Role of Cytochrome P450 Enzymes in Inflammation-induced Cardiac Hypertrophy

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In
Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta

ABSTRACT

Heart failure (HF) is typically preceded by cardiac hypertrophy (CH), which is characterized by cardiomyocytic enlargement following stress. During CH development, expression of cytochrome P450 enzymes (CYPs) and metabolism of arachidonic acid (AA) are altered. Cardiac CYPs metabolize AA into multiple biologically active eicosanoids, that are classified into hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs). HETEs are further classified as terminal, subterminal, and mid-chain HETEs. Mid-chain HETEs are a distinct group of CYP1B1-derived eicosanoids that show cardiotoxic hypertrophic properties. Inflammation is involved in CH pathophysiology, but mechanisms of inflammation-induced CH and alteration of CYP-mediated AA metabolism remain elusive. Comprehending the mechanisms of inflammation-induced CH would be invaluable in identifying the molecular entities that should be targeted for pharmacological interventions. Therefore, the objectives of the present work were to investigate the impacts of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and lipopolysaccharide (LPS) on the development of cellular hypertrophy (CeH), expression of CYP1B1, and CYP1B1-mediated AA metabolism, as well as the role of CYP1B1 in TNF-α, IL-6, and LPS -induced CeH *in vitro*. Our results show that TNF-α, IL-6, and LPS induce mRNA expression of hypertrophic markers, significantly increase cell surface area, induce CYP1B1 at mRNA, protein, and activity levels, and enantioselectively modulate CYP-mediated AA metabolism in favor of cardiotoxic mid-chain HETEs. These effects are ameliorated in the presence of CYP1B1-siRNA or trans-resveratrol. In conclusion, our results demonstrate the crucial role of CYP1B1 in TNF-α, IL-6, and LPS -induced CeH and highlight CYP1B1 as a clear target for potential therapeutic interventions for the prevention and treatment of CeH.

PREFACE

This thesis is an original work done by Mr. Mohammed ElKhatib. Section 1.1 of this thesis has been published as a review article: ElKhatib MAW, Isse FA & El-Kadi AOS. (2023). Effect of inflammation on cytochrome P450-mediated arachidonic acid metabolism and the consequences on cardiac hypertrophy, Drug Metabolism Reviews, 55:1-2, 50-74. I was responsible for collecting and summarizing data from the literature and writing the manuscript. El-Kadi AOS was the supervisory author and was involved with concept formation and manuscript composition. Sections 3.1 and 4.1 of this thesis have been submitted as ElKhatib MAW, Isse FA, Gerges SH & El-Kadi AOS. (2023). Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-induced CellularHypertrophy, Prostaglandins and Other Lipid Mediators, submitted. I was responsible for designing the research, conducting experiments and data analysis as well as the manuscript writing. Isse FA was involved in LCMS experiments and helped in reviewing the manuscript. Gerges SH contributed to reviewing the manuscript. El-Kadi AOS was the supervisory author and was involved with concept formation and manuscript composition.

This dissertation is dedicated to my parents, wife, sons; Omar & Ali, brother, and many of my friends.

Thank you for your unconditional love and support

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my supervisor and mentor Dr. Ayman El-Kadi for suggesting the original line of research presented in this thesis and for his trust, guidance, encouragement and time during my master program.

I sincerely acknowledge the members of my supervisory committee, Dr. Arno Siraki and Dr. John Ussher for their endless advice and valuable suggestions.

I would like to thank my former lab members, especially Rahmat Hidayat whose enthusiasm gave me a reason to keep working on this research project. My special appreciation is extended to my present lab colleagues, especially Mahmoud Elghiaty, Mohammed Alqahtani, Ahmed Alammari, Fadumo Isse, Sara Rasmy, Sara Helal, and Samar Gerges.

I would like to thank the Dean of the Faculty of Pharmacy and Pharmaceutical sciences, Dr. Christine Hughes and the faculty administrative and support staff for their kind advice and financial support throughout my program. Special thanks go out to Diseray Schamehorn, the Graduate Studies Coordinator in the faculty.

I am also grateful for the generous financial support provided by the University of Alberta for supporting me with the Graduate Research Assistant Fellowship, the Graduate teaching assistantships, the Mike Wolowyk graduate Scholarship, and others. In addition, this work was supported by a grant from the Canadian Institutes of Health Research [CIHR PS 168846] to Ayman O. S. El-Kadi

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AMPK-α2	Adenosine monophosphate kinase-alpha 2
Ang-II	Angiotensin-2
ANP	Atrial natriuretic peptide
AUDA	12-(3-adamantan-1-yl-ureido) dodecanoic acid
BNP	B-type natriuretic peptide
CCL4	C-C Motif Chemokine Ligand 4
CCL5	C-C Motif Chemokine Ligand 5
CH	Cardiac hypertrophy
COX	Cyclooxygenases
$cPLA_2$	cytosolic phospholipase A_2
CV	Cardiovascular
CVD	Cardiovascular diseases
CYP	Cytochrome P450 enzymes
DAC	Descending aortic constriction
DHET	Dihydroxyeicosatrienoic acids
ECs	Endothelial Cells
EETs	Epoxyeicosatrienoic acids
GPR75	G protein-coupled receptor 75

HETEs	Hydroxyeicosatetraenoic acids
HF	Heart failure
HO-1	Heme oxygenase-1
IL-1α	Interleukin-1 alpha
IL-16	Interleukin-16
IL-6	Interleukin-6
JAK2	Janus kinase 2
LAD	Left anterior descending
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTs	Leukotrienes
MMP9	Matrix metalloproteinase 9
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NO	Nitric Oxide
PGs	Prostaglandins
PKC	Protein kinase C
PPAR-γ	Peroxisome proliferator activated receptor-gamma
sEH	soluble epoxide hydrolase
SOCS3	Suppressor of cytokine signaling-3
STAT3	Signal transducer and activator of transcription 3

TGF-β1	Transforming growth factor- beta 1
TMS	Tetramethoxy stilbene
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
TRP	Transient receptor potential
α-MHC	Alpha-myosin heavy chain
β-MHC	Beta-myosin heavy chain

CHAPTER 1: INTRODUCTION

Portions of this chapter have been published in:

1-**Mohammed A. W. ElKhatib**, Fadumo Ahmed Isse & Ayman O. S. El-Kadi (2023) Effect of inflammation on cytochrome P450-mediated arachidonic acid metabolism and the consequences on cardiac hypertrophy, Drug Metabolism Reviews, 55:1-2, 50-74.

2-**Mohammed A. W. ElKhatib**, Fadumo Ahmed Isse, Samar H Gerges, & Ayman O. S. El-Kadi. Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy, Prostaglandins and Other Lipid Mediators, submitted.

1.1 Cytochrome P450 enzymes

Cytochrome P450 (CYP) enzymes are ubiquitously expressed hemoproteins that contribute to the metabolism of nearly 75% of pharmaceuticals and xenobiotics (Guengerich 2008; Roederer 2009). Although they were assumed for a long time to be biologically relevant solely to xenobiotics, multiple studies now clearly reveal that CYP enzymes are involved in metabolizing endogenous substances such as fatty acids, cholesterol, and arachidonic acid (AA) (VanRollins et al. 1984; Barbosa-Sicard et al. 2005; Fer et al. 2008; Fleming 2011).

AA is a polyunsaturated ω-6 fatty acid containing 20 carbons which is present in the phospholipids of the cell membrane and is liberated upon the action of cytosolic phospholipase A_2 ($cPLA_2$) (Sudhahar et al. 2010). The liberated intracellular AA is enzymatically metabolized into a vast array of biologically active metabolites known as "eicosanoids" (Imig 2012). Eicosanoids encompass prostaglandins (PGs) and leukotrienes (LTs) synthesized from AA by cyclooxygenases (COXs) and lipoxygenases (LOXs), respectively (Kuehl Jr and Egan 1980; Zordoky and El-Kadi 2010a). LOXs are involved in AA metabolism into hydroperoxyeicosatetraenoic acids (HPETEs), which are the precursors of lipoxins, LTs and hydroxyeicosatetraenoic acids (HETEs) (Hanna and Hafez 2018). Additionally, AA is metabolized by COXs into PGG_2 and PGH_2. It is worth mention that CYPs are crucial in PGH_2 metabolism into thromboxane A_2 and PGI_2 by CYP5A1 and CYP8A1, respectively (Meling 2016; Cho and Lee 2020).

Also, the CYP-mediated pathway generate HETEs and epoxyeicosatrienoic acids (EETs) through CYP ω-hydroxylases and CYP epoxygenases, respectively (Alsaad et al. 2013; Shoieb et al. 2020). AA epoxidation at any of the 4 cis-double bonds, with the cis conformation unchanged, gives rise to 4 EETs regioisomers (14,15-, 11,12-, 8,9-, and 5,6-(R/S)EET); epoxide function insertion at any

of the 2 flanks of AA double bonds, producing either (S,R) or (R,S) cis-EETs (VanRollins and VanderNoot 2003). Conversely, inserting hydroxyl function produces chiral carbon centres and R/S enantiomers in all HETEs except for 20-HETE (Hawkins et al. 1988). Different AA metabolism reactions are catalyzed by CYPs, such as ω-hydroxylation (producing 20-HETE), ω-n hydroxylation (producing 19-, 18-, 17-, 16-(R/S)HETEs), and bis-allylic oxidation (producing 15-, 12-, 8- and 5-(R/S)HETEs (ElKhatib et al. 2023). Figure 1.1 shows the different AA metabolites derived from COXs, LOXs, and CYPs.

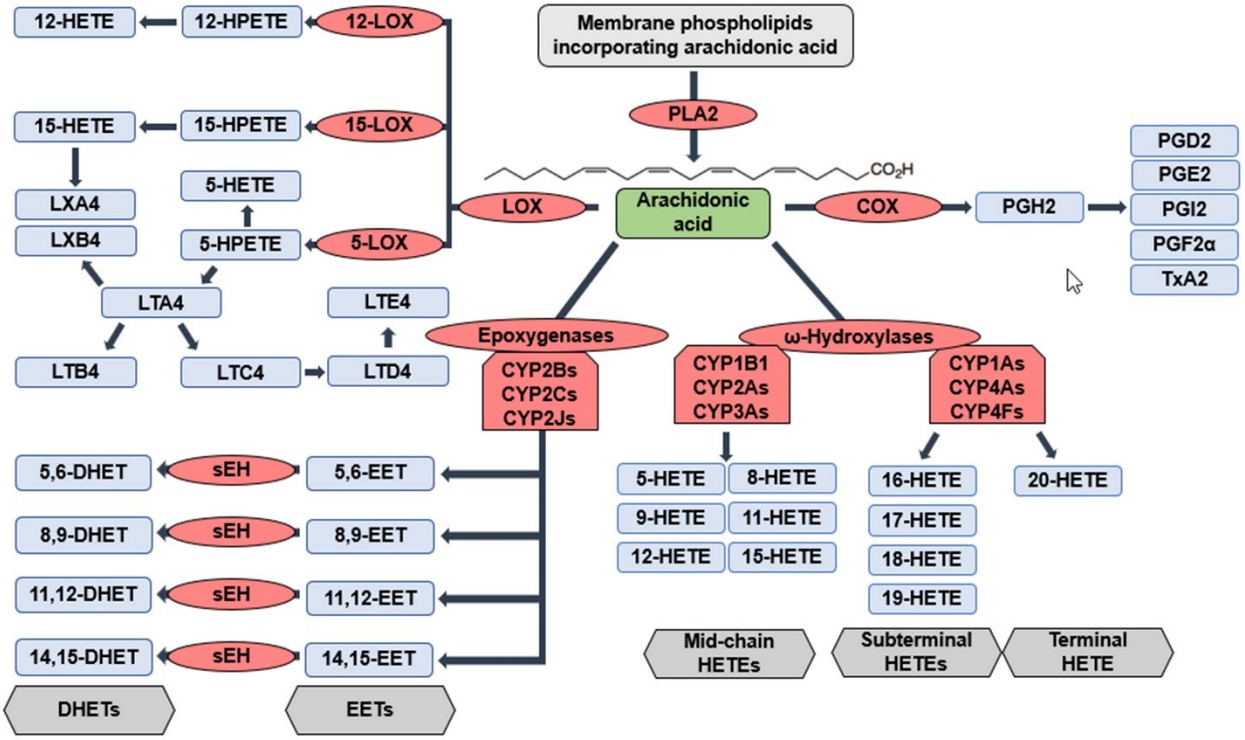


Figure 1.1. Metabolism of arachidonic acid by cyclooxygenases, lipoxygenases, cytochrome P450 epoxygenases and omega-hydroxylases.

COXs-derived AA metabolites are prostaglandins, while LOXs metabolize AA into HPETEs, HETEs, leukotrienes and lipoxins. CYP epoxygenases give rise to epoxyeicosatrienoic acids, which are later converted into dihydroxyeicosatrienoic acids via soluble epoxide hydrolase. On the other hand, CYP omega-hydroxylases produce mid-chain, subterminal, and terminal HETEs from AA.

A plethora of studies highlight the involvement of AA metabolites in the pathophysiology of cardiovascular diseases (CVDs) (Roman 2002; Sonnweber et al. 2018; Huang et al. 2020; Zhou et al. 2021). One important predictor of CV morbidity and mortality is cardiac hypertrophy (CH), which is enlargement of the cardiomyocytes following the elevation of cardiac afterload or preload (Nakamura and Sadoshima 2018).

1.2 Classifications of cardiac hypertrophy

CH can be classified into physiological or pathological according to pathophysiology. CH is regarded as pathological when it is accompanied by myocardial dysfunctions, however, physiological CH shows preserved cardiac functions (Shimizu and Minamino 2016). Also, CH can be classified into eccentric and concentric hypertrophy, indicating the different cardiac responses to stressful stimuli. Both CH phenotypes are distinguished by various molecular and structural characteristics (Khouri et al. 2010). Regarding pressure overload such as patients with hypertension, blood pressure (BP) elevation will result in concentric CH phenotype. However, increased volume such as in valvular heart disease will culminate in eccentric CH phenotype (Müller and Dhalla 2013). In pressure overload patients, an increased ratio of LV wall thickness to LV radius was demonstrated as an adaptation to increased LV end diastolic and peak systolic pressures. Conversely, volume overload patients demonstrated no changes regarding the same ratio (Grossman 1980). Physiological CH can manifest into either concentric or eccentric. Typically, research has shown that athletes involved in endurance-focused sports, such as rowing and running, manifest eccentric CH. On the other hand, participants in resistance training, such as weight lifting, demonstrate concentric CH devoid of cavity reduction or ventricular dysfunction (Barauna et al. 2007).

1.3 Classification of heart failure

If CH is not treated, it could result in heart failure (HF) among other CVDs (Tham et al. 2015). Cases of HF are globally on the rise, and HF remains the top reason for mortality and morbidity worldwide. Despite advancement in options of treatment, there are HF patients who continue to manifest symptoms, presenting a huge load on the system of health care (Inamdar and Inamdar 2016; Teerlink et al. 2017). HF is regarded as a clinical complex syndrome owing to any functional or structural derangements of ventricular ejection or filling of blood (Yancy et al. 2017). Aggressive medical interventions and early diagnosis have been widely provided, yet patients with HF demonstrate poor prognosis, and mortality rate of nearly 33% annually (Roger 2013).

HF has been classified into non-ischemic or ischemic idiopathic HF (Fox et al. 2001). While there are various HF etiologies, there is mounting research indicating that HF development is strongly associated with stimulation of different neurohormonal systems, whatever the cause is. Stimulus insults encompassing hypertension, coronary artery disease, toxins, valvular heart disease, among others induce injuries in the heart tissues, which in turn stimulate neurohormonal systems such as renin-angiotensin-aldosterone system (RAAS) and activate the sympathetic nervous system (Jackson et al. 2000). Eventually, progressive worsening of left ventricular (LV) functions ensues, leading to cardiac tissue remodeling and sustained activation of neurohormonal systems. Initially, LV dysfunctions are asymptomatic, before manifesting clinical HF syndrome distinguished by dyspnea at normal or low exertion levels, sodium and fluid retention, rehospitalization, fatigue, high mortality rate, pump failure, arrhythmias, and death (Bui et al. 2011; Ziaeian and Fonarow 2016). Thus, while initial mechanisms of myocardial injury could be different in non-ischemic and ischemic HF patients, the pathophysiology after cardiac insult is quite similar (DeFilippis et al. 2019).

In general, HF involves deranged cardiac pumping functions; the robust indicator of pump derangement is called ejection fraction (EF) and is known as the blood volume percent pumped per

beat (Sharma and Kass 2014). For a long time, the main focus of HF clinical research was on HF that is accompanied with reduced LV systolic functions, currently recognized as HFrEF (short for HF with reduced EF) (Jessup et al. 2016). Nonetheless, nearly 50% of HF patients show LVEF over 50% and constitute a different HF phenotype defined as HFpEF (short for HF with preserved EF) (Yancy et al. 2013). Along with aging, it is worth mention that patients with HFpEF typically show comorbid conditions encompassing hypertension, atrial fibrillation, metabolic syndrome, renal dysfunction, and pulmonary hypertension, however the exact contribution of each pathological condition to HF pathophysiology is still poorly understood (Desai and Fang 2008; Ter Maaten et al. 2016; Altara et al. 2017; Anderson et al. 2019; Vachiéry et al. 2019). A third HF phenotype is HFmrEF (short for HF with mid-range EF) and is characterized by EF values between 40% and 49% in line with USA and Canadian guidelines (Maddox et al. 2021; McDonald et al. 2021).

Several studies demonstrated that inflammation is a peculiar hallmark of CH and HF (Yang et al. 2012; Xu and Brink 2016; Fang et al. 2017; Anthony et al. 2019). Furthermore, our lab showed that inflammation modulate the expression of CYP enzymes and CYP-derived AA metabolites in different organs of rats treated with lipopolysaccharide (LPS) (Anwar-mohamed et al. 2010). Multiple studies demonstrated the ability of inflammation to modulate CYP expression in different organs and models (Siewert et al. 2000; Vet et al. 2011; Oni-Orisan et al. 2013; Lenoir et al. 2021; Stipp and Acco 2021).

1.4 CYP-mediated arachidonic acid metabolism

In 1980, the CYP-mediated AA metabolism was identified. Different subclasses are present under the CYP family (Capdevila et al. 1982), yet only epoxygenases and ω-hydroxylases are the most crucial AA metabolizers. The ω-hydroxylase-mediated AA metabolism gives rise to terminal HETE (20-HETE), subterminal HETEs (16-, 17-, 18-, and 19-HETE) and midchain HETEs (5-, 8-

, 12-, and 15-HETE) (El-Sherbeni and El-Kadi 2017). 20-HETE has been intensely studied and has demonstrated pro-inflammatory activities and modulation of vascular function (Tsai et al. 2017). On the other hand, the epoxygenase-mediated AA metabolism via CYP2J and CYP2C families generates EETs, such as 5,6-EET, 8,9-EET, 11,12-EET, along with 14,15-EET. Each epoxygenase can generate all four EETs from AA. The predominant eicosanoids formed in many situations are 14,15-EET and 11,12-EET (Wu et al. 1996; Capdevila et al. 2000; Zeldin 2001). There are numerous studies indicating that CYP epoxygenases in humans are relatively stereoselective and regioselective for EETs generation. For example, the main human epoxidase, CYP2C8, generates 11,12-EET and 14,15-EET in a ratio of 1.0:1.3 with very limited capacity for producing 5,6-EET and 8,9-EET (Zeldin et al. 1996; Imig 2012). Similarly, CYP2C9 produces 11,12-EET and 14,15-EET in a ratio of 1.0:2.3 with no significant production of 8,9-EET (Daikh et al. 1994; Zeldin et al. 1995). In addition, nearly 75% of CYP2J2-derived 14,15-EET and 80% of CYP2C8-derived 11,12-EET are the *R, S* enantiomer. In contrast, nearly 70% of CYP2C9-derived 14,15-EET is *S, R* (Daikh et al. 1994; Zeldin et al. 1995). Bioactive EETs are generated in the liver, blood vessels and cardiomyocytes. EETs are converted through soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs) (Campbell et al. 1996; Zeldin 2001). Due to EETs contribution to vasodilation, they are targeted for the management of CVDs such as stroke, heart failure, and hypertension (Xu et al. 2011; Imig 2018).

Enantiomers are two mirror images of a single chiral molecule. The antipodes of the same compound are characterized by being non-superimposable, and refract plane-polarized light differently (McConathy and Owens 2003). Chirality is typically known as the geometric characteristic of a molecule of being non-superimposable with its antipode (Barron 2008).

Chirality is present in many compounds in nature, and a mixture of 2 antipodes in equal amounts is named racemic mixture or racemate (McConathy and Owens 2003).

Nearly 50% of the drugs in the market are chiral, and approximately half of these chiral drugs are racemate rather than a single enantiomer (Hutt 2002). Drugs in a single enantiomer form will become more available to physicians, together with the racemate form of the marketed drugs. In such cases, it is pivotal to differentiate between the racemate form and the single enantiomer form owing to inherent differences in their indicated use, efficacy, side effects and dosages (Flockhart and Nelson 2002). The antipodes of a single chiral molecule might differ markedly in their toxicity, metabolism rate, bioavailability, metabolites, potency, excretion, and selectivity for transporters, enzymes and/or receptors (McConathy and Owens 2003). Utilizing drugs in their single-enantiomer form can lead to more selective pharmacological profile, enhanced therapeutic indices, simpler pharmacokinetics, and reduced drug interactions. For instance, one enantiomer can be responsible for therapeutic effects, while the other antipode is inactive or is involved in undesired effects. In such cases, using a single enantiomer would be preferred over using the racemate form. Clinical trials have demonstrated that (S)-omeprazole, a gastroesophageal reflux treatment that works by proton pump inhibition, and (S)-albuterol, an agonist for β_2 receptor for asthma treatment, are superior in their action compared to their racemate formulation (Flockhart and Nelson 2002). In other instances, utilizing the racemate formulation of certain chiral drugs might be critical for their therapeutic impacts, and using the single antipode approach could be less safe or effective than using the racemate form. For instance, the (-)-enantiomer of β-blocker sotalol demonstrates antiarrhythmic activity and β-blocking action, while the (+)-antipode shows antiarrhythmic properties without β-blocking actions (Kato et al. 1986; Advani and Singh 1995). Additionally in phase II studies, (R)-fluoxetine showed marked cardiac repolarization

prolongation, leading to stopping further studies on this single enantiomer (DeVane and Boulton 2002).

CYP genes express a super-family of monooxygenases, comprising nearly 57 enzymes in the human genome (Bièche et al. 2007). The CYP enzymes are best-known for metabolizing xenobiotics, such as drugs, and endogenous compounds such as biogenic amines, fat soluble vitamins, fatty acids, and steroids. The expression and activity of CYP are modulated by transcription factors, hormones, and growth factors. In fact, various subfamilies of CYP can exhibit elaborate expression patterns depending on tissue type and developmental stage. Many CYP enzymes are expressed in the heart. Table 1.1 demonstrates different CYPs expressed and their functions in the heart.

Table 1.1. Expression and functions of cardiac CYP enzymes

Isozyme	Expression	Functions	References
CYP1A1	Inducible, Moderate	Metabolism of AA (*in-vitro*). Drug metabolism eg. Verapamil.	(D Choudhary et al., 2003; Thum & Borlak, 2000)
CYP1A2	Inducible	Metabolism of AA (*in-vitro*). Production of NO via metabolism of NCX-4016.	(D Choudhary et al., 2003; Minamiyama et al., 1999)
CYP1B1	Moderate	Biosynthesis of EET and HETE (*in-vitro*).	(Chehal & Granville, 2006; D Choudhary et al., 2003)
CYP2C8	Moderate	Biosynthesis of EET. Drug metabolism eg. Verapamil.	(Chehal & Granville, 2006; Thum & Borlak, 2000)
CYP2C9	Inducible, Low	ROS production. Biosynthesis of EET. Drug metabolism eg. Verapamil.	(Chehal & Granville, 2006; Fleming et al., 2001; Thum & Borlak, 2000)
CYP2J2	High	Drug metabolism eg. Doxorubicin. Biosynthesis of EET. Production of NO via metabolism of NCX-4016.	(Chehal & Granville, 2006; Minamiyama et al., 1999; Y. Zhang et al., 2009)
CYP2B6/7	Inducible	Drug metabolism eg. Verapamil.	(Thum & Borlak, 2000)
CYP2D6	Low	Drug metabolism eg. Verapamil.	(Thum & Borlak, 2000)
CYP2E1	Low	Unknown	(Bièche et al., 2007; Thum & Borlak, 2000)
CYP3A4	Low	Unknown	(Minamiyama et al., 1999)
CYP4A1	Medium	Synthesis of 20-HETE via AA metabolism.	(Nithipatikom et al., 2004)
CYP4A2	Medium	Synthesis of 20-HETE via AA metabolism.	(Nithipatikom et al., 2004)
CYP4B1	Inducible	Drug metabolism eg. Verapamil.	(Thum & Borlak, 2000)
CYP4F12	Medium	Synthesis of 20-HETE via AA metabolism.	(Nithipatikom et al., 2004)
CYP11B2	Inducible, Low	Synthesis of Aldosterone. Induction of CH and myocardial fibrosis.	(Delcayre & Silvestre, 1999; Silvestre et al., 1998)

CYP, cytochrome P450; AA, arachidonic acid; NO, nitric oxide; EET, epoxyeicosatrienoicacids;

HETE, hydroxyeicosatetraenoic acid; CH, cardiac hypertrophy.

Nonetheless, expression of CYP2J and CYP2C enzymes can be observed in cardiomyocytes, hepatocytes, smooth muscle cells, vascular endothelial cells (ECs) and nerve cells (Enayetallah et al. 2004). It is established that various environmental and genetic factors influence CYP expression culminating in remarkable alteration in the formation of bioactive mediators.

Metabolites of AA possess a wide range of biological activities. For instance, 20-HETE is associated with myogenic tone modulation and vasoconstriction (Tsai et al. 2017). Conversely, EETs possess cardioprotective, vasodilatory, and anti-inflammatory properties and can influence migration of vascular smooth muscles, which is pivotal in atherosclerosis and vascular remodeling. It is highly likely that enantiomers of EETs and HETEs have different biological profiles (Kiss et al. 2008). The EETs intracellular levels are tightly controlled via sEH activity, which produces the corresponding DHETs. DHETs have demonstrated reduced activity compared to the EETs. Although EETs share similar biological functions, their actions differ to some extent. For instance, some EETs demonstrated better pro-angiogenic properties than others *in vitro* and *in vivo* (Wang et al. 2005; Zhang et al. 2006). Strikingly, EETs are best known for their hyperpolarizing action in some organs such as the heart. They are identified as endothelium-derived hyperpolarizing factors (EDHF), due to their role in modulating vascular functions (Campbell et al. 1996). EETs are reported to exert their vasorelaxation through a G-protein coupled receptor or transient receptor potential (TRP) channel activation (Campbell and Fleming 2010). EETs possess cardioprotective properties against hypertension, chronic non-ischemic cardiomyopathy, and acute ischemia-reperfusion injury.

A significant amount of data highlighted sex-specific variations in the outcomes and the incidence of different CVDs (Regitz-Zagrosek and Kararigas 2017; Gerges and El-Kadi 2021). Sixty years ago, it was hypothesized that sex may modulate eicosanoids levels (Monsen et al. 1962). Since

then, multiple clinical and experimental studies have shown sex differences in CYP expression and the production of various eicosanoids in the heart (Muller et al. 2007; Pace et al. 2017; Gerges and El-Kadi 2022). The mechanisms underlying sex differences in CVDs are still elusive, however, it was hypothesized that hormones and genetic factors play a role (Regitz-Zagrosek and Kararigas 2017). The phenomenon of sex-specific discrepancies contributes to altered eicosanoid production and hence influences the cardiac function (von Jeinsen et al. 2017). One study reported that males show a greater risk of developing CVDs, such as ischemic heart diseases, hypertension and CH compared to same-age females, and women in post-menopausal stage demonstrate a greater risk than pre-menopausal women (Vitale et al. 2009). Also, findings from human and experimental studies demonstrate that the incidence of CH is more common in males than in females, and that females lose this advantage following menopause (Regitz-Zagrosek et al. 2010; Wu et al. 2020).

1.5 Role of CYP-mediated arachidonic acid metabolism in cardiac hypertrophy

1.5.1 EETs and CH

The impact of EETs on CH has been discussed previously (Alsaad et al. 2013; Wang et al. 2013). Inhibition of sEH reversed CH development mediated by chronic pressure overload (Xu et al. 2006). Moreover, reports from our lab indicated that isoproterenol-elicited CH in RL-14 cells, evidenced by increased hypertrophic markers such as BNP and ANP, can be prevented by inhibiting sEH (Althurwi et al. 2013; Althurwi et al. 2015).

EETs derived from CYP2J2 demonstrated protective effects in CH models (Alsaad et al. 2013). In a rat model of isoproterenol-mediated CH, hypertrophy was established following 72 hours of isoproterenol treatment. EETs levels were reduced in the hypertrophic hearts, and CYP2J2

overexpression restored levels of EETs and inhibited CH development via NF-κB-dependent mechanism (Althurwi et al. 2015). Another study on rat cardiomyocytes (H9c2) highlighted that the expression of CYP enzymes was elevated following isoproterenol treatment, which was responsible for increased cellular surface area. Remarkably, 14,15-EET markedly ameliorated isoproterenol-mediated CH (Mandy et al. 2013). This is supported by another report detailing the involvement of CYP2J2 overexpression and 11,12-EET in ameliorating Ang II-induced CH by stimulating adenosine monophosphate kinase-alpha 2 (AMPK-α2) (Wang et al. 2016).

Inflammation is a pivotal pathological player in CH and HF. It is recognized that inflammation exists in chronic and acute HF, with poorer prognosis associated with increased levels of pro-inflammatory mediators. It was unveiled that EETs possess anti-inflammatory properties (Inceoglu et al. 2007). In 1999, Node and colleagues were the first to publish the anti-inflammatory properties of EETs against various inflammatory mediators, such as IL-1α and TNF-α, via NF-κB inhibition (Node et al. 1999). Both 14,15-EET (Morin et al. 2008) and 11,12-EET (Bystrom et al. 2011) inhibited in vitro stimulation of NF-κB. Also, Ang II-treated mice demonstrated CH with NF-κB stimulation, whereas CYP2J2 transgenic mice exhibited ameliorated CH with reduced nuclear translocation of NF-κB p65 (He et al. 2015). It is well known that PPAR-γ contributes to the anti-inflammatory properties of EETs. Importantly, EETs have been reported to be PPAR-γ ligands and to elevate the transcription activity of PPAR-γ in 3T3-L1 preadipocytes and endothelial cells (Liu et al. 2005). This was proven by the fact that the anti-inflammatory properties of EETs can be inhibited via PPAR-γ antagonist in vitro and in vivo (Liu et al. 2005; He et al. 2015). Furthermore, a PPAR-γ-independent cascade was also implicated in Ang II-induced inflammation. A recent study highlighted that Ang II triggered JAK2/STAT3 cascade with subsequent

inflammation and aortic fibrosis. CYP2J2 overexpression stimulated SOCS3 (Suppressor Of Cytokine Signaling 3) expression, which inhibited JAK2/STAT3 activation (Zhou et al. 2016).

The rate limiting enzyme involved in heme catabolism is heme oxygenase-1 (HO-1). It contributes to the attenuation of inflammation and CH. Due to possessing similar properties, researchers began to uncover the links between EETs and HO-1. Various studies highlighted that HO-1 expression could be induced via EETs (Sacerdoti et al. 2007; Li et al. 2009; Aliwarga et al. 2020). In obesity-induced cardiomyopathy, deteriorated cardiac functions were detected, whereas EET analog administration reduced pro-inflammatory adiponectin expression and hampered HF development. These effects may be attributed to HO-1 induction through EET analog (Cao et al. 2017).

Several studies demonstrated that sEH inhibition was a prime target to inhibit inflammation (Schmelzer et al. 2005). One study showed that sEH inhibitor, AUDA, prevented inflammation development in mice through NF-κB inhibition (Liu et al. 2005). Similarly, these effects were noted in CH models. A study by Stevenson and his team investigated the inflammatory mediators such as IL-16, CCL4, CCL5, and MMP9 in ischemic cardiomyopathy, and revealed upregulated inflammatory cytokines in ischemic hearts at both protein and mRNA levels. Moreover, in a model of left anterior descending (LAD) ligation-induced HF, both inflammatory cytokine CCL5 and sEH expression were significantly increased. The treatment with sEH inhibitors attenuated the inflammatory cytokines expression (Stevenson et al. 2019). In a different HF model instigated via LPS, cardiac functions were deteriorated based on echocardiography and hemodynamic analysis, whereas sEH deficiency ameliorated LPS-mediated cardiac dysfunction. Also, it was highlighted that sEH deficiency reduced MCP-1 and TNF-α levels upregulated by LPS (Samokhvalov et al. 2019), which indicated that these cardioprotective properties of EETs in HF were involved in inhibiting inflammatory responses.

1.5.2 HETEs and CH

1.5.2.1 20-HETE and CH

Current findings regarding the contribution of 20-HETE in CH and HF require further investigations. Notably, 20-HETE may exert pro-inflammatory response by eNOS uncoupling mechanism (Cheng et al. 2010), NF-κB activation (Ishizuka et al. 2008), and increasing ROS generation (Singh et al. 2007). Figure 1.2 shows the postulated mechanisms of 20-HETE pro-inflammatory functions in the cardiomyocytes.

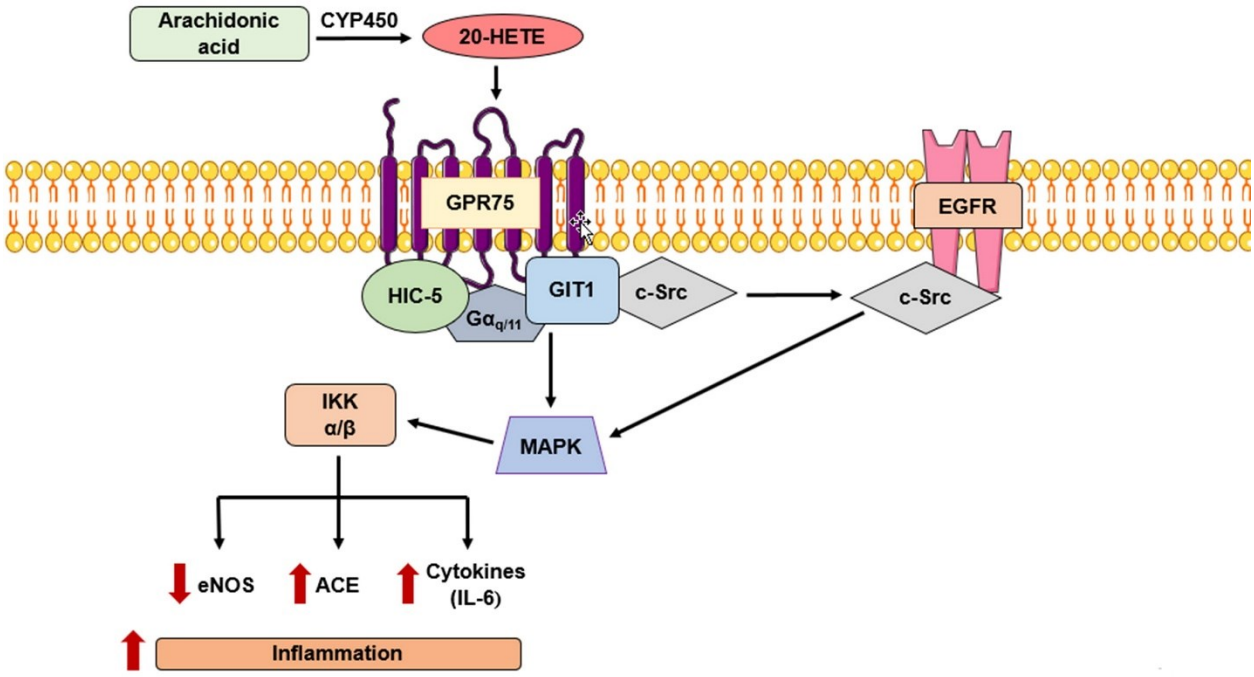


Figure 1.2. Postulated mechanisms of 20-HETE pro-inflammatory bioactions in the cardiomyocytes.

20-HETE binds to its receptor GPR75 in cardiac cells, triggering a signaling pathway that is mediated by a $G\alpha_{q/11-}$ and GIT1-induced c-Src stimulation and EGFR phosphorylation which is followed by stimulation of MAPK (ERK1/2) and IKKβ-NF-κB resulting in ACE induction, uncoupling of eNOS and production of inflammatory cytokines

In addition, 20-HETE may initiate pro-hypertrophic cascades through triggering translocation of nuclear factor of activated T-cells (NFAT) by a calcineurin and Rho-kinase-dependent pathway (Yaghi and Sims 2005), or through Ras/MAPK (Muthalif et al. 1998). In cardiomyocytes, 20-HETE was demonstrated to induce NADPH oxidase-mediated superoxide generation by PKC-reliant mechanism (Zeng et al. 2010). Similarly, 20-HETE can trigger L-type calcium channel in the heart through the same mechanism (Zeng et al. 2010). The cardioprotective effects of inhibiting 20-HETE in the context of ischemia-reperfusion injury are diminished upon blocking K_{ATP} channels (Gross et al. 2004). 20-HETE mediates apoptosis in neonatal rat cardiomyocytes via several mechanisms encompassing increased Bax expression, elevated caspase-3 activity, and altering mitochondrial membrane potential (Bao et al. 2011).

Recently, it has been demonstrated that 20-HETE is a ligand for one of the orphan receptors, namely G protein-coupled receptor 75 (GPR75) (Garcia et al. 2017). GPR75 has been shown to be expressed in different tissues, including liver, lung, spleen, kidney (Ignatov et al. 2006), and vascular smooth muscle cells in the aorta, endothelium and brain (Garcia et al. 2017; Gonzalez-Fernandez et al. 2020). GPR75 can be stimulated via different stimuli encompassing pro-inflammatory eicosanoids and cytokines, which play a key role in the pathophysiology of certain disorders. (Khan and He 2017; Alavi et al. 2018; Pascale et al. 2021). Following 20-HETE binding to GPR75, it stimulates c-Src/EGFR and $G\alpha_{q/11}$/PLC/PKC pathways which mediate its effects on vascular function and blood pressure (Garcia et al. 2017).

It has also been claimed that CYP4F and CYP4A are upregulated in animal models of CH-associated HF. In rats, CYP4F and CYP4A were elevated in isoproterenol-induced CH (Zordoky et al. 2008; Althurwi et al. 2015). Also, levels of 20-HETE were demonstrated to be increased in Ang II-induced CH (Elkhatali et al. 2015). Notably, pretreatment with an inhibitor of ω-

hydroxylase to hamper 20-HETE formation was partly beneficial against CH development (Alsaad et al. 2013).

In an acute model of doxorubicin-mediated cardiotoxicity, 24-hour doxorubicin treatment increased the expression of CYP4A1, CYP4A3, CYP4F1, and CYP4F4 (Zordoky et al. 2010). Though cardiac functions were not assessed, doxorubicin's connection to acute and chronic HF has been well investigated, when employed as a chemotherapeutic agent (Levis et al. 2017).

According to angiogenesis and arteriogenesis studies, 20-HETE effects could be phenotype-specific, in that risk factors of CVDs such as metabolic syndrome, hypertension, diabetes, and obesity can modify expressed CYP4A localization, elevate levels of 20-HETE, and aggravate 20-HETE effects. Interestingly, a study highlighted a link between increased levels of 20-HETE, diabetes, and myocardial ischemia in humans (Issan et al. 2013). Another report demonstrated reduced infarcted size in Langendorff-perfused diabetic rat hearts but not healthy ones (Yousif et al. 2009). These two studies point out a significant impact of diabetes on effects of 20-HETE and potential efficiency of 20-HETE inhibitors. Thus, including animal models that show those risk factors in prospective studies may be invaluable.

1.5.2.2 19-HETE and CH

It has been found that 19-HETE constitutes the main subterminal HETE produced in the rat heart, and its production was reduced in a descending aortic constriction model of CH (El-Sherbeni and El-Kadi 2014). Hence, the cardioprotective effect of 19-HETE was demonstrated in RL-14 cells *in vitro* and in Ang II-induced CH model *in vivo*. Notably, 19-HETE has prevented CH development in RL-14 cardiomyocytes as exhibited by reduced hypertrophic markers. Furthermore, a well-recognized hepatic CYP2E1 inducer, isoniazid, showed marked alterations in

cardiac CYP-induced AA metabolism, including increased levels of cardioprotective 19-HETE. Importantly, these alterations were linked to attenuated Ang II-mediated CH, suggesting a potential therapeutic outcome of isoniazid and similar cardiac 19-HETE inducers on CH (Elkhatali et al. 2015). It is worth mentioning that the S-enantiomer of 19-HETE exhibited more cardioprotective properties against Ang II-induced CH compared to the R-enantiomer (Shoieb and El-Kadi 2018). This cardioprotective effect of 19-HETE may be attributed to its ability to noncompetitively inhibit CYP1B1 (Shoieb et al. 2019a) or acting as a functional antagonist of 20-HETE (Cheng et al. 2008). Moreover, it was found that new synthetic 19(R/S)-HETE analogs demonstrate noncompetitive inhibitory effect on the activity of CYP1B1 and CYP1A1 (Shoieb et al. 2021) which are the main enzymes involved in the production of deleterious mid-chain HETEs.

1.5.2.3 Current knowledge regarding 16, 17 and 18-HETE

With respect to 16-HETE, its production via hydroxyl group incorporation onto the 16th carbon atom of AA produces a chiral center giving rise to 16(R)-HETE and 16(S)-HETE enantiomers (Powell and Rokach 2015). Previously, it has been documented that 16-HETE exerts multiple physiological functions and is implicated in various diseases (Shoieb et al. 2019b). In the rabbit kidneys, 16-HETE was found to be contributing to tubular transport, regulation of renal perfusion, along with vasodilation (Ivanov et al. 2004). Also, 16-HETE is involved in inhibiting the aggregation and adhesion of human leukocytes (Bednar, Gross, Balazy, et al. 2000), which could be beneficial in the management of acute ischemic stroke where neutrophil stimulation exacerbates brain injury (Bednar et al. 1997). Further, 16-HETE exerts pivotal functions in the murine intestine including transport of intestinal electrolytes, intestinal motility regulation, as well as anti-inflammatory properties (Tsao et al. 2000). Interestingly, elevated 16-HETE levels are linked to intracytoplasmic sperm injection failure (Ciepiela et al. 2015). It has been postulated that 16-HETE

could be beneficial in central blood pressure as a potential therapeutic target or diagnostic marker as its elevation could be a cardioprotective response in patients plagued with central hypertension (Caligiuri et al. 2016). Furthermore, 16(R)-HETE has demonstrated specific and potent inhibition of polymorphonuclear functions *in vitro* along with reducing increased intracranial pressure in a thromboembolic stroke model (Bednar, Gross, Russell, et al. 2000). Moreover in the platelet membranes, 16-HETE has been detected as a major AA metabolite, yet its functions require further investigation (Zhu et al. 1995). It is worth note that 16-HETE profile could help to follow the non-alcoholic fatty liver disease development (Maciejewska et al. 2015).

Turning to 17-HETE, it is the major urinary HETE that is limited to phosphatidylethanolamine and neutral lipids of renal medullary and cortical lipids (Carroll et al. 1997). One study found that 17-HETE is a potent inhibitor of ATPase activity in the proximal tubules without affecting 70-pS K^+ channels in the rat kidney (Wang and Lu 1995). It has been stated that the brain levels of 17-HETE were significantly increased following acute treatment of C57Bl/6 mice with arsenite, suggesting that 17-HETE may exert a pivotal role during acute arsenite toxicity (Anwar-Mohamed et al. 2014).

Regarding 18-HETE, it was found almost entirely in the neutral lipid part of the renal cortex (Carroll et al. 1997). It is intriguing to know that a direct association is established between increased 18-HETE levels and insulin resistance in the microvasculature. Also, it has been revealed that elevated levels of 18-HETE are correlated with aberrated vascular recruitment in the skeletal muscles. Subsequently, 18-HETE may be involved in insulin resistance (Chadderdon et al. 2016). In spontaneously hypertensive rats, enhanced phenylephrine vasoconstriction in the renal interlobar arteries has been ascribed to vasoregulatory response due to reduced vascular CYP2E1-

generated 18(R)-HETE (Zhang et al. 2005). This calls for further studies illuminating the roles of 17-HETE and 18-HETE in health and diseases, especially CVDs.

1.5.2.4 Mid-chain HETEs and CH

Multiple studies are in favor of the contribution of mid-chain HETEs to CH development. In this regard, mid-chain HETEs were found to be elevated following pressure overload-mediated CH (El-Sherbeni and El-Kadi 2014). As a CH model, the descending aortic constriction (DAC) is clinically invaluable as the development of CH takes longer time periods (Patten and Hall-Porter 2009). The formation of mid-chain HETEs was associated with increased CYP1B1 protein expression. The contribution of CYP1B1 to mid-chain HETEs production was ascertained via the recombinant CYP1B1 ability to generate mid-chain HETEs (Choudhary et al. 2004; El-Sherbeni and El-Kadi 2014). Similar to DAC model, our lab reported elevated mid-chain HETEs in Ang II-treated rats. Of note, rats pretreated with tetramethoxy stilbene (TMS), which is a specific inhibitor of CYP1B1, were protected from Ang II-mediated CH (Elkhatali et al. 2017). Critically, the cardioprotection against CH correlated with significant reduction in mid-chain HETE levels proposing a pivotal involvement of mid-chain HETEs in CH and also affirms that CYP1B1 is crucial in mid-chain HETEs formation. Interestingly, inhibition of CYP1B1 by treatment with CYP1B1-siRNA or with a chemical inhibitor like trans-resveratrol was previously found to significantly ameliorate isoproterenol and Ang II-induced CeH, respectively, through lowering the levels of CYP1B1 and its associated cardiotoxic midchain HETEs (Maayah et al. 2017; Shoieb and El-Kadi 2020). It is worth mention that trans-resveratrol was effective in ameliorating cardiac remodeling after myocardial infarction (Burstein et al. 2007).

For 12-HETE, it has been shown to induce renal vasoconstriction, fibrosis, and cellular hypertrophy (Kayama et al. 2009). Additionally, increased formation of 12-HETE has been observed in spontaneously hypertensive rats and essential hypertension patients (Sasaki et al. 1997; González-Núñez et al. 2001). In cardiac fibroblasts, the 12-LOX overexpression was utilized as a model to study the hypertrophic properties of 12-HETE (Wen et al. 2003). 12-LOX overexpression induced cell size enlargement, collagen expression, fibronectin and cell protein content due to increased production of 12-HETE (Wen et al. 2001). In rat cardiac fibroblasts, overexpression of 12-LOX resulted in cellular hypertrophy. This is confirmed by hematoxylin and eosin (H&E) staining, which showed that nucleoli mean number and long nuclei axis were significantly increased in cells transfected with 12-LOX (Wen et al. 2001; Wen et al. 2003).

In cardiomyocytes, it has been demonstrated that 15-HETE enhance the sensitivity of β-adrenergic response induced by isoproterenol and thus has been suggested to be involved in HF through triggering cardiac fibrosis (Wallukat et al. 1994; Levick et al. 2007; Kayama et al. 2009; L. Zhang et al. 2014). It has been demonstrated that norepinephrine evoked its hypertrophic effects by stimulating the production of 15-HETE and 12-HETE (Parmentier et al. 2001). From a mechanistic point of view, 15-HETE is significantly deposited into the cellular pool of phosphatidylinositol. The phosphatidylinositol containing 15-HETE is converted into diacylglycerol substituted with 15-HETE. This particular diacylglycerol could modulate PKC (Wallukat et al. 1994). 15-HETE triggered adventitial fibrosis and phenotypic changes in fibroblasts dependent on TGF-β1 cascade (L. Zhang et al. 2014). This is in agreement with a previous study with baicalein, an inhibitor of 12/15-LOX, which ameliorated cardiac fibrosis observed in spontaneously hypertensive rats (Kong et al. 2011). Additionally, wogonin and baicalein, which are 12/15-LOX inhibitors, have been documented to reduce collagen deposition following Ang II treatment (Kong et al. 2010).

Regarding 5-HETE, it has been reported to exert pro-inflammatory and vasoconstrictive functions (Burhop et al. 1988). Also, it has been demonstrated that it contributes to Ang II-mediated hypertrophy (Revermann et al. 2011). In this regard, 5-LOX inhibitor, LP105, attenuated Ang II-mediated hypertrophy in mice lacking ApoE. This was exhibited by the LP105 ability to reduce heart-to-body weight ratio, decrease heart rate, as well as inhibit the Ang II-induced increased aortic diameter and weight (Revermann et al. 2011). Another study reported that selenium was able to ameliorate diabetic CH via 5-LOX and 5-HETE downregulation (Dhanya et al. 2014).

Our lab was the first to report the ability of 8-HETE to instigate CH in RL-14 cells, indicated by elevated expression of hypertrophic markers ANP and BNP. Both NF-κB and MAPK are implicated in 8-HETE-mediated CH (Maayah, Abdelhamid, et al. 2015). Though the previous reports have demonstrated the possible hypertrophic properties of mid-chain HETEs in the heart, no study employed human cardiomyocytes to investigate the mid-chain HETEs-mediated CH. Hence, our lab was first to report the contribution of mid-chain HETEs to cellular hypertrophy by utilizing RL-14 cells, which are human ventricular cardiomyocytes (Maayah, Abdelhamid, et al. 2015; Maayah and El-Kadi 2016a). Mid-chain HETEs evoked cellular hypertrophy via stimulating CH markers ANP, BNP, β-MHC, and α-MHC in concentration and time-dependent fashion. Also, mid-chain HETEs induced enlargement of cardiomyocytic size compared to controls. This offered the first insight that mid-chain HETEs can cause cellular hypertrophy in human cardiomyocytes (Maayah and El-Kadi 2016a).

1.6 Role of inflammation in the pathogenesis of cardiac hypertrophy

Inflammation was demonstrated to be a distinguished hallmark of CH (Yang et al. 2012). Fibrosis, inflammatory cascades stimulation such as NF-κB, elevated expression of cytokines encompassing

TNF-α, IL-1β, IL-6, and IL-1RA, and infiltration of inflammatory cells such as T-lymphocytes and macrophages are all prominent characteristics of pathological CH (Erten et al. 2005; Kuusisto et al. 2012). The exact role of inflammation in CH is elusive, yet it is assumed to aggravate this condition. For instance, IL-6 was exhibited to trigger CH in vivo and in vitro (Hirota et al. 1995; Hilfiker-Kleiner et al. 2010). Moreover, macrophage-derived microRNA-155, which is stimulated by INF-γ, TNF-α, and LPS, induces CH and HF (Heymans et al. 2013). Targeting inflammatory mediators and cell receptors was also demonstrated to modulate CH development and might sustain cardiac functions (Heymans et al. 2009; Velten et al. 2012). Figure 1.3 summarizes inflammation-induced signaling pathways promoting CH.

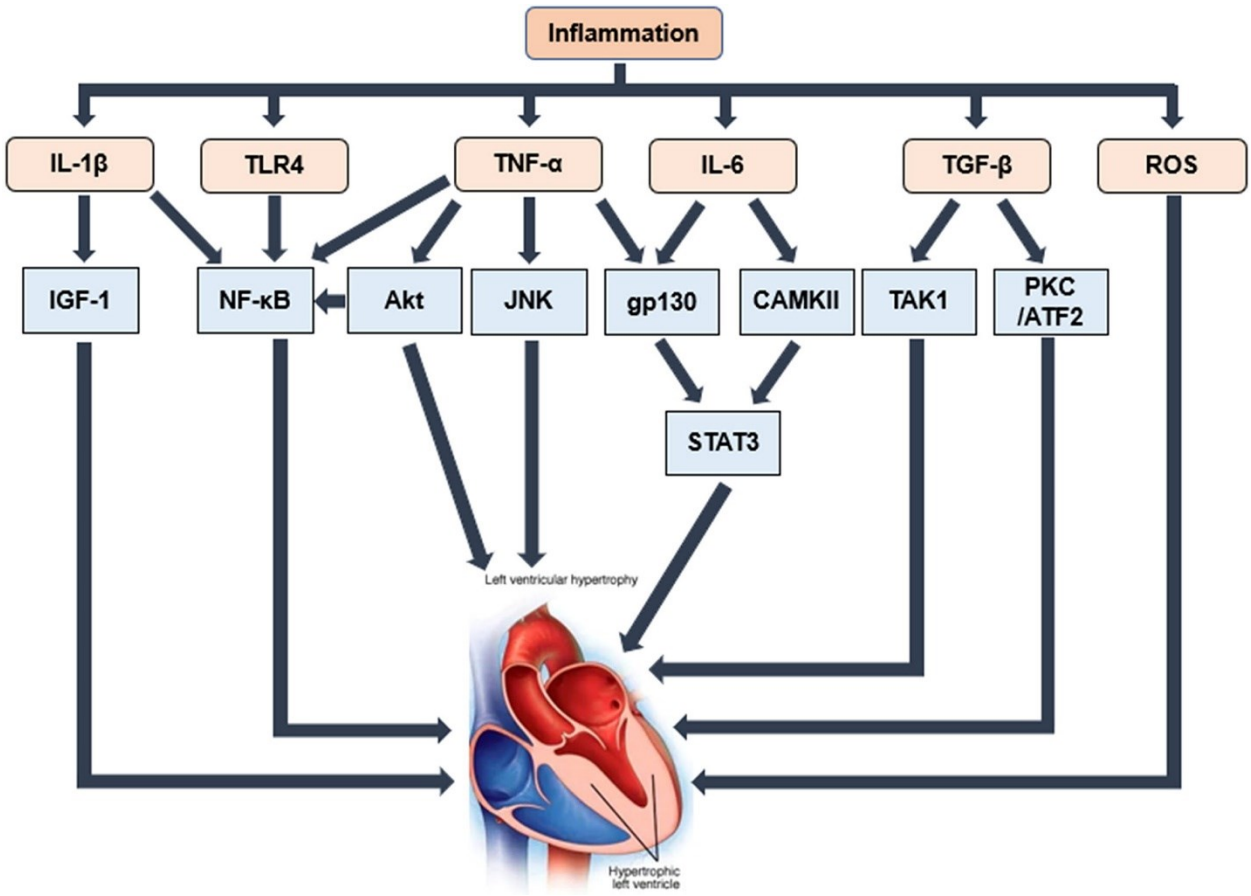


Figure 1.3. Signals promoting cardiac hypertrophy induced by inflammation.

Cytokines in the inflamed heart may modulate several signaling mechanisms converging toward either STAT3 or NF-κB thereby leading to cardiac hypertrophy. TAK1: transforming growth factor beta-activated kinase 1. PKC: protein kinase C. ATF2: activating transcription factor 2. CAMKII: $Ca^{2+/}$calmodulin-dependent protein kinase II. JNK: c-Jun N-terminal kinase. IGF1: insulin-like growth factor 1

The contribution of inflammatory cells to CH is well established. Macrophages (Mϕ) are mononuclear phagocytes that exert pivotal functions in tissue repair and remodeling, and regulation of adaptive and innate immunity (Murray and Wynn 2011; Takeda and Manabe 2011). In the heart, the two Mϕ phenotypes that are present are pro-inflammatory M1 and anti-inflammatory M2 (Mosser and Edwards 2008; Takeda and Manabe 2011). Regarding M1, it exacerbates cardiac inflammation through cytokine release and enhanced apoptosis, as well as its implication in cardiac remodeling (Takeda and Manabe 2011; Van den Akker et al. 2013; Fernández-Velasco et al. 2014). On the other hand, M2 attenuates inflammation and induces cardiac reparative cascades and angiogenesis (Van den Akker et al. 2013). A robust association between Mϕ and CH was created, however, reports have demonstrated that depletion of Mϕ exacerbates cardiac dysfunction following CH, proposing a pivotal undetermined contribution to both CH development and outcomes (Takeda and Manabe 2011). In summary, inflammation is a potential intervention target in CH development for discovering novel therapeutic agents that can improve the cardiac functions (Heymans et al. 2009; Hofmann and Frantz 2013).

1.6.1 Role of tumor necrosis factor alpha

TNF-α is regarded as one of the highly potent cytokines that promotes inflammation, thus it was chosen as the primary target in strategies focusing on cytokines. At present, inhibitors of TNF-α are clinically employed as anti-inflammatory medications to treat patients suffering systemic inflammatory diseases. Nearly one million patients use TNF-α antagonists, making them the most profitable medication globally with annual sales of 25 billion USDs (Monaco et al. 2015). Regarding HF, clinical findings highlighted that TNF-α inhibitors were not beneficial and even may aggravate HF outcomes. Nevertheless, the reasons underlying these unexpected results require further investigations.

TNF-α is expressed in humans through the *TNFA* gene present on chromosome 6 (Shiina et al. 2017). The *TNFA* gene contains 200 nucleotide promoters that can bind to various transcription factors, culminating in increased transcription flexibility to a wide array of stimuli, which also relies on cell type (Falvo et al. 2010).

Following transcription, TNF-α biosynthesis is regulated mostly via a binding competition between two RNA-binding proteins, namely stabilizing factor human antigen R (HuR) and tristetraprolin (TTP). Dephosphorylated TTP binds to mRNA causing its degradation. In contrast, phosphorylated TTP cannot bind effectively to mRNA, providing the ideal conditions for HuR to bind to mRNA for effective translation. Stimuli that promote inflammation modulate TTP activity and HuR translocation to cytoplasm from the nucleus, thusly promoting TNF-α biosynthesis. Notably, activity of TTP is modulated via p38 MAPK, which regulates target genes of TTP post-transcriptionally and the binding of NF-κB to TTP promoter, which enhances its translation (Tiedje et al. 2012; Chen et al. 2013; Clark and Dean 2016). Mice lacking TTP elevates TNF-α expression leading to cachexia, growth retardation, autoimmune response and arthritis (Taylor et al. 1996). Multiple inflammatory mediators are implicated in modulating TNF-α biosynthesis, such as IL-6, IL-1β, LPS, tissue trauma, IFN-γ, and hypoxia (Chung and Benveniste 1990; Kohanawa 2006; Li et al. 2011).

After translation, TNF-α is produced as a 17 kDa type II transmembrane protein, which has one transmembrane segment that is uncleavable and harboring the protein to cell membrane. This membrane type of TNF-α (mTNF-α) can act as a ligand. The extracellular segment of mTNF-α, through TNF-α cleaving enzyme (TACE), can be cleaved to produce soluble TNF-α (sTNF-α) (Moss et al. 1997). Both sTNF-α and mTNF-α congregate as homotrimers that are noncovalently bound and biologically active (Grell et al. 1995).

There are two TNF-α receptors (TNFRs), which are TNFR1 and TNFR2. TNFRs are transmembrane glycoproteins containing extracellular domains that bind TNF-α (MacEwan 2002). Often, TNFRs are present on the cell membrane, yet they can be produced in soluble forms which can neutralize sTNF-α activity. Apart from the constitutive expression of TNFR1 in most cells, TNFR2 expression is typically triggered by pro-inflammatory stimuli and is limited to immune cells, albeit its upregulation in cardiomyocytes and endothelial cells is reported (Al-Lamki et al. 2009; Cabal-Hierro and Lazo 2012). Stimulation of TNFR1 or TNFR2 relies on the bioavailability of both sTNF-α and mTNF-α. While sTNF-α mainly triggers TNFR1, TNFR2 is mostly stimulated via mTNF-α (Wajant et al. 2003). The activation of TNFR1 and TNFR2 induces a specific molecular response leading to different effector productions in the affected cell. Moreover, mTNF-α contributes to reverse signaling, thus it must be perceived as a receptor as well (Ardestani et al. 2013). Subsequently, TNFRs function as mTNF-α ligands. Notably, TNFR2 typically activates mTNF-α-mediated reverse signaling (Ardestani et al. 2013).

1.6.1.1 TNFR1 signaling:

Following ligand stimulation, multiple adaptor proteins are recruited via TNFR1, encompassing TNF-α receptor associated factor 2 (TRAF2), TNFR-associated death domain (TRADD), receptor-interacting protein kinase (RIPK), MAPK activating death domain (MADD), inhibitors of apoptosis proteins (IAPs) and Fas-associated death domain (FADD) (Wajant et al. 2003; Cabal-Hierro and Lazo 2012). The nascent complex of TNFR1/TRADD/TRAF2/RIPK/IAPs triggers MAPKs, predominantly inhibitor of kappa B kinases (IKKs), p38 and JNK. MAPKs transmit the signal to the nucleus via activation protein-1 (AP-1) among other transcription factors that attach to the distinct DNA motifs of target genes. NF-κB is activated through IKKs-induced degradation of inhibitor of kappa B (IκB) complex and subsequent liberation of p50 subunit to enter the nucleus

and modulate gene expression. It is worth mentioning that these IKK and MAPK-mediated responses are involved in cell survival and pro-inflammatory cytokine expression among other processes (Cabal-Hierro and Lazo 2012; Liu et al. 2017). On the other hand, TNFR1 internalization can occur resulting in production of TNFR1/TRADD/FADD complex after binding to pro-caspase-8. Caspase-8 activation induces a proteolytic cascade mediating cellular apoptosis. Strikingly, TNF-α-mediated apoptosis is triggered via MAPK/JNK as well (Schneider-Brachert et al. 2013). Additionally, TNFR1 can trigger necroptosis via mitochondrial fission. Notably, necroptosis is not reliant on other caspases, with the process taking place under conditions of depletion or inhibition of caspase-8. TNF-α-induced necrosome production is heavily dependent on mixed lineage kinase domain like (MLKL) pseudokinase, RIPK1 and RIPK3 (Vanlangenakker et al. 2011; Remijsen et al. 2014).

1.6.1.2 TNFR2 signaling:

In contrast with TNFR1, TNFR2 cannot bind to TRADD and trigger caspase-dependent apoptosis owing to the lack of intracellular death domain. Alternatively, TNFR2 activation recruits IAPs and TRAF2, inducing IKK-mediated canonical NF-κB cascade. Additionally, TNFR2, IAPs and TRAF2 aggregate with NF-κB-inducing kinase (NIK) to for a complex with subsequent liberation and activation of NIK. Activated NIK mediates non-canonical NF-κB cascade via IKKα which forms an active p52 subunit (Wajant and Siegmund 2019). Also, TNFR2 can trigger PI3K/Akt cascade, resulting in modulation of various downstream mediators (S. Yang et al. 2018). TNFR2-dependent stimulation of PI3K/Akt and NF-κB cascades predominantly contributes to cell survival and proliferation. In cells exhibiting both TNFRs, TRAF2 mediates the cross-talk between TNFR2 and TNFR1 (Cabal-Hierro and Lazo 2012; Borghi et al. 2016). As extensive activation of TNFR2 causes TRAF2 breakdown, this negatively modulates transcription factors together with the

immune response, yet augments TNFR1-reliant caspase-induced necroptosis and apoptosis (Wajant et al. 2003; Guo et al. 2017).

1.6.1.3 Reverse signaling of mTNF-α:

Not only does mTNF-α function as TNFRs ligand, but it also transmits a reverse signaling to the cells expressing mTNF-α. Mechanistically, the reverse signaling of mTNF-α is activated via TNFR2 manifested by the neighboring cells (Miller et al. 2015). Moreover, soluble TNFR2 or specific antibodies against TNF-α can trigger the reverse signaling of mTNF-α (Van den Brande et al. 2003; Zhang et al. 2017). Despite the lack of kinase activity on mTNF-α intracellular domain, TNFR binding to mTNF-α can trigger MAPKs and JNK signaling cascades along with nuclear downstream mediators of transcription. Strikingly, the reverse signaling of mTNF-α modulates the expression of several inflammatory cytokines in addition to various immune functions (Rossol et al. 2007). Still, the contribution and mechanisms underlying the reverse signaling of mTNF-α require further investigation.

1.6.1.4 Role of TNF-α in hypertrophic cardiomyopathy:

It is common to establish murine models of hypertension via partial aortic occlusion or sustained Ang II infusion with the aid of osmotic minipumps. Within these models, elevated cardiac blood pressure triggers a compensatory mechanism that promotes left ventricular thickening and negatively impacts myocardial relaxation. Subsequently, cardiac tissue exhibits interstitial fibrosis and CH. Findings from an aortic banding-induced pressure overload murine model highlight the implication of TNF-α-TNFR1 axis in hypertensive cardiomyopathy instigation, indicated by the association of progressive CH with elevated levels of TACE, TNF-α and TNFR1in the heart (Kassiri et al. 2005; Sun et al. 2007). Additionally, mice lacking *Tnfa* demonstrated sustained

cardiac functions along with lower inflammation response, left ventricular remodeling and CH in multiple studies (Kassiri et al. 2005; Sun et al. 2007; Awad et al. 2010; Kishore et al. 2011). This phenotype was ascribed to abolished superoxide production mediated by TNF-α/PI3K in cardiac fibroblasts and cardiomyocytes (Awad et al. 2010), modulated metalloproteinases expression and activity (Kassiri et al. 2005), attenuated cardiac inflammation, and reduced cardiomyocytic apoptosis (Sun et al. 2007). Uniquely, in this hypertensive model, tissue inhibitor of metalloproteinase (TIMP)-3 modulated TNF-α levels and cardiac TACE activity (Kassiri et al. 2005). The underlying mechanism is induced via TNFR1. Hence, *Tnfrsf1a*-knockout mice are partly protected against transverse aortic constriction (TAC)-mediated CH and exhibit enhanced survival rates (Nan et al. 2017). Also, mice without TRADD, an adaptor protein for TNFR1, demonstrated abrogated fibrosis with enhanced cardiac functions, indicating a pivotal role of the TNFR1-TRADD- induced cell death in CH (Wu et al. 2017). Strikingly, the pathological cascade of TNFR1 seems to be counteracted via TNFR2. Following blood pressure elevation mediated through TAC, *Tnfrsf1b*-null mice demonstrated lower survival rates and increased CH, and TNFR2-mediated cardioprotection has been associated with its mitochondrial effects (Nan et al. 2017). Furthermore, mice lacking TRAF2 showed aggravated HF, pathogenic remodeling and cardiomyocytic necroptosis (Guo et al. 2017).

Similar findings were generated in a different CH model. In Ang II osmotic minipump model, mice without *Tnfrsf1a* and *Tnfa* exhibited significantly abrogated CH (Sriramula et al. 2008; Mayr et al. 2016). Thorough analysis showed decreased immunofibrotic aberrations in the cardiac tissues of *Tnfrsf1a*-null mice, yet no protective outcome on diastolic dysregulation was observed (Mayr et al. 2016). On the other hand, *Tnfrsf1b*-knockout mice infused with Ang II showed fibrosis along

with negligible changes in pro-fibrotic genes' expression (Duerrschmid et al. 2013). Therefore, it seems that TNF-α-TNFR1 is implicated in the CH development in this model.

Alternatively, sustained infusion of isoproterenol (a β-adrenergic agonist) induces CH along with diastolic dysfunction. In this model, TNFR1-null mice exhibited abrogated inflammation response, yet this was inadequate to prevent isoproterenol-mediated CH. On the other hand, TNFR2-null mice demonstrated elevated pro-inflammatory response together with aggravated CH (Garlie et al. 2011). Notably, *in vitro* findings affirmed that TNF-α could exacerbate isoproterenol-mediated CH, yet extraordinarily, this effect was absolutely abolished via anti-TNFR2 antibody (Keck et al. 2019).

Interestingly, chronic ethanol consumption develops a unique model of murine alcoholic cardiomyopathy exhibiting left ventricular structural and fibrotic aberrations. Regarding this model, mice demonstrated TNFR1-mediated increase in TNF-α serum levels, pro-inflammatory cytokines and cardiac ROS formation along with left ventricular dysfunction (Nakashima et al. 2019). Also, elevated levels of TNF-α have been highlighted in a model of Adriamycin-mediated cardiomyopathy in rats. Animals with increased serum levels of TNF-α demonstrated worsened cardiac functions and increased rate of mortality (Tang et al. 2019).

In summary, data from CH models indicate that targeting TNF-α might impede CH development. It is worth mentioning that TNFR1 is implicated in pathogenic TNF-α cascade in a plethora of CVDs.

1.6.1.5 TNF-α signaling in CVDs

The heart comprises three major types of cells: cardiac stromal cells (typically fibroblasts), microvascular endothelial cells, and cardiomyocytes. Furthermore, in the healthy heart, cardiac

macrophages constitute a small yet crucial cell population. Also, following heart insult, immune cells (inflammatory lymphocytes and monocytes) infiltrate cardiac tissue. Strikingly, most cardiac cell types produce TNF-α, yet immune cells are a pivotal source of TNF-α in multiple CVDs. TNF-α signaling cascade is involved in a plethora of biological processes such as apoptosis, inflammation, cardioprotection and regeneration. Not only do stimulation of molecular cascades and cell type dictate the actual TNF-α effect, but also inducible TNFR2 expression is critical. As highlighted by animal models, the chronic stimulation of TNFR1 mediates the detrimental TNF-α effects, whereas TNFR2 activation bestows cardioprotective effects.

1.6.1.6 Pathogenic TNF-α mechanisms:

Endothelial cells stimulation is regarded as one of the most detailed pro-inflammatory mechanisms. TNF-α-induced activation of endothelial cells elevates adhesion molecules expression, which modulate inflammatory immune cells adhesion into cardiac tissue. Interestingly, both TNFRs are crucially involved in the diapedesis process in this mechanism (Chandrasekharan et al. 2007). Additionally, TNF-α is reported to elevate ROS levels and reduce nitric oxide generation in blood vessels, with subsequent endothelial dysfunction and ultimately atherogenesis (Lee et al. 2017). In this regard, TNF-α-mediated ROS formation relies on triggering NADH oxidase (De Keulenaer et al. 1998; Gao et al. 2007). Further, TNF-α is implicated in atherosclerotic plaques development in endothelial cells by elevated LDL transcytosis (Brånén et al. 2004; Y. Zhang et al. 2014) along with modulating foam cell production and macrophage scavenger receptor activity (Hsu and Twu 2000). Notably, TNFR2 signaling cascade mediates detrimental effects through increased proliferation of smooth muscles and macrophages (Nicolaou et al. 2017). Within cardiomyocytes, TNF-α induces CH and apoptosis (Nakashima et al. 2019). Endogenous TNF-α is implicated in CH and elevated protein synthesis (Yokoyama et al. 1997) via the NF-κB-

induced ROS production (Higuchi et al. 2002). This process in cardiomyocytes is TNFR1-dependent, and is also involved in negative modulation of cellular contractility and calcium handling (Defer et al. 2007). Furthermore, TNF-α-mediated superoxide formation was revealed to be reliant on PI3K-induced triggering of NADPH oxidase along with its modulation of various matrix metalloproteinases (MMPs) production (Awad et al. 2010). Additionally, TNF-α-induced oxidative stress contributes to mitochondrial DNA insult via the sphingomyelin–ceramide cascade (Suematsu et al. 2003), which was also involved in triggering cardiomyocytic apoptosis (Krown et al. 1996). Further, TNFR1-induced cardiomyocytic apoptosis can be initiated via RIP1–RIP3–MLKL cascade stimulation through apoptosis signal-regulating kinase 1 in a NF-κB-independent manner (Al-Lamki et al. 2009; Guo et al. 2017).

Another type of cardiac cells that can be stimulated by TNF-α is cardiac fibroblast. Following stimulation, cardiac fibroblasts take part in the inflammatory process via producing MCP-1 and MCP-3, which modulate TNF-α formation and monocyte recruitment (Lindner et al. 2014). In addition, TNF-α promotes ROS generation and MMP expression in cardiac fibroblasts through PI3Kγ stimulation (Awad et al. 2010). Also, TNF-α mediates cardiac fibrosis via increased proliferation of cardiac fibroblasts and fibronectin formation (Jacobs et al. 1999). Furthermore, TNF-α is implicated in the transformation of cardiac fibroblasts into pathological myofibroblasts (Porter et al. 2004). It is worth mention that abnormal collagen deposition, pro-fibrotic genes expression and subsequent pathological cardiac remodeling is TNFR1-dependent (Porter et al. 2004; Duerrschmid et al. 2013). Notably, TNFR1 is critical in the maturation of fibroblasts from myeloid cells (Duerrschmid et al. 2015). In brief, all these findings highlight numerous pathological effects of TNF-α in various cardiac cells.

1.6.1.7 Cardioprotective TNF-α mechanisms

The majority of cardioprotective mechanisms are TNFR2-dependent. Strikingly, repression of pathological TNFR1 signaling cascade activation is regarded as one of the most critical functions of TNFR2-mediated signaling. Studies implied that TNFR2 knockdown promotes the activity of downstream effectors such as MAPK p38 (Garlie et al. 2011) and NF-κB (Hamid et al. 2009) along with increased formation of IL-6 and IL-1β (Monden et al. 2007). Interestingly, the cardioprotective mechanism of TNFR2 has been shown to counteract the detrimental effects in cardiomyocytes instigated by TNFR1 signaling. Subsequently, TNFR2 stimulation hampered cardiomyocytic apoptosis and induced cell cycle entry through the activation of endothelial/epithelial tyrosine kinase (Al-Lamki et al. 2009) and improved oxidative stress resistance (Defer et al. 2007; Keck et al. 2019). In addition, TNFR2 is a key player in the cardiomyocytes differentiation from stem cells (Aker et al. 2003) along with cell cycle initiation in cardiac stem cells (Al-Lamki et al. 2013). Also, TNFR2 signaling is implicated in immunosuppression. In regulatory T cells, TNFR2 stimulation promotes their expansion (Wang et al. 2018) while halting differentiation of effector T cells (Miller et al. 2015). Furthermore, TNFR2 signaling is critical in recruiting myeloid suppressor cells (Ba et al. 2017), which confer cardioprotective effects during heart failure (Zhou et al. 2018). It is worth note that immunomodulatory role of TNF-α in the context of heart failure is not well comprehended, mainly attributed to its pro-inflammatory effect and stimulation of endothelial cells in the early phase of inflammation.

1.6.2 Role of Toll-like receptor 4 agonists

The innate immune system is induced via cytosolic and transmembrane receptors, called pattern recognition receptors (PRRs). These receptors are responsible of identifying pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) originating from

stressed cells, and microorganism-associated molecular patterns (MAMPs) secreted by intestinal microbiota (Mackey and McFall 2006; Mann 2011). PRRs are typically expressed by endothelial cells, cardiomyocytes, and macrophages. Following the induction of PRRs via MAMPs, DAMPs, or PAMPs, a plethora of signaling pathways are activated to modulate the genetic expression of pro-inflammatory cytokines (Mann 2011). Notably, the major cardiac PRRs comprise Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors, the nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome, C-type lectin receptors, and pentraxins (Mann 2011; Frantz et al. 2018; Toldo and Abbate 2018).

As a member of PRRs, TLRs recognize PAMPs secreted by different bacteria and viruses. These PAMPs induce inflammatory response (Vallejo 2011; O'neill et al. 2013). In addition, TLRs recognize DAMPs, which are secreted by host cells after cellular insult (Lee and Seong 2009; O'neill et al. 2013). For the time being, TLRs are involved in modulating cardiovascular diseases (CVDs), specifically Toll-like receptor 4 (TLR4) which has been demonstrated to act as an inflammatory protein. For instance, several reviews have highlighted its therapeutic potential (Hofmann et al. 2011; Vallejo 2011; Kuzmich et al. 2017) and it was identified as a pivotal inflammatory protein in the development of CH and hypertension (Singh and Abboud 2014).

The first mammalian TLR to be characterized was human TLR4, which initiate inflammatory response upon binding to DAMPs or PAMPs. In fact, DAMPs and PAMPs act as endogenous or exogenous ligands that initiate TLR4 signaling and instigate inflammatory response in a plethora of pathological conditions (Bianchi 2007; Vallejo 2011). Prior to ligand binding, TLR4 forms a complex with its co-receptors, which is crucial to instigate inflammatory response. Interestingly, lipopolysaccharide (LPS) is the most investigated PAMP. Previous reports have demonstrated that LPS forms a complex with LPS binding protein (LBP), and the LPS/LBP complex binds to cluster

of differentiation 14 (CD14). This induces LPS transfer to a different complex comprising TLR4 and myeloid differentiation protein 2 (MD2), a TLR4 co-receptor. Subsequently, the formation of LPS/TLR4/MD2 complex initiates multiple inflammatory signaling pathways (Kim and Kim 2017; Ryu et al. 2017). To initiate intracellular signaling, TLR4 forms a complex with its ligands and co-receptors on the cell membrane. Strikingly, TLR4 is the only TLR family member that transmits the activation signal via two intracellular signaling pathways. One is mediated by TIR-domain containing adaptor-inducing interferon-β (TRIF), and the other is dependent on myeloid differentiation protein 88 (MyD88) (Kawasaki and Kawai 2014).

Regarding the MyD88-mediated pathway, MyD88 employs multiple adaptor proteins involved in signal transduction, with subsequent activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways. These pathways activate transcription factor NF-κB together with activator protein-1 (AP-1), resulting in the production of various inflammatory chemokines and cytokines, including interleukin-1β (IL-1β), MCP1, TNF-α, and IL-6 (O'neill et al. 2013; Kawasaki and Kawai 2014). Concurrently, other downstream mediators of MyD88, such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) (Laird et al. 2009) and Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CaMK II) (Singh et al. 2012; Rusciano et al. 2019) can also be induced to stimulate the production of inflammatory cytokines mediated via NF-κB. Studies have highlighted that MyD88 is implicated in the regulation of several pathways, including MAPK, Ca^{2+}/CaMK II, PI3K/Akt, and NF-κB pathways, which in turn modulate transcription of inflammatory cytokines.

On the other hand, in the TRIF-mediated pathway, the extracellular complex of TLR4 undergoes endocytosis, with subsequent migration of TRIF to cytoplasmic receptors. Similar to the MyD88-

mediated pathway, TRIF activates the transcription factor and promotes the expression of genes induced by type I interferons (IFNs) (Kawasaki and Kawai 2014; Ullah et al. 2016).

1.6.2.1 TLR4 signaling pathways involved in cardiac hypertrophy

1.6.2.1.1 TLR4/MyD88/NF-κB cascade

TLR4/MyD88/NF-κB signaling pathway is strongly associated with CH. Notably, NF-κB is a transcription factor in the TLR4-dependent signaling cascade that can be induced to enter the nucleus and stimulate the genetic expression of various pro-inflammatory cytokines in addition to mediating the subsequent inflammatory response. Thus, it functions as a pivotal regulatory factor in the pathogenesis of CH (Li et al. 2004; Gupta et al. 2008). Several studies reported that prevention of NF-κB translocation to the nucleus has the ability to halt the development of CH (Ahmed et al. 2018; Nalban et al. 2020), and similar results were obtained in a hypertrophic model via NF-κB silencing with the aid of small interfering RNA (Gupta et al. 2008). MyD88 is regarded as the canonical adaptor contributing to TLR4-dependent inflammatory cascades. Inhibition of MyD88 resulted in reduction of hypertrophic response, while MyD88 overexpression culminated in induction of NF-κB cascade and promoting apoptosis of cardiomyocytes in a murine model of pressure overload-mediated CH (Li et al. 2005; Ha et al. 2006).

Other than the established pressure overload model, numerous studies have highlighted different models to induce CH. Importantly, induction of TLR4/MyD88/NF-κB cascade was observed in hypertrophic cardiomyocytes. For instance, a study employing a model of unilateral renal ischemia/reperfusion injury demonstrated that elevated serum inflammatory cytokines and activity of TLR4 promotes CH, and TLR4 knockout slows CH progression and electrical dysfunction (Trentin-Sonoda et al. 2015). Additionally, chronic intermittent hypoxia has been demonstrated to

promote CH in animal models, while the inhibition of TLR4/MyD88/NF-κB cascade rectifies cardiac derangement (Yuan et al. 2014). Up until now, a significant body of evidence has linked pathological hypertension with TLR4/MyD88/NF-κB cascade in the brain, whereas sympathetic nervous system (SNS) and RAS play a crucial role in blood pressure regulation. Indeed, Angiotensin II (Ang II) is a crucial RAS effector, and plays a pivotal role in increasing plasma norepinephrine (NE) levels in CH and hypertensive models. Nonetheless, TLR4 inhibition in the brain was shown to decrease NF-κB activity, suppress inflammatory cytokines, and attenuate NE levels and Ang II-mediated hypertensive response (Dange et al. 2014). Also, blockade of TLR4 or Ang II type 1 receptor in the brain of spontaneously hypertensive rats produced similar results (Dange et al. 2015; Li et al. 2016). Altogether, these findings indicate that TLR4/MyD88/NF-κB cascade is a link between SNS, RAS and hypertension, outlining a new therapeutic target for hypertension and hypertension-induced CH.

Additionally, several mediators associated with TLR4/MyD88/NF-κB cascade have been illuminated. For instance, insulin resistance is a known causative factor of CH and diabetes. One study demonstrated that retinol-binding protein 4 (RBP4) may be a crucial mediator of the vicious cycle of CH and insulin resistance (Gao et al. 2016). Indeed, RBP4 deficiency rectified cardiomyocytic expression of glucose transporter 4 (GLUT4) and reduced hypertrophic response to pressure overload, while knockdown of MyD88 or TLR4 impaired RBP4-mediated insulin resistance and CH (Gao et al. 2016). All in all, these results highlight that RBP4, via TLR4-induced cascade, not only impairs cardiomyocytic expression of GLUT4 but also stimulates production of pro-inflammatory mediators and CH (Gao et al. 2016). These studies indicate a strong correlation between TLR4/MyD88/NF-κB and CH.

1.6.2.1.2 TLR4/MyD88/MAPK cascade

MAPKs are highly conserved serine-threonine kinases which are key players in the development of different human diseases through modulating various cellular activities like cell proliferation, survival, differentiation and inflammation (Kim and Choi 2015). It comprises four well-known pathways, namely p38 MAPK, the extracellular signal-related kinases (ERK1/2), ERK5, and c-Jun N-terminal kinase (JNK). Functionally, TLR4 recruits MyD88 stimulating MAPKs, which modulate induction of various transcription factors, like AP-1, therefore playing a part in pro-inflammatory cytokines expression (Yang et al. 2016). Certainly, elevated activity of nearly all myocardial MAPKs was identified in pressure overload-mediated CH model where they have various effects that ultimately induce CH (Muslin 2008). For the time being, MAPKs are well-characterized downstream mediators in the TLR4 cascade and have been investigated in CVDs. The role of cardiac TLR4/MyD88/ERK cascade has also been established. For example, LM8-induced MyD88 inhibition attenuates inflammatory cytokines expression and infiltration of immune cells in obese mouse models, therefore producing beneficial results for CH and fibrosis (Liu et al. 2020).

A multitude of studies have investigated the contribution of TLR4/MAPK in CH utilizing genetic knockout mice or pharmacological inhibitors, which modulate p38 MAPK, JNK and ERK1/2. One study demonstrated that reduced expression of TLR4 blunted phosphorylation levels of p38 MAPK and ERK1/2 in a spontaneously hypertensive rat model, which rectified vascular inflammation and CH (Romero et al. 2016). A recent study highlighted that knockdown of MD2 (a co-receptor of TLR4) in mice resulted in resistance to Ang II-mediated CH together with blunted ERK phosphorylation and cardiac inflammation, indicating that TLR4/ MD2/ ERK cascade is pro-hypertrophic in the heart (Han et al. 2017). Additionally, preventing HSP70 (a ligand of TLR4) binding to TLR4 was shown to reduce pressure overload-mediated CH and fibrosis through

modulating p38 MAPK and ERK activity (Cai et al. 2010). Strikingly, TLR4 inhibition exhibited no effect on ERK1/2 activity after HMGB1 (a ligand of TLR4) stimulation in mechanical stress-mediated CH *in vitro* (Zhang et al. 2019), indicating that different ligands may stimulate TLR4 downstream cascades. Also, a novel modulatory mechanism for TLR4/MAPK cascade has been recognized. Interestingly, downregulation of MD1 was observed in patients suffering hypertrophic cardiomyopathy, while MD1 overexpression in the hearts of mice exhibited beneficial effects against CH mediated by pressure overload or obesity. Not to mention, MD1 binding to TLR4 inhibits MAPK activation, consequently attenuating hypertrophic response (Xiong et al. 2017; Shen et al. 2019). Hence, MD1 seems to be a promising target to prevent CH. In addition, various tyrosine kinases are stimulated following TLR4 activation. For instance, the knockdown of RIP2, which has tyrosine kinase-mediated activation of MAPKs, blunts CH develepment following aortic banding. The beneficial effect obtained from RIP2 knockdown in mice is associated with TLR4/MAPKs cascade inhibition, since RIP2 knockdown decreased the phosphorylation levels of p38 MAPK, JNK and ERK1/2 (Zhao et al. 2017). Overall, these findings indicate that TLR4-induced activation of MAPKs contributes to CH.

1.6.2.1.3 TLR4/MyD88/CaMK II cascade

A different potential mechanism of CH development is via TLR4 modulation of CaMK II, suggesting that TLR4 downstream mediators are more intricate than previously presumed and include inflammasome stimulation or CaMK II contribution. CaMK II is a serine/threonine kinase which is extensively expressed in cardiac cells and modulates cardiac structure together with electrical activity. Following stimulation, CaMK II functions as a downstream mediator that induces inflammation and oxidative stress which develops CH (Singh and Anderson 2011), and derangement of ion channels which promotes arrhythmias.

In a CH model, CaMK II stimulation increased the expression of complement factor B (CFB), a pro-inflammatory gene, via NF-κB cascade in mice and cardiomyocytes (Singh et al. 2009), with subsequent induction of cardiomyocytic cell membrane injury. Moreover, CaMK II inhibition or CFB knockdown in mice improved myocardial insult and inflammation following myocardial infarction (MI) (Singh et al. 2009). Similarly, silencing a specific CaMK II isoform, CaMK IIδB, prevented CH and reduced expression of NF-κB and CFB without affecting inflammatory cytokines expression (TRENTIN-SONODA et al. 2019), highlighting that CaMK II is truly a pivotal modulator of inflammatory response in TLR4 cascade. A study exhibited that MyD88 blockade remarkably improves survival rate and attenuates CaMK II expression and CH (Singh et al. 2012). Lately, several studies indicated that CaMK II as downstream mediator of TLR4 cascade (Singh et al. 2009; Zhong et al. 2018; TRENTIN-SONODA et al. 2019) contributes to CH development in the context of hyperlipidemia. In addition, TLR4 inhibition *in vitro* rectified expression levels of CH and fibrotic genes (Zhong et al. 2018). On the other hand, stimulation of TLR4/MyD88 cascade promotes CaMK II activity, hence contributing to left ventricular remodeling in the context of chronic pressure overload (Peng et al. 2017; Shuai et al. 2019), highlighting that TLR4/MyD88/CaMK II cascade induced inflammation associated with CH. Furthermore, the molecular mechanisms responsible for CaMK II-induced inflammation involved in obesity-mediated CH suggests a crosstalk linking CaMK II to various cascades such as NF-κB and MAPKs cascades (Zhong et al. 2018).

In contrast, CaMK II modulates oxidative stress by unsettling the balance established between antioxidants and oxidants in the heart, whereas inhibition of CaMK II via KN93 or Myr-AIP triggers nuclear factor-like 2 cascade increasing antioxidant genes expression, such as NADPH

quinone acceptor oxidoreductase 1 and heme oxygenase-1. In turn, this decreases ROS generation in H9c2 cells treated with palmitate, hence halting CH (Zhong et al. 2018).

Aside from contributing to CH, CaMK II can induce pivotal changes in electrical activity, causing elevated vulnerability to arrhythmias. In the context of hyperlipidemia, CaMK II stimulation downregulates a multitude of ion channels protein, including Kv4.3, Kv4.2, Kv2.1, Kv1.5 and Cav1.2, and culminates in extended action potential duration, and detrimental ion channels remodeling (Zhong et al. 2017; Shuai et al. 2019). Moreover, CaMK II stimulation impairs homeostasis of intracellular calcium in mice fed with high fat diet (Zhong et al. 2017). Altogether, these findings illustrate the modulation of heart electrical activity through TLR4/MyD88/CaMK II cascade. To sum up, CaMK II plays a pivotal role in cardiac electrical and structural remodeling.

1.6.2.1.4 TLR4/MyD88/PI3K/Akt cascade

The PI3K/Akt cascade has become one of the major key players in CH development. This cascade was shown to be contributing to both pathological and physiological CH, with subsequent changes in angiogenesis, production of inflammatory cytokines, and cardiomyocytic morphology and survival (Aoyagi and Matsui 2011).

The modulation of the PI3K/Akt cascade through growth hormone (GH)/insulin-like growth factor (IGF) axis is accompanied by physiological hypertrophy inducing adaptive CH, exhibited by increased cardiomyocytic size and angiogenesis, hence maintaining cardiac functions (Weeks et al. 2017). A plethora of studies indicate that PI3K/Akt cascade is regarded as negative feedback modulator of TLR4 cascade during cardiac inflammation with the purpose of curbing the pro-inflammatory process (Pourrajab et al. 2015; Yang et al. 2016; Ahmed et al. 2018; Chao et al. 2019). Recently, it was demonstrated that erythropoietin has a beneficial role in CH and

myocardial fibrosis via triggering PI3K/Akt cascade, with subsequent downregulation of TLR4 expression (Liu et al. 2018). Remarkably, PI3K/Akt cascade also negatively modulates the heart, likely via triggering various PI3K isoforms, especially p110α and p110γ. To put it simply, PI3K (p110α) induces adaptive CH, whereas PI3K (p110 γ) induces maladaptive CH (Aoyagi and Matsui 2011). Also, it was highlighted that the effect of Akt-induced CH is heavily reliant on the duration of Akt stimulation (Oka et al. 2014). Some studies have linked stimulation of PI3K/Akt cascade to pathological CH. For example, Isorhamnetin, an anti-inflammatory agent, was demonstrated to prevent pressure overload-mediated CH via blocking PI3K/Akt cascade (Gao et al. 2017). Moreover, one downstream mediator of PI3K/Akt cascade, mammalian target of rapamycin (mTOR), has shown pro-hypertrophic action (Xu and Brink 2016). Earlier, it was demonstrated that rapamycin-mediated mTOR inhibition is associated with attenuated CH through NF-κB cascade downregulation (Zhang et al. 2015). In addition, rapamycin has shown auxiliary CH improvement in mice lacking TLR4 (Ha et al. 2005).

Another study demonstrated that TLR4 upregulation leads to the loss of beneficial effects obtained following the inhibition of PI3K/Akt/mTOR cascade in Ang II-mediated CH (Li et al. 2018). Thus, one CH mechanism is triggered through PI3K/Akt/mTOR cascade after TLR4 activation, causing a detrimental cardiac effect. In summary, the contribution of PI3K/Akt cascade to cardiac diseases does not only depend on duration of Akt stimulation and isoforms of PI3K, but also on its upstream effector. Nonetheless, whether TLR4/MyD88 in association with PI3K/Akt cascade modulates myocardial inflammation requires further studies.

1.6.2.1.5 TLR4/TRIF cascade

TLR4 relays signals via the TRIF-dependent cascade, also identified as the MyD88-independent cascade. It is regarded as the main signaling cascade in the development of CVDs. As a matter of fact, inhibiting the TLR4/TRIF cascade mediated a protective effect on the heart in murine models of heart ischemia/reperfusion injury (J. Yang et al. 2018; Li et al. 2019).

Despite the dearth of comprehensive data, multiple studies have highlighted that TRIF-dependent cascade aggravates CH. One study demonstrated that TRIF-dependent cascade, not MyD88-dependent cascade, is a pivotal determinant in CH and hypertension (Singh et al. 2015). Certainly, in Ang II-mediated CH model, CH was shown to improve in TRIF-null mice, while being exacerbated in mice lacking MyD88 in comparison with wild type mice via obtaining the heart weight to body weight ratio (Singh et al. 2015; Singh et al. 2019). Moreover, the inflammation in mice lacking MyD88 was elevated, with increased expression of cardiac TLR4 and TRIF, suggesting that MyD88-dependent cascade acts as negative modulator of the pro-inflammatory effect induced via TRIF (Singh et al. 2015). Further studies confirmed that TRIF cascade activation promotes the expression of type I IFNs which are crucial for Ang II-mediated CH and hypertension, while the TLR3-induced TRIF cascade mediates CH and hypertension following Ang II infusion, yet the TLR4/TRIF cascade is only essential for CH (Singh et al. 2019). Nevertheless, it is not obvious whether there are similar findings in CH models mediated by obesity or pressure overload. A recent study demonstrated that TLR4 antagonist, LPS-RS, diminishes TRIF expression, but not MyD88, in hyperoxia-induced CH model (Mian et al. 2019). Overall, these results indicate that TLR4/TRIF cascade is implicated in CH.

1.6.3 Role of Interleukin 6

Previously, it was thought that only leukocytes express interleukins (ILs). Later, different studies indicated that ILs are expressed by various cell types. Essentially, ILs are involved in immune cells differentiation, activation, maturation, proliferation, adhesion, and migration. Additionally, they exert anti-inflammatory and pro-inflammatory effects (Akdis et al. 2011). Notably, some ILs are pro-inflammatory such as IL-1, IL-6, IL-12, IL-17, IL-18 and IL-23 (Cuneo and Autieri 2009; Duvallet et al. 2011; Su et al. 2012), while others are anti-inflammatory such as IL-4, IL-6, IL-10, IL-13, IL-19 and IL-35 (Cuneo and Autieri 2009; Woodward et al. 2010; Scheller et al. 2011; Li et al. 2012; Yilma et al. 2012). It is noteworthy that IL-6 possesses both pro-inflammatory and anti-inflammatory effects (Rose-John 2012).

IL-6 is known to be a pleiotropic cytokine that is involved in both adaptive and innate immune systems (Jones 2005). If innate-to-adaptive immunity transition is somehow aberrated, it will instigate chronic repercussions on autoimmunity and inflammation (Hoebe et al. 2004). The IL-6-mediated acute response, which is mainly cardioprotective, to chronic signaling inducing pathological autoimmunity and inflammation is one example the multiple aspects of IL-6 (Kaplanski et al. 2003).

1.7 Impact of inflammation on myocardial CYP, EETs and HETEs

1.7.1 Inflammation and CYP epoxygenases

Few studies investigated how inflammation modulates the expression of myocardial CYPs. Findings from a rat model of LPS-induced acute inflammation for 24 hours highlighted reduced cardiac mRNA expression of CYP1A1, CYP2C11, CYP2E1, CYP4F1, and CYP4F5. Also, elevated cardiac mRNA expression of CYP1B1, CYP4A1, and CYP4A3 was observed. Furthermore, cardiac protein expression of CYP2C11, CYP2E1, and CYP4A was comparable to

the mRNA expression levels (Anwar-mohamed et al. 2010). It is likely that inflammation reduced CYP1A1 expression via NF-κB stimulation (Ke et al. 2001; Tian 2009; Zordoky and El-Kadi 2009). On the other hand, it was assumed that inflammation-mediated induction of CYP1B1 expression is hormonal-dependent (Malaplate-Armand et al. 2003; Umannová et al. 2007). Inflammation is also implicated in increased 20-HETE generation in inflamed murine cardiac microsomes through inducing CYP4A and CYP1B1 (Roman 2002; Choudhary et al. 2004). It is likely that 20-HETE generation is a compensatory mechanism by the inflamed cardiac tissue to avert systemic hypotensive shock (Tunctan et al. 2008). Moreover, 20-HETE generation coincided with reduced EETs expression in the context of acute inflammation, highlighting impaired CYP-mediated AA metabolism (Anwar-mohamed et al. 2010). Further studies are required to investigate the inflammation-induced modulation of CYP-mediated AA metabolism in human heart utilizing *in vitro* cardiac cell lines, such as RL-14 (Maayah, Elshenawy, et al. 2015) and AC16 cells (Zhou et al. 2017).

Several reports from our lab indicated that mid-chain HETEs, such as 5-, 8-, 12-, and 15-HETE, are pro-hypertrophic in RL-14 cells. The mechanism underlying 8-HETE-induced CH involves the participation of NF-κB and ERK1/2 cascades (Maayah, Abdelhamid, et al. 2015), which are typically induced in inflammation (Marchant et al. 2012; Chen et al. 2017). Similar findings were obtained from RL-14 cells treated with 5-HETE, 12-HETE, and 15-HETE (Maayah and El-Kadi 2016a). On the other hand, ERK1/2 and NF-κB contribute to cardioprotective effects of 2-methoxyestradiol in AAC rats and isoproterenol-induced CH in RL-14 cells (Maayah et al. 2018). In summary, these findings create a clear association between inflammation and imbalanced CYP-mediated AA metabolism in favor of cardiotoxic metabolites, namely mid-chain HETEs and 20-HETE.

The impact of inflammation-mediated downregulation of hepatic and extrahepatic CYP enzymes on metabolism of xenobiotics has been reported in animal and human models (Morgan 2001; Renton 2004; Sunman et al. 2004; Aitken et al. 2006; Aitken and Morgan 2007; Morgan 2009). Inflammation-mediated reduction in CYP expression appears to be mediated by inflammatory mediators such as TNF-α, INF-γ, INF-α, IL-1, and IL-6 (Kim et al. 2010; Burgess et al. 2012). In addition to this, it has been found that cytokines possess enzyme-specific impacts on CYPs expression due to differences in cytokines-regulated CYPs (Aitken and Morgan 2007).

Several transcription-dependent mechanisms are involved in inflammation-mediated reduction in CYPs activity based upon inflammatory stimuli type, response time point, and specific CYP gene. For the majority of CYPs investigated, a pre-translation mechanism, encompassing reduced levels of CYP mRNA with consequent reduction in CYP proteins, contributes to the inflammatory impact observed for the alterations in CYPs expression. Clearly, NF-κB plays a pivotal role in CYP regulation through interacting with their signaling pathways (Aitken et al. 2006; Morgan 2009). For instance, inflammation-induced suppression of CYP1A2 and CYP1A2 transcription is attributed to mutual antagonistic interaction of NF-κB with aryl hydrocarbon receptor (AhR) through binding of NF-κB to AhR and subsequent functional suppression of both of them (Ke et al. 2001). Other proposed mechanisms for inflammation-mediated CYP downregulation include NF-κB interaction with constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Beigneux et al. 2002; Kim et al. 2003; Aitken et al. 2006; Morgan 2009). Although pretranscriptional repression is the major factor in downregulation of CYPs by inflammation, multiple post-transcription mechanisms are also implicated in this phenomenon, for example, inflammation-induced NO production via inducible NO synthase (iNOS) which downregulates CYP2B6 and CYP2B1 (Aitken et al. 2008; Lee et al. 2008).

Previous *in vivo* reports have shown that LPS-mediated innate immune response stimulation leads to downregulation of CYP epoxygenases culminating in altered epoxygenases-derived AA metabolites in rat heart and liver (Anwar-mohamed et al. 2010; Theken et al. 2011). These results provide a critical notion that signifies the involvement of inflammation-mediated CYP downregulation which could have potential effects in modulation of xenobiotics metabolism and endogenous eicosanoid formation.

1.7.2 Inflammation and EETs

A mounting body of in vitro and in vivo studies has affirmed the hypothesis that EETs demonstrate a broad anti-inflammatory activity during chronic and acute inflammation (Spector and Norris 2007; Campbell and Fleming 2010; Deng et al. 2010; Pfister et al. 2010; Sudhahar et al. 2010; Imig 2012; Campbell et al. 2017; Shi et al. 2022; Zhang et al. 2022). Figure 1.4 demonstrates the mechanisms underlying the anti-inflammatory properties of EETs.

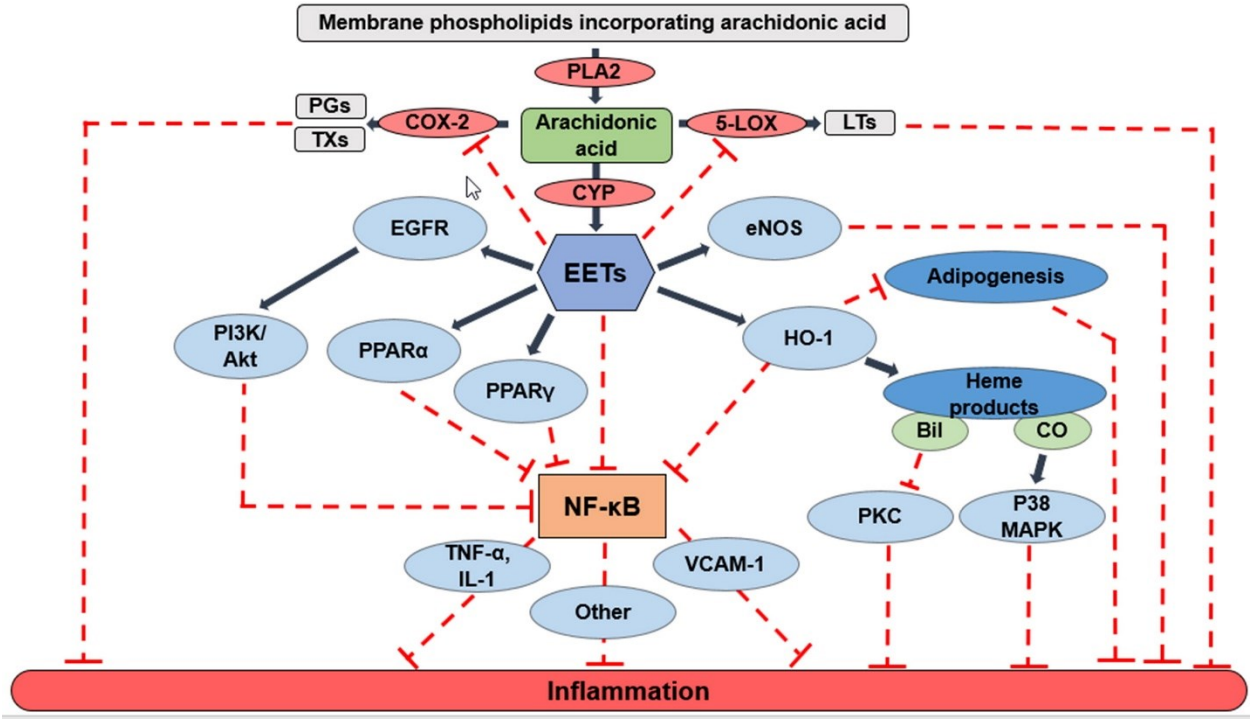


Figure 1.4. The mechanisms involved in the anti-inflammatory effects of EETs.

Through EETs-mediated inhibition of COX-2 and consequently in the production of TXs/PGs, EETs may reduce the inflammation. EETs-induced inhibition of 5-LOX and its products is involved in inhibition of inflammation. Also, EETs stimulate a number of pathways that converge at NF-κB inhibition, leading to reduction of inflammation. Arrows represent stimulatory influence and dash lines represent inhibitory influence.

There is a significant difference among the EETs regioisomers regarding the anti-inflammatory effect, for instance, 14,15-EET is devoid of activity, whereas the most potent is 11,12-EET, and less activity was reported for 8,9-EET and 5,6-EET (Campbell 2000; Xu et al. 2011). The complex mechanisms by which EETs modulate vascular inflammation have been documented by multiple studies and can be classified under two categories. The first is limiting macrophage stimulation and hampering platelets adhesion, while the second is inhibiting endothelial stimulation and leukocytic adhesion.

In human ECs EETs, 11,12-EET represses expression of cytokine-mediated ECs adhesion molecules and inhibit leukocyte adhesion to arterial wall, mainly through inhibition of NF-κB activity. The EETs-mediated NF-κB inhibition occurs either directly or via stimulation of epidermal growth factor receptor (EGFR) and peroxisome proliferator-activated receptor (PPAR) (Node et al. 1999; Falck et al. 2003; Liu et al. 2005; Moshal et al. 2008; Deng et al. 2010; Norwood et al. 2010; Xu et al. 2011; Zhao et al. 2012). Furthermore, other mechanisms including stimulation of HO-1 and endothelial NOS (eNOS) expression, along with inhibition of 5-LOX and COX-2 are implicated in EETs-mediated anti-inflammatory functions via hampering EC stimulation.

1.7.2.1 Inflammation and NF-κB

NF-κB is a mammalian nuclear transcription factor which is ubiquitously expressed and modulates the expression of different genes involved in a plethora of pathological processes including cell survival, apoptosis, proliferation, and inflammatory responses (May and Ghosh 1998; McKay and Cidlowski 1999; Bottex-Gauthier et al. 2002; Aggarwal 2004; Sethi et al. 2008; Prasad et al. 2010). Considering inflammation, NF-κB stimulation instigates activation of ECs via increased expression of cell adhesion molecules (CAMs), endothelial chemokines and cytokines with

consequent leukocyte adhesion to the endothelium. Different stimuli can activate NF-κB including oxidants, drugs, toxins, LPS, and inflammatory cytokines such as IL-1 and TNF-α (Prasad et al. 2010).

Inactive NF-κB resides in the cytoplasm of every cell as a heterogenous group of hetero- or homodimeric transcription factors comprising different subunits (Aggarwal 2004). These subunits belong to the reticuloendotheliosis (Rel) protein family. The whole five Rel proteins possess the Rel homology domain (RHD) comprising nearly three hundred amino acids which is involved in DNA binding, dimerization, and interacting with NF-κB inhibitor (IκB). These five proteins are NF-κB1 (comprising p50 with its precursor p105), NF-κB2 (comprising p52 with its precursor p100), Rel A (p65), Rel B, and c-Rel. Commonly, the first two proteins are generated as large precursors (p100 and p105) which are further transformed into smaller proteins with transcriptional activity, while the latter three are produced as active transcriptional factors (May and Ghosh 1998; McKay and Cidlowski 1999; Bottex-Gauthier et al. 2002; Aggarwal 2004; Sethi et al. 2008; Prasad et al. 2010).

It is worth note that the two signaling cascades that activate NF-κB are referred to as canonical along with non-canonical cascades (Vandenabeele et al. 2010). In these cascades, stimulation of the complex of IκB kinase (IKK) is a shared regulatory step that takes place in response to stimuli encompassing inflammatory cytokines.

The canonical (or classical) cascade of NF-κB activation occurs for dimers that comprise p50 and p65 which are normally inactive via specific inhibitors known as IκB in the cytoplasm. In the canonical cascade, the IKK complex consists of two catalytic subunits (IKKβ and IKKα) with a regulatory subunit (IKKγ). The activated IKK complex induces IκB phosphorylation with its

subsequent targeting for ubiquitination and proteasomal degradation. This in turn liberates NF-κB dimers p65-p50 which undergo nuclear translocation and DNA binding. Stabilization of IKK complex occurs through linear ubiquitin chain assembly complex (LUBAC), whose function is addition of ubiquitin to IKKγ.

On the other hand, the non-canonical cascade contributes to stimulation of p100/Rel B complexes. Regarding non-canonical cascade, the IKK complex possessing IKKα dimers is triggered via NF-κB-inducing kinase (NIK)-evoked phosphorylation. Then, activated IKKα induces p100 phosphorylation to undergo proteolysis giving rise to p52, which dimerizes with other subunits of NF-κB and translocates to the nucleus (Vandenabeele et al. 2010).

Following nuclear translocation, NF-κB dimers that are transcriptionally active attach to particular recognition sequences located in the promoter portion of various inflammation-relevant genes including adhesion molecules and COX-2 (May and Ghosh 1998; Prasad et al. 2010).

1.7.2.2 EETs as inhibitors of NF-κB

Node and his team were the first to document the vascular anti-inflammatory functions of EETs (Node et al. 1999). Several studies elucidated that CYP2J2 overexpression and elevation of physiological levels of EETs ameliorated inflammation by inhibiting cytokines-mediated ECs expression of adhesion molecules via a mechanism involving IKK and NF-κB suppression. Since then, a substantial number of animal and human reports have demonstrated evidence that CYP-generated epoxides can modulate various steps of NF-κB cascade which operate in both the nucleus and cytoplasm. The modulation is EET, cytokine, endothelium and species-specific (Brash 1999; Node et al. 1999; Fleming et al. 2001; Falck et al. 2003; Liu et al. 2005; Moshal et al. 2008; Deng et al. 2010; Norwood et al. 2010; Xu et al. 2011; Zhao et al. 2012). EETs modulate NF-κB-

evoked activation of pro-inflammatory genes via NF-κB binding, Rel A nuclear translocation, degradation of IκB, and suppressing the activity of IKK complex (Deng et al. 2010).

In addition, administration of 11,12-EET in human umbilical vein endothelial cells (HUVECs) caused significant reduction of TNF-α-mediated ECs expression of ICAM-1, E-selectin, VCAM-1 (Node et al. 1999). The most potent suppressor of VCAM-1 upregulation was 11,12-EET, yielding nearly 70% suppression of TNF-α-mediated VCAM-1 expression, with less suppression observed for 5,6-EET and 8,9-EET and no anti-inflammatory properties was reported for 14,15-EET (Node et al. 1999). Another study supported this finding by illuminating that 11,12-EET can ameliorate TNF-α-mediated NF-κB stimulation in HUVECs (Fleming et al. 2001). Moreover, in human saphenous vein ECs, 11,12-EET has been demonstrated to inhibit TNF-α-mediated VCAM-1 expression (Falck et al. 2003). These results highlight that CYP-generated epoxides suppress cytokine-mediated leukocytic adhesion and endothelial stimulation partly via inhibiting NF-κB stimulation, and hence are crucial modulators of vascular inflammation.

Evidence highlights that EETs-mediated NF-κB inhibition may play a key role in other anti-inflammatory cascades. For instance, inflammation is suppressed following direct activation of Ang II type 2 receptors (AT_2R). This is because 11,12-EET functions as a second messenger in AT_2R-induced anti-inflammatory effects. Direct activation of AT_2R elevates CYP2J/CYP2C-derived 11,12-EET generation culminating in altered NF-κB activity and diminished IL-6 expression (Rompe et al. 2010; Matavelli et al. 2011).

1.7.2.3 EETs, PPAR and EGFR

Accumulating findings highlight that epoxides-mediated NF-κB inhibition is partly mediated via stimulation of several established effectors encompassing epidermal growth factor receptor

(EFGR) and peroxisome proliferator-activated receptors (PPARs) (Liu et al. 2005; Ng et al. 2007; Norwood et al. 2010; Zhao et al. 2012).

Typically, PPARs are classified under nuclear receptor superfamily that are ligand-stimulated transcription factors. PPARs functions are modulated depending on the exact shape of ligand-binding domain evoked via ligand binding as well as number of co-repressor and co-activator proteins whose presence can inhibit or activate receptor functions, respectively (Yu and Reddy 2007). A plethora of natural ligands, such as eicosanoids and fatty acids, along with synthetic ligands including glitazones and fibrates, may induce PPARs activity (Delerive et al. 2001; Bocher et al. 2002). Various subtypes of PPARs have been documented to modulate expression of genes involved in glucose homeostasis, cell differentiation, cell proliferation, along with lipoproteins and lipid metabolism (Delerive et al. 2001; Bocher et al. 2002). In the arterial wall, PPAR-γ and PPAR-α can suppress inflammation via inhibition of NF-κB cascade. Further in cultured ECs, triggering PPAR-γ and PPAR-α inhibit NF-κB-mediated expression of inflammation-relevant molecules including endothelin-1, ICAM-1, and VCAM-1 (Pasceri et al. 2000; Wang et al. 2002; Liu et al. 2005; Ng et al. 2007; Norwood et al. 2010; Zhao et al. 2012). Chiefly, PPAR-α is expressed in the kidney, liver, and heart, while PPAR-γ is predominantly expressed within adipose tissue. Yet, both PPAR-γ and PPAR-α have been illuminated to be expressed within vascular ECs and smooth muscle cells (SMCs) (Bishop-Bailey 2000).

Recently, CYP-generated EETs and their subsequent hydration metabolites, DHETs, have been recognized as novel activators of PPAR-γ and PPAR-α. While 11,12-EET and 14,15-DHET have been the most robust PPAR-α activator (Ng et al. 2007), it seems there is no regioisomeric preference regarding PPAR-γ activation across the 4 EETs (Liu et al. 2005). Taken together, these

findings highlight that PPAR-γ and PPAR-α play a key role in EETs-induced anti-inflammatory effects majorly by NF-κB inhibition.

Another mechanism involved in EETs-mediated NF-κB inhibition is stimulation of EGFR and PI3K-dependent Akt signaling cascades. Recently, a study by Zhao and his team demonstrated that overexpression of CYP2J2 and EETs ameliorated TNF-α-mediated suppression of EGFR-induced stimulation of PI3K/Akt cascade in rat cardiomyocytes (Zhao et al. 2012). This result indicates that the inhibitory role of CYP-generated epoxides in TNF-α-mediated cardiac insult can lead to a potential therapeutic target in inflammation and hence might protect the heart from the harmful effects following the activation of inflammatory cascades.

1.7.2.4 EETs and eNOS

There is evidence highlighting a functional crosstalk between CYP-generated EETs and eNOS expression along with NO synthesis cascades (Chen et al. 2005; Hercule et al. 2009). Both CYP epoxygenases overexpression and addition of exogenous EETs have been correlated with a potent increase in eNOS activity and expression, with subsequent increased NO production in ECs (Wang et al. 2003; Chen et al. 2005; Jiang et al. 2007). Cell signaling cascades which are involved in EETs-induced overexpression of eNOS in ECs encompass PKC cascade, PI3K/Akt cascade, and MAPK cascade (Wang et al. 2003; Jiang et al. 2007)., 11,12-EET has been demonstrated to induce activity of eNOS in human platelets (Jiang et al. 2007). Despite these results, the anti-inflammatory properties of EETs-mediated eNOS expression and NO production are still elusive.

1.7.3 Inflammation and HETEs

Recent studies have demonstrated that inflammation could markedly reduce the CYP epoxygenase expression in the liver, kidney, and heart, while increase CYP ω-hydroxylase expression.

Therefore, CYP epoxygenases-induced EETs production is decreased with an increased formation of pro-inflammatory 20-HETE. These alterations could impact the progression and onset of different diseases via inflammatory response (Anwar-mohamed et al. 2010). One *in vivo* study reported that salidroside can reprogram CYP4A-induced AA metabolism in macrophages during treatment of gouty arthritis caused by monosodium urate crystal. This study highlighted that salidroside could decrease the generation of IL-1β and TNF-α via CYP4A downregulation which ameliorates inflammation and induces macrophages polarization independent from M1 phenotype (Liu et al. 2019). Another study illuminated that retinoic acid stimulates corneal gene expression of *CYP4B1* with subsequent production of pro-inflammatory 12-hydroxyeicosanoic acid (Ashkar et al. 2004).A recent study employing a rat model of LPS-induced septic shock highlighted that 20-HETE analog, 5,14-HEDGE, prevents LPS-induced hypotension and tachycardia through activating GPR75 signaling cascade (Tunctan et al. 2022). In a rat model of LPS-mediated inflammatory infection and insult, hepatic mRNA expressions of CYP4F5 and CYP4F4 were reduced by 40 and 50%, respectively, while the levels of PGs and LTs were elevated. On the contrary, CYP4F upregulation was associated with reduced levels of PGs and LTs, thus ameliorating inflammation (Cui et al. 2003). CYP4F upregulation-mediated reduction in PGs and LTs may be justified by CYP4F-induced metabolism of PGs and LTs or metabolic shunting among COXs, LOXs, and CYPs. Furthermore, in a murine model of traumatic brain injury, inflammatory cells in the airways underwent migration, and further secondary insult could be ameliorated through decreasing LTB_4 by stimulating CYP4F-mediated LTB_4 degradation, which paved novel methods to treat post-traumatic pulmonary inflammation (Kalsotra, Zhao, et al. 2007). The main hepatic LTB_4 hydroxylase, CYP4F2, may contribute to the regulation of LTB_4 levels in the liver and circulation (Johnson et al. 2015). Besides LTB_4, hydroxyeicosanoic acid and LXA_4 have been

demonstrated to undergo degradation in rat hepatocytes through recombinant CYP4F-medaited ω-hydroxylation. Pro-inflammatory mediators, including TNF-α, IL-6, and IL-1β, stimulate CYP4Fs through STAT3 signaling. On the other hand, IL-10, the anti-inflammatory mediator, suppresses CYP4F expression (Kalsotra, Anakk, et al. 2007).

1.8Rationale, hypotheses, and objectives

1.8.1 Rationale

CH is a composite condition which is defined as increased cardiac muscle mass due to stress. Pathological CH is correlated with aberrated cardiac morphology. This pathological condition is developed owing to stress stimuli, such as hypertension-induced pressure overload, and is known to be implicated in the development of HF and various cardiac pathologies (Kahan and Bergfeldt 2005; Bernardo et al. 2010). HF is regarded as a worldwide pandemic and a global leading cause of mortality and morbidity (Inamdar and Inamdar 2016; Teerlink et al. 2017). According to research, more than 600,000 Canadians are afflicted with HF and 50,000 new cases are annually diagnosed, with HF being responsible for nearly $2.8 billion yearly in health expenditure (Cameron et al. 2016). Unveiling new treatments that have the ability to halt CH development is considered indispensable to prevent one of the pivotal risk factors of HF and will give room for improving drug therapies (Olson and Molkentin 1999).

Mounting evidence propose that pathological CH is robustly associated with altered CYP-induced AA metabolism in the heart (Malik et al. 2012; El-Sherbeni and El-Kadi 2014). Several AA metabolites are implicated in CH pathogenesis, especially 20-HETE and mid-chain HETEs, encompassing 15-, 12-, 11-, 9-, 8-, and 5-HETE. Mid-chain HETEs are members of the

eicosanoids family that demonstrate clear cardiac biological functions. These lipid compounds are derived from AA by the actions of CYP- or LOX-induced bis-allylic oxidation (Nayeem 2018).

Cardiac CYP1B1 is constitutively expressed and is shown to be involved in the development of many CVDs, encompassing hypertension, atherosclerosis, and CH (Song et al. 2016; Anderson and Mazzoccoli 2019). Mid-chain HETEs are the main metabolites of CYP1B1-derived AA biotransformation. Along with CYP1B1, they have been highlighted as a possible target to develop drugs that treat CVDs (Choudhary et al. 2004; Li et al. 2017). Cardiac CYP1B1 overexpression has been previously shown in various CH models (Elkhatali et al. 2017; Maayah et al. 2017). For example, CH in rats instigated by abdominal aortic constriction was correlated with marked CYP1B1 protein induction and increased levels of its derived mid-chain HETEs metabolites (Maayah et al. 2018). In contrast, strategies including CYP1B1 knockdown or pharmacological inhibition have been implemented to protect against CH induced experimentally (Jennings et al. 2010; Zhang et al. 2020).

Inflammation was shown to be a critical hallmark of CH (Yang et al. 2012). Infiltration of inflammatory cells such as macrophages and T-lymphocytes, increased cytokines expression such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α), along with fibrosis and NF-κB activation are all notable features in pathological CH (Erten et al. 2005; Kuusisto et al. 2012). Results from our lab demonstrated decreased cardiac mRNA expression of CYP2E1, CYP2C11, CYP1A1, CYP4F5, and CYP4F1 in a murine model of LPS-mediated acute inflammation for 24 h. Conversely, mRNA expression of cardiac CYP4A1, CYP4A3, and CYP1B1 was shown to be increased, together with the cardiotoxic 20-HETE (Anwar-mohamed et al. 2010).

Despite achieving great strides in comprehending different aspects of CH, there is lack of substantial research on inflammation-induced CH and its mechanisms, including its effect on cardiac CYP-mediated AA metabolism, and the role of CYP1B1 in this process. In addition, no previous studies have investigated the modulation of CYPs in inflammation-induced CH in human cardiomyocytes, such as AC16 and RL-14 cells. Therefore, we proposed that TNF-α, IL-6, and LPS-induced CeH is dependent on CYP1B1 and its cardiotoxic mid-chain HETEs metabolites, and inhibition of CYP1B1 bestows cardioprotective impacts in the context of TNF-α, IL-6, and LPS-induced CeH.

1.8.2 Hypotheses

1- Inflammation alters the expression of cardiac CYP1B1 enzyme.
2- Inflammation alters CYP1B1-derived AA metabolism in an enantioselective manner.
3- Inflammation instigates CeH through induction of CYP1B1 and its dependent cardiotoxic mid-chain HETEs metabolites.
4- Inhibition of CYP1B1 bestows cardioprotective effects in inflammation-induced CeH.

1.8.3 Objectives

1- To examine the impact of inflammation on cardiac CYP1B1 and CeH markers in AC16 cells.
2- To characterize the role of cardiac CYP1B1 in enantioselective metabolism of AA in normal and inflammation-induced hypertrophic heart.
3- To investigate whether cardiac CYP1B1 inhibition bestows cardioprotection against inflammation-induced CH.

CHAPTER 2: MATERIALS AND METHODS

Portions of this chapter have been published in:

1- **Mohammed A. W. ElKhatib**, Fadumo Ahmed Isse, Samar H Gerges, & Ayman O. S. El-Kadi. Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy, Prostaglandins and Other Lipid Mediators, submitted.

2.1. Chemicals

Human adult ventricular cardiomyocytes (AC16 cells) were procured from Sigma-Aldrich (SCC109). Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F- 12) was obtained from Gibco, Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS), trans-resveratrol (3,4′,5-trihydroxy-trans-stilbene), and (R)-lipopolysaccharides (LPS) from *Escherichia coli* O111:B4 were purchased from Sigma-Aldrich. High-Capacity cDNA RT (Reverse-Transcription) Kit and TRIzol were from Invitrogen Co. (Carlsbad, CA, USA). Applied Biosystems supplied the SYBR® Green PCR Master Mix (Foster City, CA, USA). Integrated DNA Technologies developed and supplied the primers for real-time PCR analysis (Coralville, IA, USA). Immun-Blot® PVDF membrane was procured from Bio-Rad Laboratories (Hercules, CA, USA). Anti-GAPDH and recombinant anti-CYP1B1 polyclonal antibodies were from Abcam (Toronto, ON). The Anti-rabbit IgG HRP-linked secondary antibodies and anti-mouse IgG HRP-linked secondary antibodies were from Cell Signaling (Massachusetts, United States). Chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). CYP1B1-siRNA was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). P450-Glo™ CYP1B1 assay system was procured from Promega Co. (Madison, WI). The human (R)-TNF-α recombinant protein, human IL-6 recombinant protein, 4-nitrophenol, molecular probes wheat germ agglutinin, Alexa Fluor 488 Conjugate and Invitrogen ProLong Gold Antifade, 4′,6-diamidino-2-phenylindole (DAPI) were from Thermo Fisher Scientific (Edmonton, Canada).

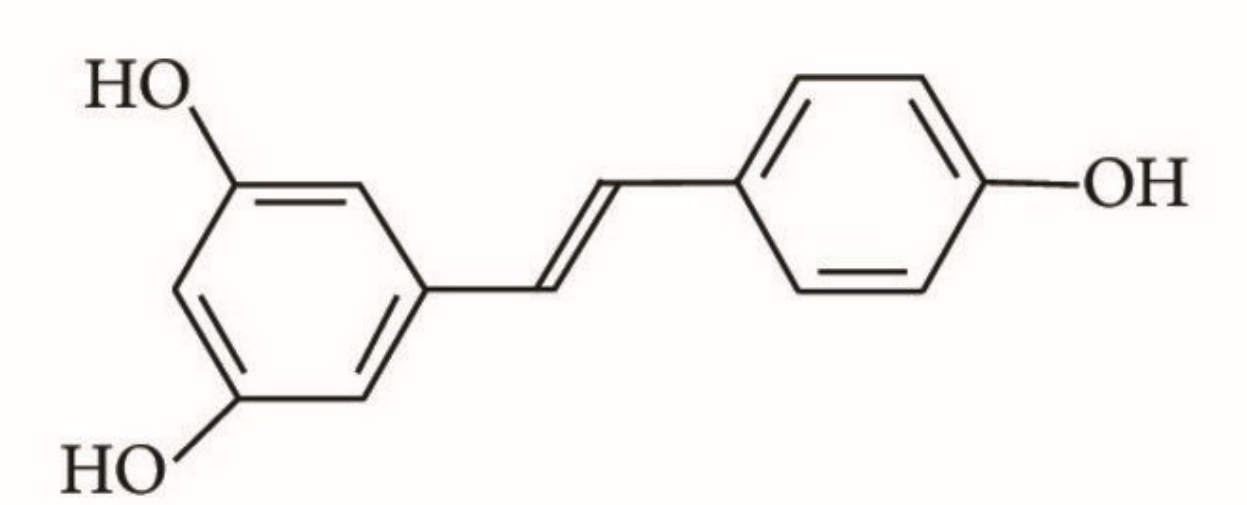


Figure 2.1. The chemical structure of trans-resveratrol.

Chemical structure of trans-resveratrol (3,4′,5-trihydroxy-trans-stilbene).

2.2. Cell culture

AC16 cells were sustained with DMEM/F-12 media containing phenol red supplemented with 1% penicillin-streptomycin, 12.5% FBS, and 20 mM l-glutamine. The cells were cultured in a 75 cm^2 culture flasks at 5% CO_2 and 37°. Each 75 cm^2 flask had nearly 7.5×10^6 cells.

2.3. Cell treatment

Cells were cultivated in complete media. Upon reaching confluency state, cells were plated and prepared for treatment. The cells were grown in DMEM/F12 serum-free media (SFM) for 6 h before treatment addition.

2.4. Measurement of cell viability

MTT assay was performed by examining the ability of living cells to convert the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide into its insoluble formazan crystalline. To reach sufficient confluency, cells were plated in 96-well plates for 24 h. Then different concentrations of TNF-α (0.1, 1, 5, 10, 25, 50, and 100 ng/mL), IL-6 (0.1, 1, 10, 20, 30, 50, and 100 ng/mL), and LPS (0.1, 1, 5, 10, 25, 50, and 100 µg/mL) were added to the cells for 24 h. A volume of 100 µL of 1.2 mM MTT reagent in SFM was added to each well of the plate and maintained at 37 °C for 2 h. The purple formazan crystalline precipitates were solubilized with 200 µL of DMSO. The absorbance was recorded using the Bio-Tek Synergy H1Hybrid Multi-Mode Microplate Readers at a wavelength of 570 nm (Bio-Tek Instruments, Winooski, VT, USA).

2.5. Measurement of cell surface area

Phase-contrast imaging was carried out using a Zeiss Axio Observer Z1 inverted microscope and a 20X objective lens to measure the relative alteration in cell surface area in response to compounds treatments as a marker for cellular hypertrophy. AC16 cells were plated on 6 well plate. Mount cover slips were put in the plates before adding the cell suspension. Cells were then treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL) in the presence and absence of CYP1B1-siRNA (25 nM) or trans-resveratrol (10 µM) (Shoieb and El-Kadi 2020) for 24 h. After the treatment, cells were washed with 1x PBS (pH 7.4) three times gently followed by fixation with 4% paraformaldehyde. Then, 10 µg/ml molecular probes wheat germ agglutinin (WGA) was added to the cells and kept in a dark for 2 h. The washing step was repeated this time using a shaker (3 times washing; each for 5 min) under dark. The cover slips that had the stained cells were put on a glass slide with antifade 4′,6-diamidino-2-phenylindole (DAPI) and imaged with an inverted

microscope. Using Zeiss AxioVision software, version 4.8, the surface area was calculated for each cell by measuring the complete boundary of the cell. Thirty cells were included in the analysis for each group.

2.6. Transfecting AC16 cells with CYP1B1-siRNA

AC16 cells were plated onto 6-well cell culture plates. Each well of cells was transfected with CYP1B1-siRNA at the concentration of 25 nM using siRNA transfecting reagent according to manufacturer's instructions (Santa Cruz). siRNA against CYP1B1 (sc-44546) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After 48 h of transfection, the experimental medium was added to the cells, followed by treatment with TNF-α, IL-6, and LPS for 24 h.

2.7. RNA extraction and cDNA synthesis

AC16 cells were cultured in 12-well plates in complete DMEM/F12 until confluency, after which cells were cultured in SFM for 6 h. Then, cells were treated with treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL) for 24 h. Total RNA was extracted from the cells using the TRIzol reagent as previously reported, according to the manufacturer's instructions (Invitrogen®), (Shoieb et al. 2022). The purity of RNA was > 1.80. The High-Capacity cDNA reverse transcription kit (Applied Biosystems) was employed to carry out first-strand cDNA synthesis according to the manufacturer's instructions.

2.8. Quantification of mRNA expression

The cDNA went through PCR amplification, real-time PCR was used to quantify CYP1B1, ANP, α-MHC, β-MHC, and β-actin genes expression with the help of Applied Biosystems Quant Studio

5 Real-Time PCR System. Human primer sequences utilized are highlighted in Table 1. Analysis of relative fold gene expression (ΔΔ CT) was employed to examine the data from real-time PCR and the reference gene (β-actin) was utilized to normalize the expression level of the target genes.

Table 2.1. Human primer sequences utilised for real time- PCR reactions.

Gene	Forward primer	Reverse primer
CYP1B1	TTCGGCCACTACTCGGAGC	AAGAAGTTGCGCATCATGCTG
α-MHC	GCCCTTTGACATTCGCACTG	GGTTTCAGCAATGACCTTGCC
β-MHC	TCACCAACAACCCCTACGATT	CTCCTCAGCGTCATCAATGGA
ANP	GGAGCCTGCGAAGGTCAA	TATCTTCGGTACCGGAAGCTGT
β-actin	ACCAGTTCCTGAATGGCTGC	GGC TGCACTCCACCATTTCT

2.9. Protein extraction

AC16 cells were cultured in 6-well plates and treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h. Afterward, cell lysates were collected using lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μL/mL of protease inhibitor cocktail. Cell homogenates were prepared by incubating the cell lysates on ice for 1 h, with sporadic vortexing every 10 min. Then, cell homogenates were centrifuged at 12,000g for 10 min at 4°C. Supernatant of total cellular lysate was collected and stored at −80°C. Afterward, the Lowry method was used to determine the protein concentration by using bovine serum albumin as a standard (Lowry et al., 1951).

2.10. Western blot analysis

Western blot analysis was performed according to previously detailed assay (Shoieb and El-Kadi, 2018b). Briefly, proteins from each group were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), samples underwent electrophoresis at 120 V for 2 h and separated proteins were transferred onto Immun-Blot® PVDF membranes. Afterwards, protein membranes were blocked overnight at 4 °C using blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base, 5% skim milk, 2% bovine serum albumin, and 0.5% Tween 20. Following blocking, the blots were subjected to washing cycles 3 times for 30 min with Tris-buffered saline (TBS)–Tween-20. The blots were subsequently incubated for 2 h at 4 °C with primary antibodies (rabbit anti-rat CYP1B1 (1:1000) and goat anti-rat GAPDH (1:200)) in TBS solution (0.05% (v/v) Tween-20, 0.02% sodium azide). Incubation with secondary antibodies (peroxidase-conjugated IgG) in blocking solution was performed for 30-45 min at room temperature. Visualization of the bands was carried out using the enhanced chemiluminescence method according to the manufacturer's guide (GE Healthcare Life Sciences). ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij) was used to quantify the intensity of the protein bands in relation to the signals acquired from GAPDH loading control. Data, given in the figures, are represented as relative protein intensity (%) + SEM, as compared to the control group. **2.11. Assessment of CYP1B1 enzyme activity**

P450-Glo™ CYP1B1 assay system kit was used to determine the enzymatic activity of CYP1B1, according to manufacturer's protocol. Briefly, AC16 cells were plated in 48-well plate then exposed to TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL) for 48 h. Thereafter, cells were washed once using SFM before incubation with luciferin CEE substrate (100 µM) for 3 h at 37 °C. Then, luciferin detection reagent was freshly prepared and added to the cells. The plate was

left to stand in room temperature for 20 min before reading luminescence signals. Bio-Tek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instrument, Winooski, VT, USA) was used to record the luminescence signals at integration time 0.25-1.00 second/well.

2.12. Incubation of AC16 cells with arachidonic acid

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h, followed by incubation with AA (50 μM) for 3 h. AA metabolites were then exposed to single liquid-liquid extraction with ethyl acetate and dried using speed vacuum (Savant SpeedVac SPD130DLX, NY, USA). Extracted AA metabolites were determined using liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Shimadzu LCMS-8050, Kyoto, Japan). Chromatographic peak integration was performed using LabSolutions software version 5.91 (Shimadzu, Kyoto, Japan).

2.13. Liquid chromatography-tandem mass spectrometry

A validated LC-MS/MS method was used to separate and quantify the hydroxy-metabolites of arachidonic acid (Isse et al., 2023). Briefly, gradient elution with organic phase: acetonitrile, methanol, and isopropyl alcohol (88:6:6, v/v) + 0.1% acetic acid and aqueous phase: water + 0.1% acetic acid was the LC operating condition. A chiral stationary phase column REFLEC C-AMYLOSE A column (5μm, 250 × 4.6 mm) (Regis Technologies Inc., Morton Grove, Illinois, USA) was used for the enantiomer separation. The MS was operated under negative electrospray\ ionization (ESI-).

2.14. Data and statistical analysis

Data are presented as mean ± standard error of mean (SEM). Student's *t* test or one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test was carried out to assess which treatment group(s) showed a significant difference compared to control group. The result was considered statistically significant when p value< 0.05. Analysis was performed using GraphPad Prism (version 8.0; GraphPad software, San Diego, CA).

CHAPTER 3: RESULTS

Portions of this chapter have been published in:

1- **Mohammed A. W. ElKhatib**, Fadumo Ahmed Isse, Samar H Gerges, & Ayman O. S. El-Kadi. Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy, Prostaglandins and Other Lipid Mediators, submitted.

3.1 Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy

3.1.1 Effect of TNF-α, IL-6, and LPS on cell viability

AC16 cells were treated with different concentrations of TNF-α, IL-6, and LPS for 24 h, then MTT assay was used to determine the cell viability. As shown in Figure 3.1A, B and C, TNF-α, IL-6, and LPS did not significantly affect cell viability compared to untreated cells at the tested concentrations.

3.1.2 Effect of TNF-α, IL-6, and LPS on cellular hypertrophic markers and cell surface area

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h, and the expressions of cellular hypertrophic markers, α-MHC, β-MHC, and ANP were measured using real-time PCR. Our results showed that the treatments significantly increased the mRNA expression levels of all the hypertrophic markers. As shown in Figure 3.1D, TNF-α, IL-6, and LPS induced mRNA expression of ANP by 100% ± 0.1751, 500% ± 0.6990 and 400% ± 0.3623, respectively. In a similar trend, β/α-MHC ratio was induced by TNF-α, IL-6, and LPS by 300% ± 0.6436, 600% ± 2.809 and 1400% ± 2.917, respectively (Figure 3.1E). To establish a link between cellular hypertrophy and the regulation of hypertrophic markers in response to TNF-α, IL-6, and LPS, the relative changes in cell surface area were evaluated. As shown in Figure 3.1F, treatment of AC16 cells with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) resulted in a considerable increase in cell surface area by 76% ± 1244 pixel^2, 88% ± 1587 pixel^2, and 79% ± 1253 pixel^2, respectively compared to control.

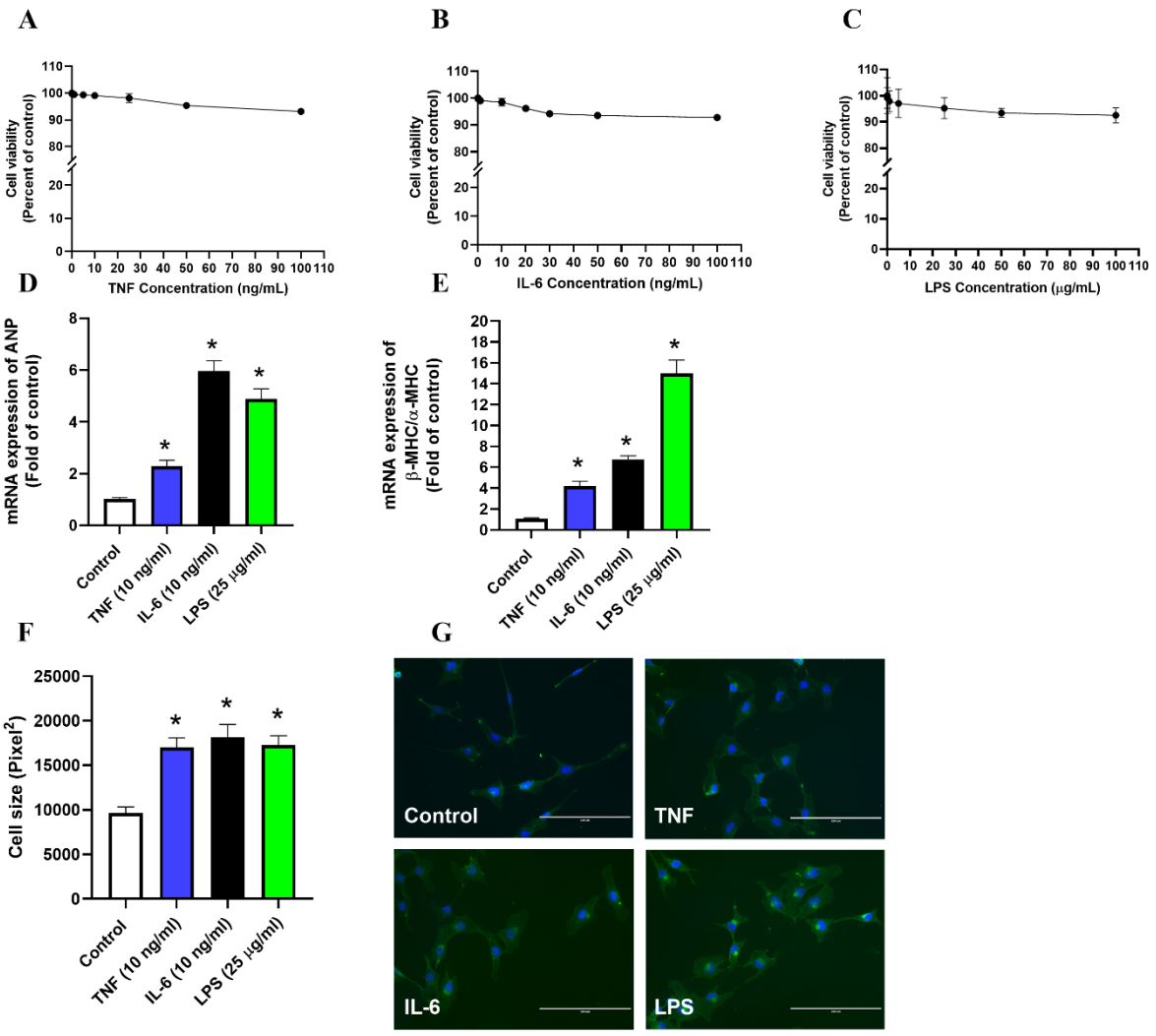


Figure 3.1. Effect of TNF-α, IL-6, and LPS on cell viability and hypertrophic indicators in AC16 cells.

AC16 cells were treated with increasing concentrations of TNF-α (A), IL-6 (B), and LPS (C) for 24 h and cell viability was measured using MTT assay. Data are represented as the percentage of untreated control (set as 100%) mean ± SEM (n = 6); D and E, AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h. Then, mRNA levels of atrial natriuretic peptide (ANP) and beta over alpha myosin heavy chain ratio (β-MHC/α-MHC) were quantified using real time-PCR, respectively. The quantified mRNA levels were normalized to β-

actin housekeeping gene. Data are represented as fold of control mean ± SEM (n = 6). *p < 0.05 compared to control. F, Quantification of cell surface area of AC16 cells treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h using Zeiss Axio Vision software. Data are represented as fold change mean ± SEM (n = 30). t test was performed. *p < 0.05 versus control. G, Representative images of AC16 cells stained with WGA (green) and DAPI (blue), scale bar = 200 μm from pictures taken with Zeiss Axio Observer Z1 inverted microscope.

3.1.3 Effect of TNF-α, IL-6, and LPS on CYP1B1 gene and protein expression

To investigate the effect of TNF-α, IL-6, and LPS on CYP1B1 expression in AC16 cells, cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL) for 24 h. Thereafter, the mRNA and protein expression levels of CYP1B1 were determined by real-time PCR and Western blot analysis, respectively. In comparison with the control, TNF-α, IL-6, and LPS significantly increased the mRNA expression of CYP1B1 by about 170% ± 0.3310, 110% ± 0.2880, and 140% ± 0.2761, respectively (Figure 3.2A). Furthermore, TNF-α, IL-6, and LPS led to 151% ± 21.48, 232% ± 7.130, and 251% ± 25.18 increase in CYP1B1 protein expression, respectively (Figure 3.2B).

3.1.4 Effect of TNF-α, IL-6, and LPS on CYP1B1 activity

To examine the effect of TNF-α, IL-6, and LPS on CYP1B1 activity, cells were treated with TNF, IL-6 and LPS for 48 h. As shown in Figure 3.2C, TNF-α, IL-6, and LPS significantly increased CYP1B1 activity by 40% ± 5.688, 24% ± 5.308, and 59% ± 6.928, respectively in comparison to control.

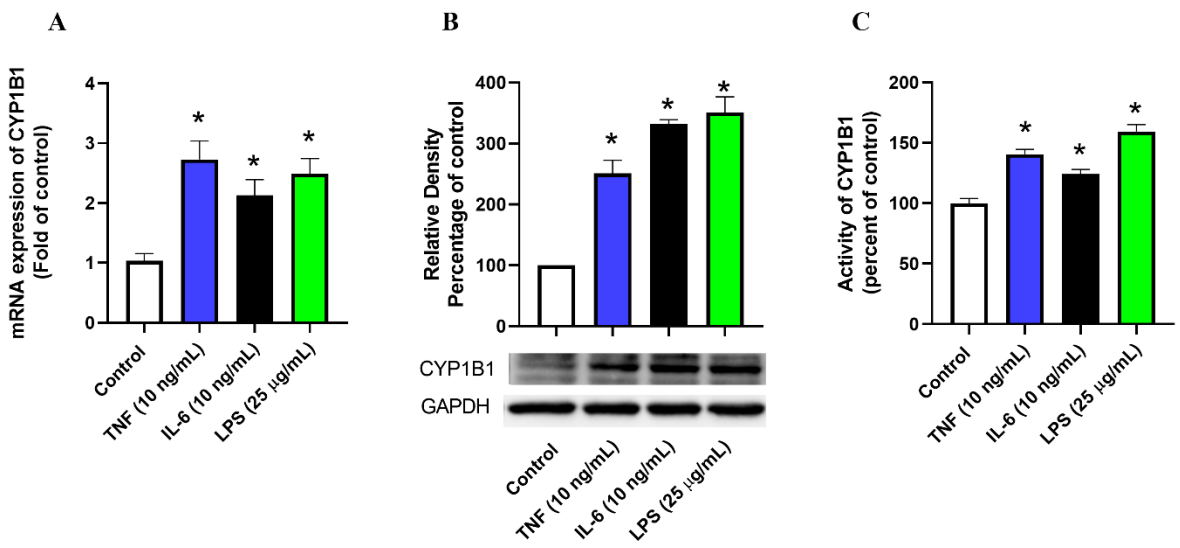


Figure 3.2. Effect of TNF-α, IL-6, and LPS on CYP1B1 gene expression, protein levels, and catalytic activity in AC16 cells.

AC16 cells were treated with TNF (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL). A, CYP1B1 mRNA levels were quantified after 24 h using real time-PCR. mRNA level was quantified and normalized to β-actin housekeeping gene. Data are represented as fold of control mean ± SEM, Student's *t* test, n=6 independent experiments per group. B, CYP1B1 protein levels were determined after 24 h by Western blot analysis (100 µg protein). Protein levels were detected using the enhanced chemiluminescence method and were normalized to the signals obtained for GAPDH protein and quantified using ImageLab software. Data are represented as percentage of control mean ± SEM, *p < 0.05 compared with control group, Student's *t* test, n=3 independent experiments per group. C, CYP1B1 catalytic activity level was determined after 48 h using a specific kit. Data are represented as a percentage of control mean ± SEM, *p < 0.05 compared to control group, Student's *t* test, n=8 independent experiments per group.

3.1.5 Effect of TNF-α, IL-6, and LPS on midchain (R/S)-HETE metabolite concentrations

To examine whether TNF-α, IL-6, and LPS alter the production of midchain (R/S)-HETEs and 20-HETE, AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h, followed by incubation with AA (50 μM) for 3 h. Our results demonstrate that TNF-α, IL-6, and LPS were able to significantly increase the concentrations of 5(R)-HETE and 5(S)-HETE by 133% ± 0.057 μg/mL, 125% ± 0.016 μg/mL, and 84% ± 0.015 μg/mL, and 158% ± 0.019 μg/mL, 164% ± 0.042 μg/mL, and 107% ± 0.018 μg/mL, respectively, compared to control cells (Figure 3.3A). Notably, the concentration of 5(R)-HETE was significantly higher than 5(S)-HETE at all treatments (Figure 3.3A).

Conversely, the concentration of 8(S)-HETE was remarkably higher compared to 8(R)-HETE after TNF-α, IL-6, and LPS treatment (Figure 3.3B). Further, 8(S)-HETE concentration was significantly elevated by nearly 129% ± 0.014 μg/mL, 125% ± 0.019 μg/mL, and 83% ± 0.012 μg/mL, while 8(R)-HETE concentration was markedly increased by 92% ± 0.016 μg/mL, 104% ± 0.006 μg/mL, and 78% ± 0.013 μg/mL after TNF-α, IL-6, and LPS treatment, respectively (Figure 3.3B).

Both 9(R/S)- and 11(R/S)-HETE showed similar trends after treatment. Only TNF-α and LPS treatments resulted in significantly higher concentrations of the S enantiomers (Figure 3.3C). The concentration of 9(S)-HETE was significantly increased by 116% ± 0.008 μg/mL, 106% ± 0.012 μg/mL, and 85% ± 0.010 μg/mL upon treatment with TNF-α, IL-6, and LPS, respectively, while 9(R)-HETE concentration was significantly increased by roughly 132% ± 0.018 μg/mL, 155% ± 0.013 μg/mL, and 60% ± 0.019 μg/mL, respectively (Figure 3.3C). As for 11-HETE, the R-enantiomer concentration was significantly elevated by 142% ± 0.014 μg/mL, 181% ± 0.008

μg/mL, and 116% ± 0.006 μg/mL following TNF-α, IL-6, and LPS, respectively, while the S enantiomer was increased by nearly 118% ± 0.011 μg/mL, 103% ± 0.011 μg/mL, and 81% ± 0.021 μg/mL, respectively (Figure 3.3D).

Furthermore, the concentrations of 12(S)-HETE and 12(R)-HETE were markedly increased by 44% ± 0.018 μg/mL, 23% ± 0.027 μg/mL, and 27% ± 0.036 μg/mL, and 46% ± 0.013 μg/mL, 27% ± 0.039 μg/mL, and 30% ± 0.034 μg/mL following TNF-α, IL-6, and LPS treatments, respectively (Figure 3.3E). Lastly, TNF-α, IL-6, and LPS treatments significantly elevated 15(R)-HETE concentration by roughly 47% ± 0.046 μg/mL, 27% ± 0.038 μg/mL, and 45% ± 0.044 μg/mL, respectively, and 15(S)-HETE concentration by 57% ± 0.049 μg/mL, 43% ± 0.037 μg/mL, and 48% ± 0.028 μg/mL, respectively (Figure 3.3F).

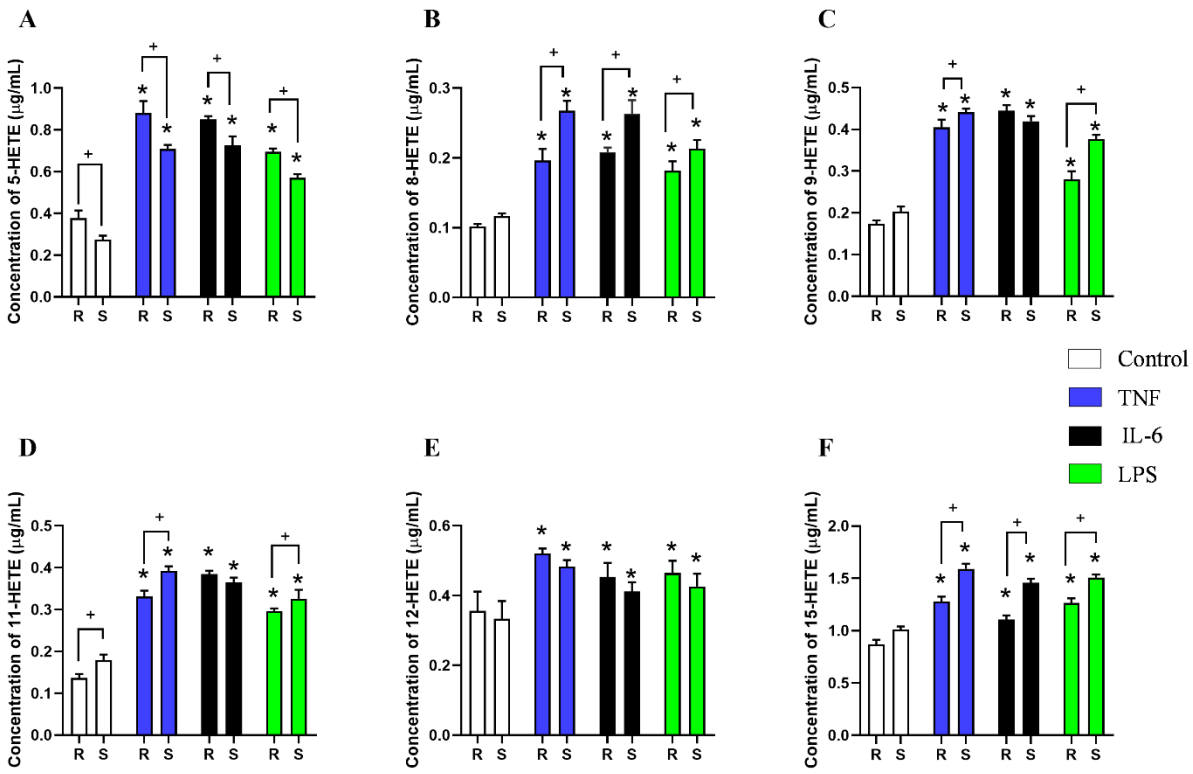


Figure 3.3. Effect of TNF-α, IL-6, and LPS on mid-chain (R/S) HETEs metabolites concentration in AC16 cells.

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 µg/mL) for 24 h, followed by incubation with AA (50 µM) for 3 h. Then, midchain (R/S)-HETEs metabolites concentrations were measured using LC-MS/MS (A-F). Data are represented as mean ± SEM (n=6). *$p < 0.05$ compared to respective control. +$p < 0.05$ compared to respective enantiomer in the same treatment.

3.1.6 Effect of TNF-α, IL-6, and LPS on 20-HETE metabolite concentrations

Our results demonstrate that 20-HETE concentration was markedly elevated by 60% ± 0.012 µg/mL, 37% ± 0.012 µg/mL, and 50% ± 0.016 µg/mL after TNF-α, IL-6 and LPS, respectively compared to controls (Figure 3.4).

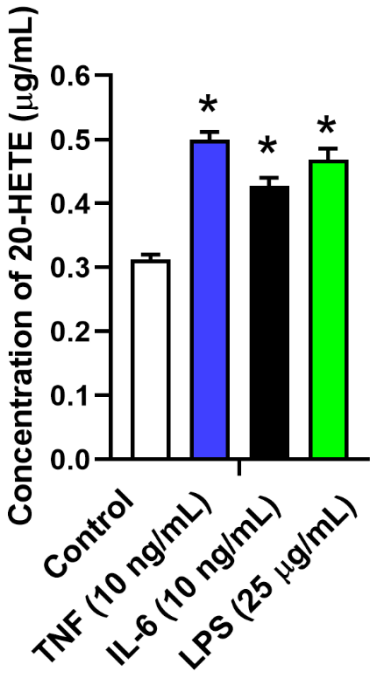


Figure 3.4. Effect of TNF-α, IL-6, and LPS on terminal 20-HETE metabolite concentration in AC16 cells.

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h, followed by incubation with AA (50 μM) for 3 h. Then, terminal 20-HETE metabolite concentrations were measured using LC-MS/MS. Data are represented as mean ± SEM (n=6). *p < 0.05 compared to respective control.

3.1.7 Effect of CYP1B1 inhibitor, trans-resveratrol, and CYP1B1-siRNA on TNF-α, IL-6, and LPS-mediated cellular hypertrophy

To examine whether TNF-α, IL-6, and LPS-induced cellular hypertrophy in AC16 cells is a CYP1B1-dependent mechanism, cells were treated with CYP1B1-siRNA (25 nM) or trans-resveratrol (10 μM) together with the inflammatory mediators. Again, treatment of cells with TNF-α, IL-6, and LPS significantly induced the mRNA expression of ANP, β/α-MHC ratio, and CYP1B1 (Figure 3.5 and Figure 3.6). Concomitant treatment with 10 μM trans-resveratrol significantly blunted the TNF-α, IL-6, and LPS-mediated induction of ANP gene expression by 165% ± 0.1362, 471% ± 0.6272, and 200% ± 0.3220, respectively (Figure 3.5A). In addition, cotreatment with trans-resveratrol markedly reduced the β/α-MHC ratio induction by nearly 777% ± 0.5093, 879% ± 0.5634, and 872% ± 0.5562 compared to TNF-α, IL-6, and LPS, respectively (Figure 3.5B). Treatment of AC16 cells with trans-resveratrol alone significantly lowered the mRNA expression of CYP1B1 by 43% ± 0.1107 compared to untreated cells, and cotreatment with the inflammatory mediators significantly blunted CYP1B1 mRNA levels by 216% ± 0.3133, 192% ± 0.2627, and 217% ± 0.2517 compared to TNF-α, IL-6, and LPS-treated cells, respectively (Figure 3.5C).

Similarly, cells treated with CYP1B1-siRNA significantly blunted the TNF-α, IL-6, and LPS-mediated induction of ANP gene expression by 109% ± 0.2477, 493% ± 0.4040, and 374% ± 0.3967, respectively (Figure 3.6A). In addition, CYP1B1-siRNA treatment markedly reduced the induction of β/α-MHC ratio by nearly 200% ± 0.4960, 600% ± 0.3788, and 1100% ± 1.273 compared to TNF-α, IL-6, and LPS, respectively (Figure 3.6B). Finally, CYP1B1-siRNA markedly blunted the mRNA expression of CYP1B1 by 72% ± 0.0718 compared to untreated cells, and its combination with the inflammatory mediators significantly lowered CYP1B1 mRNA

expression by 249% ± 0.1675, 312% ± 0.2052, and 392% ± 0.3131 compared to TNF-α, IL-6, and LPS alone, respectively (Figure 3.6C). These results indicate a possible role for CYP1B1 in the mechanism underlying TNF-α, IL-6, and LPS-induced cellular hypertrophy.

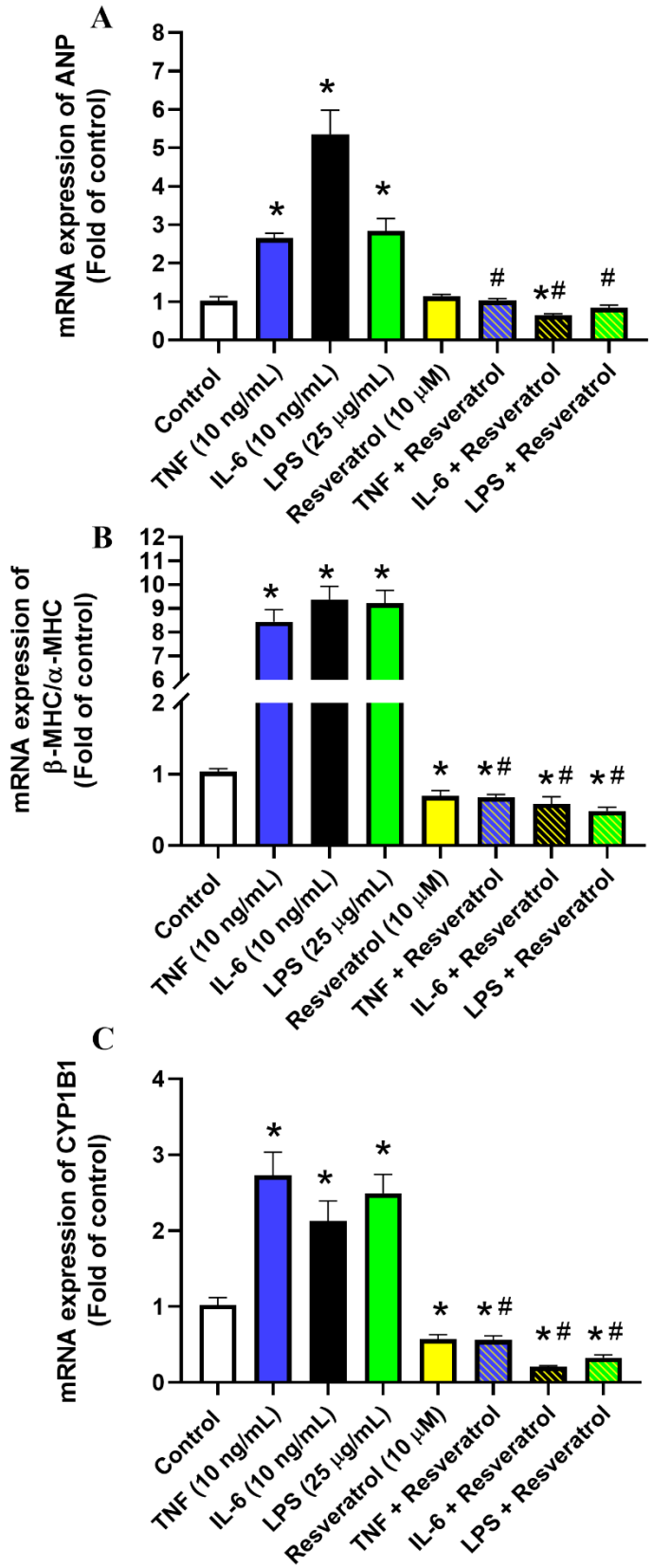
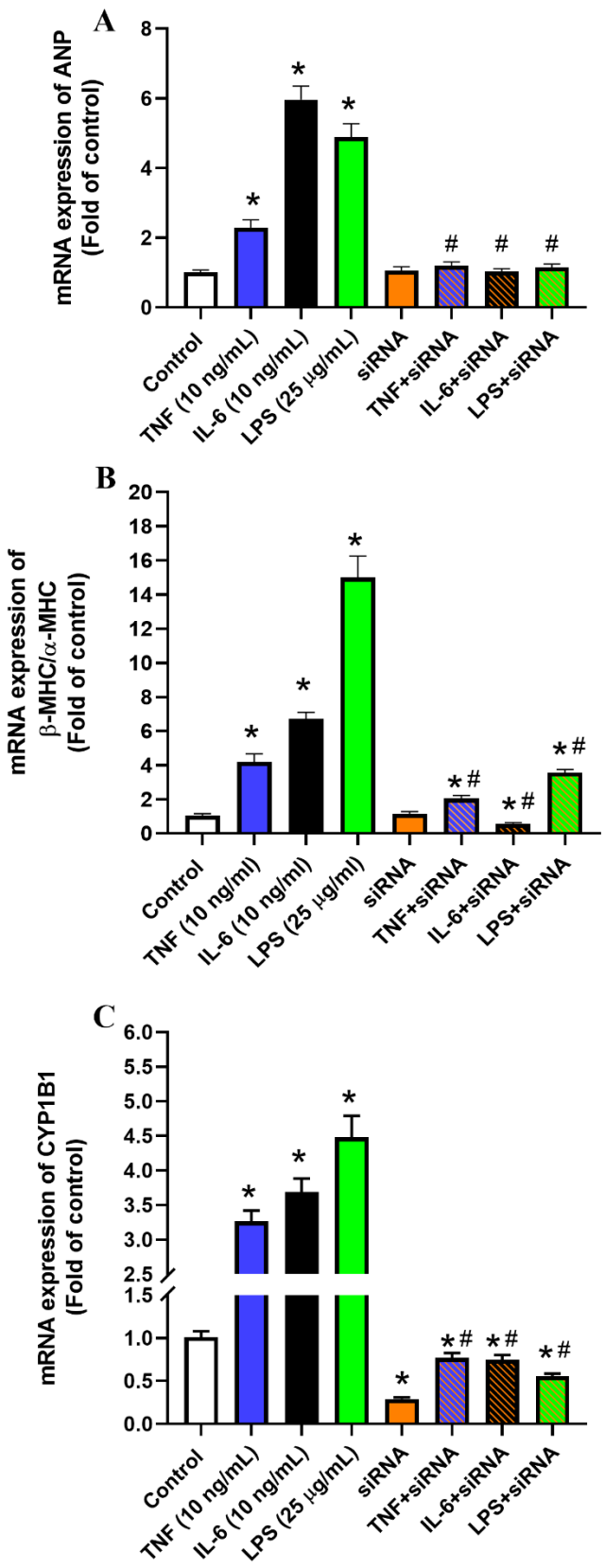

A
mRNA expression of ANP
(Fold of control)
B
mRNA expression of
β-MHC/α-MHC
(Fold of control)
C
mRNA expression of CYP1B1
(Fold of control)
Control
TNF (10 ng/mL)
IL-6 (10 ng/mL)
LPS (25 μg/mL)
Resveratrol (10 μM)
TNF + Resveratrol
IL-6 + Resveratrol
LPS + Resveratrol

Figure 3.5. The effect of trans-resveratrol on TNF-α, IL-6, and LPS-induced increase in the mRNA expression of hypertrophic markers and CYP1B1 in AC16 cells.

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) in the presence and absence of 10 μM trans-resveratrol for 24 h. Then, mRNA levels of atrial natriuretic peptide (ANP) (A), beta over alpha myosin heavy chain ratio (β-MHC/α-MHC) (B), and CYP1B1 (C) were quantified using real time-PCR. mRNA level was quantified and normalized to β-actin housekeeping gene. Data are represented as fold of control mean ± SEM. *$p < 0.05$ compared to control. #$p < 0.05$ compared to respective treatment without trans-resveratrol.


A
mRNA expression of ANP
(Fold of control)
0
2
4
6
8
*
*
*
#
#
#
Control
TNF (10 ng/mL)
IL-6 (10 ng/mL)
LPS (25 μg/mL)
siRNA
TNF+siRNA
IL-6+siRNA
LPS+siRNA
B
mRNA expression of
β-MHC/α-MHC
(Fold of control)
0
2
4
6
8
10
12
14
16
18
20
*
*
*
*#
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Control
TNF (10 ng/ml)
IL-6 (10 ng/ml)
LPS (25 μg/ml)
siRNA
TNF+siRNA
IL-6+siRNA
LPS+siRNA
C
mRNA expression of CYP1B1
(Fold of control)
0.0
0.5
1.0
1.5
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
*
*
*
*
*#
*#
*#
Control
TNF (10 ng/mL)
IL-6 (10 ng/mL)
LPS (25 μg/mL)
siRNA
TNF+siRNA
IL-6+siRNA
LPS+siRNA

Figure 3.6. Effect of CYP1B1-siRNA on TNF-α, IL-6, and LPS-induced increase in the mRNA expression of hypertrophic markers and CYP1B1 in AC16 cells.

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h in the presence and absence of 25 nM CYP1B1-siRNA. Then, mRNA levels of atrial natriuretic peptide (ANP) (A), beta over alpha myosin heavy chain ratio (β-MHC/α-MHC) (B), and CYP1B1 (C) were quantified using real time-PCR. mRNA level was quantified and normalized to β-actin housekeeping gene. Data are represented as fold of control mean ± SEM. *$p < 0.05$ compared to control. #$p < 0.05$ compared to respective treatment without CYP1B1-siRNA.

3.1.8 Effect of CYP1B1 inhibitor, trans-resveratrol, and CYP1B1-siRNA on TNF-α, IL-6, and LPS-mediated increase in cell surface area

To further confirm the role of CYP1B1 in TNF-α, IL-6 and LPS-induced CH, AC16 cells were exposed to TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) in the presence of CYP1B1-siRNA (25 nM) or trans-resveratrol (10 μM) for 24 h. Investigating the cell surface area revealed that both CYP1B1-siRNA and trans-resveratrol (10 μM) reversed the increase in cell surface area induced by TNF-α, IL-6, and LPS to normal control values (Figure 3.7A). These results, along with previous ones, highlight the crucial role of CYP1B1 in TNF-α, IL-6, and LPS-induced CH.

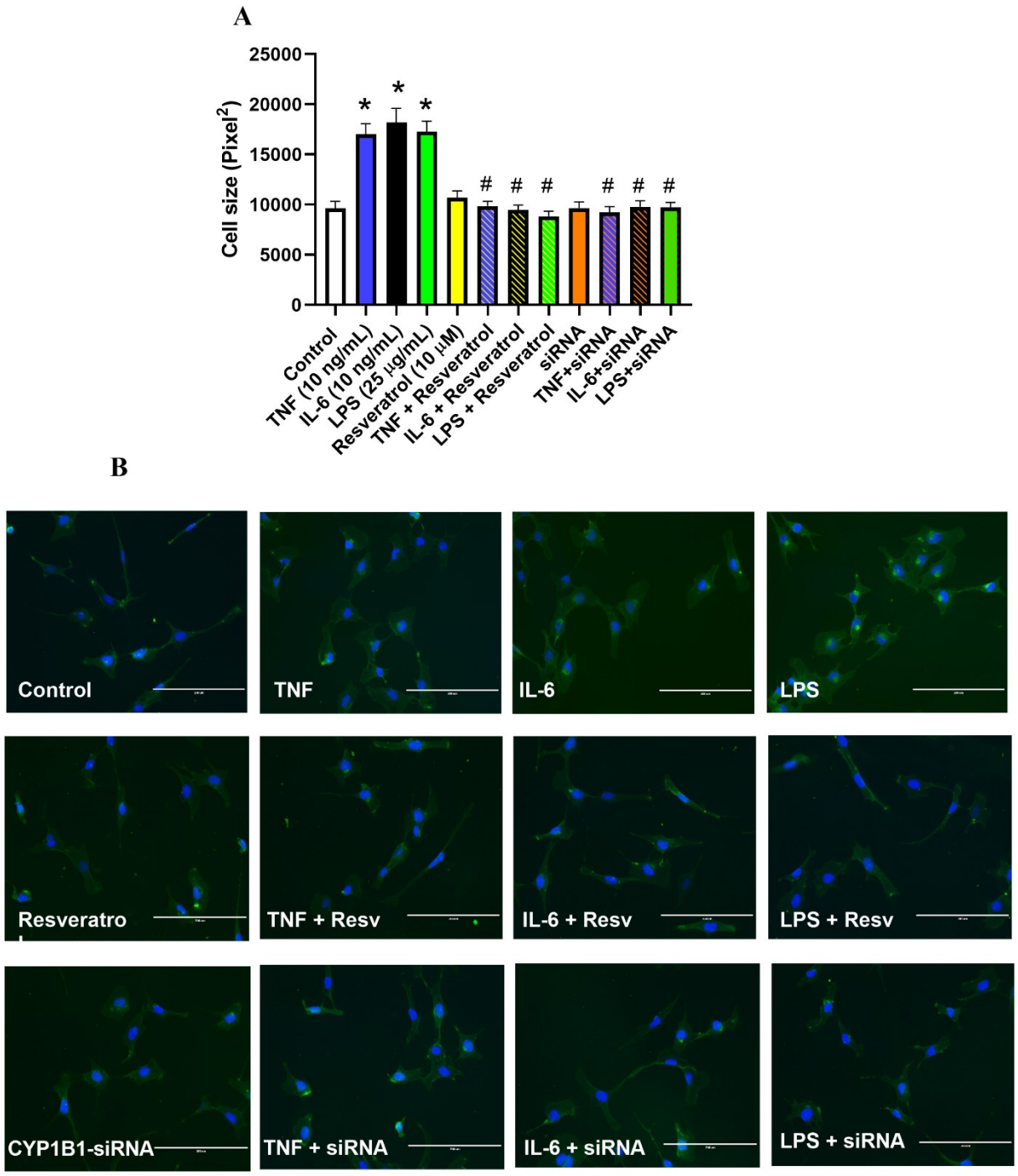

Figure 3.7. Effect of trans-resveratrol and CYP1B1-siRNA on TNF-α, IL-6, and LPS-induced increase in cell surface area in AC16 cells.

AC16 cells were treated with TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) for 24 h in the presence and absence of trans-resveratrol (10 μM) and CYP1B1-siRNA (25 nM). A, Cell surface area in AC16 cells was quantified using Zeiss Axio Vision software. Data are represented as mean ± SEM, (n=30). *$p < 0.05$ compared to control group, #$p < 0.05$ compared to respective treatment. B, Representative images of AC16 cells stained with WGA (green) and DAPI (blue), scale bar = 200 μm from pictures taken with Zeiss Axio Observer Z1 inverted microscope.

CHAPTER 4: DISCUSSION

Portions of this chapter have been published in:

1- **Mohammed A. W. ElKhatib**, Fadumo Ahmed Isse, Samar H Gerges, & Ayman O. S. El-Kadi. Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy, Prostaglandins and Other Lipid Mediators, submitted.

4.1 Cytochrome P450 1B1 is Critical in The Development of TNF-α, IL-6, and LPS-Induced Cellular Hypertrophy

Countless studies were conducted to comprehend the link between inflammation and CeH (Wang et al. 2004; Sriramula et al. 2008; Chang et al. 2011; Singh et al. 2012; Magi et al. 2015; Fang et al. 2017). Several inflammatory mediators including TNF-α, IL-6, and LPS were previously shown to be key players in the induction of CeH (Sano et al. 2000; Sriramula et al. 2008; Magi et al. 2015). For instance, Ang II-mediated CeH was markedly attenuated in TNF-α knockout mice compared to wild-type counterparts, indicating a mechanistic role for TNF-α in CH (Sriramula et al. 2008). In addition, IL-6 trans-signaling was involved in the induction of various CeH markers, such as c-fos and BNP, along with increased cell size *in vitro* (Szabo-Fresnais et al. 2010). Moreover, CeH was induced in H9c2 cells treated with LPS owing to altered calcium homeostasis (Magi et al. 2015).

Cardiac CYP enzymes, particularly CYP1B1, and their produced AA metabolites were also established in various studies to be largely involved in the development of CeH (Alsaad et al. 2013; El-Sherbeni and El-Kadi 2014; Maayah and El-Kadi 2016b). Regardless, no study has examined the impact of inflammatory mediators on CYP enzymes and the potential role of CYP enzymes and their AA metabolites in CeH induction in human cardiac cell lines such as RL-14 and AC16 cells. Thus, in this study, we aimed to examine the impact of TNF-α, IL-6, and LPS on CYP enzymes and CYP-mediated enantioselective AA metabolism in AC16 cells, and to investigate the potential role of CYP1B1 in inflammation-mediated CeH.

A mounting number of studies investigated the impact of TNF-α on the CV system (Bryant et al. 1998; Kaur et al. 2006; Haudek et al. 2007; Cacciapaglia et al. 2011; Urschel and Cicha 2015;

Dhiman et al. 2021). In humans, an association was unveiled between survival and serum TNF-α levels in HF patients (Torre-Amione et al. 1996). Transgenic mice overexpressing cardiac TNF-α demonstrated cardiac dilatation, aberrated calcium homeostasis, ventricular arrythmia, and premature death (Kubota et al. 1997; Kadokami et al. 2000; Li et al. 2000; London et al. 2003). Although the administration of anti-TNF-α antibody or soluble TNF receptor abolished HF development in experimental animals (Kubota et al. 2000; Kadokami et al. 2001), these anti-cytokines were not beneficial in chronic HF patients (Anker and Coats 2002). Several signaling pathways triggered by TLR-4 activation are known to contribute to CeH development, including TLR4/MyD88/NF-κB, TLR4/MyD88/MAPK, TLR4/MyD88/CaMK II, and TLR4/MyD88/PI3K/Akt (Xiao et al. 2020). TNF-α mediates its cardiac effects via binding to two specific receptors on the cell surface of cardiomyocytes, namely TNFR1 and TNFR2 (Torre-Amione et al. 1995). Most of the detrimental effects of TNF-α are mainly mediated by TNFR1, including cell death and negative inotropic effects (Torre-Amione et al. 1995; Shen and Pervaiz 2006). Conversely, TNFR2 induction was shown to bestow cardioprotective effects (Higuchi et al. 2004; Ramani 2004).

Various studies have shown the importance of IL-6 in CeH pathophysiology (Sano et al. 2000; Diaz et al. 2009; Szabo-Fresnais et al. 2010; Chang et al. 2011; Mir et al. 2012; Fang et al. 2017). IL-6 is an inflammatory cytokine secreted by different cell types, including lymphocytes and macrophages (Mohamed-Ali et al. 1998). Adult cardiomyocytes were shown to produce IL-6 through a β-adrenergic receptor-mediated pathway (Szabo-Fresnais et al. 2010). IL-6 deletion played a critical role in mitigating pressure overload-mediated CeH (Zhao et al. 2016). IL-6 mediates its functions through classical IL6R signaling (Yudkin et al. 2000) and trans-signaling (Szabo-Fresnais et al. 2010).

LPS is also known to induce CeH (Liu et al. 2008; Chowdhury et al. 2013; Chen et al. 2014; Magi et al. 2015; Chao et al. 2019). LPS is a bacterial toxin that instigates CeH through activating Toll-like receptor-4 (TLR-4) (Poltorak et al. 1998; Chow et al. 1999) expressed by cardiomyocytes (Frantz et al. 1999). LPS was implicated in apoptotic induction in adult rat ventricular cardiomyocytes (Li et al. 2002) and reduced cardiac contractility in adult rabbit left ventricular cardiomyocytes (Yasuda and Lew 1997).

In this study, we investigated the impact of TNF-α (10 ng/mL), IL-6 (10 ng/mL), and LPS (25 μg/mL) on AC16 cells. Our results indicate that TNF-α, IL-6, and LPS induce CeH, as evidenced by increased cell surface area and mRNA expression of hypertrophic markers, namely ANP and β /α-MHC ratio. These results are in line with previous studies performed on various cell lines (Yokoyama et al. 1997; Condorelli et al. 2002; Higuchi et al. 2002). The mechanisms underlying CeH induced by TNF-α, IL-6, and LPS are still elusive. In this regard, studies demonstrated the possible involvement of NF-κB (Kawamura et al. 2005), reactive oxygen species (Higuchi et al. 2002), TNFR1 signaling (Li et al. 2000), disruption of TNFR2 signaling (Higuchi et al. 2004), TNF-α converting enzyme (Wang et al. 2009), TNFR associated factor 2 (Sack et al. 2000), protein kinase C (Baines et al. 1999), stress activated protein kinases (Bogoyevitch et al. 1996), TLR-4 (Vallejo 2011), myeloid differentiation protein 2 (Kim and Kim 2017), mitogen-activated protein kinase (O'neill et al. 2013), gp130 (Hirota et al. 1995), and signal transducer and activator of transcription 3 (Mir et al. 2012), among others.

Some studies have investigated the effect of inflammatory cytokines on CYP1B1 expression in different organs. For example, TNF-α was found to increase CYP1B1 expression in hepatic cells and rat liver epithelial cells (Piscaglia et al. 1999; Umannová et al. 2007; Šmerdová et al. 2014). IL-1β was found to enhance CYP1B1 expression in a dose-dependent manner in astroglial cells (Malaplate-Armand et al. 2003; Anwar-mohamed et al. 2010). LPS-induced systemic inflammation in rats was found to differentially alter CYP enzymes expression and their mediated AA metabolism in different organs. Interestingly, it was associated with a significant elevation in cardiac CYP1B1 expression, as well as formation of 20-HETE (Malaplate-Armand et al. 2003; Anwar-mohamed et al. 2010). In agreement, our results showed that TNF-α, IL-6, and LPS induce CYP1B1 expression at mRNA, protein, and activity levels in AC16 cells. Several studies from our lab highlighted that different *in vitro* and *in vivo* models of CeH are characterized by an increase in CYP1B1 expression, including angiotensin and pressure overload-induced hypertrophy (Anwar-mohamed et al. 2010; Maayah et al. 2017; Alsaad 2018; Shoieb and El-Kadi 2020; Isse et al. 2023). The mechanisms underlying CYP1B1 expression are still under investigation; however, studies pointed out the involvement of translational stability, transcriptional processing, AhR-mediated and non-AhR-mediated pathways (Murray et al. 2001).The detrimental role of CYP1B1 in CeH originates from its involvement in AA metabolism into cardiotoxic mid-chain HETEs and production of superoxide radicals (Morgan 2001; Maayah and El-Kadi 2016b). Interestingly, inflammatory mediators like TNF-α and LPS are known to increase the activity of phospholipase A2 enzyme, thereby increasing AA release from cellular membrane phospholipids (Mohri et al. 1990; Lee et al. 2013). Thus, they will result in more AA being available to CYP1B1, which they also increase as demonstrated by our results and previous studies (Piscaglia et al. 1999; Malaplate-

Armand et al. 2003; Umannová et al. 2007; Anwar-mohamed et al. 2010; Šmerdová et al. 2014), finally leading to increased AA metabolism by CYP1B1 producing midchain HETEs.

Multiple studies have unveiled the deleterious role of mid-chain HETEs, namely 15-, 12-, 11-, 9-, 8-, and 5-HETE, in CVDs development (Nozawa et al. 1990; Cyrus et al. 1999; Jenkins et al. 2009). For instance, our lab highlighted that mid-chain HETEs instigate CeH in RL-14 cells via NF-κB and MAPKs-dependent mechanisms, as they stimulate the phosphorylation of extracellular signal-regulated kinase and induce the binding of NF-κB to its responsive element, thereby leading to the transcription of several hypertrophy genes (Maayah, Abdelhamid, et al. 2015; Maayah and El-Kadi 2016b; Maayah and El-Kadi 2016a; Elkhatali et al. 2017). Interestingly, inhibition of CYP1B1 by treatment with CYP1B1-siRNA or with a chemical inhibitor like trans-resveratrol was previously found to significantly ameliorate isoproterenol and Ang II-induced CeH, respectively, through lowering the levels of CYP1B1 and its associated cardiotoxic midchain HETEs (Maayah et al. 2017; Shoieb and El-Kadi 2020). In this study, we analyzed the produced AA metabolites from AC16 cells treated with TNF-α, IL-6, and LPS. Our results revealed for the first time that mid-chain HETEs concentrations were all significantly elevated compared to controls after inflammatory mediator treatment, with a clear enantioselective induction of 5(R)-, 8(S)-, 9(S)-, 11(S)-, 12(R)-, and 15(S)-HETE. Notably, both 11(S)- and 15(S)-HETE were previously found to enhance the sensitivity of rat neonatal cardiomyocytes to isoproterenol (Wallukat et al. 1994). The contribution of LOXs in mid-chain HETEs production was not considered as this study was focused mainly on the impact of CYP1B1 on AA metabolism in the context of TNF-α, IL-6, and LPS -induced CeH.

Additionally, analysis of terminal 20-HETE demonstrated an increase in its concentration in response to TNF-α, IL-6, and LPS treatment. 20-HETE is known to be derived from AA by the

actions of CYP4A (Mccarthy et al. 2005), CYP4F (Alexanian et al. 2012), and CYP1B1 (Zaabalawi 2022). These results are in line with previous findings showing increased production of 20-HETE in different inflammatory models (Ishizuka et al. 2008; Anwar-mohamed et al. 2010; Tunctan et al. 2012; Garcia et al. 2017; Hamers et al. 2022).

TNF-α, IL-6, and LPS-mediated CeH was significantly ameliorated following the inhibition of CYP1B1 using trans-resveratrol or CYP1B1-siRNA, as evidenced by reversal of mRNA expression of hypertrophic markers and CYP1B1, along with normalized cell surface area. Indeed, trans-resveratrol showed promising effects regarding amelioration of CeH in various models (Chan et al. 2008; Zordoky and El-Kadi 2010b; Matsumura et al. 2018; Shoieb and El-Kadi 2019; Shoieb and El-Kadi 2020). CYP1B1-siRNA, which is used experimentally to specifically silence CYP1B1 gene expression, has also demonstrated the pivotal role played by CYP1B1 in mediating CeH in several studies (Maayah et al. 2017; Zhang et al. 2020). The results obtained from trans-resveratrol and CYP1B1-siRNA unveil the involvement of CYP1B1 in TNF-α, IL-6, and LPS -induced CeH and establish CYP1B1 as a potential therapeutic target for mitigation of CeH mediated by TNF-α, IL-6, and LPS (El-Sherbeni and El-Kadi 2016).

In summary, the pathogenesis of CeH is complex and involves numerous factors including inflammation. In this study, we provide unmistakable evidence that TNF-α, IL-6, and LPS induce CYP1B1-dependent CeH in AC16 cells. TNF-α, IL-6, and LPS increased cell surface area and expression of hypertrophic markers. Further, TNF-α, IL-6, and LPS enhanced the mRNA, protein, and activity levels of CYP1B1. Moreover, TNF-α, IL-6, and LPS showed enantioselective induction of cardiotoxic mid-chain HETEs, along with terminal 20-HETE. CYP1B1 is crucial in mediating TNF-α, IL-6, and LPS -induced CeH as its inhibition resulted in amelioration of TNF-

α, IL-6, and LPS -mediated CeH. This study sheds light on CYP1B1 as a potential therapeutic target for the prevention and treatment of TNF-α, IL-6, and LPS -induced CeH.

Limitations: we acknowledge that the described effects of TNF-α, IL-6, and LPS were solely investigated in an *in vitro* model of CeH. It is challenging to address the separate effects of each inflammatory mediator on cardiac CYP1B1 in an *in vivo* model of CH. We suggest that each inflammatory mediator could be tested in an *in vivo* model of cardiac hypertrophy such as LPS-induced CH experimental model in rats.

4.2 Summary and general conclusions

In the current work, our goal was to investigate the impacts of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and lipopolysaccharide (LPS) on the development of CeH, expression of CYP1B1, and CYP1B1-mediated AA metabolism, as well as the role of CYP1B1 in TNF-α, IL-6, and LPS-induced CeH *in vitro*.

Our results show that TNF-α, IL-6, and LPS induce mRNA expression of hypertrophic markers, significantly increase cell surface area, induce CYP1B1 at mRNA, protein, and activity levels, and enantioselectively modulate CYP1B1-mediated AA metabolism in favor of cardiotoxic mid-chain HETEs. These effects are ameliorated in the presence of CYP1B1-siRNA or trans-resveratrol.

In conclusion, our results demonstrate the crucial role of CYP1B1 in TNF-α, IL-6, and LPS -induced CeH and highlight CYP1B1 as a clear target for potential therapeutic interventions for the prevention and treatment of CeH.

4.3 Future Research directions

The findings of the current work have mainly focused on comprehending how TNF-α, IL-6, and LPS-induced CeH impacts the expression of cardiac CYP1B1 and the production of its derived AA metabolites in an enantioselective manner. Also, it highlighted the critical importance of CYP1B1 in mediating the TNF-α, IL-6, and LPS-induced CeH. However, further research is required to confirm these findings *in vivo*. This can open up the path to investigate the role of other cardiac CYPs in inflammation-induced CeH, such as CYP2C11 and CYP2E1. Therefore, we propose the following intriguing points:

1. To investigate the impact of TNF-α, IL-6, and LPS-induced CeH on the expression of cardiac CYPs, encompassing CYP2C11 and CYP2E1 *in vitro*.
2. To confirm the impact of TNF-α, IL-6, and LPS-induced CeH on CYP1B1 *in vivo* using an animal model, such as LPS-treated rats model.
3. To examine the impact of TNF-α, IL-6, and LPS-induced CeH on CYP1B1-derived AA metabolites in an enantioselective point of view in the presence of CYP1B1-siRNA or trans-resveratrol. The impact of trans-resveratrol and CYP1B1-siRNA on mid-chain HETEs should be confirmed.
4. To explore the mechanisms by which TNF-α, IL-6, and LPS-induced CeH modulates the expression of CYP1B1 among other enzymes.
5. To unveil the mechanisms underlying the modulation of CYP1B1-derived AA metabolites by TNF-α, IL-6, and LPS-induced CeH.

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