypertension and cardiac hypertrophy in Ren-2 transgenic rats (Certikova Chabova, et al., 2010). Similarly, Chabova and his reserch group showed that 1-aminobenzotriazole reduced development of hypertension in male heterozygous Ren-2 transgenic rats (Chabova, et al., 2007). They also showed that $CoCl_2$, another inhibitor of CYP activity, attenuated the blood pressure in adult Ren-2 transgenic rats with established hypertension (Chabova, et al., 2007). Recently, it has been shown that a selective CYP1B1 inhibitor prevented both Ang-II- and deoxycorticosterone-induced hypertension and cardiac hypertrophy (Jennings, et al., 2010; Sahan-Firat, et al., 2010). However, to date, it has been difficult to develop CYP ω-hydoxylase inhibitors of sufficient selectivity and adequate pharmacokinetic properties that could enable the transition of these compounds toward the human clinical trials.

In conclusion, the balance between the detrimental 20-HETE and the cardioprotective EETs is crucial in maintaining the cardiovascular homeostasis. This balance is disrupted in cardiac hypertrophy and HF. Therefore, strategies to correct this imbalance by decreasing the 20-HETE and/or increasing the EETs are potential therapeutic

interventions to prevent and/or treat pathological cardiac hypertrophy and HF.

1.6 DOXORUBICIN-INDUCED CARDIOTOXICITY

1.6.1 Types of doxorubicin-induced cardiotoxicity

There are two types of doxorubicin (DOX)-induced cardiotoxicity i.e. acute and chronic cardioxticity. The acute toxicity usually starts immediately after administration of DOX where it can be manifested by mild arrhythmias and hypertension (Takemura & Fujiwara, 2007; Y. W. Zhang, Shi, Li, & Wei, 2009). It usually affects 11% of cancer patients. On the other hand, the chronic cardiotoxicity usually starts after completion of cumulative dose regimen and affects 1.7% of cancer patients (Von Hoff, et al., 1979). This chronic cardiotoxicity is reported to be dose-dependent and to be irreversible. In addition, this cardiotoxicity causes the enlargement of the heart chamber and thinning of the ventricles' walls leading to dilated cardiomyopathy (Buja, Ferrans, Mayer, Roberts, & Henderson, 1973; Takemura & Fujiwara, 2007).

1.6.2 Doxorubicin pharmacokinetics and its distribution in the heart tissue

DOX is highly distributed in the heart tissue where the distribution is attributed to the high binding affinity of DOX to cardiolipin, which is localized in the inner membrane of mitochondria (Nicolay, et al., 1984; Terasaki, Iga, Sugiyama, & Hanano, 1982). Based on the pharmacokinetic profile, DOX has a volume of distribution of 15.4 l/Kg at the steady state (Mross, Mayer, Hamm, Burk, & Hossfeld, 1990). The maximum concentration of DOX is 2.3 μg/ml in cancer patients receiving 60 mg/m^2 IV dose (Bronchud, et al., 1990). The half-life of DOX can be classified into initial distribution half-life which is

4.2 min and the elimination half-life which is 9.87 hr (Bronchud, et al., 1990). Therefore, DOX has a slow elimination half-life attributed to renal and biliary metabolism.

1.6.3 Mechanisms of doxorubicin-induced cardiotoxicity

Mechanistically, the anticancer mechanisms of DOX are mediated through intercalation with DNA, inhibition of macromolecule synthesis, induction of oxidative stress, DNA cross-linkage, inhibition of topoisomerase 2, and induction of apoptosis (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). These anticancer mechanisms are quite different from the cardiotoxic mechanisms induced by DOX administration. However, the exact cardiotoxic mechanisms are still not fully understood. In literature, several reports linked this cardiotoxicity to generation of oxidative stress (free radical/doxorubicinol formation), disruption of mitochondria, and disruption of apoptotic cascades (Nakamura, et al., 2000; Takemura & Fujiwara, 2007; Ueno, et al., 2006).

With regard to generation of free radicals and doxorubicinol metabolites, metabolism of DOX is associated with formation of semiquinone intermediate metabolite with great tendency to form free radicals and to increase ROS leading to oxidative stress (Neilan, et al., 2007; Wallace, 2003). Directly, DOX has the ability to initiate respiratory failure through destruction of mitochondria, which eventually increase the oxidative stress (Lebrecht & Walker, 2007). Consequently, the level of antioxidant enzymes is reduced, which is originally low in the heart tissue (Doroshow, 1983; Doroshow, Locker, Baldinger, & Myers, 1979). In addition, it is well reported that DOX decreases energy reservoir (ATP/PCr) in animal models and patients due to compromised mitochondrial functions (Tokarska-Schlattner, Wallimann, & Schlattner, 2006; Tokarska-Schlattner, et

al., 2005; Tokarska-Schlattner, Zaugg, Zuppinger, Wallimann, & Schlattner, 2006).

With regard to cell deaths, there are three types of DOX-induced cardiomyocyte death including autophagy, necrosis, and apoptosis (Y. W. Zhang, et al., 2009). A recent study demonstrated that autophagy contributes in DOX-induced cell death through depletion of GATA-4 (Kobayashi, et al., 2010). Also, other studies demonstrated that necrosis mediates cell death where DOX has been reported to increase necrotic cells and inflammatory cell infiltration (Y. W. Zhang, et al., 2009). However, the main contributor in DOX-induced cell death is the disruption of apoptotic cascades (Y. W. Zhang, et al., 2009). Both directly and indirectly, DOX treatment was shown to be able to induce apoptosis in cardiomyocytes. Directly, DOX increases the calcium concentration in the mitochondria, which causes the rupture of mitochondria and the release of cytochrome c to induce apoptosis known as the caspase-dependent mechanism (Childs, Phaneuf, Dirks, Phillips, & Leeuwenburgh, 2002; Kim, et al., 2006). On the other hand, DOX can induce the apoptosis in a caspase-independent mechanism by inducing DNA lesion which, in turn up-regulates p53 and Bax (J. Liu, Mao, Ding, & Liang, 2008).

1.6.4 Current treatment approaches of doxorubicin-induced cardiotoxicity

Protection against DOX cardiotoxicity represents an important challenge to prevent detrimental effects of DOX on the heart functions while maintaining the same anticancer efficacy. Several studies showed promising results with regard to reduced myocardial damage where many efforts are focused on the modulation of DOX dosage regimen (Creech, Catalano, & Shah, 1980; Erttmann, Erb, Steinhoff, & Landbeck, 1988). Also, the use of free iron chelators was effective approach to protect the heart by preventing the

depletion of mitochondrial DNA in chronic DOX cardiotoxicity of rat models (Hasinoff, 1990; Lebrecht, et al., 2007; Schroeder & Hasinoff, 2005). For instance, inhibition of iron–related ROS generation by dextrazone was shown to be effective in reducing the DOX cardiotoxicity (Seifert, Nesser, & Thompson, 1994). In addition, DOX-induced cardiotoxicity was effectively reduced using the transgenic mice with elevated level of antioxidant enzymes (Simunek, et al., 2009). However, the use of N-acetylcysteine was not effective to protect against the DOX cardiotoxicity (Ladas, et al., 2004).

The prophylactic use of resveratrol for two weeks before DOX treatment was effective to reduce the generation of ROS and to improve the activity of glutathione/catalase enzymes (Tatlidede, et al., 2009). Also, the use of sildenafil in combination with DOX was reported to increase ROS in cancer cells while maintaining less ROS levels in normal cells (Di, et al., 2010). The early use of β-receptor antagonists with ACE inhibitors was shown to be an effective approach to improve the contraction of the myocardium (Tallaj, et al., 2005). Recent studies also showed that prophylactic treatment with erythropoietin protects against apoptosis induced by DOX (Ramond, Sartorius, Mousseau, Ribuot, & Joyeux-Faure, 2008).

1.7 RATIONALE, HYPOTHESIS, AND OBJECTIVES

1.7.1 Rationale

Doxorubicin (DOX, adriamycin) is an effective anti-neoplastic agent commonly used to treat different types of cancer such as ovarian, thyroid, gastric, breast, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, and sarcomas (Weiss, 1992; Arcamone et al., 2000; Cortes-Funes and Coronado, 2007). However, the clinical use of this drug is

limited due to cardiotoxicity, which might proceed to irreversible heart failure (Swain et al., 2003; Christiansen and Autschbach, 2006; Outomuro et al., 2007; Carvalho et al., 2009). Furthermore, it is well documented that CYP enzymes are considered as one of the major metabolic pathways for AA (Roman, 2002). Also, it has been demonstrated that there is an increase in gene expression of CYP and sEH enzymes during several CVDs (Thum & Borlak, 2002). Moreover, there is a strong correlation between the pathogenesis of CVDs and the alteration of CYP-mediated AA metabolism (Zordoky & El-Kadi, 2008b). However, few studies have investigated the effects of drug-induced cardiotoxicity on expression of CYP, sEH, and CYP-mediated AA metabolism. Taking into consideration the detrimental effects of drug-induced cardiotoxicity, DOX cardiotoxicity has been recognized as a leading cause of drug-induced heart failure, which limits the beneficial anticancer effects of DOX in cancer patients (Christiansen & Autschbach, 2006; Outomuro, Grana, Azzato, & Milei, 2007).

Data from our laboratory demonstrated that acute DOX toxicity altered expression of CYP enzymes and CYP-mediated AA metabolism with a significant decrease in the cardioprotective metabolites, EETs and a significant increase in the cardiotoxic metabolite 20-HETEs (Zordoky, Anwar-Mohamed, Aboutabl, & El-Kadi, 2010). However, the clinical situation involves chronic administration of the drug where the cumulative dose is the only currently used predictor of cardiotoxicity. Therefore, we investigated the effect of chronic DOX treatment on expression of the cardiac CYP enzymes and AA metabolism in male SD rats. A comprehensive understanding of the effects of chronic DOX treatment is urgently needed to promote the effective and rational use of several therapeutic agents that can treat and/or protect against DOX-induced HF.

1.7.2 Hypothesis

1) Chronic DOX treatment modulates cardiac expression and activity of CYP and sEH enzymes leading to imbalance between cardiotoxic and cardioprotective metabolites that cause progressive cardiotoxicity.

2) CYP ω-hydroxylase inhibitor (HET0016) confers protection against DOX-induced hypertrophy *in vitro* using cardiac-derived H9c2 cells.

3) sEH inhibitor (t-AUCB) confers protection against DOX-induced hypertrophy *in vitro* using cardiac-derived H9c2 cells.

1.7.3 Objectives

1) To determine the effects of chronic DOX treatment on the cardiac expression of CYP and sEH enzymes and the CYP-mediated AA metabolism in male SD rats.

2) To examine whether inhibition of CYP ω-hydroxylase enzymes confers protection against DOX cardiotoxicity in cardiac-derived H9c2 cells *in vitro*.

3) To examine whether inhibition of sEH enzyme confers protection against DOX cardiotoxicity in cardiac-derived H9c2 cells *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Materials. Applied Biosystems (Foster City, CA) was the source for High-Capacity cDNA Reverse Transcription Kit, SYBR Green Super Mix, and 96-well optical reaction plates with optical adhesive films. Integrated DNA Technologies Inc. (San Diego, CA) was the synthesizer for real time-PCR primers according to previously published sequences. Sigma-Aldrich (St. Louis, MO) was the source for AA, 4-hydroxybenzophenone, and DOX hydrochloride. Cayman Chemical (Ann Arbor, MI) was the provider for arachidonic acid metabolites 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, 19-HETE, and 20-HETE. Liquid chromatographic-electron spray ionization-mass spectrometry (LC-ESI-MS) reagents were at HPLC-grade. EM Scientific (Gibbstawn, NJ) was the source for acetonitrile and water (HPLC grade). Bio-Rad Laboratories (Hercules, CA, USA) were the source for Acrylamide, JVW-bis-methylene-acrylamide, ammonium persulphate, p-mercaptoethanol, glycine, nitrocellulose membrane (0.45*um)* and TEMED (N,N,N',N'-tetramethylethylenediamine). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences, Piscataway, NJ, USA. Rabbit polyclonal primary antibodies of CYP2E1 and CYP2C11 were purchased from Abeam (Cambridge, UK). Sigma-Aldrich Chemical Co. was the source for anti-goat IgG peroxidase secondary antibody (St. Louis, MO). Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) was the source for goat anti-rabbit IgG with horseradish peroxidase secondary antibody, sEH rabbit polyclonal primary antibody and actin goat polyclonal primary antibody. Dr. Darryl Zeldin (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC) was the donor of

CYP2J primary antibody. Other primary and secondary antibodies used for western blot were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, Canada).

2.2 Methods

2.2.1 Experimental models

2.2.1.1 In vivo

Since discovery of anthracyclines, the early efforts were devoted to design experimental models that allow rational development of new anthracycline with reduced cardiotoxicity while maintaining the same anticancer efficacy. Interestingly, there are two approaches named the short- and the long-term models that have been extensively used to observe and to study the changes in the heart functions (Robert, 2007a). These models are useful for the detection of cardiotoxicity and for the evaluation of cardioprotective agents (Herman & Ferrans, 1986). These models usually utilize different animal species such as rabbits, rats, and mice.

Of importance, using the long-term observational model over a period of weeks was essential to study the morphological and/or the physiological changes in the heart. This model was observed in rabbit where daunorubicin was given every week for 3 months (Maral, et al., 1967). Furthermore, several studies were able to monitor the progression of cardiotoxicity for a longer period (~18 weeks) in rabbits (Jaenke, 1974; Simunek, et al., 2005). Although species such as rats and mice models are frequently preferred, the major disadvantage of these species in long-term models was attributed to nephrotoxicity,

which might occur before the cardiotoxicity (Robert, 2007b).

On the other hand, the short-term models aimed to observe the changes accompanied each DOX injection with a great interest on the alteration occurred in the left ventricle's functions. Among short-term models, the isolated rats hearts were utilized to perform the continuous monitoring of the left ventricle after perfusion of the heart (Pouna, et al., 1995). Also, these models have been used to study the combined cardiotoxic effects associated with combined administration of anthracyclines (Platel, Pouna, Bonoron-Adele, & Robert, 2000). The general requirements for the short-term models are treatment of SD rats with DOX every other day, assessment of toxicity, and harvesting the animals after 2 weeks (Pouna, et al., 1996).

Indeed, chronic DOX cardiotoxicity has been studied in rat models and has been shown to be dependent on dose, schedule, period, and how the DOX was administered (Kharin, Krandycheva, Tsvetkova, Strelkova, & Shmakov, 2012). Furthermore, several lines of evidence have revealed that DOX cardiotoxicity is induced by using a high cumulative dose of DOX over a period of two weeks, which has been characterized by increased mortality rate of experimental rats (Kazachenko, et al., 2008; Siveski-Iliskovic, Kaul, & Singal, 1994; Tong, Ganguly, & Singal, 1991). These studies confirm the occurrence of congestive HF after 6 IP injections of DOX with a cumulative dose of 15 mg/kg over a period of two weeks. For instance, it has been reveled that two weeks of 6 IP DOX injections were enough to cause ascites, depressed cardiac function, elevated left ventricular end-diastolic pressure, congested liver, myocardial cell damage, reduced myocardial glutathione peroxidase activity, and increased lipid peroxidation in experimental rat models (Siveski-Iliskovic, et al., 1994). Also, another study were

successful in showing that a cumulative dose of 15 mg/kg divided into 6 IP injections over a period of two weeks decreased left ventricle contractility, decreased eccentric cardiac remodeling, and led to chronic HF in rats (Kazachenko, et al., 2008). Taken together, these findings and evidences demonstrate sufficient background for using two weeks period of DOX IP injections to induce chronic DOX cardiotoxicity in experimental rats.

Therefore, in the present study, we decided to allow a washout period of two weeks after the last DOX injection in male SD rats. This period was intended to investigate the long-term cardiotoxic effects of DOX and to make sure that DOX is washed out from the plasma as well as from the tissues, so that the observed effects will be due to its long-term toxic effects not due to DOX itself.

2.2.1.2 In vitro

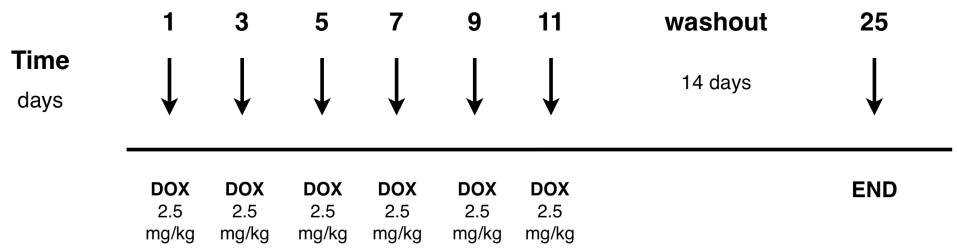
H9c2 cells were used as an *in vitro* model that has been proven to be reliable to study cellular mechanisms involved in drug-induced cardiotoxicity (Q. M. Chen, Tu, Wu, & Bahl, 2000). The H9c2 cell line is an embryonic rat ventricles cell line having the morphological characteristics of embryonic cardiomyocytes (Hescheler, et al., 1991; Kimes & Brandt, 1976). This cell line differs from the freshly isolated cardiomyocytes since H9c2 cells do not have gap junction and T tubules (Hescheler, Meyer et al. 1991). It is well reported that most of H9c2 cells (~95%) are mononucleated cells containing the L-type calcium channels (Sipido & Marban, 1991). As compared with primary cardiomyocytes, H9c2 cells are still expressing specific cardiac markers and are good model to observe the changes in gene expression of CYP and sEH enzymes (Zordoky &

El-Kadi, 2007). However, the major disadvantages of H9c2 cells are related to dedifferentiation of these cells over passages since they possess both the skeletal and the cardiac mucsle properties.

With regards to H9c2 experiments, the effect of DOX on cell viability was determined previously (Zordoky & El-Kadi, 2008a). Using the MTT assay, it was clear that treatment of H9c2 with DOX in micro-range doses (1–10 μM) maintain about 90% cell viability, which eliminates the possibility that the changes in gene expression are due to reduced cell viability.

2.2.2 Animals and treatment

Our experimental procedures and animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Charles River Canada (St. Constant, QC, Canada) was the source for male SD rats weighing 250-300 g. Multiple intraperitoneal (IP) injections were given to animals for a cumulative dose of 15 mg/kg of DOX divided into six injections within two weeks (n = 7). Control group of same weight received the same volume of normal saline volume (n = 7). A washout period was allowed to elimination of the drug after 14 days of the last injection, animals were euthanized under isoflurane anesthesia. Throughout the treatment period, all animals were allowed free access to food and water.



2.2.3 Tissue isolation

The hearts, liver, and kidney were harvested and immediately frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

2.2.4 RNA extraction and cDNA synthesis

Frozen tissues (heart, liver, and kidney) were thawed and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantification of RNA was determined by measuring the absorbance at 260 nm. The RNA quality was determined by measuring the absorbance at 260/280 ratio. Thereafter, the High-Capacity cDNA reverse transcription kit (Applied Biosystems) was used to synthesize first-strand cDNA according to the manufacturer's instructions. From each sample, 1.5 μg of total RNA was added to a mix of 2.0 μl 10X RT buffer, 0.8 μL 25X dNTP mix (100mM), 2.0 μL 10X RT random primers, 1.0 μl MultiScribe TM 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 sec, and finally cooled to 4°C.

2.2.5 Quantification by real time-polymerase chain reaction (PCR)

Specific mRNA expression was quantitatively analyzed by real time-PCR and by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). Reaction mix of 25-µl in volume contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green Universal Master mix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. The primers were chosen from previously published studies, which are listed in Table 2.1 (Anwar-mohamed, Zordoky, Aboutabl, & El-Kadi, 2010). To test for the contamination of any assay reagents, no-template controls were incorporated onto the same plate. The plate was sealed with an optical adhesive cover. Thereafter, thermocycler was used and conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. To confirm the specificity of the primers and the purity of the final PCR product, the dissociation curves were performed by the end of each cycle.

2.2.6 Real time-PCR data analysis

The relative gene expression method was used to analyze the real time-PCR data as described in Applied Biosystems User Bulletin No.2. The fold change in gene expression normalized to the endogenous reference gene (β-actin) and relative to the untreated control of the same time point was used. **Table 2.1** Primer sequences used for real time PCR reactions.

Table 2.1 Primer sequences used for real time-PCR reactions

Gene	Forward Primer	Reverse Primer
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2B1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
CYP2J3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
CYP4A1	TTGAGCTACTGCCAGATCCCAC	CCCATTTTTGGACTTCAGCACA
CYP4A2	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATT
CYP4A3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCAT TCATGG CAATC
CYP4F1	CCCCCAAGGCTTTTTGATG	GAGCGCAACGGCAGCT
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
CYP4F5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
BNP	CAGAAGCTGCTGGAGCTGATAAG	TGTAGGGCCTTGGTCCTTTG
Bax	CCCACCAGCTCTGAACAGTTC	GTGTCTCCCCAGCCATCCT
β-MHC	CGCTCAGTCATGGCGGAT	GCCCCAAATGCAGCCAT
EPHX2	CACATCCAAGCCACCAAGCC	CAGGCCTCCATCCTCCAG
IL-6	ATATGTTCTCAGGGAGATCTTGGAA	GTGCATCATCGCTGTTCATACA
iNOS	GTGCTAATGCGGAAGGTCATG	GCTTCCGACTTTCCTGTCTCAGTA
p53	CAGCTTTGAGGTTCGTGTTTGT	ATGCTCTTCTTTTTTGCGGAAA
TNF	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
β-actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA

2.2.7 Microsomal preparation

Microsomal protein was prepared from the heart tissues as described previously (Aboutabl, et al., 2009). In brief, heart tissues were washed in ice-cold KCL (1.15% w/v), cut into pieces, and homogenized separately in cold sucrose solution (1g of tissue in 5 mL of 0.25 M sucrose). Microsomal protein was separated by differential ultracentrifugation from the homogenized tissues. Thereafter, the final microsomal pellet was reconstituted in cold sucrose and stored at -80 °C. Lowry method using bovine serum albumin as a standard (Lowry, Rosebrough, Farr, & Randall, 1951) was used to determine the concentration of heart microsomal protein.

2.2.8 Western blot analysis

Western blot analysis was performed using a previously described method (Gharavi and El-Kadi, 2005). Briefly, 5-40 μg microsomal protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in blocking solution containing 0.15 M sodium chloride, 3 mM KC1, 25 mM Tris-base (TBS), 5% skimmed milk, 2% bovine serum albumin and 0.5% Tween-20. After blocking, the blots were incubated with primary polyclonal rabbit anti-rat CYP2E1 for 4 hr. Goat anti-rat sEH was incubated for 12 hr. In addition, rabbit anti-rat CYP2C11, rabbit anti-mouse CYP2J, mouse anti-rat CYP4A, and rabbit anti-rat actin were incubated for 2 hr at 4°C. The primary antibodies were prepared in TBS solution containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for CYP2E1 and sEH was carried out for 2 hr at room

temperature. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for CYP2C11, CYP2J, and actin, or goat anti-mouse IgG secondary antibody for CYP4A, or donkey anti-goat IgG secondary antibody for sEH was carried out for 1 hr at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensities of the protein bands were quantified, relative to the signals obtained for actin, using Image J software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

2.2.9 Microsomal incubation

Incubation of heart microsomes (1 mg protein/ml) were performed in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH=7.4) at 37 °C in a shaking water bath (50 rpm). Five minutes period of pre-equilibration was allowed. After that, 1 mM NADPH was added to initiate the reaction where the AA was added to a final concentration of 50 μM and incubated for 30 min. Termination of the reaction was performed by adding 600 μL of ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. To extract AA metabolites, 1 ml ethyl acetate was added twice and dried using speed vacuum (Savant, Farmingdale, NY). Accordingly, the homogenization of heart tissues (1 g) was performed in the incubation buffer and centrifuged at 10,000 g for 30 min. After adding the acetonitrile, the supernatant was extracted twice with 1 ml ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). The LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method was used to analyze extracted arachidonic acid metabolites as described previously (Aboutabl, et al., 2009). In brief, the mass spectrometer was used in negative

mode with single ion recorder acquisition. The nebulizer gas was acquired from an in house high purity nitrogen source. The source temperature was set at 150 °C and the voltages of the capillary and the cone were 3.51 KV and 25 V, respectively. The samples (10 μL) were separated on reverse phase C18 column (Kromasil, 250 x 3.2 mm) using linear gradient mobile phase system with a mobile phase of water/acetonitrile with 0.005% acetic acid at flow rate of 0.2 mL/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min, and held for 5 min.

2.2.9.1 LC-ESI-MS and separation of CYP-mediated arachidonic acid metabolites

The separation and quantification of CYP-derived AA metabolites were performed using LC-ESI-MS. Using the negative ionization mode, we were able to detect the most abundant ion corresponding to the m/z = $[M-1]^-$. For EETs, the most abundant ions corresponding was at m/z = 319 ion. For DHETs, the most abundant ions corresponding was at m/z = 337 ion. For 20-HETE, the most abundant ions corresponding was at m/ z = 319 ion. Using the reverse phase C18 HPLC column and linear gradient mobile phase, we were also able to separate different AA metabolites. Based on the authentic standards, the elution time of EETs (14,15-, 11,12-, 8,9-, and 5,6-) was at 26.33, 28.56, 29.38, and 30.09 min, respectively. For DHETs (14,15-, 11,12-, 8,9-, and 5,6-), the elusion time was at 11.35, 12.58, 13.59, and 14.73 min, respectively. For 20-HETE, the elusion time was at 15.07 min.

2.2.10 Cytosolic soluble epoxide hydrolase activity assay

sEH activity was measured using Morisseau and Hammock method with modifications. 14,15-EET was used as the natural substrate (Morisseau C, 2007). Briefly, the cytosolic fraction was diluted to 0.4 mg/mL with sodium phosphate buffer (0.076 M, pH 7.4) supplemented with BSA (2.5 mg/mL). After preincubation for 5 min at 37°C, the assay was initiated by the addition of 14,15-EET (final concentration of 14,15-EET is 2 μg/mL). The mixture was incubated at 37°C for 5 min. Then the reaction was terminated by the addition of 600 μL ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. 14,15-EET and its corresponding 14,15-DHET were extracted by 1 mL ethyl acetate twice and dried using speed vacuum (Savant, Farmingdale, NY). Extracted 14,15-EET and its metabolite were analyzed using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer) method as described previously (Anwar-Mohamed, et al., 2012b).

2.2.11 Cell culture and treatments

H9c2 cells (American Type Culture Collection, Manassas,VA) were maintained in DMEM, without phenol red, supplemented with 0.45% glucose, 0.15% sodium bicarbonate, 0.11% sodium pyruvate, 10% fetal bovine serum, 20 μM L-glutamine, 100 IU/mL penicillin, 10 μg/mL streptomycin, and 25 ng/mL amphotericin B. Cells were grown in 75-cm^2 tissue culture flasks at 37^{o}C in a 5% CO_2 humidified incubator. For analysis of mRNA, cells were grown at a density of 1–1.5 x 10^6 cells per well in a 6-well tissue culture plate.

2.2.11.1 Treatment of H9c2 cells with soluble epoxide hydrolase inhibitor (t-AUCB) and ω-hydroxylase inhibitor (HET001)

To investigate the cardioprotective effect of sEH and ω-hydroxylase inhibitors, the cardiac-derived H9c2 cells were plated onto 6-well cell culture plates and on 60-80% confluence, the cells were treated with either the vehicle or 100 nM t-AUCB or 50 nM HET0016 for 24 hr. Thereafter, the cells were treated with 1 μM DOX in the presence or absence of t-AUCB or HET0016 for additional 6 hr.

2.2.12 Statistical analysis

Our data are presented as mean ± standard error of the mean (SEM). Control and DOX-treated measurements were compared using Student's t-test. In H9c2 experiment, comparison of 4 different groups was performed using one-way ANOVA test. A result was considered statistically significant where $p < 0.05$.

CHAPTER 3

RESULTS

3.1 CHRONIC DOXORUBICIN-INDUCED CARDIOTOXICITY MODULATES CARDIAC CYTOCHROME *CYP* GENE EXPRESSION AND ARACHIDONIC ACID METABOLISM IN MALE SPRAGUE DAWLEY RATS

3.1.1 Effect of chronic DOX treatment on the apoptotic, inflammatory, and hypertrophic markers

The gene expression of apoptotic, inflammatory, and hypertrophic markers were determined to confirm the occurrence of cardiotoxicity induced by chronic DOX administration in male SD rats. Both gene expression of p53 and Bax were significantly increased in DOX-treated rats to 1.7 and 1.9 fold, respectively as compared to the control group 14 days after last injection of DOX (Fig. 3.1A). The inflammatory marker IL-6 was also significantly induced to 1.8 fold as compared to control group (Fig. 3.1A). Furthermore, to investigate the effect of chronic DOX treatment on the hypertrophic markers, gene expression of atrial natriuretic peptide (ANP) and myosin heavy chain beta (β-MHC) were measured relative to control group. The chronic DOX treatment caused a significant induction of ANP and β-MHC to 3.5 and 2.7 fold, respectively (Fig. 3.1B). In addition, the ratio of β-MHC to α-MHC was also significantly induced to 1.6 fold of control group (Fig. 3.1B). However, there was no change in gene expression of brain natriuretic peptide (BNP).

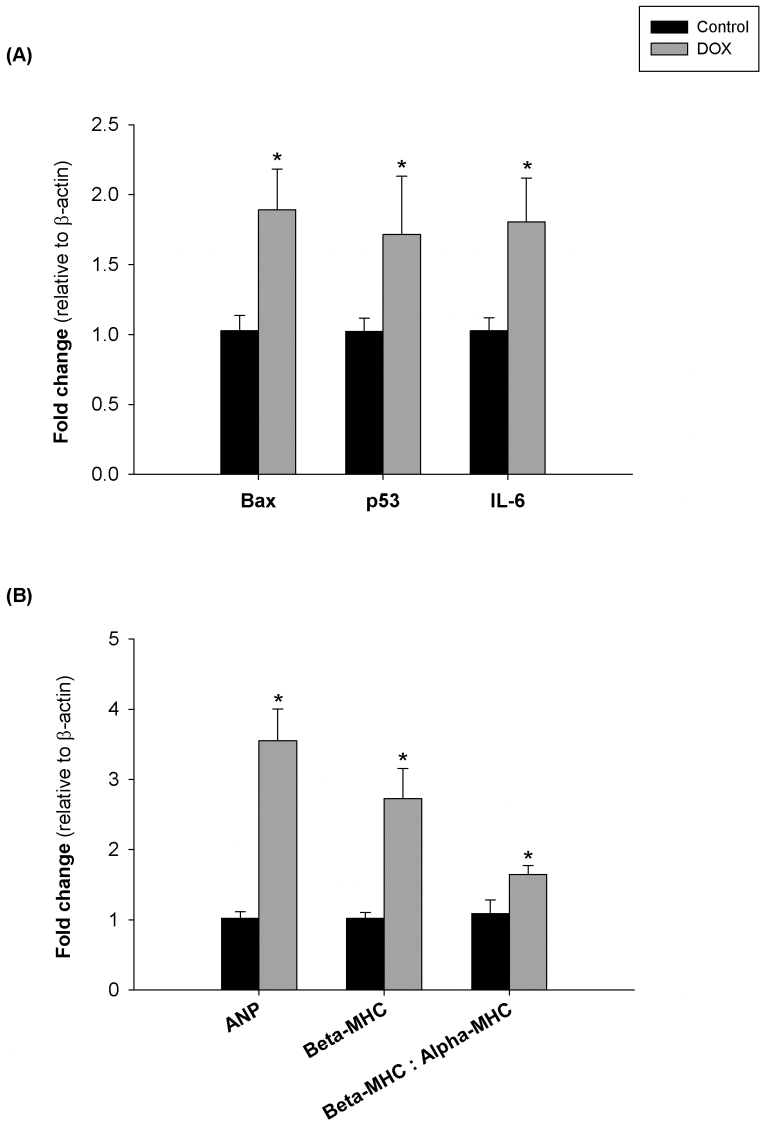


Figure 3.1 Effect of chronic DOX treatment on the apoptotic (A) and hypertrophic markers (B). Total RNA was isolated from the heart of control and DOX-treated rats after 14 days of last injection of DOX. Apoptotic and inflammatory markers (Bax, p53, and IL-6) (A) and hypertrophic markers (ANP and β-MHC) (B) gene expressions were determined by real-time PCR. The β-MHC:α-MHC ratio was calculated (B). Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

3.1.2 Effect of chronic DOX treatment on CYP gene expression

To investigate the effect of chronic DOX treatment on expression of several CYP genes within the heart tissue, total RNA were extracted from the heart of both control and DOX-treated groups. Thereafter, reverse transcription was performed and followed by real-time PCR. The chronic DOX treatment significantly induced *CYP2E1* gene expression in the heart to 1.8 fold of the control group. On the other hand, there was no significant change in gene expression of *CYP1A1, CYP1B1, CYP2B1, CYP2C11,* and *CYP2J3* as compared to control group. **Figure 3.2** shows the effect of chronic DOX treatment on gene expression of CYP1 and CYP2 families. Furthermore, the chronic DOX treatment caused a significant induction in the gene expression of ω-hydroxylase enzymes *CYP4A3, CYP4F1,* and *CYP4F5* to 2, 1.6, and 2 fold, respectively as compared to control group (Fig. 3.3). However, there was no significant change in gene expression of *CYP4A1* and *CYP4F4* (Fig. 3.3).

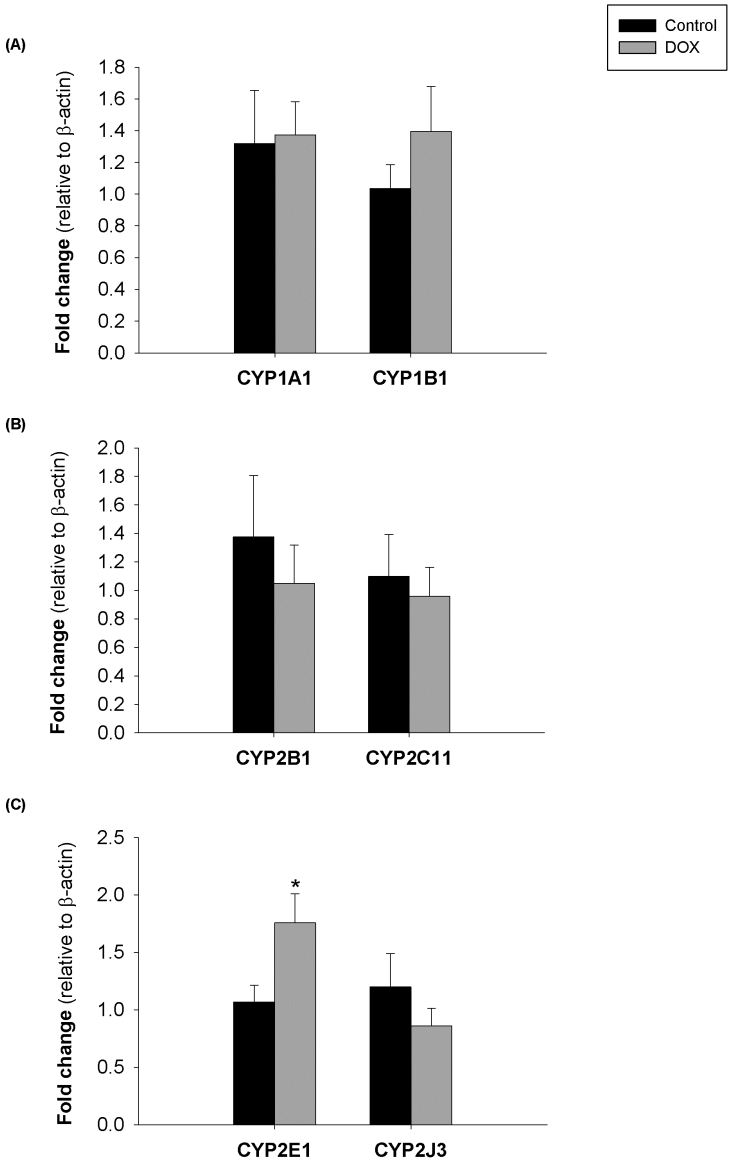


Figure 3.2 Effect of chronic DOX treatment on CYP1 family (A) and CYP2 family (B,C) gene expression. Total RNA was isolated from the heart of control and DOX-treated rats after 14 days of last injection of DOX. CYP1A1, CYP1B1, CYP2E1, and CYP2J3 gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

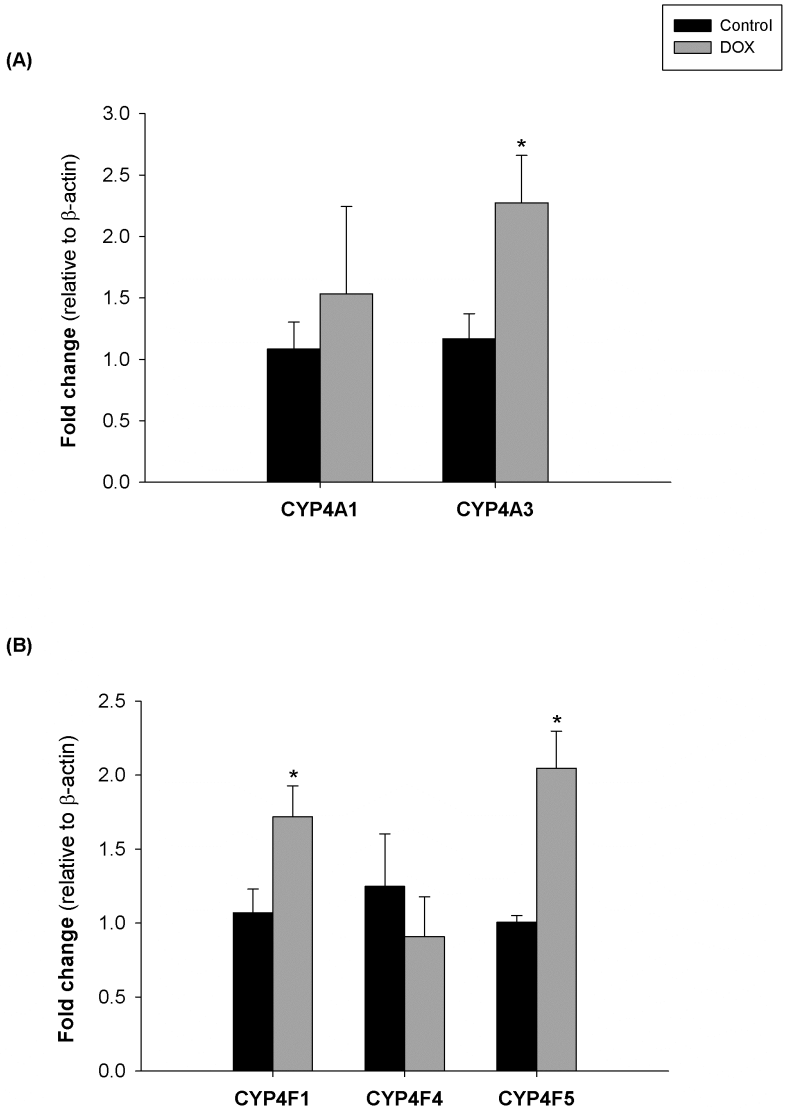


Figure 3.3 Effect of chronic DOX treatment on CYP4A family (A) and CYP4F family (B) gene expression. Total RNA was isolated from the heart of control and DOX-treated after 14 days of last injection of DOX. CYP4A1, CYP4A3, CYP4F1, and CYP4F5 gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

3.1.3 Effect of chronic DOX treatment on CYP-mediated arachidonic acid metabolism

The heart microsomes of control and DOX-treated rats were incubated with 50 μM AA for 30 min to examine the effect of chronic DOX treatment on the formation of CYP-derived AA metabolites. Our results showed that formation of 5,6-, 8,9-, 11,12-, and 14,15-EET did not change as compared to control group (Fig. 3.4A). In addition, enzymatic conversion of EETs to DHETs was measured. As compared to control group, formation of 11,12-, and 14,15-DHET was significantly increased to 1.9 and 1.3 fold, respectively (Fig. 3.4B).

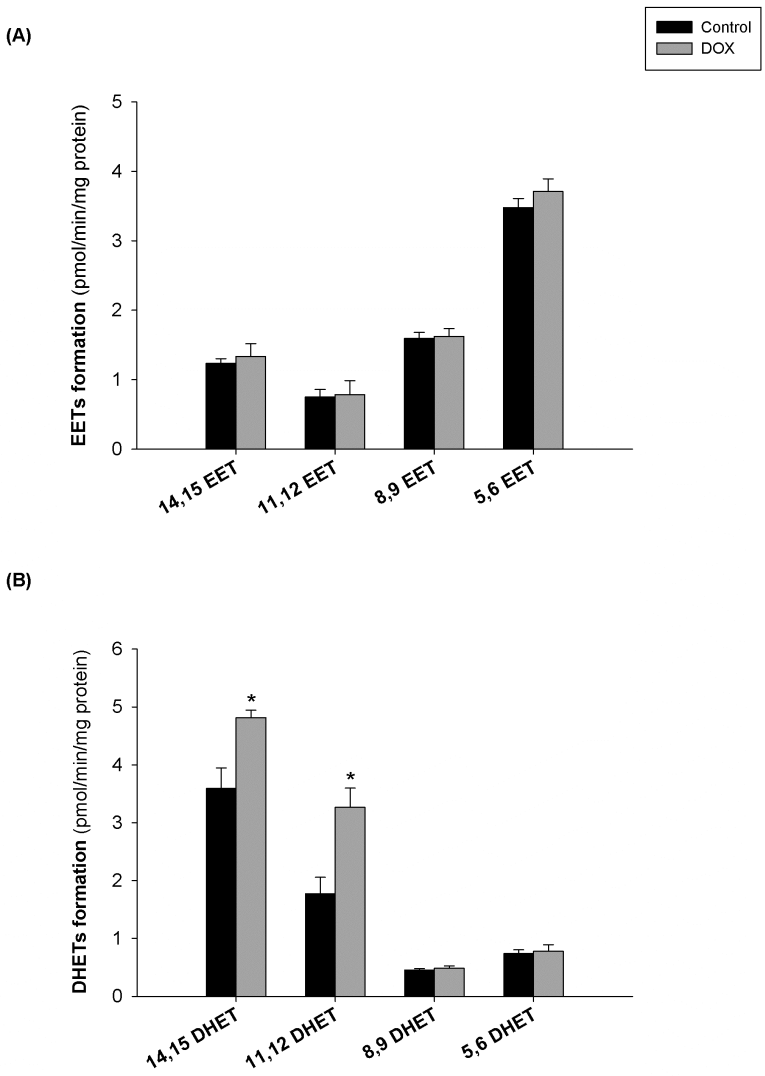


Figure 3.4 Effect of chronic DOX treatment on EETs (A) and DHETs formation (B). Heart microsomes of control and DOX-treated rats were isolated after 14 days of last injection of DOX and incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

To investigate the effect of chronic DOX treatment on the total epoxygenase activity, the sum of all the products of epoxygenase enzymes, i.e. the total EETs and DHETs, was measured in the control and DOX–treated rats 14 days after the last injection of DOX. As compared to control group, there was a trend of increase but did not reach the statistical significance change in the total epoxygenase activity in heart microsomes of DOX-treated rats (Fig. 3.5A). On the other hand, there was a significant increased in 20-HETE formation in the DOX-treated rats as compared to the control group (Fig. 3.5B). Therefore, the ratio of 20-HETE formation to the total EETs was calculated to confirm our finding. Of interest, the ratio was significantly increased to 1.6 fold as compared to the control group (Fig. 3.5C). With regard to ω-1 hydroxylase activity, there was no change in ω-1 hydroxylase activity where the formation of 19-HETE was not changed in the DOX-treated rats as compared to the control group (Fig. 3.5D).

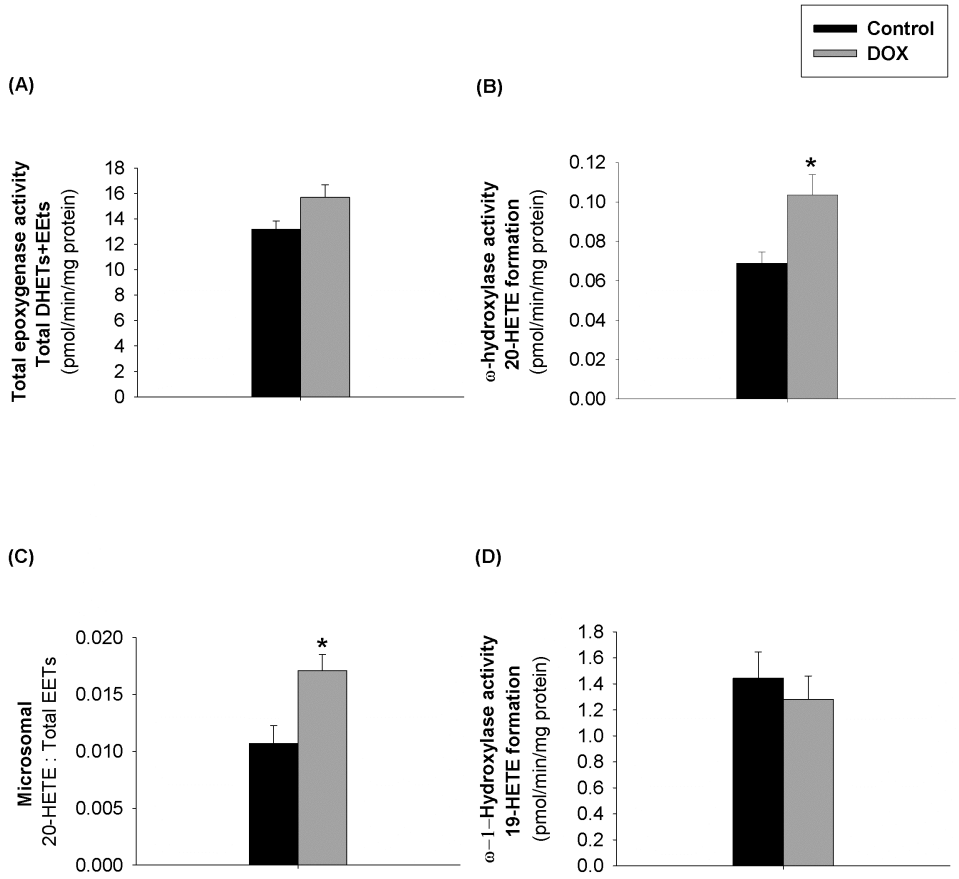


Fig. 3.5 Effect of chronic DOX treatment on epoxygenase (A), ω-hydroxylase activity (B), ratio of 20-HETE formation to total EETs (C), and ω-1 hydroxylase activity (D). (A) Epoxygenase activity was determined from the sum of EETs and DHETs formation. (B) ω-hydroxylase activity was determined from the 20-HETE formation. (C) The ratio of 20-HETE:total EETs. (D) ω-1 hydroxylase activity was determined from the 19-HETE formation. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

3.1.4 Effect of chronic DOX treatment on *EPHX2* gene expression and sEH activity

The sEH enzyme is an important enzyme involved in conversion of EETs to biologically less active metabolites DHETs. Therefore, the gene expression of *EPHX2* gene, which is the gene encoding sEH, was determined 14 days after the last injection of DOX. Interestingly, chronic DOX treatment caused a significant induction in the gene expression of *EPHX2* within the heart tissue to 1.9 fold (Fig. 3.6A). Similar to the induction seen at the mRNA level, there was also a significant increase in sEH activity in the heart microsomes to 1.6 fold (Fig. 3.6B). In agreement with the induction of sEH activity in the microsomal fraction, sEH activity was also induced to 1.3 fold in heart cytosols of DOX-treated rats as compared to control group (Fig. 3.6C).

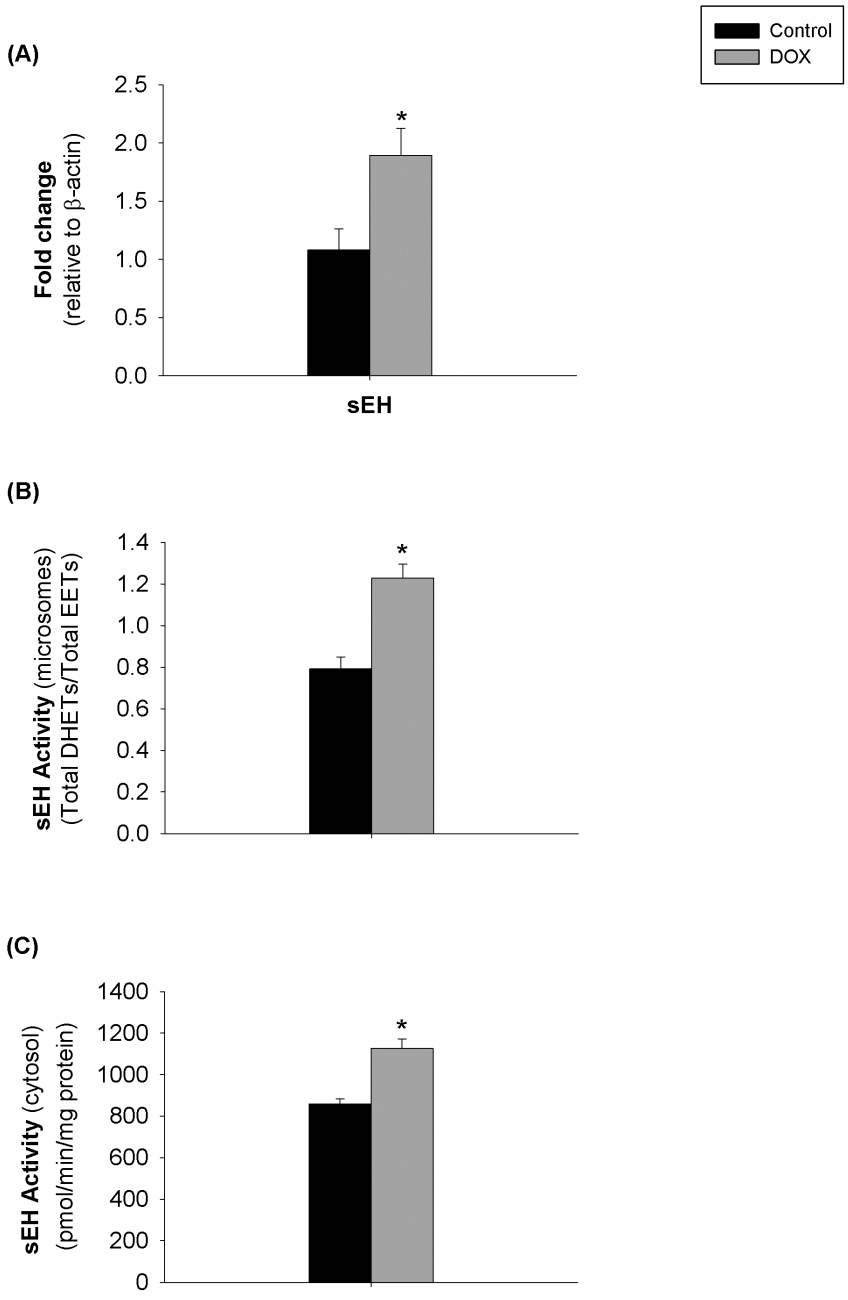

(A)
Control
DOX
Fold change
(relative to β-actin)
2.5
2.0
1.5
1.0
0.5
0.0
*
sEH
(B)
sEH Activity (microsomes)
(Total DHETs/Total EETs)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
(C)
sEH Activity (cytosol)
(pmol/min/mg protein)
1400
1200
1000
800
600
400
200
0
*

Fig. 3.6 Effect of chronic DOX treatment on EPHX2 gene expression (A), sEH activity (microsomes) (B), and sEH activity (cytosols) (C). (A) Total RNA was isolated from the hearts of control and DOX-treated rats after 14 days of last injection of DOX. EPHX2 gene expression was determined by real-time PCR (n = 7). (B) Heart microsomes of control and DOX-treated rats were isolated after 14 days of last injection of DOX and incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1ml of ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC-ESI-MS for metabolite determination. sEH activity was calculated as the ratio of total DHETs/ total EETs. (C) Heart cytosols of control and DOX-treated rats were isolated after 14 days of last injection of DOX and sEH activity was measured by the addition of 14,15-EET (final concentration of 14,15-EET is 2 μg/mL) to the cytosolic fractions. After incubation for 5 min at 37^{o}C, the reaction was terminated by the addition of ice-cold acetonitrile. The corresponding 14,15-DHET were extracted by ethyl acetate, dried and reconstituted by acetonitrile and measured using LC-ESI-MS. Results are presented as mean ± SE (n = 7 rats) * $P < 0.05$ compared with control.

3.1.5 Effect of DOX treatment on CYP and sEH protein expression

Microsomal protein was prepared from heart of control and DOX-treated rats after 14 days of DOX last injection to examine whether the changes of CYP and *EPHX2* gene expression were translated into protein. Thereafter, CYP2C11, CYP2E1, CYP2J3, CYP4A, and sEH protein levels were determined using Western blot analyses relative to β-actin as a loading control. Of interest, chronic DOX treatment caused a significant induction of CYP2E1, CYP4A, and sEH by about 1.6, 1.7, and 1.8 fold, respectively. On the other hand, there was no significant change in the protein expression of CYP2C11 and CYP2J3 (Fig. 3.7).

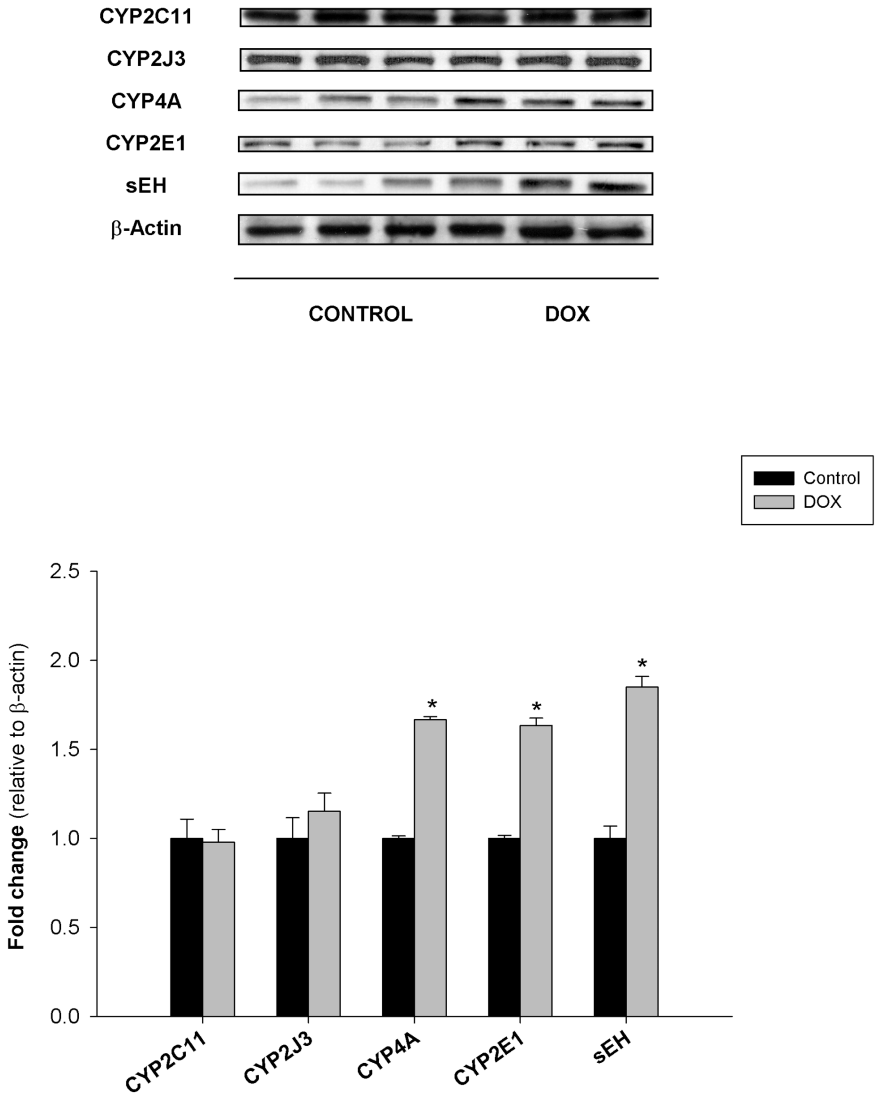


Fig. 3.7 Effect of chronic DOX treatment on CYP and sEH protein expression. Heart microsomal protein was isolated from the heart of control and DOX-treated animals 14 days after last injection of DOX. Microsomal protein was separated on a 10% SDS–PAGE. CYP2C11, CYP2J, CYP4A, CYP2E1, and sEH proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of CYP and sEH protein normalized to the endogenous control (mean ± SE, n = 3). The results are expressed as fold change of the control values taken as 1 fold. * $P < 0.05$ compared with control.

3.1.6 Effect of soluble epoxide hydrolase inhibitor (t-AUCB) on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells

To confirm reversal and/or prevention of cardiotoxicity induced by DOX treatment, H9c2 cells were treated with sEH inhibitor (t-AUCB) for 24 hr prior to treatment with DOX. Thereafter, DOX was added for 6 hr and then mRNA was isolated. Our results showed that inhibition of sEH significantly reversed the DOX-induced ANP and BNP mRNA to 0.3 and 0.4 fold, respectively as compared to DOX-treated cells (Fig. 3.8A).

3.1.7 Effect of ω-hydroxylase inhibitor (HET0016) on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells

To confirm reversal and/or prevention of cardiotoxicity induced by DOX treatment, H9c2 cells were treated with HET0016 for 24 hr prior to treatment with DOX. Thereafter, DOX was added for 6 hr and then mRNA was isolated. Our results showed that inhibition of ω-hydroxylase significantly reverses the DOX-induced ANP and BNP mRNA to 0.5 fold, as compared to DOX-treated cells (Fig. 3.8B).

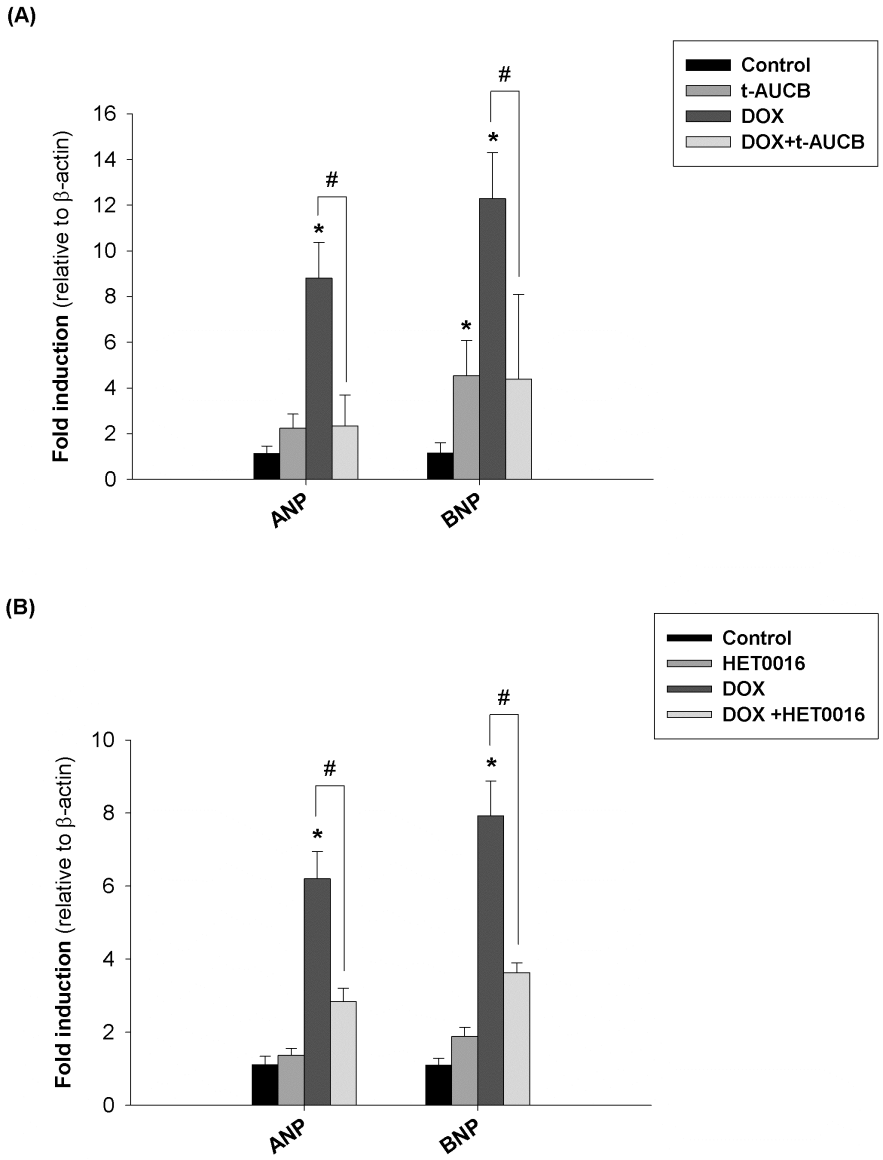


Fig. 3.8 Effect of soluble epoxide hydrolase inhibitor (t-AUCB) (A) and ω-hyroxylase inhibitor (HET0016) (B) on DOX-mediated induction of ANP and BNP mRNA in H9c2 cells. Cells were treated with t-AUCB or HEET0016 for 24 hr. Thereafter, cells were treated with DOX for 6 hr and then total RNA was isolated from the H9c2 cells of control and DOX-treated cells. Hypertrophic markers (ANP and BNP) gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 4). * $P < 0.05$ compared to control. # as compared to control group.

3.1.8 Effect of chronic DOX treatment on the expression of CYP, sEH, and inflammatory markers genes in the kidney and liver

Total RNA was isolated from the kidney and the liver of DOX-treated and control rats to determine the effect of chronic DOX toxicity on gene expression of CYP, sEH, and inflammatory markers in the other tissues. Using the real time-PCR, the expressions of several genes were determined after 14 days of last injection of DOX. Of interest, there was no significant induction gene expression of sEH within the liver or the kidney.

In the liver, the chronic DOX treatment significantly induced the gene expression of the inflammatory markers TNFα and IL-6 to 2.2 and 4.5 fold, respectively. In addition, there was significant induction in gene expression of *CYP1A1*, *CYP1A2*, *CYP1B1*, and *CYP4F5* to 2.8, 1.9, 2.5, and 1.8 fold, respectively. Also, there was a significant inhibition in the gene expression of *CYP2B1, CYP2B2,* and *CYP2C11* to 0.5, 0.5, and 0.7 fold, respectively. However, there was no significant change in gene expression of *CYP2E1*, *CYP2J3*, *CYP4A1*, *CYP4A3*, *CYP4F1*, and *CYP4F4*. **Figure 3.9** shows the effect of chronic DOX treatment on gene expression of inflammatory markers, CYP, and sEH in the liver.

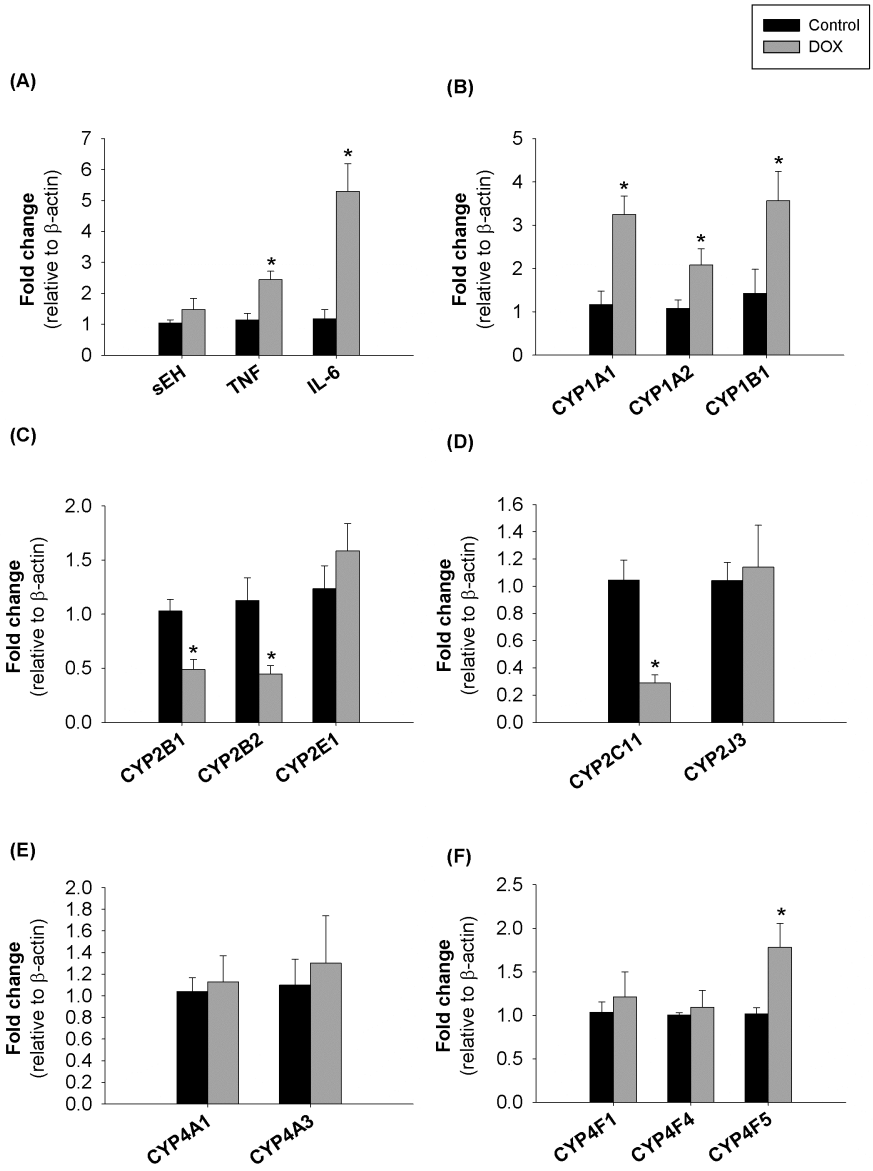


Fig. 3.9 Effect of chronic DOX treatment on gene expression of inflammatory markers, CYP, and sEH in the liver (A-F). Total RNA was isolated from the liver of control and DOX-treated rats after 14 days of last injection of DOX. TNFα, IL-6, CYP1A1, CYP1A2, CYP1B1, CYP2B1, CYP2B2, CYP2C11, CYP2E1, CYP2J3, CYP4A1, CYP4A3, CYP4F1, CYP4F5, and sEH gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

In the kidney, there was induction in gene expression of TNFα and IL-6 to 2.9 and 4.7 fold, respectively as compared to the control group. Also, there was a significant induction in the gene expression of *CYP1B1, CYP2J3,* and *CYP4F5* to 1.8, 1.6, and 1.7 fold, respectively. Also, there was a significant inhibition in gene expression of *CYP2B2* to 0.74 fold as compared to the control. However, there was no significant change in the gene expression of *CYP1A1, CYP1A2, CYP2B1, CYP2C11, CYP2E1, CYP4A1, CYP4A3, CYP4F1,* and *CYP4F4*. **Figure 3.10** shows the effect of chronic DOX treatment on gene expression of inflammatory markers, CYP, and sEH in the kidney.

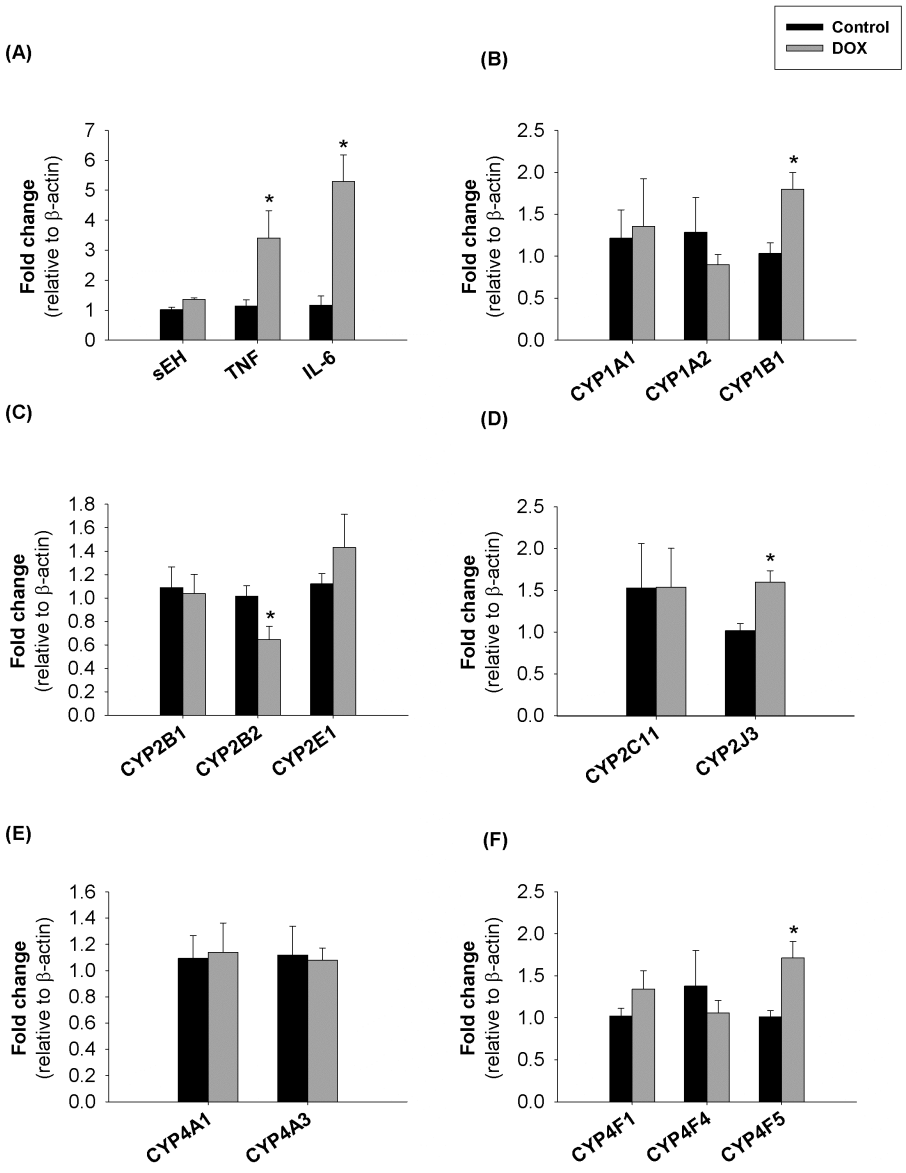


Fig. 3.10 Effect of chronic DOX treatment on gene expression of inflammatory markers, CYP, and sEH in the kidney (A-F). Total RNA was isolated from the kidney of control and DOX-treated rats after 14 days of last injection of DOX. TNFα, IL-6, CYP1A1, CYP1A2, CYP1B1, CYP2B1, CYP2B2, CYP2C11, CYP2E1, CYP2J3, CYP4A1, CYP4A3, CYP4F1, CYP4F5, and sEH gene expressions were determined by real-time PCR. Results are presented as mean ± SE (n = 7 rats). * $P < 0.05$ compared to control.

Table 3.1 Gene expressions of CYP and sEH enzymes in the heart, kidney, and liver (N=7 rats)

Gene	Organ		
	Heart	Kidney	Liver
TNFα	↑	↑	↑
IL-6	↑	↑	↑
CYP1A1	↔	↔	↑
CYP1A2	↔	↔	↑
CYP1B1	↔	↑	↑
CYP2B1	↔	↔	↓
CYP2B2	↔	↓	↓
CYP2C11	↔	↔	↓
CYP2E1	↑	↔	↔
CYP2J3	↔	↑	↔
CYP4A1	↑	↔	↔
CYP4A3	↑	↔	↔
CYP4F1	↑	↔	↔
CYP4F5	↑	↑	↑
sEH	↑	↔	↔

CHAPTER 4

DISCUSSION

4.1 GENERAL DISCUSSION

Drug-induced cardiotoxicity is considered as a predisposing factor leading to HF (Maxwell & Jenkins, 2011). In addition, CYP and sEH enzymes expressed within the heart tissue are subjected to changes during several CVDs leading to altered CYP-mediated AA metabolism (Zordoky & El-Kadi, 2008b). Of importance, DOX is considered as an effective anticancer agent whose major limiting side effect is cardiotoxicity. This cardiotoxicity is mediated through generation of free radicals/doxorubicinol metabolites, disruption of mitochondrial, alteration of cellular energetic, and initiation of apoptotic cascades (Nakamura, et al., 2000; Takemura & Fujiwara, 2007; Ueno, et al., 2006). Although these mechanisms have been proposed to describe the mechanisms by which DOX induces cardiotoxicity, these mechanisms are still not fully understood. Also, since these cardiotoxic mechanisms are quite different from the anticancer mechanisms, there is still a hope to discover strategies that treat or protect against the DOX cardiotoxicity while maintaining the same anticancer efficacy (Carvalho et al., 2009).

Recently, data from our lab demonstrated that acute DOX cardiotoxicity induces the cardiac expression of CYP and sEH enzymes *in vivo* in male SD rats as well as *in vitro* in the cardiac-derived H9c2 cells (Zordoky, et al., 2010; Zordoky & El-Kadi, 2008a). However, the clinical situation requires chronic drug administration where the cumulative dose is the only currently used predictor of cardiotoxicity. Therefore, we investigated for the first time the effects of chronic DOX treatment on expression of the cardiac CYP, sEH, and CYP-mediated AA metabolism in male SD rats.

In the present study, we hypothesized that chronic DOX treatment modulates cardiac expression of CYP and sEH enzymes leading to altered CYP-mediated AA metabolism. Therefore, the effect of chronic DOX treatment on cardiac expression of CYP and sEH enzymes was studied. Also, the effect of chronic DOX treatment on CYP-mediated AA metabolism was investigated. In addition, to further show the role of CYP and sEH enzymes in development and/or progression of DOX cardiotoxicity, the effects of sEH inhibitor, t-AUCB and ω-hydroxylase inhibitor, HET0016 on hypertrophic markers induced by DOX treatment were determined in cardiac H9c2 cells. Finally, the gene expression of CYP and sEH enzymes was investigated in the kidney and the liver.

First, chronic DOX treatment was induced by multiple IP injections of 15 mg/kg cumulative dose divided into six injections within two weeks. A washout period of 14 days was allowed to ensure elimination of the drug to make sure that the observed effects are related to the chronic rather than the acute effect of DOX treatment. Thereafter, several apoptotic, inflammatory, and hypertrophic markers have been used to confirm the occurrence of chronic DOX cardiotoxicity. These markers include: p53, Bax, IL-6, ANP, and β-MHC. Interestingly, our results showed that there was a significant induction in gene expression of hypertrophic markers ANP and β-MHC. Furthermore, the ratio of β-MHC:α-MHC was significantly induced.

In agreement with our results, several studies have supported our findings that DOX cardiotoxicity selectively induces the hypertrophic marker ANP in different animal model such as dogs (S. Chen, Garami, & Gardner, 1999; Rahman, et al., 2001). Also, in agreement with our results, Zordoky et al have shown that ANP is induced in cardiac

cells line H9c2 (Zordoky & El-Kadi, 2008a). However, in contrary to the chronic model, those hypertrophic markers were inhibited in acute DOX cardiotoxicity or in *in vitro* studies using the neonatal cardiomyocytes (Rahman, et al., 2001; Zordoky, et al., 2010). These differences might be attributed to the differences in model, dose, and duration of treatment.

Regarding the cardiac gene expression of CYP enzymes after the chronic DOX treatment, there was a quite difference in their expression from the acute DOX model (Zordoky, et al., 2010). In contrary to the acute DOX model, our result showed that there was a significant induction in the cardiac gene expression of CYP2E1 with no change in gene expression of CYP1A1, CYP1B1, CYP2C11, and CYP2J3. These CYP enzymes are involved in the formation of CYP-mediated AA metabolites to different extents. CYP1A1 is involved in formation of HETE metabolites while CYP1B1 is involved in formation of EETs and HETEs (Choudhary, et al., 2004). Also, EETs can be produced by major CYP epoxygenases such as CYP2B1, CYP2J3, and CYP2C11 (Laethem, Halpert, & Koop, 1994; Ng, et al., 2007). However, CYP2E1 is involved in formation of 19-HETE (Laethem, et al., 1993). In agreement with our results, there was no change in gene expression of CYP2B1 in H9c2 cells and in the acute DOX model.

Furthermore, the cardiac gene expression of CYP ω-hydroxylases CYP4A3 and CYP4F1 were significantly induced similar to the acute model. These ω-hydroxylases are mainly involved in the formation of 20-HETE. However, there was no change in gene expression of CYP4A1 and CYP4F4 that was induced in the acute DOX model (Zordoky, et al., 2010). In contrary to the acute DOX model, our results showed that cardiac gene expression of CYP4F5 was significantly induced. Furthermore, the chronic DOX

cardiotoxicity induced protein expression of CYP2E1, CYP4A, and sEH without significant change in protein expression of CYP2C11 and CYP2J3.

To investigate the effect of the aforementioned changes in CYP gene expression on CYP-mediated AA metabolism, AA was incubated with microsomes extracted from the heart tissue and the CYP-derived AA metabolites were analyzed by LC-MS. Of importance, our results showed that there is no significant change in formation of 5,6-, 8,9-, 11,12-, and 14,15-EET relative to the control group. In addition, there was no significant increase in epoxygenase activity, which was estimated by calculating the sum of total EETs and DHETs. However, there was a significant increase in formation rates of 14,15-, and 11,12- DHETs. Therefore, it was necessary to investigate the effects of chronic DOX cardiotoxicity on expression and activity of sEH enzyme which is responsible for the enzymatic conversion of EETs to less biologically active metabolites DHETs (Imig, et al., 2002).

Of interest, our results showed that chronic DOX treatment significantly induced the gene expression of *EPHX2*. This finding was translated to a significant increase in the sEH activity within the heart microsomal and cytosolic fractions as compared to control group, which resulted in higher formation rates of DHETs. In agreement with these findings, we have previously shown that acute DOX cardiotoxicity induced cardiac gene expression of *EPHX2* both *in vivo* and *in vitro* (Zordoky, et al., 2010).

Despite the induction of the ω-1 hydroxylase CYP2E1 gene expression, there was no significant change in 19-HETE formation within the heart of DOX-treated group. This discrepancy between the gene expression and the enzymatic activity of CYP2E1 may be

attributed to regulation of CYP2E1 by different post-transcriptional mechanisms and to relative low basal levels of CYP2E1 in the heart. Similar to our finding, several studies have reported that the increase in CYP2E1 expression did not correlate with the rate of 19-HETE formation (Amet, Zerilli, Goasduff, Dreano, & Berthou, 1997; Poloyac, et al., 2004).

With regards to 20-HETE formation, our findings showed that CYP ω-hydroxylase activity was increased after 14 days of the last DOX injection as compared to the control group. This finding is matching the increase in cardiac gene expression of major CYP ω-hydroxylase enzymes i.e. CYP4A3, CYP4F1, and CYP4F5. Also, it was accompanied by a significant increase in the formation of cardiotoxic metabolite 20-HETE.

Of importance, the overproduction of 20-HETE has been extensively implicated in development and/or progression of several cardiovascular diseases (Bao, et al., 2011; Certikova Chabova, et al., 2010). Taking into consideration the detrimental effects of 20-HETE, the elevation in 20-HETE levels might be another potential mechanism by which the chronic DOX treatment causes the progressive cardiotoxicity. Although several studies have showed that 20-HETE induces endothelial dysfunction by activating several signaling cascades within the endothelial cells (Cheng, et al., 2010), these signaling cascades also exist within the heart where the detrimental effects of 20-HETE might be attributed to modulation of these intracellular targets. Within the vasculature, 20-HETE was reported to induce endothelial dysfunction through uncoupling of endothelial nitric oxide synthase (eNOS). Also, 20-HETE was reported to reduce nitric oxide by promoting the dissociation of heat shock protein from the eNOS. Both effects were shown to be reversed by inhibition of tyrosine kinase and MAPK/ERK cascades suggesting a direct

involvement of 20-HETE in development of endothelial dysfunction. Of interest, Randriamboavonjy and his group (Randriamboavonjy, et al., 2003) have also shown that 20-HETE is capable to phosphorylate PKCα and Rho-kinase pathway, which inhibit endothelial derived relaxation factor-mediated coronary arteries relaxation. In summary, 20-HETE has been reported to activate several signaling cascades within non-cardiac cells such as NFAT (Yaghi & Sims, 2005), NF-κB (Ishizuka, et al., 2008), MAPKs (Sun, et al., 1999), Rho/ROCK (Randriamboavonjy, et al., 2003), and PI3K (Stec, et al., 2007). These signaling cascades are also existed within the cardiomyocytes, which might be potential targets for 20-HETE in the heart. Taken together, these evidences illustrate the proposed mechanisms by which 20-HETE would be directly involved in development and/or progression of DOX cardiotoxicity.

Furthermore, a recent study has shown a direct stimulating effect of 20-HETE on L-type Ca^{2+} channel within the heart leading to contractile dysfunction in rat cardiomyocytes (Zeng, et al., 2010). Of interest, inhibition of ω-hydroxylases by the selective inhibitor HET0016 has shown to be effective to protect against BaP-induced cardiac hypertrophy in rats (Aboutabl, et al., 2009). Also, reversing of 20-HETE's effects was shown to be a feasible approach achieved by using the inhibitors of 20-HETE formation leading to reduced apoptosis (Lv, et al., 2008; Yousif, et al., 2009). Therefore, CYP ω-hydroxylase inhibitors might represent another potential strategy to avoid progression of the chronic DOX cardiotoxicity. In the current study, we demonstrated that CYP ω-hydroxylase inhibitor HET0016 significantly reversed the DOX-mediated induction of the hypertrophic markers ANP and BNP in cardiac derived H9c2 cells. This finding showed

the involvement of CYP ω-hydroxylase enzymes and CYP mediated AA metabolites in DOX cardiotoxicity.

On the other hand, several studies have demonstrated that cardioprotective metabolites EETs have opposing effects to those mediated by cardiotoxic metabolite 20-HETE (Elbekai & El-Kadi, 2006; Roman, 2002). EETs have been shown to inhibit NF-κB, to inhibit cardiomyocyte apoptosis (Batchu, et al., 2011b), to decrease ROS (L. Liu, et al., 2011), and to show anti-inflammatory properties (Norwood, et al., 2010). Furthermore, EETs are reported to activate some pathways that are cardioprotective, such as PI3K (p110α) (Batchu, et al., 2011b). Of importance, the level of EETs can be elevated by two approaches i.e. inhibition and/or knockdown of sEH enzyme. For instance, silencing of sEH using the small interfering RNA in cultured rat cardiomyocytes was shown to be an effective approach to reduce the apoptosis induced by DOX (Du, et al., 2011). Furthermore, targeting sEH enzyme activity with a specific sEH inhibitor is also considered as a feasible approach to reduce the metabolic conversion of EETs to less biologically active metabolite DHETs. This approach has been shown to be effective against cardiac hypertrophy in the murine model of thoracic aortic constriction resulting in a complete resolution of cardiac hypertrophy (Harris, et al., 2008). Therefore, elevation of EETs level by using the sEH inhibitors might represent another potential strategy to avoid progression of the chronic DOX cardiotoxicity. In the current study, we also demonstrated that sEH inhibitor t-AUCB significantly reversed the DOX-mediated induction of the hypertrophic markers ANP and BNP in H9c2 cells. The modest increase in BNP gene expression after treatment with t-AUCB might be attributed to the cardioprotective effect of EETs, which is mediated through BNP (Chaudhary, et al.,

2009). Of interest, this finding showed the involvement of sEH enzymes and CYP-mediated AA metabolites in DOX cardiotoxicity.

According to the previous findings, the opposing effects of 20-HETE and EETs are also reported to be mediated through several signaling cascades that have been linked to development and/or progression of cardiovascular toxicity such as NF-κB, oxidative stress, apoptosis, and inflammatory cytokines (Elbekai & El-Kadi, 2006; Ishizuka, et al., 2008). Therefore, the ratio of 20-HETE formation to total EETs formation was calculated and this ratio was significantly induced by chronic DOX treatment suggesting that imbalance between 20-HETE and EETs might be involved in the pathogenesis and/or progression of chronic DOX cardiotoxicity.

In addition, to determine to which extent those changes in gene expression of CYP and sEH enzymes are specific to the heart, gene expression of these enzymes was determined in the kidney and the liver. Of interest, the chronic DOX treatment specifically modulated gene expression of CYP and sEH enzymes in an organ and enzyme specific manner. With regard to CYP enzymes, CYP1B1 was induced in the liver and kidney, which is in agreement with the acute DOX model (Zordoky, Anwar-Mohamed, Aboutabl, & El-Kadi, 2011). In the liver, this induction seems to be attributed to the activation of the AhR and to the DOX-induced inflammation within the liver since the induction was observed in gene expression of CYP1A1 and CYP1B1. This finding is in agreement with a recent work from our lab indicating that lipopolysaccharide-induced inflammation induces hepatic *CYP1B1* in SD rats (Anwar-mohamed, et al., 2010).

However, in the kidney, the induction in gene expression of CYP1B1 seems to be attributed to DOX-induced inflammation since the CYP1A1 was not induced. In

agreement with acute model, there was inhibition of CYP2B1 and CYP2C11 gene expression in the liver. This inhibition is attributed to inflammation where several studies have shown that inflammation is involved in down-regulation of those enzymes (Iber, et al., 1999; Li-Masters & Morgan, 2001). In contrary to the acute model, our results showed that there was a significant induction in gene expression of CYP2J3 with no significant change in CYP2B1 and CYP2C11 in the kidney. Also, in contrary to acute study, there was no significant change in the gene expression of CYP2E1 in the liver and kidney.

With regard to major CYP ω-hydroxylases, our results showed that there was no significant change in CYP4A1, CYP4A3, CYP4F1, and CYP4F4 in the liver and kidney. However, there was a significant induction in the gene expression of CYP4F5 in the liver and kidney. Regarding sEH gene expression, there was no significant change within the liver and kidney. These findings further confirm the specificity of chronic DOX effects on cardiac sEH, which suggests its pivotal role in cardiotoxicity induced by the chronic DOX administration.

4.2 General Conclusions

In the present work, we have investigated the effects of chronic DOX treatment on CYP-mediated AA acid metabolism in male SD rats. Of interest, the chronic DOX model modulates the gene expression of CYP and sEH enzymes in an organ-specific manner. The chronic DOX treatment caused a significant induction in cardiac gene expression of CYP2E1, CYP4A3, CYP4F1, CYP4F5, and sEH in the heart of DOX-treated rats 14 days after last DOX injection. This alteration was accompanied by an increase in formation of 20-HETE and DHETs. Furthermore, the involvement of these enzymes and metabolites in DOX cardiotoxicity was confirmed by using the cardiac derived H9c2 cells to induce hypertrophic markers at the therapeutic concentrations of DOX where the inhibitors of CYP ω-hydroxylases and sEH reversed the DOX-induced hypertrophic markers i.e. ANP and BNP.

In short, the chronic DOX administration significantly modulates cardiac expression of CYP ω-hydroxylase and sEH enzymes and their activity leading to imbalance between CYP-mediated cardiotoxic and cardioprotective pathways. Therefore, sEH and CYP ω-hydroxylase enzymes might be considered as novel targets to treat and/or to protect against chronic DOX cardiotoxicity.

4.3 Future Directions

Both CYP ω-hydroxylase and sEH enzymes were shown to be involved in the development of DOX-induced cardiotoxicity. However, further studies are required to transfer these findings from the bench to the bed-side.

1) To examine whether inhibition or knockdown of sEH and CYP ω-hydroxylase enzymes confers protection against DOX-induced cardiotoxicity *in vivo*.

2) To elucidate the mechanisms involved in DOX-induced alteration of sEH and CYP ω-hydroxylase and subsequent cardiotoxic effects.

3) To investigate whether 20-HETE potentiates detrimental effects of DOX *in vivo* and *in vitro*.

4) To investigate whether EETs protects against DOX cardiotoxicity *in vivo* and *in vitro*.

CHAPTER 5

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