University of Alberta

Role of Cytochrome P450 (CYP) Metabolites of Arachidonic Acid in the Regulation of cAMP in HEK293 Cells

By

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To my family

ABSTRACT

Cytochrome P450 epoxygenases metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs), which in turn are converted to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). EETs are known to modulate a number of vascular and renal functions but the exact signaling mechanism(s) of these EET-mediated effects remains unknown. **Purpose:** To investigate the role of EETs and DHETs in regulating cAMP production via adenylyl cyclase (AC) in a human embryonic kidney cell line (HEK 293). **Method:** HEK 293 cells were treated with vehicle, forskolin, epinephrine, 11,12-EET, 11,12-DHET, as well as potential pathway and G-protein inhibitors to assess changes in cAMP production. **Results:** Co-administering 11,12-EET with forskolin or epinephrine effectively eliminated the increased cAMP levels observed in cells treated with forskolin alone. The inhibitory effect of EETs on forskolin-mediated cAMP production was abolished when cells were treated with a sEH inhibitor (*t*AUCB). 11,12-DHET also negated the effects of forskolin, suggesting the inhibitory effect observed in EET-treated cells could be attributed to the downstream metabolites, DHETs. In contrast, inhibition of phosphodiesterase IV (PDE4) with rolipram eliminated the effects of EETs or DHETs, and Gαi with pertussis toxin also resulted in enhanced cAMP production. **Conclusion:** Our data suggest that DHETs regulate cAMP production via PDE4 and Gαi protein. Moreover, they provide novel evidence as to how EET-mediated signaling may alter G-protein coupling in HEK 293 cells.

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AA	arachidonic acid
AC	adenylyl cyclase
BK_{Ca}	Ca^{2+}-sensitive K^+ channels
cAMP	cyclic adenosine monophosphate
DHET	dihydroxyeicosatrienoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
EET	epoxyeicosatrienoic acid
FBS	fetal bovine serum
GDPβS	guanosine 5'-O-(2-thiodiphosphate) trilithium salt
HEK 293	human embryonic kidney cell line
H89	5-isoquinolinesulfonamide
PDE	phosphodiesterase
PKA	protein kinase A
PTX	pertussis toxin
sEH	soluble epoxide hydrolase
*t*AUCB	trans-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid

Chapter 1
INTRODUCTION

1.1. Overview

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the Western world, and, according to the World Health Organization will be a major cause of death worldwide by 2020 (Murray and Lopez 1997). Ischemic heart disease accounts for the majority of CVD in the industrialized world (Ferdinandy, Schulz et al. 2007). There are many contributing and/or risk factors associated with ischemic heart disease, including hypertension, diabetes mellitus, atherosclerosis, genetic background and age. The heart is a vital organ in the body, thus it is important to keep it functional. Cardioprotection describes "all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage"(Kubler and Haass 1996).

Extensive research has been performed investigating the underlying mechanisms for cardioprotection, with the goal of decreasing mortality and morbidity associated with ischemic heart disease. This thesis focuses on one aspect in a complex array of protective mechanisms: Understanding how the arachidonic acid (AA) metabolites, epoxyeicosatrienoic acids (EETs), regulate cAMP levels. Cytochrome P450 (CYP) epoxygenase, CYP2J2, an abundant isoform in cardiomyocytes, metabolizes AA to EETs, which have been demonstrated to improve postischemic recovery of left ventricular function (Seubert, Yang et al. 2004). While the intercellular signaling pathways remain both elusive and complex for EETs, work in the present study focuses on regulation of cAMP within a renal cell model system. Investigators have shown that EETs activate Ca^{2+}-sensitive K^+ channels (BK_{Ca}) in vascular smooth muscle cells, resulting in hyperpolarization of the resting membrane potential and vasodilation of the coronary circulation (Campbell, Gebremedhin et al. 1996; Campbell and Fleming 2010). Other studies demonstrate that EETs provide a cardioprotective effect through activation of the mitochondrial

ATP-sensitive K^+ channel (Seubert, Yang et al. 2004; Nithipatikom, Endsley et al. 2006). Further evidence demonstrates that 11,12-EET increases intracellular cAMP content in heart myocytes (Xiao 2007). In this context, EETs are thought to be associated with G-protein stimulatory unit (Gαs) activation (Node, Ruan et al. 2001), which in turn will lead to activation of AC. Interestingly, activation of adenosine receptors, a G-protein-coupled receptor (GPCRs) thought to have an important role in the cardioprotection, is coupled to EET release upstream of AC activation (Carroll, Doumad et al. 2006). Evidence reveals that activation of Gαs in vascular endothelial cells mediates induction of tissue-type plasminogen activator gene transcription by EETs (Node, Ruan et al. 2001). On the other hand, EET cardioprotective effects in dogs were associated with an increase cAMP level via an inhibition of phosphodiesterase III activity (Sanada, Kitakaze et al. 2001). Indeed, there is much evidence to support the hypothesis that increasing EET levels would have beneficial effects on renal and cardiovascular function in disease states. Relevant to the current thesis, is the strong correlation between EETs and cAMP production. As such, the studies undertaken try to explore the mechanistic pathway of how EETs regulate cAMP levels in a Human Embryonic Kidney (HEK293) cell line.

1.2. G protein- coupled receptors

Seven transmembrane G protein-coupled receptors (7TM GPCRs) are an important part of cellular biology and organ function, representing one of the largest families of proteins in the human genome (Kostenis 2006). GPCRs function to transduce a wide variety of extracellular signals into a cell and have key roles in regulating cell activity and physiology. GPCRs mediate a number of essential events in cardiovascular function (Tang and Insel 2004; Lattin, Zidar et al. 2007). The activation of α- and β-adrenergic, muscarinic, angiotensin II or endothelin receptors is central to cardiac contractility, vascular resistance, and the development of the cardiovascular

system (Penela, Murga et al. 2006). GPCR malfunction has role in a wide range of diseases including cancer, diabetes and hyperthyroidism and cardiovascular (Barki-Harrington, Perrino et al. 2004), as such a large proportion of drugs on the market target these receptors (Worth, Kreuchwig et al. 2011). GPCRs have long been a useful target for a number of therapeutic agents. Such drugs include agents that work at adrenergic, histaminergic, muscarinic, and serotonergic receptors (Iyengar 1993). Understanding the signaling mechanisms of these receptors in the context of the heart and kidney is likely to provide a better therapeutic approach towards treatment of cardiovascular and kidney diseases.

1.2.1. Classification of GPCRs

Over 800 GPCRs have been cloned to date and over 1000 are suspected in the human genome (Penn, Pronin et al. 2000). In brief GPCRs can be classified into six main classes based on sequence homology and functional similarity: Class A (or 1) Rhodopsin-like, Class B (or 2) Secretin receptor family, Class C (or 3) Metabotropic glutamate/pheromone, Class D (or 4) Fungal mating pheromone receptors, Class E (or 5) Cyclic AMP receptors, and Class F (or 6) Frizzled/Smoothened (Attwood and Findlay 1994; Foord, Bonner et al. 2005; Bjarnadottir, Gloriam et al. 2006). The beta-adrenergic receptors (β-AR) are a family of GPCRs that have an important role in cardiac function and disease (Caron and Lefkowitz 1993). There are three main types of β-AR in mammalian tissues: β1-, β2- and β3-AR, which have demonstrated tissue specificity. β1-AR are the predominant receptor type found in the heart, β2-AR is the main receptor in respiratory system and β3-AR has a larger role in the adipose tissues. Furthermore, a putative β4-AR is believed to exist in tissues such as fat and heart, which are known to express high levels of β1-AR (Granneman 2001; Skeberdis 2004). Malfunction of β-AR is associated with heart diseases, for example, altered α and β adrenergic receptor signaling in vivo is

associated with cardiac hypertrophy and failure. Changes in the β-AR system such as receptor down-regulation, uncoupling from G-proteins, receptor internalization and receptor degradation may account for some of the abnormalities of contractile function in this disease. Also an increase in the level of Gαi appears to be involved in attenuating the β-AR signal (Chakraborti, Chakraborti et al. 2000; Barki-Harrington, Perrino et al. 2004).

GPCRs are activated by an external signal in the form of a ligand or other signal mediator. Ligand binding creates a conformational change within the receptor, activating the G protein. The specific downstream pathway activated or inhibited is dictated by the specific type of G protein-coupled to the receptor. G proteins are a family of complex molecules that possess the ability to interact with a variety of compounds. G proteins (guanine nucleotide-binding proteins) are a family of proteins involved in transmitting chemical signals outside the cell, and causing changes inside the cell. They communicate signals from many hormones, neurotransmitters, and other signaling factors. G proteins can regulate metabolic enzymes, ion channels, transporters and a multitude of other targets that regulate cellular function (Neves, Ram et al. 2002). G proteins are made up of α, β, and γ subunits, four main classes of G proteins can be distinguished: Gαs, which activates AC; Gαi, which inhibits AC; Gq, which activates phospholipase C; and G_{12} and G_{13}, of unknown function (Hamm 1998).

Most GPCRs are known to function via interaction with and activation of heterotrimeric G proteins (Kostenis 2006). The majority of known GPCRs preferentially couple to members of the Gi family, and Gi appears to be the most abundantly expressed heterotrimeric G protein in most cell types (Billington and Penn 2003). Upon ligand binding to the receptors at cell membrane there is dissociation of Gα unit from G betagamma unit. Gα will in sequence bind to and activate/inhibit AC enzyme which in turn will control formation of cAMP from ATP.

Conversely, interaction with Gα subunits of the Gαi type inhibits AC from generating cAMP. Thus, a GPCR coupled to Gαs will counteract the actions of a GPCR coupled to Gαi and vice versa.

1.3. G alpha i (Gαi) and G alpha s (Gαs) subunits

1.3.1 Role of different subunits in the cells activation and inhibition of AC

Gαs activation is well known to activate all 10 ACs isoforms except AC IX and will induce intracellular cAMP production, while inhibiting Gαs result in less cAMP production. Alternatively, GPCR interaction with Gα subunits of the Gαi type inhibits AC from generating cAMP. Thus, a GPCR coupled to Gαs will counteract the actions of a GPCR coupled to Gαi, and vice versa. The specificity with which G protein subunits interact with receptors and effectors defines the range of responses a cell is able to make to an external signal. Thus, the G proteins act as a critical control point that determines whether a signal spreads through several pathways or is focused to a single pathway (Neer 1994).

1.3.2. Role of G protein subunits inhibitors and activators

G protein subunit is an important component in the cell signaling. For example, the β1-AR is coupled only to G protein stimulatory unit (Gαs) and induces cAMP production, whereas the β2-AR can interact with both G protein stimulatory and inhibitory units Gαs and Gαi, respectively (Rockman, Koch et al. 2002). In the human heart β1-ARs expression is around 70% while β2-ARs expression is only 30%. Activation of β-adrenergic receptors activates AC, which leads to increased production of cAMP. However, myocardial β-AR mediate increases in heart rate and contractility in response to changes in sympathetic tone. This pathway involves increased cAMP levels. 11,12-EET increases intracellular cAMP content in heart myocytes. β_2 AR stimulation with isoproterenol results in a transient pulse of cAMP, reaching a maximal

effect at a concentration of ~10 μM and persisting for less than 5 min (Violin, DiPilato et al. 2008). cAMP promotes phosphorylation of several key proteins by PKA, which affects force development in cardiac muscle (Burns, Zhao et al. 1996; Houslay and Milligan 1997).

1.4. Adenylyl cyclase classification, function and tissue specificity

AC is an enzyme that catalyzes the conversion of ATP to adenosine-3',5' cyclic monophosphate (cAMP) and pyrophosphate. AC can be divided into membrane-bound and cytosolic forms (Wittpoth, Scholich et al. 1999). To date, nine distinct membrane-bound AC forms (types I–IX; ACI–ACIX) and one cytosolic (soluble) AC form have been cloned and characterized (Iyengar 1993; Sunahara, Dessauer et al. 1997; Geng, Wang et al. 2005). The AC enzyme is composed of at least three distinct protein components: a catalytic subunit which converts the substrate adenosine triphosphate (ATP) to cyclic AMP; a guanine nucleotide-binding protein which mediates hormonal activation; and a hormone receptor (Rodbell 1980; Hamm 1998). Mammalian ACs consists of two homologous cytoplasmic domains ($C_1$and C_2), each following one transmembrane domain (M_1 and M_2) (Taussig and Gilman 1995; Sunahara, Dessauer et al. 1996). The two cytoplasmic domains form the catalytic core; forskolin binds and activates these core domains directly (Scholich, Wittpoth et al. 1997; Yan, Huang et al. 1998).

HEK293 cells were derived by transformation of primary cultures of human embryonic kidney (HEK) cells with sheared adenovirus (Ad) 5 DNA (Graham, Smiley et al. 1977). The HEK cell line originated from epithelium and has been extensively used as an expression tool for recombinant proteins (Thomas and Smart 2005). Pertinent to the present thesis, the immortalized HEK-293 cells endogenously express GPCRs and related signaling proteins as well AC type IV, V, VI, VII and IX (Defer, Best-Belpomme et al. 2000; Atwood, Lopez et al. 2011). AC types V

and VI are the predominant isoforms detected in cardiomyocytes (Tobise, Ishikawa et al. 1994). All mammalian AC (except AC XI and soluble AC) are regulated by G proteins and by the labdane diterpene compound called forskolin. The localization of these enzymes, either to specific intracellular sites or to complexes within the cytosol, is believed to be responsible for generating gradients of cAMP. Elevation of cAMP levels occurs via two main mechanisms, either increased production or decreased breakdown. cAMP production can be stimulated by agonist stimulation of AC enzyme or inhibition of intracellular phosphodiesterase enzymes (PDE), which are responsible for the cAMP breakdown.

1.5. Forskolin and its role in controlling cAMP in the cells.

Forskolin is a chemical found in the roots of the plant Plectranthus barbatus (Coleus forskohlii) (Kreutner, Chapman et al. 1985). Interestingly, this plant has been used since ancient times to treat heart disorders such as high blood pressure and chest pain (angina) (Tirapelli, Ambrosio et al. 2010). Forskolin potently induces cAMP production via activation of AC. Forskolin can directly activate all AC isoforms except AC IX (Yan, Huang et al. 1998; Nowak and Zawilska 1999) and increase intracellular cAMP content independent of the βAR (Hearse, Zucchi et al. 1986). Research has demonstrated that forskolin, working as an inotropic agent in isolated perfused rat and guinea pig hearts, is thought to act by stimulation of AC independently of the βAR. In both species, forskolin increases heart rate, left ventricular pressure and tissue cAMP levels in a concentration-dependent manner (Buschmans, Hearse et al. 1985; Hearse, Zucchi et al. 1986; England and Shahid 1987). Forskolin is known to stimulate cAMP production either by direct activation of AC (Nowak and Zawilska 1999) or via activation of Gαs (Harry, Chen et al. 1997). In both situations it leads to accumulation of cAMP. Forskolin also promotes the formation of the catalytic core of most mammalian adenylyl cyclases by binding to C1a and

C2a at sites different from those involving Gas (Xiao, Huang et al. 1998; Yan, Huang et al. 1998).

1.6. Phosphodiesterases (PDE) expression and activity

Phosphodiesterases (PDEs) break down cAMP and/or cGMP, acting as the major determinants of cyclic nucleotide levels and cAMP degradation pathway. Selective PDE inhibitors are currently in clinical use for treatment of many clinical diseases (Francis, Blount et al. 2011). PDE tissue expression is an important issue in the PDE activity. PDE is comprised of 11 families of enzymes expressed in most tissues and in HEK 293 cells, PDE4 activity constitute about 60 % of endogenously expressed isoforms (Francis, Blount et al. 2011). Specific PDE activators and inhibitors have been used in research to investigate the importance of PDE in controlling cAMP production. It has previously been established that rolipram (a competitive selective PDE4 inhibitor) produces a greater increase in cAMP than does AC activation using forskolin or isoproterenol (Stevens, Creighton et al. 1999). Direct stimulation of AC using forskolin or inhibition of PDE4 using rolipram increased cAMP and revealed Ca^{2+} inhibition of AC6. PKA-mediated activation of PDE4 is an important mechanism for shaping GPCR-mediated signals, as an activation of PDE4 results in breakdown of cAMP (Terrin, Di Benedetto et al. 2006; Willoughby, Wong et al. 2006). Collectively, there are many factors which are responsible for the cAMP production and breakdown in the cells and can affect the cell response according to the cAMP level.

1.7. Arachidonic acid synthesis and metabolism

AA is a polyunsaturated fatty acid that can be released by phospholipases in response to several stimuli such as ischemia and/or stress (Seubert, Zeldin et al. 2007). Upon release from

cell membranes, AA can be converted to a range of eicosanoids by three principal classes of enzymes: cyclooxygenase, lipoxygenase and cytochrome P450 enzymes (Figure.1.1).

Cytochrome P450 (CYP) monooxygenase system pathway consists of two important branches that are involved in AA metabolism: ω-hydroxylase convert arachidonic acid to hydroxyeicosatetraenoic acids (HETEs) and epoxygenases convert it to epoxyeicosatrienoic acids (EETs). Epoxidation of AA by CYP enzymes generates 5,6-, 8,9-, 11,12- and 14,15-EETs which are beneficial to the heart as they reduce the ischemic-reperfusion injury (Karmazyn 1989), while CYP mediated hydroxylation of AA produces 20-hydroxyeicosatetraenoic acid (20-HETE) which has detrimental effects in the heart during ischemia, pro-inflammatory effects during reperfusion and potent vasoconstrictor effects in the coronary circulation (Seubert, Zeldin et al. 2007). Because many CYP isozymes are also capable of metabolizing arachidonic acid to biologically active products (Capdevila, Chacos et al. 1981; Schwartzman, Ferreri et al. 1985), CYP enzymes are often described as the third pathway of arachidonic acid metabolism (ie, in addition to cyclooxygenases and lipoxygenases)(Fleming 2001).

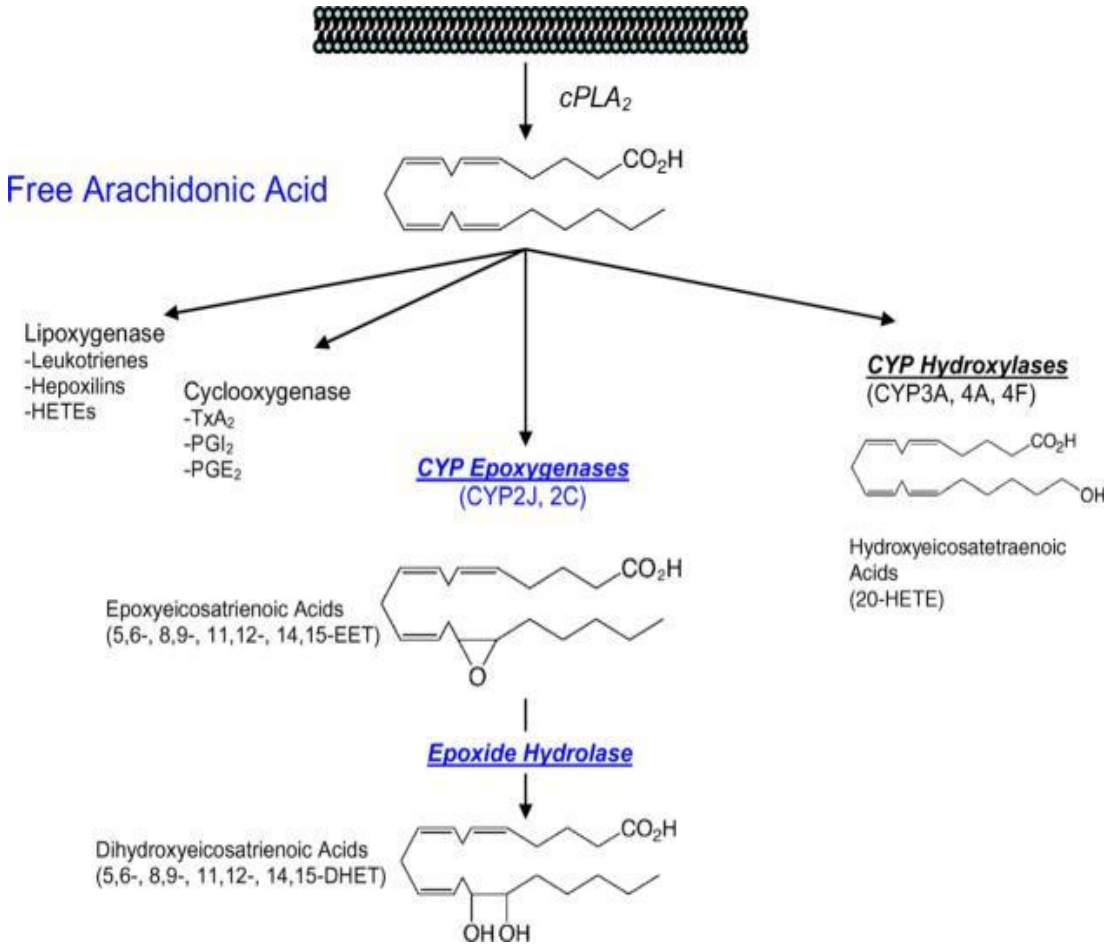


Figure 1-1: CYP epoxygenase and hydroxylase mediated metabolism of arachidonic acid. From: Seubert JM, et al. Prostaglandins Other Lipid Mediat. 2007 Jan;82(1-4):50-59.

1.7.1. Cyclooxygenase (COX) pathway

There are two isozymes of prostaglandin endoperoxide (PGH) synthase (cyclooxygenase) called PGH synthase-1 and -2, commonly knowna as COX I and II, respectively. Both isozymes catalyze the conversion of AA to PGH2, the committed step in the formation of both prostacyclin and thromboxane A2 (TX A2) (Smith, Meade et al. 1994). COX2 isoform can be induced by inflammatory stimuli (Choi, Aid et al. 2009), so it has been considered as a target for non-steroidal anti-inflamatory drugs such as Rofecoxib. Metabolism by COX isozymes gives rise to the 2-series of metabolites prostaglandins (PGs) and thromboxanes (TXs). PGs are a group of

eicosanoids which have important biological roles such as regulating the contraction and relaxation of smooth muscle tissue (Ruan, Zhou et al. 2011). COX metabolizes AA to a number of vasoactive products, with PGI_2 and PGE_2 being two major products with potent vasodilator activity (Moncada and Vane 1978)**.** COX mediated metabolism within platelets will generate compounds that causes blood clotting and constriction of blood vessels, known as thromboxanes. Two key TXs are TXA2, which is active but is very unstable undergoes hydrolysis to form TXB2, which is inactive (Michel, Silvestre et al. 2006). TXA2 is released from activated platelets, monocytes, and damaged vessel wall, and causes platelet aggregation, vasoconstriction, and hypertrophy of vascular smooth muscle (Kinsella 2001). TXA2 and its receptor (TP) are known mediators of unstable coronary disease (Fitzgerald, Roy et al. 1986), acute myocardial infarction (Dorn, Liel et al. 1990), reocclusion after coronary thrombolysis (Fitzgerald and Fitzgerald 1989) and ischemia of multiple organs (Ogletree 1987).

1.7.2. Lipoxygenase (LO) pathway

AA is metabolized via LO to the products LO-12 and LO-15. Arachidonate 12-lipoxygenase introduces molecular oxygen at carbon 12 of AA to generate a 12-hydroperoxy derivative (Yoshimoto and Takahashi 2002). The 12-LOX pathway of AA metabolism is known to be stimulated within myocardium *via* hypoxia or ischemia (Sexton, McDonald et al. 2007) and there is now a substantial body of evidence demonstrating that eicosanoids derived *via* the 12-LOX pathway are protective against the damaging effects of myocardial ischemia/reperfusion (I/R) injury (Chen, Glasgow et al. 1999; Sexton, McDonald et al. 2007). In rabbits and humans the major product is 15-HPETE, and hence the enzyme is designated a 15-LOX. The lipoxygenases (LOXs) convert AA to primary oxidation products are either reduced by

glutathione peroxidases to corresponding hydroxy derivatives to form hydroxyeicosatetraenoic acids (HETEs) or metabolized into secondary oxidized lipids including leukotrienes, lipoxins and hepoxilins, which act as lipid mediators (Brash 1999; Kuhn and O'Donnell 2006).

1.7.3. Cytochrome P450 pathway

Though several CYP isoforms that can generate EETs in humans, the predominant CYP isoforms, CYP2C8, CYP2C9 and CYP2J2, are expressed in the endothelium (Zeldin 2001; Larsen, Gutterman et al. 2006). Cytochrome P450 (CYP) epoxygenases include isozymes from the CYP1A, CYP2B, CYP2C and CYP2J sub-families, which catalyze the formation of four regioisomeric active metabolites (5,6-, 8,9-, 11,12-, and 14,15-EET) (Seubert, Zeldin et al. 2007; Katragadda, Batchu et al. 2009). CYP2J2, CYP2C8, and CYP2C9 are the predominant epoxygenase isoforms involved in EET formation. CYP2J and CYP2C gene families in humans are abundantly expressed in the endothelium, myocardium, and kidney (Zhang, El-Sikhry et al. 2009).

1.8. CYP epoxygenases

In humans, the CYP epoxygenases CYP2J and CYP2C are expressed in the cardiovascular system, namely the endothelium, vascular smooth muscle and cardiomyocytes, where they have a role in mechanisms such as protection against ischemia-reperfusion injury and inhibition of pro-inflammatory transcription factors (Spiecker and Liao 2005). A single nucleotide polymorphism found within the promoter region of CYP2J2 resulting in decreased production of EETs has been associated with coronary artery disease in a German cohort of patients (Spiecker and Liao 2006).

CYP2C isoforms are responsible for the majority of EET biosynthesis in human liver and kidney, whereas CYP2J isoforms have been proposed to be the predominant enzymes responsible for epoxidation of endogenous arachidonic acid in the rat and human heart (Wu, Chen et al. 1997). CYP2C8 genetic variations have been shown to be associated with higher risk of coronary heart disease in smokers (Lee, North et al. 2007). However, CYP2C9 is primarily localized to liver and the vascular endothelium (Halpert, Naslund et al. 1983). Evidence suggests that CYP2C9 can be associated with detrimental effects such as mitochondrial damage and worsen ischemic perfusion injury in the cardiovascular system via producing reactive oxygen species (Fichtlscherer, Dimmeler et al. 2004; Chehal and Granville 2006).

More than 90% of the plasma EETs can be found esterified to phospholipids of circulating lipoproteins and they are released upon exposure to cellular stress such as ischemia (VanRollins, Kaduce et al. 1996). EETs are present in similar amounts in heart, endothelium, and human plasma with concentrations ranging from 0.1 to 0.3μM (Node, Huo et al. 1999). EETs can be metabolized by multiple pathways, including epoxide hydration, phospholipid esterification and β-oxidation (Spector and Norris 2007). Epoxide hydrolases comprise a family of enzymes important in detoxification and conversion of lipid signaling molecules, namely EETs, to their supposedly less active form dihydroxyeicosatrienoic acids (DHETs). There are at least five epoxide hydrolase forms, including 5,6-oxide hydrolase, hepoxilin A(3) hydrolase, leukotriene A(4) hydrolase, soluble (sEH), and microsomal epoxide hydrolase (mEH) (Fretland and Omiecinski 2000). Intracellular levels of the EETs are regulated by the activity of the CYP epoxygenases that generate them as well as by the sEH (Chiamvimonvat, Ho et al. 2007). EETs are rapidly metabolized by sEH (*EPHX2*) to DHETs (Yu, Xu et al. 2000). Hydrolysis of the epoxide group of EET regioisomers by sEH leads to the generation of DHETs which limits many

of the biological actions of EETs (Karara, Wei et al. 1992). sEH activity within the tissues depends on expression within those cells, importantly, sEH is endogenously expressed in HEK293 cells. Genetic deletion of the sEH gene (*EPHX2*) as well as the pharmacological inhibition of the enzyme increase plasma EET levels and potentiates their effects (Chiamvimonvat, Ho et al. 2007). Many studies have been carried out to investigate the role sEH inhibitors and their application in the treatment of clinical conditions such as treatment of high blood pressure (Honetschlagerova, Sporkova et al. 2011; Sporkova, Kopkan et al. 2011). sEH enzyme inhibitors, such as trans-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*AUCB) have been utilized to study EET effects and found to be effective in limiting cardiac ischemia-reperfusion injury in mice (Chaudhary, Abukhashim et al. 2010).

Multiple polymorphisms in *EPHX2* have been recently reported (Sandberg, Hassett et al. 2000; Przybyla-Zawislak, Srivastava et al. 2003). An association between sEH (*EPHX2)* polymorphisms and subclinical atherosclerosis has been previously reported (Fornage, Boerwinkle et al. 2004); however, a recent study in humans (Danish population) published last year by Lee and co-workers (Lee, Dahl et al. 2010) suggested that genetically reduced sEH activity is not a major risk factor for ischemic stroke, myocardial infarction, or ischemic heart disease.

1.8.1. Intracellular targets of EETs signaling pathway

The concentration of circulating EETs and DHETs may be increased in certain diseases. Clinical studies demonstrate that an enhanced urinary excretion of DHETs accompanies hypertension (Catella, Lawson et al. 1990) and atherosclerosis (Braden, Knapp et al. 1991). The intracellular balance between epoxyeicosatrienoic, dihydroxyeicosatrienoic and

hydroxyeicosatetraenoic acids influences the biological response to these eicosanoids and alterations in their levels have recently been associated with certain pathological conditions (Kroetz and Zeldin 2002). Increased EETs in the heart activate various cardioprotective pathways leading to improved contractile function and reduced damage to the heart (Seubert, Yang et al. 2004; Gross, Gauthier et al. 2008). The most potent of these CYP products are 11,12- and 14,15-EET (Seubert, Sinal et al. 2006). Thus, CYP2J2 and arachidonic acid-derived metabolites likely play important roles in regulating cardiovascular functions and malignancy under physiological and/or pathological conditions.

EETs also have important biological functions in tissues such as kidney and gastrointestinal system. However, renal EETs possess a broad and contrasting spectrum of biological activities including vasodilatation and vasoconstriction (Schwartzman, Ferreri et al. 1985). In the kidney, some EETs (11,12- and 14,15-EET) cause vasodilation, while others (5,6-EET) can cause vasoconstriction (McGiff, Steinberg et al. 1996). EET-induced vasodilation is associated with an increased open state of calcium-activated potassium channels and hyperpolarization of the vascular smooth muscle (Campbell, Gebremedhin et al. 1996). Evidence suggests that CYP-450-dependent HETEs and EETs influence vascular tone (Harder, Campbell et al. 1995) and may be important in the progression of hypertension in animals models. EETs also increase sodium excretion (Maier and Roman 2001) and decrease cortical renin release (Henrich, Falck et al. 1990) in addition to modulating vascular diameter. EETs and/or their metabolites, DHETs, have been implicated in a variety of physiologically important processes, including the regulation of renal tubular Na^+ and K^+ fluxes, water permeability and potent systemic vasodilators and intrarenal vasoconstrictors (McGiff and Carroll 1991; Harder,

Campbell et al. 1995; Makita, Falck et al. 1996), thus implicating the regulation of EETs as a potential anti-diuretic and anti-natriuretic therapy controlling renal function.

EETs possess many potent biological effects, such as vasodilation in coronary circulation (Larsen, Miura et al. 2006), protection against ischemia-reperfusion injury (Seubert, Yang et al. 2004), as well as anti-inflammatory, thrombolytic and angiogenic properties (Node, Ruan et al. 2001; Liu, Yang et al.). In endothelial cells, EETs activate mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathways, increase intracellular cAMP levels, upregulate expression of nitric oxide synthase, and protect against hypoxia-reoxygenation injury (Node, Ruan et al. 2001; Yang, Graham et al. 2001; Wang, Wei et al. 2005). EET-mediated effects are dependent upon both concentration and site of action. For example, EET-mediated cAMP production in guinea pig mononuclear (GPM) cells occurs in a concentration-dependent manner (Wong, Lai et al. 2000). The exact mechanism(s) by which EETs and DHETs function remains unclear (Capdevila, Chacos et al. 1981). One proposed mechanism is that EETs function as an endothelium-derived hyperpolarizing factors (EDHFs), which relaxes vascular smooth muscle by hyperpolarization of the plasma membrane (Campbell, Gebremedhin et al. 1996). The vasodilatory action of EETs seems to be attributed to activation of large conductance Ca^{2+}-activated K channels (BK_{Ca}) (Fukao, Mason et al. 2001). BK_{Ca} channels play a fundamental role in the regulation of membrane potential in smooth muscle. Previous studies suggest that 11,12-EETs can relax blood vessels by activating BK_{Ca} channels in the smooth muscle via ADP-ribosylation of the G protein Gαs with a subsequent membrane delimited action on the channel (Fukao, Mason et al. 2001). Although a specific receptor has not been identified for EETs, experimental evidence suggests that such a membrane-bound receptor may exist. A high-affinity binding site for 14,15-EET was identified in human U937

cells (Wong, Lai et al. 1997) and GPM cells, Scatchard analysis of saturation binding studies yielded a dissociation constant (Kd) of 5.7 x 10(-9) M, and maximum number of binding sites (Bmax) of 2.4 pmol/mg membrane protein. (Wong, Lin et al. 1993).

EETs have potent vasodilatory properties while 20-hydroxyeicosatetraenoic acid (20-HETE) is a potent vasoconstrictor. Both effects are mediated through actions on large conductance Ca^{2+}-activated K^+ channels (Kroetz and Zeldin 2002). Interestingly, there is species and tissues specificity of EET action, at endothelial and smooth muscle cells, cardiac and non-cardiac tissues**.** In the coronary artery, the endothelium produces EETs; however, 20-HETE is not made by the coronary smooth muscle (Rosolowsky and Campbell 1993; Rosolowsky and Campbell 1996)**.** In contrast, CYP 2C produces vasodilatory EETs in the endothelium while CYP 4A produces the vasoconstrictor 20-HETE in the smooth muscle. However, EETs are known as potent vasoconstrictors in the pulmonary circulation (Keseru, Barbosa-Sicard et al. 2008). This was correlated with sEH expression in the pulmonary vasculature; however, decrease in sEH expression is intimately linked to pathophysiology of hypoxia-induced pulmonary hypertension.

Experiments performed in mouse neonatal cardiomyocytes suggest that the catecholamine-dependent increase of cardiac contraction rate is regulated predominantly by the activation of the cAMP pathways (Devic, Xiang et al. 2001). Consistent with this notion are reports that suggest EETs work through receptors in the cells to increase cAMP level, for example; adenosine 2A receptor (A2AR) vasodilation of rat preglomerular microvessels can be mediated by EETs via a cAMP/PKA pathway (Carroll, Doumad et al. 2006). Data demonstrated that activation of A2AR was coupled to EET release, upstream of AC activation, where EETs stimulated mono-ADP-ribosyltransferase resulting in Gαs protein activation (Carroll, Doumad et al. 2006). EET have antihypertensive and anti-inflammatory properties and play a role in the maintenance of renal

vascular function (Zhao, Yamamoto et al. 2004). Within the vasculature system, EETs released from endothelium are transferred to smooth muscle cells where they cause hyperpolarization and relaxation (Campbell and Fleming 2010). In contrast, hepatic EETs, particularly 11,12-EET, cause vasoconstriction of the porto-sinusoidal circulation and may participate in the pathophysiology of portal hypertension of cirrhosis (Sacerdoti, Gatta et al. 2003).

The role of EETs in protection against ischemia-reperfusion injury comes from studies that increase the levels of EETs (CYP 2J2 is abundant in heart and active in the biosynthesis of EETs and CYP2J2 have been shown to produce increases in postischemic function (Seubert, Yang et al. 2004; Nithipatikom, Moore et al. 2006)) or prevent EETs removal (sEH enzyme is responsible for conversion of EET to its metabolite (DHET), thus, inhibition of sEH by t-AUCB significantly improved postischemic left ventricular developed pressure (LVDP) recovery and reduced the infarct size after ischemia and reperfusion, as compared with control (Chaudhary, Abukhashim et al. 2010)). Transgenic mice with cardiomyocyte specific overexpression of human CYP2J2 have an improvement in functional recovery after 20 min of global ischemia and 40 min of reperfusion (Seubert, Yang et al. 2004)**.** Moreover, knockout of sEH in mice results in protective effects against ischemia-reperfusion injury in the heart (Seubert, Sinal et al. 2006) and brain (Zhang, Otsuka et al. 2008). However, the exact mechanism by which EETs prevent or protect against injury remains elusive. Evidence suggests that EET-mediated increase in cAMP content within cardiomyocytes is correlated to cardioprotection after ischemia in canine hearts (Asanuma, Kitakaze et al. 2001). Interestingly, EETs enhance the L-type Ca^{2+}- current (ICa,L) in rat cardiomyocytes via intracellular cAMP-dependent mechanism (Xiao, Huang et al. 1998). Together, these data suggest that a potential mechanism for EET-mediated protection involves regulation and/or enhancement of cAMP production.

Investigators have suggested that the signal transduction mechanism of 14,15-EET begins with the binding of the receptor, which leads to the increase of intracellular cAMP levels and the activation of PKA, and finally with the down regulation of 14,15-EET receptor binding (Wong, Lai et al. 1997). However, no known receptor has been identified. It has been well established that EETs can activate ion channels, such as L-type calcium channel, through modulation of intracellular cAMP and PKA protein levels (Xiao 2007). We and others have demonstrated EET-mediated reduction in postischemic infarction and improved contractile function involves PI3K pathway (Bodiga, Zhang et al. 2009; Chaudhary, Abukhashim et al. 2010). Consistent with our data, Dhanasekaran et al. reported 14,15 EET-mediated activation of multiple anti-apoptotic targets through PI3K/Akt survival signaling (Dhanasekaran, Gruenloh et al. 2008). Interestingly, data from our lab suggested that EETs have an important intracellular action at the mitochondria, where they limit damage to function following ischemic injury (Katragadda, Batchu et al. 2009).

1.8.2 Role of EETs in cancer tissue/cells

Eicosanoids play a regulatory role in inflammation and, therefore, they have proved to be involved in different pathological states such as cancer, atherosclerosis, arthritis and cardiovascular or immunological diseases (Ferreiro-Vera, Mata-Granados et al.). Previous studies demonstrated a role for CYP2J2-derived EETs in promoting the neoplastic phenotype of cancer cells, which increased CYP2J2 expression or activity was associated with increased tumor growth and lung metastasis (Jiang, Ning et al. 2007). EETs have been reported to induce angiogenesis and promote cell growth and multiplication and this might be an important issue in the case of the cancer cells. The anti-apoptotic effects of CYP epoxygenase overexpression were significantly attenuated by the inhibition of the phosphoatidylinositol 3-kinase (PI3K)/AKT and MAPK signaling pathways (Jiang, Ning et al. 2007). Indeed, regulation of EET levels is

dependent upon the tissue and whether more EET bioavailability will be cytoprotective or detrimental.

1.8.3 EET regioisomers and stereo-specificity

The biological properties of eicosanoids are derived from the enzymatic, regio-, and stereoselective oxygenation of AA. There are four regioisomers of EET which include 5,6-, 8,9-, 11,12- and 14,15-EET and there are association with tissue EET regioisomers specificity. Many studies have shown that the different EET stereo-specificity with different biological activity in the tissues. Of the 5,6-, 8,9-, 11,12-, and 14,15-EET that can be generated from arachidonic acid, the (R),(S) or the (S),(R) enantiomers can be formed. Differences in biological activity have been reported in as much as the R,S-isomer of 11,12-EET was reported to be the active enantiomer and a potent dilator of small renal arteries at a concentration of 1–100 nmol/L (Zou, Fleming et al. 1996). Also it has been reported that P-450 2C23 product, the 11(R),12(S)-EET, as the major rat kidney epoxygenase metabolite and the renal P-450 isoform regulated by excess dietary salt intake Interestingly, the 11(R),12(S)-EET relaxes the rat renal artery while the 11 (S),12(R)-EET isomer is inactive (Zou, Fleming et al. 1996).

1.9 Role of DHET in the cells

Data from intact human blood vessels indicate that conversion of 11,12- and 14,15-EET to the corresponding DHET metabolites is the predominant pathway for metabolism and that sEH inhibition can modulate EET metabolism in vascular tissue (Fang, Weintraub et al. 2004). While the dihydroxy- metabolites of EETs are the main bi-product, relatively little is known regarding any physiological function. DHETs have been shown to activate BKCa channels in coronary artery myocytes producing vasodilation, as well data demonstrate DHETs are inhibitors

of the hydroosmotic effect of arginine vasopressin (AVP) in the kidney (Hirt, Capdevila et al. 1989). AVP's hydroosmotic effect is mediated through its intracellular second messenger, cAMP. It has been reported that 11,12-DHET is more potent than its parent compound 11,12-EET in relaxing canine coronary microvessels and the effects of 11,12-DHET reached a steady state in about 4 min (Oltman, Weintraub et al. 1998). Finally, studies on toad urinary bladders have shown that EETs and DHETs inhibit vasopressin-induced osmotic water flow (Schlondorff, Petty et al. 1987).

1.10 Putative EET receptor antagonist

Data from studies utilizing a putative EET receptor antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) have suggested that EET-mediated effects might occur through a specific receptor (Gross, Gauthier et al. 2008). However, to date, no specific EET receptor(s) has been identified. For example, we previously demonstrated that perfusion of isolated mice heart with 14,15-EEZE, prior to ischemia abolished EET-mediated cardioprotective responses (Chaudhary, Abukhashim et al. 2010). Thus, the identification and functional characterization of the EET receptor(s) and development of specific EET receptor agonists and antagonists would greatly aid future research in this field and might lead to the development of novel therapeutics for vascular protection.

1.11. Thesis objective

The central goal of my thesis was to investigate how EETs regulate cAMP production. Two questions were addressed in this study: (1) Do EETs enhance cAMP levels within the cell?; and (2) What is the role of DHETs in regulating cAMP? HEK 293 cells were used to investigate the mechanistic role of EETs and its metabolite, DHET, in regulating forskolin-induced cAMP production.

1.12. Hypothesis

CYP-derived metabolites of arachidonic acid, EETs, regulate forskolin-induced cAMP production via G proteins in HEK293 cells.

1.13. Thesis structure

This work has been prepared in three chapters: Introduction, results and discussion. Chapter 1; provides a general introduction and background of the research project, chapter 2; contains the research project and results which have been published in Cell and Toxicology Journal Oct 2011, and chapter 3; is the overall thesis discussion and future directions.

Chapter 2

REGULATION OF FORSKOLIN INDUCED cAMP PRODUCTION BY CYTOCHROME P450 EPOXYGENASE METABOLITES OF ARACHIDONIC ACID IN HEK293 CELLS

A version of chapter 2 has been published. Cell Biol Toxicol. 2011 Oct; 27(5):321-32

2.1 Introduction

Activation of adenylyl cyclase results in the production of cyclic adenosine monophosphate (cAMP), which is an important intracellular second messenger. cAMP signaling leads to a diverse array of cellular responses with distinct downstream effects and as such correct and accurate delivery is imperative for appropriate cellular response (Zaccolo 2009). While the basic mechanism of cAMP-mediated signaling, involving response to hormonal and neurotransmitters binding to GPCRs, has been well established, our understanding of the complex regulation required for specific responses remains incomplete (Adderley, Sprague et al. 2010). Emerging evidence has demonstrated that factors such as phosphodiesterase, protein kinases and cellular compartmentalization play a key role in shaping distinct cAMP signals (Dessauer 2009).

Epoxyeicosatrienoic acids (EETs) are derived from the metabolism of AA by cytochrome P450 epoxygenases, resulting four regioisomers, 5,6-, 8,9-, 11,12- and 14,15-EET (Falck, Schueler et al. 1987; McGiff and Carroll 1991). Modulation of EET levels can occur by conjugation, chain elongation, β-oxidation, and esterification, resulting in reincorporation into phospholipid membranes (Fang, Kaduce et al. 2001; Fang, Weintraub et al. 2004; Larsen, Miura et al. 2006; Falck, Kodela et al. 2009). However, the predominant pathway of inactivation is the formation of vicinal diol compounds by soluble epoxide hydrolase (sEH) (Pain T 2000; Newman, Morisseau et al. 2005). EETs are important components of many intracellular signaling pathways, for example, EETs activate Ca^{2+}-sensitive K^+ channels (BK_{Ca}) in vascular smooth muscle cells, resulting in hyperpolarization of the resting membrane potential and vasodilation of the coronary circulation (Campbell, Gebremedhin et al. 1996; Campbell and Fleming 2010). While the mechanisms triggering the intracellular signaling effects of EETs

remain uncertain, evidence suggests that EET-mediated events increase cAMP content in certain cell types. EET-mediated enhancement of L-type Ca^{2+}- current in rat cardiomyocytes occurs via increased intracellular cAMP content (Xiao, Huang et al. 1998). Moreover, 11,12-EET activation of BK_{Ca} channels and tissue plasminogen activator expression is mediated by the Gαs-component of a heterotrimeric GTP binding protein (Li, Chen et al. 1999; Node, Ruan et al. 2001). In contrast, data demonstrate that dihydroxyeicosatrienoic acids (DHETs), the metabolites of EETs, have inhibitory effects on cAMP signaling that can impede such processes as hydroosmotic regulation (Schlondorff, Petty et al. 1987; Hirt, Capdevila et al. 1989).

In the present study, we examine the effects of EETs and their diol metabolites, DHETs, on forskolin- or epinephrine-induced activation of adenylyl cyclase in HEK 293 cells. We demonstrate that DHETs are responsible for the observed changes in cAMP levels and inhibit production via phosphodiesterase (PDE4) and Gαi proteins. Thus, our data provide evidence of a role for endogenous fatty acids in regulating cAMP levels in HEK 293 cells.

2.2 Materials and Methods

2.2.1. Cell culture and chemicals

Human embryonic kidney cells (HEK 293) cells (American Type Culture Collections, Manassas, VA) were cultured in 75cm ^{2} flasks at 37°C in an humidified atmosphere of 5% CO_2/ 95% air. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution (10,000U/ml penicillin, 10mg/ml streptomycin, and 25μg/ml amphotericin B) (Gibco, Invitrogen, Carlsbad, CA). Stock solutions of 11,12-EET, 11,12-DHET (Cayman Chemicals, USA) and 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE generous gift from Dr. John Falck) were prepared in 100% ethanol and stored at -80^{o}C. Forskolin was dissolved in dimethylsulfoxide (DMSO); pertussis toxin (PTX), SQ 22,536 (adenylyl cyclase inhibitor) and guanosine 5'-O-(2-thiodiphosphate) trilithium salt (GDPβS) were dissolved in deionized water (Sigma-Aldrich). Epinephrine (CalBiochem/EMD) and trans-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*AUCB) (generous gift from Dr. Bruce Hammock) were dissolved in DMSO. 5-isoquinolinesulfonamide (H89) and rolipram were dissolved in deionized water (Sigma-Aldrich). One day prior to experiments, cells were seeded (2x10^5 cells/well) into 6-well microtitre plates with DMEM containing 10% FBS and 1% penicillin-streptomycin (Gibco, Invitrogen, Carlsbad, CA). All experiments were performed using cells with passage numbers between 17-31.

2.2.2. Cellular protocols

We established a concentration-response relationship for EET-induced cAMP production in HEK 293 cells to confirm published literature. HEK 293 cells were incubated with 0, 0.001, 1 or 10 μM of 11,12-EET for 5 or 30 min. Intracellular cAMP levels were measured by using

commercially available kit (Mediomics). The fluorescent signal was determined using a spectrofluorometric BioTek Synergy2 plate reader (480±20 nm excitation, 520±20 nm emission). Determinations were routinely done in triplicate and for each set of incubations a reaction blank without drug (control) was used. The total amount of cAMP present was calculated from a standard curve and expressed as picomoles per 500,000 cells per well.

To investigate whether EETs caused an additive effect, cells were co-treated with forskolin (50 μM) and either of 11,12-EET (0.001, 0.01, 0.1, 1 or 10 μM) or 11,12-DHET (0.001, 0.01, 0.1 or 1μM) for 5 or 30 min. To determine the effect of diol metabolites, cells were co-treated with forskolin (50 μM), 11,12-EET (1 μM) and *t*AUCB or directly with 11,12-DHET (0.01, 0.1 or 1 μM). Inhibition experiments were performed by co-treating cells with 11,12-EET (1 μM) or 11,12-DHET (1 μM) and either SQ 22,536 (10 μM, adenylyl cyclase inhibitor), H89 (10 μM, PKA inhibitor) or 14,15-EEZE (1 μM, putative EET receptor antagonist). To determine the role of Gαs, cells were co-treated with forskolin (50μM), 11,12-EET (1 μM) or 11,12-DHET (1 μM) and GDPβs (1 μM). To investigate the involvement of Gαi towards cAMP production, cells were pretreated for 12h with pertussis toxin (200 ng/ml), followed by co-treatments with forskolin (50 μM) and 11,12-EET (1 μM) or 11,12-DHET (1μM).

To evaluate the role of phosphodiesterase (PDE), we pretreated cells with rolipram (10 μM, PDE4 inhibitor) for 10 min prior to co-treatment with forskolin (50 μM) and 11,12-EET or 11,12-DHET (1 μM). In separate experiments, PDE activity was assessed using a commercially available kit (Mediomics). Briefly, cells were co-treated with either rolipram (10 μM) or forskolin (50 μM) and 11,12-DHET (1 μM) for 5 min. Following cell lysis, PDE activity in 5

μug total protein was determined by the depletion of cAMP from the reaction mix using a spectrofluorometric BioTek Synergy2 plate reader (480±20 nm excitation, 520±20 nm emission).

Determinations were routinely done in triplicate and for each set of incubations a reaction blank without drug (control) was used. PDE relative activity (RA) was calculated using the formula: RA=(Fs-Fpc)/(Fnc-Fpc), where Fs is the fluorescence signal from the sample, Fpc is the positive control and Fnc is the negative control. Results are then expressed as the percentage increase above vehicle control.

2.2.3. Immunoblotting

Sub-cellular fractions were prepared from HEK293 cells and proteins were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Batchu, Law et al. 2009). Immunoblots were then probed with antibodies to phosphorylated and total AKT (1:1000), (Cell Signalling Technology, Inc. USA), sEH (1:1000, generous gift from Dr. Bruce Hammock, UC Davis) and GAPDH (1:1000) (Santa Cruz Biotech., USA). Relative band intensities were expressed in arbitrary units assessed using Image J software (USA, NIH, Bethesda, MD).

2.2.4. Statistical analysis

Statistical significance was determined using the unpaired Student's t-test and one-way ANOVA followed by a Duncan's test to assess multiple group comparisons where applicable. Results were considered significant if $p < 0.05$.

2.3 Results

2.3.1. Effects of EETs on cAMP levels

Consistent with published reports, we observed that incubating HEK 293 cells with 11,12-EETs for 5 minutes did not cause a significant increase in cAMP levels until concentrations of 10 μM were utilized, which then resulted in an approximately 4 to 5-fold increase above vehicle controls (Fig.2.1 A). Incubation with 11,12-DHET failed to increase cAMP levels (Fig.2.1 A). In contrast, co-treatment of 11,12-EETs with forskolin resulted in a significant dose-dependent inhibition of forskolin-induced cAMP production (Fig.2.1 B). Based on the initial concentration-response data, all subsequent studies were conducted using 1 μM of 11,12-EET. Increasing the incubation time of 11,12-EET and forskolin from 5 min to 30 min resulted in a greater inhibition of forskolin-induced cAMP production (1.5-fold vs. 4-fold inhibition, respectively) (Fig.2.2 A). While the different time points indicated the same inhibitory trends, we found that EET-induced cAMP inhibition is time-dependent and more prominent at 30 min. Therefore, subsequent experiments were done with a 30 min incubation time.

To investigate whether the EET-mediated inhibition of cAMP production was attributed to an interaction with forskolin, we co-treated HEK 293 cells with 11,12-EET and a different AC activator, epinephrine (10 μM), as a positive control. Consistent with the above results, we observed an approximately 5-fold inhibition of epinephrine-induced cAMP production by co-treatment with 11,12-EET (Fig.2.2 B). We and others have demonstrated that EET-mediated events result in activation of Akt; evidence demonstrates that cAMP can activate Akt (Sable, Filippa et al. 1997; Jiang, Ning et al. 2007; Dhanasekaran, Gruenloh et al. 2008; Chaudhary,

Batchu et al. 2009; Batchu, Lee et al. 2011). Therefore, we assessed the effects of EETs on epinephrine or forskolin-induced phosphorylation of Akt. Cells treated with 11,12-EET, epinephrine or forskolin for 30min had increased expression of phospho-Akt (Fig.2.2 C). Interestingly, co-incubation of 11,12-EET with epinephrine or forskolin resulted in a moderate reduction in phospho-Akt expression levels (Fig.2.2 C). Further experiments looked at the effect of SQ 22,536 (200 μM) as a negative control to inhibit AC, and, as expected, the results showed that there was a significant inhibition of forskolin-induced cAMP production (25.3±1; 11.7±1.3; 7.35±1.2 pmol cAMP/500,000; forskolin, forskolin+11,12-EET, forskolin+SQ,22,536, respectively).

2.3.2. DHET inhibition of cAMP production

Increased inhibition of forskolin-mediated cAMP production by 11,12-EET over the longer 30 min time course suggested the potential involvement of an EET metabolite such as the vicinal diol, 11,12-DHET. In order to determine whether the cAMP inhibitory effect was mediated via the parent compound or its metabolite (DHET), we inhibited the conversion of EET to DHET by sEH with the specific inhibitor *t*AUCB (0.1 μM). Our data demonstrated that inhibition of sEH activity with *t*AUCB significantly increased 11,12-EET-mediated (1 μM) cAMP production over controls by 3-fold (Fig. 3 A). Interestingly, co-treatment of cells with forskolin, 11,12-EET and *t*AUCB resulted in an 8-fold increase in cAMP production compared to control (Fig.3A), suggesting DHETs were involved in inhibiting cAMP. To directly investigate the role of these metabolites, we treated cells with 11,12-DHET. The data demonstrated that 11,12-DHET caused a dose-dependent decrease in forskolin-induced cAMP production (Fig.2.3 B). Immunoblot analysis demonstrated that expression of sEH in HEK293 cells was not affected by any of the treatment groups (Fig.2.3 C).

2.3.3. EET-mediated regulation of cAMP

G protein subunits, Gαs and Gαi, are important elements in intracellular signaling pathways involved in regulating chemical signals such as cAMP. Previously published data has demonstrated that EET-mediated events involve Gαs coupled signals (Li, Chen et al. 1999; Node, Ruan et al. 2001). An important consideration in this study was to investigate the role of Gα proteins, which was accomplished by blocking Gα subunits with a non-specific inhibitor, GDPβS. Cells were pretreated with GDPβS (1μM) for 10 min before being stimulated with 11,12-EET (1 μM) and/or forskolin (50 μM). Inhibition of Gα subunits prevented 11,12-EET-mediated effects on forskolin-induced cAMP production (Fig.2.4 A). Further experiments were performed to investigate the role of Gαi protein by pretreating cells with pertussis toxin (200 ng/ml, 3-4 hours) before cell stimulation with 11,12-EET and/or forskolin. Inhibition of Gαi protein by pertussis toxin prevented the EET-mediated effects on cAMP production; no significant difference was noted between forskolin used alone or when added simultaneously with EET (Fig.2.4 B).

Evidence shows that protein kinase A (PKA) events are involved in switching intracellular signals from Gs to Gi to initiate downstream events that can turn off cAMP production (Daaka, Luttrell et al. 1997). In the present study, HEK 293 cells were pretreated for 10 min with the PKA inhibitor, H89, prior to co-treatment with 11,12-EET and/or forskolin (50 μM) for 30 min. Treating cells with H89 (10 μM) failed to prevent the inhibition of forskolin-mediated cAMP production by 11,12-EET suggesting the inhibitory effects were not attributable to PKA (Fig.2. 4 C).

Finally, we investigated the role of phosphodiesterase (PDE) in the downstream regulation of cAMP, by treating HEK 293 cells with rolipram (PDE4 inhibitor, 10 μM) for 10 min prior to co-treatment with 11,12-EET and/or forskolin for 30 min. We observed that rolipram treatment blocked the 11,12-EET-mediated inhibition of forskolin-induced cAMP production (Fig.2.4 D).

2.3.4. DHET- mediated regulation of cAMP

Next, we investigated the potential mechanisms of 11,12-DHET-mediated inhibition. Cells were pretreated with GDPβS (1 μM) for 10 min before being stimulated with 11,12-DHET (1 μM) and/or forskolin (50 μM). As with the EETs, the inhibition of Gα subunits prevented 11,12-DHET-mediated effects on forskolin-induced cAMP production (Fig.2.5 A). Similar experiments were also carried out using Gαi inhibitor, pertussis toxin, and PKA inhibitor, H89, showing the 11,12-DHETs showed similar effects as 11,12-EETs (Fig. 2.5 B,C).

Next, we investigated role of phosphodiesterase (PDE) in the 11,12-DHET-mediated effects. Pretreated of cells with rolipram (10μM) for 10 min prior to co-treatment with 11,12-DHET and/or forskolin for 30 min blocked the 11,12-DHET-mediated inhibition of forskolin-induced cAMP production (Fig.2.5D). Analysis of enzymatic activity demonstrated that co-treatment of 11,12-DHET and forskolin for 30min significantly increased HEK 293 PDE activity (Fig.2.5 E); however, we found that this increased PDE activity was blocked by rolipram (Fig.2.5 E).

Finally, to confirm our observations could be attributed to EETs and DHETs, we conducted experiments in the presence of 14,15-EEZE. Interestingly, this putative pan-EET

receptor antagonist abrogated the effect both 11,12-EET and 11,12-DHET had toward forskolin-induced cAMP production after 30 minutes (Fig.2.6).

2.4 Discussion

Our studies, consistent with other reports, show that 11,12-EET enhances cAMP response in a concentration- and time-dependent manner in HEK 293 cells. Interestingly, our data demonstrated that 11,12-EET inhibited forskolin and epinephrine-induced cAMP production in HEK 293 cells. The inhibitory effect of 11,12-EET was abolished when cells were co-treated with a sEH inhibitor, *t*AUCB, supported by data demonstrating that 11,12-DHET inhibited forskolin-induced cAMP production. Inhibition of Gαi with pertussis toxin, or PDE4 with rolipram, abolished the inhibitory effects of 11,12-EET and 11,12-DHET on forskolin-induced cAMP production. Taken together, our data suggest a regulatory mechanism in which EETs will activate or augment cAMP production to a certain level, and then, subsequent conversion to DHETs will inhibit cAMP production.

Both forskolin and epinephrine are known to activate AC and increase cAMP in different cells, including HEK 293 cells (Insel and Ostrom 2003). We hypothesized that the addition of exogenous EETs would result in synergistic effect with forskolin. However, the addition of 11,12-EET resulted in significant inhibition of forskolin-induced cAMP production. Our results demonstrated that co-treatment with the sEH inhibitor, *t*AUCB, resulted in a loss of the inhibitory effect of EET toward forskolin; moreover, it significantly increased EET-mediated cAMP production. Consistent with previous studies that show HEK 293 cells possess active sEH enzymes, we demonstrate significant protein expression (Barbosa-Sicard, Fromel et al. 2009). Collectively, this suggests that the inhibitory effect of 11,12-EET on cAMP production might be due to its conversion to downstream active metabolites (11,12-DHET). To address this question directly, we demonstrated that co-treatment of 11,12-DHET inhibited forskolin-induced cAMP production.

In order to determine the relative importance of EETs and DHETs in this model, we utilized 14,15-EEZE, which has been shown to be inhibit both EETs and DHET-mediated EET effects within the cardiovascular system (Falck, Krishna et al. 2003; Gauthier, Jagadeesh et al. 2003; Kopf, Gauthier et al. 2011). Interestingly, 14,15-EEZE abolished 11,12-DHET-mediated inhibition of forskolin-induced cAMP production. The antagonistic effects are consistent with a recent report demonstrating 14,15-EEZE can antagonize relaxation effects of all three regioisomers, 8,9-, 11,12- and 14,15-DHET (Kopf, Gauthier et al. 2011). While there is no known receptor these data confirm the inhibitory effect of forskolin-induced cAMP production was attributed to 11,12-DHET.

How do EET-mediated events regulate forskolin-induced cAMP production? Intracellular concentrations of cAMP are tightly controlled through regulation of AC and removal by PDE hydrolysis. Early evidence has shown that increased cAMP levels can result in PKA activation which will negatively feedback to decrease G-protein interactions decreasing production (Daaka, Luttrell et al. 1997). In the present report, extracellular application of H89 did not significantly alter the EET-mediated cAMP inhibition. Therefore, it appears that the PKA-dependent reduction of cAMP levels does not play a significant role in the EET-mediated inhibition. Control of intracellular cAMP production can occur by a 'switching' process in which receptor coupling to Gαs switches them to Gαi proteins after agonist stimulation (Daaka, Luttrell et al. 1997). Gαi activation is typically associated with the inhibition of Gαs stimulated AC activity and thus reduced cAMP generation (Barnes 1998; Johnston and Watts 2003; Watts and Neve 2005). While there is no known EET receptor identified to date, previous data showed that EETs activate Gαs but not Gαi in endothelial cells and are known to stimulate the ADP-ribosylation of Gαs in vascular smooth muscle cells (Fang, Weintraub et al. 1999; Devic, Xiang et al. 2001).

Our data, demonstrating a reduction of the DHET-inhibitory effect by GDPβS and PTX suggest that following enhanced intracellular cAMP production, 11,12-DHET will activate Gαi to work as a negative control mechanism to reduce intracellular levels. However, it remains unknown whether 11,12-DHET directly activates Gαi subunits or whether it initiates a other mechanisms following activation of the Gαs subunit, resulting in inhibition of cAMP production.

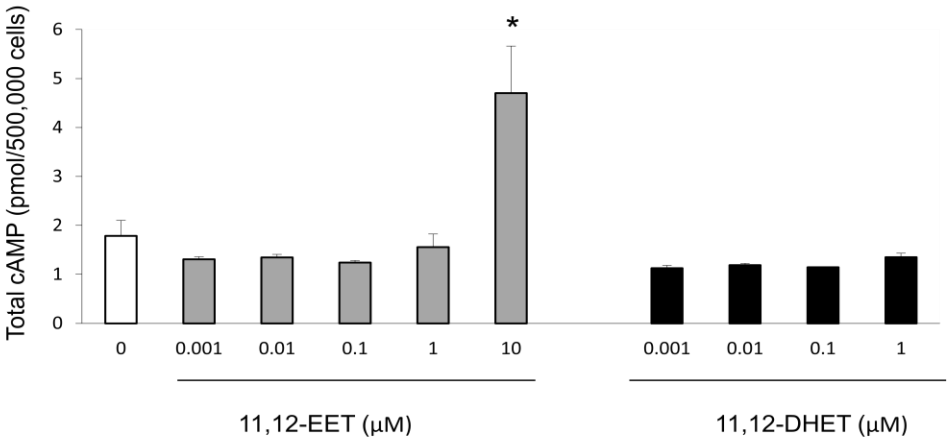
The PDE4 isoform is known to play an important role in controlling the intracellular cAMP pool by targeting certain proteins, such as arrestins, SRC family tyrosyl kinases (Houslay and Adams 2003). Therefore, another possible mechanism by which EET-mediated events inhibit cAMP production might be through PDE activation. In fact, HEK 293 cells treated with EET or DHET, forskolin and rolipram exhibited a marked increase in cAMP stimulation, suggesting that activation of PDE4 by DHET does play a role in the observed effects. While our data does not conclusively demonstrate that 11,12-DHET can directly activates PDE activity, the enhanced activation of PDE observed with the co-treatment of DHET and forskolin supports this notion. It remains unknown how 11,12-DHET can enhance PDE activity but would suggest it works as feedback mechanism limiting production of cAMP.

There is a growing amount of literature demonstrating EETs act as intracellular signaling molecules having important physiological roles in maintaining tissue homeostasis (Spector and Norris 2007). It is generally considered that the conversion to DHETs, via sEH, is a process that reduces EET bioactivity. While there is limited information regarding the intracellular role of DHETs, studies have shown they possess biological activity. For example, in kidney, DHETs are associated with relaxation of the preglomerular vasculature, calcium mobilization, stimulation of ADP-ribosylation and inhibition of the hydrosmotic action of arginine vasopressin (Hirt, Capdevila et al. 1989; Seki, Hirai et al. 1992; Fang, Weintraub et al. 1999; Imig 2005).

Furthermore, evidence has suggested that DHETs are highly potent activators of large conductance Ca^{2+} activated-K^{+} channels in smooth muscle cells from rat coronary arteries, where they might act as endogenous vasodilators (Lu, Katakam et al. 2001). Our findings are consistent with Schlondorff and co-workers who reported the inhibitory effect of DHET on forskolin-mediated enhance adenylyl cyclase activity in toad bladder (Schlondorff, Petty et al. 1987).

In conclusion, we report a novel response system in HEK 293 cells, suggesting that EETs and DHETs work as endogenous regulators of cAMP production. In this system, EETs will activate and/or enhance cAMP production to a maximal response, and then subsequent production of the DHET metabolite, by soluble epoxide hydrolase, will work as a negative controller to limit cAMP production. Our data provide potential insight into the regulation of AC and G-protein coupling by endogenous fatty acids in HEK 293 cells.

A.



B.

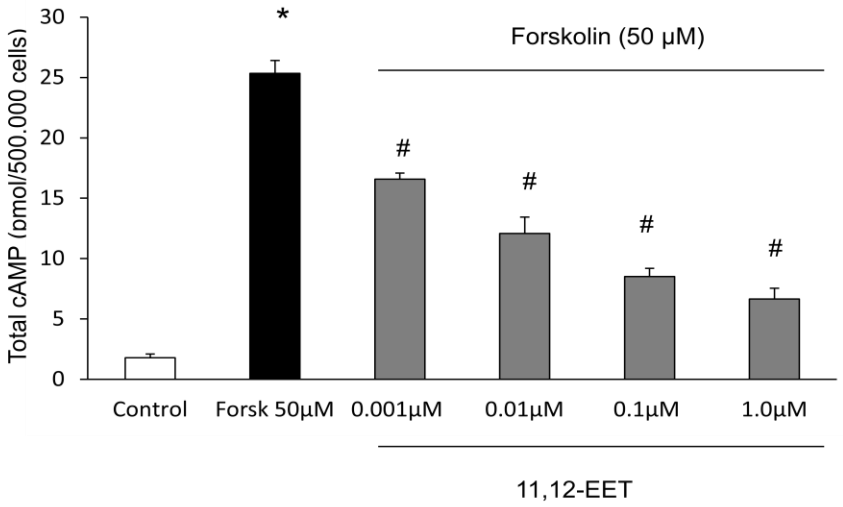


Figure 2-1: Effect of 11, 12-EET on cAMP production

(A) HEK 293 cells were incubated for 30 min with vehicle, 11,12-EET (0.001, 0.01, 1 or 10 μM), or its metabolite 11,12-DHET (0.001, 0.01 or 1 μM). Total cAMP levels were determined by extrapolation from a standard curve. Values represent mean±SEM; * p <0.05 vs. control; N=5-7, repeat experiments. (B) HEK 293 cells were incubated for 30 min with vehicle, forskolin (50 μM, AC activator), or forskolin (50 μM) and 11,12-EET (0.001, 1 or 10 μM). Values shown are mean±SEM; N= 3; '*'p <0.05 vs. control; '#' p <0.05 vs. forskolin alone.

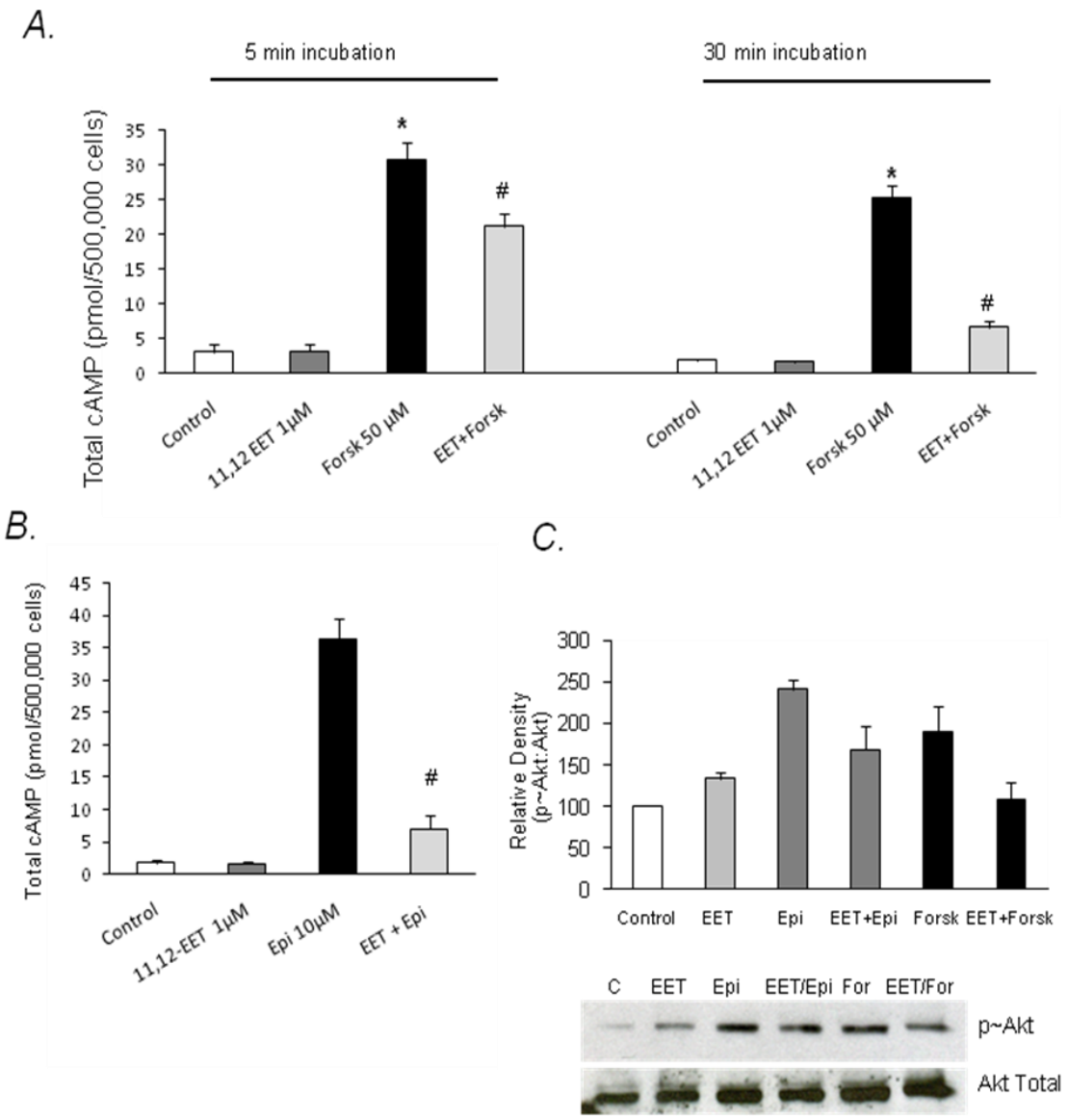


Figure 2-2: Time-dependent effect of 11,12-EET on cAMP production

(A) HEK 293 cells were incubated for 5 or 30 min with vehicle, 11,12-EET (1 µM), forskolin (50 µM) or 11,12-EET with forskolin. Total cAMP levels were determined by extrapolation from a standard curve. Values shown are mean±SEM; N=3; '*' p <0.05 vs. control; '#' p <0.05 vs. forskolin alone. (B) HEK 293 cells were incubated for 30 min with vehicle, 11,12-EET (1 µM), epinephrine (10 µM, AC activator) or 11,12-EET with epinephrine. Values shown are mean±SEM; N=3; '*' p <0.05 vs. control; '+' p <0.05 vs. epinephrine alone. (C) HEK 293 cells were incubated for 30 min with vehicle, 11,12-EET (1 µM), epinephrine (10 µM) or 11,12-EET with epinephrine The phosphorylation state of Akt was analyzed by immunoblotting. The histogram represents the ratio of phorphorylated protein to total protein measured by densitometry. Values represent the mean±SEM; N= 3; '*' p <0.05 vs. control.

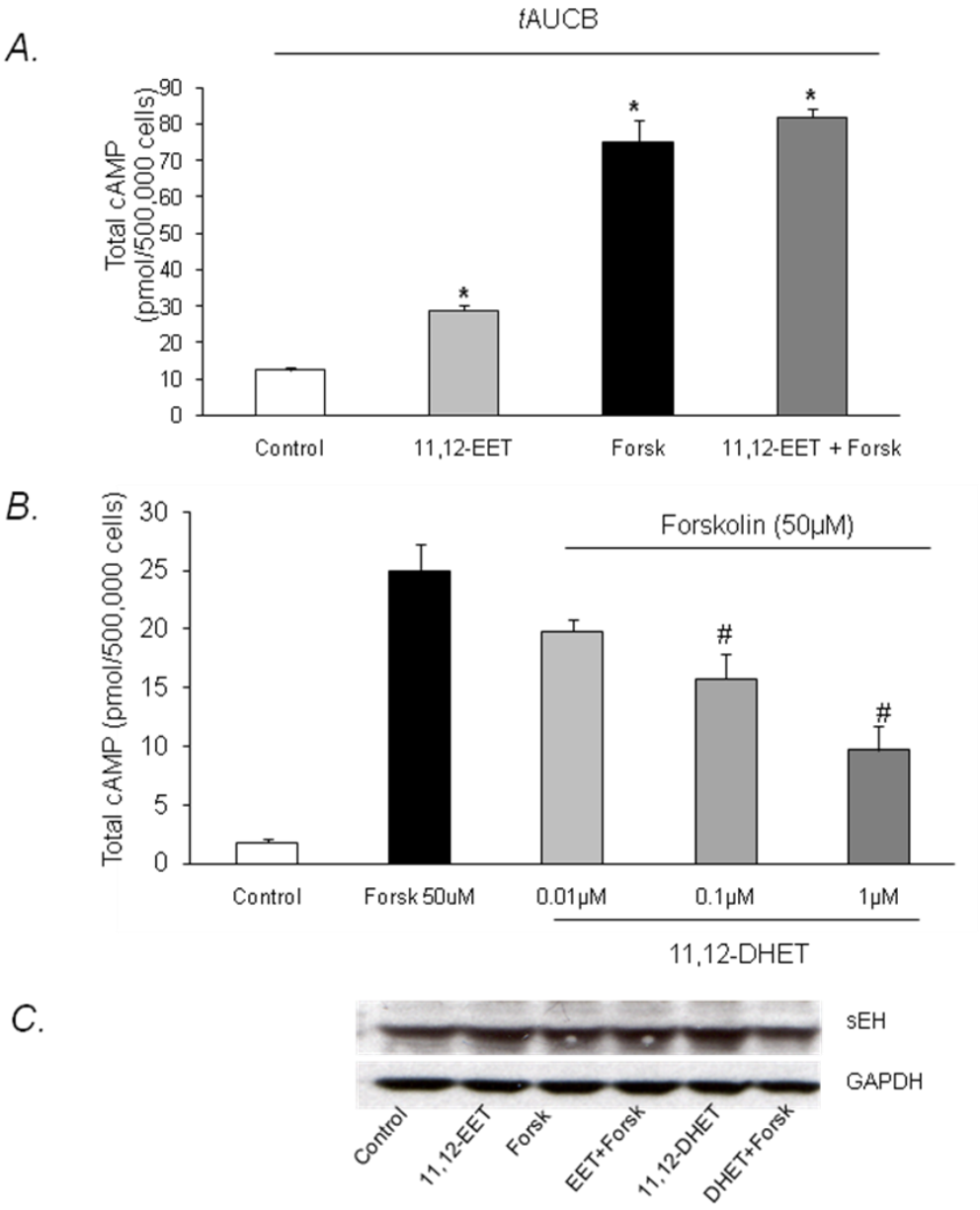


Figure 2-3: Role of EET metabolites in regulating cAMP levels

(A) HEK 293 cells were incubated for 5 min with vehicle, 11,12-EET (1 μM), forskolin (50 μM), in the presence of *t*AUCB (0.1 μM, sEH inhibitor). Total cAMP levels were determined by extrapolation from a standard c urve. Values shown are mean±SEM; N= 3; '*' $p<0.05$ vs. control (no *t*AUCB); '#'$p<0.05$ vs. respective forskolin alone. (B) HEK 293 cells were incubated for 30 min with vehicle, 11,12-DHET (0.01, 0.1 or 1 μM), forskolin (50 μM) or 11,12-DHET with forskolin. Values shown are mean±SEM; N=7; '*' $p<0.05$ vs. control; '#'$p<0.05$ vs. forskolin. (C) HEK 293 cells were incubated for 30 min with vehicle, 11,12-EET (1 μM), forskolin (50 μM), 11,12-EET with forskolin, 11,12-DHET (1 μM) or 11,12-DHET with forskolin. sEH expression was analyzed by immunoblotting.

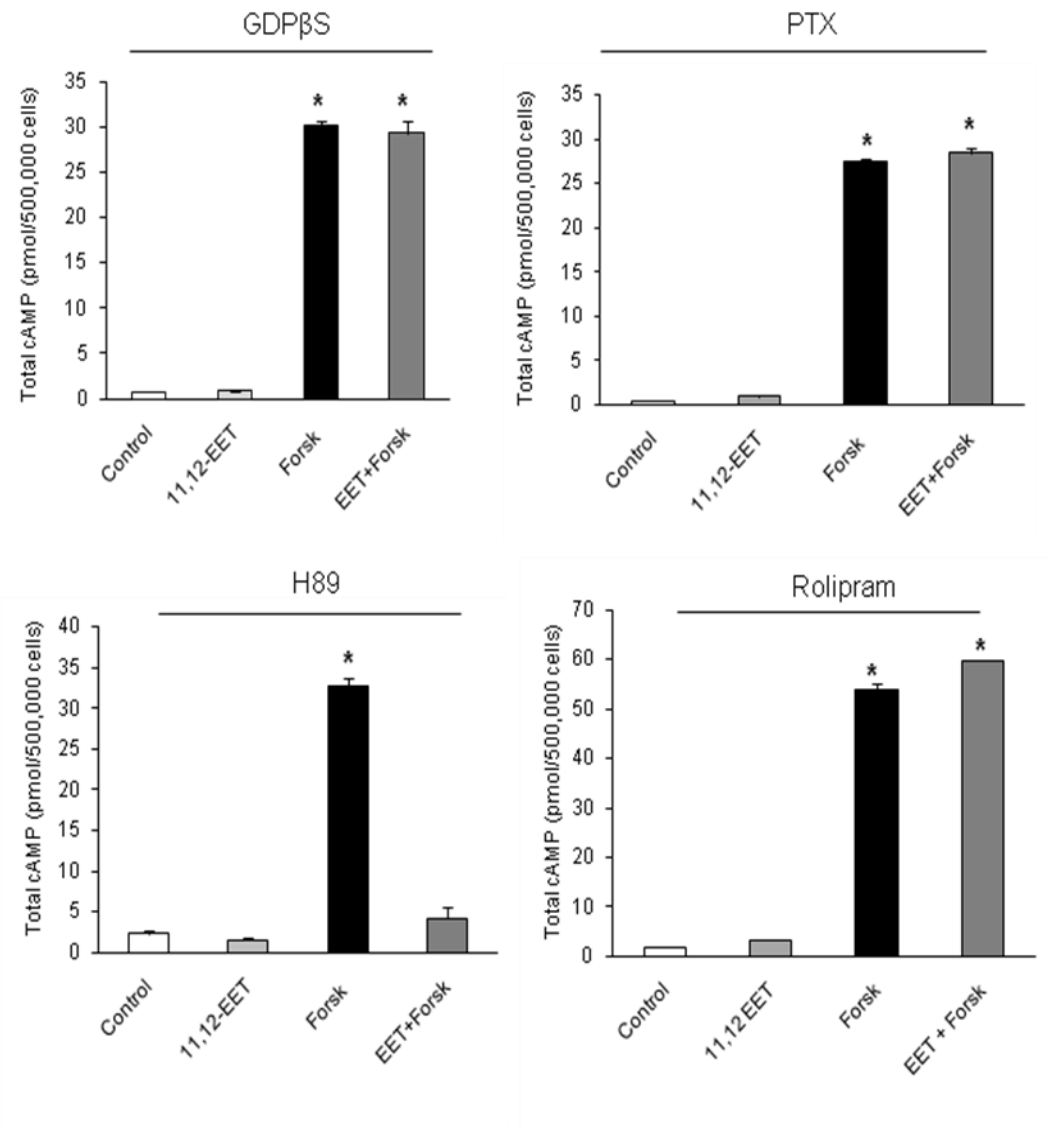


Figure 2-4: Regulation of cAMP production in EET-treated cells

(A) HEK 293 cells were pretreated with GDPβS (1 μM, Gs inhibitor) for 10 min and then incubated for 30 min with vehicle, 11,12-EET (1 μM), forskolin (50 μM) or 11,12-EET with forskolin. Total cAMP levels were determined by extrapolation from a standard curve. Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control. (B) HEK 293 cells were pretreated with PTX (200 ng/mL) for 4 hours and then incubated for 30 min with vehicle, 11,12-EET (1μM), forskolin (50μM) or EET with forskolin. Values shown are mean±SEM; N=6; '*' $p<0.05$ vs. control. (C) HEK 293 cells were incubated for 30 min with vehicle, 11,12-EET (1 μM), forskolin (50 μM) or EET with forskolin and co-treated with H89 (10 μM, PKA inhibitor). Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control; '#' $p<0.05$ vs. forskolin alone. (D) HEK 293 cells incubated for 30 min with vehicle, 11,12-EET (1 μM), forskolin (50 μM) or EET with forskolin and co-treated with rolipram (10 μM, PDE4 inhibitor). Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control.

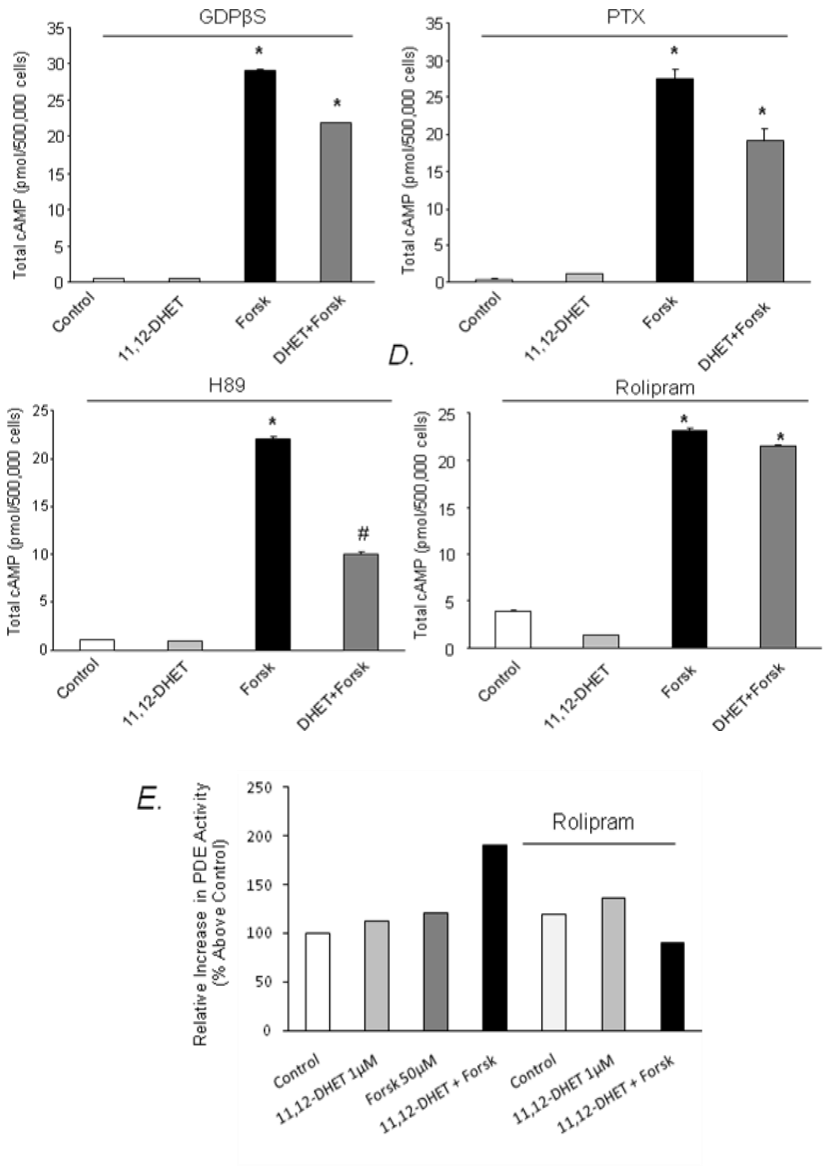


Figure 2-5: Regulation of cAMP production in DHET-treated cells

(A) HEK 293 cells were pretreated with GDPβS (1 μM, Gαs inhibitor) for 10 min and then incubated for 30 min with vehicle, 11,12-DHET (1 μM), forskolin (50 μM) or 11,12-DHET with forskolin. Total cAMP levels were determined by extrapolation from a standard curve. Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control. (B) HEK 293 cells were first pre-treated with PTX (200 ng/mL) for 4 hours and then incubated for 30 min with vehicle, 11,12-DHET (1 μM), forskolin (50 μM) or DHET with forskolin. Values shown are mean±SEM; N=6; '*' $p<0.05$ vs. control. (C) HEK 293 cells incubated for 30 min with vehicle, 11,12-DHET (1 μM), forskolin (50 μM) or DHET with forskolin and co-treated with H89 (10 μM, PKA inhibitor). Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control; '#' $p<0.05$ vs. forskolin alone. (D) HEK 293 cells incubated for 30 min with vehicle, 11,12-DHET (1μM), forskolin (50 μM) or DHET with forskolin and co-treated with rolipram (10 μM, PDE4 inhibitor). Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control. (E) HEK 293 cells incubated for 30 min with vehicle, forskolin (50μM), 11,12-EET (1 μM), 11,12-DHET (1 μM), 11,12-EET with forskolin or 11,12-DHET with forskolin. Values shown are mean±SEM; N=3; '*' $p<0.05$ vs. control.

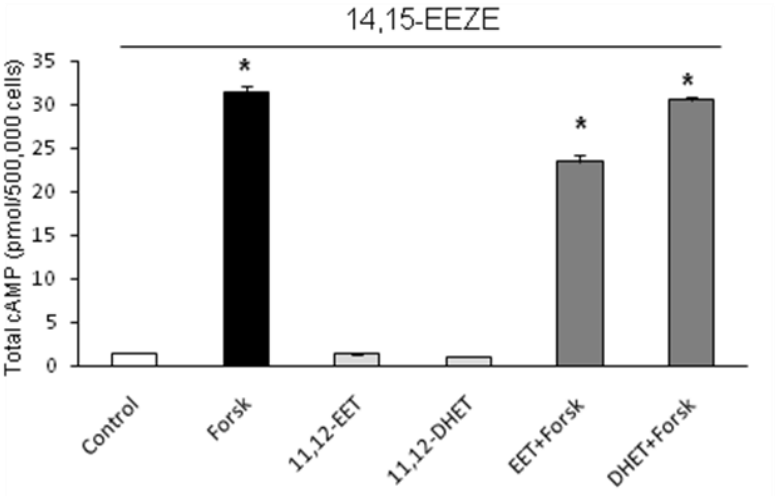


Figure 2-6: PDE activity in DHET-treated cells

HEK 293 cells were incubated for 5 min with vehicle, 11,12-DHET (1 μM), forskolin (50 μM) or 11,12-DHET with forskolin, with or without rolipram (10 μM, PDE4 inhibitor). Relative PDE activity levels in cell lysates were determined with respect to controls.

Chapter 3

Discussion and Future Directions

3.1. Discussion

GPCRs are critical links in relaying information from the extracellular space to the intracellular environment (Xin, Tran et al. 2008). Two important signal transduction pathways involving the GPCRs are the cAMP signal pathway and the phosphatidylinositol signal pathway (Gilman 1987). Mechanisms for the regulation of cAMP production, beyond receptor-mediated changes in synthesis rates via G protein activation, have been studied in different cell lines. PDEs provide the sole means of degrading cAMP in cells (Beavo and Brunton 2002). Within the heart, PDE4 is the isoform that plays an important role in controlling intracellular cAMP pools by targeting certain proteins, such as arrestins and SRC family tyrosyl kinases (Houslay and Adams 2003). However, the activity of AC can be regulated by both extracellular stimuli (via GPCR receptor activation) and intracellular stimuli (Cooper, Mons et al. 1995; Hanoune and Defer 2001; Patel, Du et al. 2001). Activation of β2ARs with a potent agonist, such as isoproterenol, has been demonstrated in HEK293 cells to trigger transient increases in cAMP signals which are followed by a rapid decline suggesting a tightly regulated intracellular mechanism. The decline in cAMP levels has been associated with a corresponding activation of PDE (Seibold, Williams et al. 2000). Recent evidence has been reported demonstrating an active role for PDE in regulating cAMP levels. PDE activation or inhibition is significantly impacts cellular function in relation with the end product of cAMP. For example, siRNA knockdown of β-arrestin 2 and PDE4D impeded the down regulation of cAMP signals in HEK-293 cells (Willoughby, Baillie et al. 2007).

Response to forskolin is also strongly influenced by the activation of AC by the heterotrimeric G-protein, Gs. Gs-promoted enhancement of AC activity in response to forskolin occurs not only when cells are incubated with exogenously administered agonists that activate

GPCR but also by agonists that can be endogenously released by cells (Insel and Ostrom 2003). Collectively, generation of cAMP occurs upon ligand binding to Gs protein–GPCRs and the consequent activation of a family of transmembrane ACs localized at the plasma membrane. GPCR signaling is dynamically regulated by multiple feedback mechanisms in which might causing desensitization. Desensitization to agonist could be explained by progressive alteration of AC and/or PDE activity (Violin, DiPilato et al. 2008).

The current thesis investigates potential underlying mechanisms of how EETs regulate cAMP levels. The data show a significant increase in cAMP formation in HEK293 cells occurring in a concentration and time-dependent manner. Interestingly, co-administration of EETs with forskolin resulted in significant inhibition of forskolin-induced cAMP production instead of a predicted additive effect. To explore the underlying mechanism we blocked EET metabolism with *t*AUCB (sEH inhibitor) and found increased cAMP accumulation, which suggested EETs were not involved in the inhibition. Data demonstrated that inhibition of cAMP production with DHET strongly suggested involvement of the EET metabolite in regulating cAMP levels. Moreover, inhibition of Gi with PTX abolished the inhibitory effect suggesting the role of Gαi protein.

Previous studies in intact human blood vessels indicate that conversion to DHET is the predominant pathway for 11,12- and 14,15-EET metabolism in vascular tissue (Fang, Weintraub et al. 2004). EETs hydrolysed into DHETs via sEH are generally considered to be less bioactive products; as such few studies have demonstrated significant biological effects of DHETs in the cells and tissues. It has been reported that 11,12-DHET is more potent than its parent compound 11,12-EET in relaxing canine coronary microvessels (Oltman, Weintraub et al. 1998). The importance of sEH is in many biological function is reflected by its ubiquitous expression levels;

moreover, sEH deletion is thought to reduce survival after cardiac arrest (Hutchens, Nakano et al. 2008) and genetic variations has been linked to a higher incidence of stroke in rats and humans (Seubert, Sinal et al. 2006; Corenblum, Wise et al. 2008; Gschwendtner, Ripke et al. 2008).

Data from this thesis suggest EETs and the dihydroxy metabolite, DHET, work as endogenous regulators of cAMP levels in HEK293 cells via PDE. Our data show that 11,12-EET inhibits forskolin-induced cAMP production significantly, and suggest that EETs might work through one of the following pathways: (1) rapid conversion to its metabolite11,12-DHET which activates PDE, leading to breakdown of cAMP; or, (2) activation of Gαi which in turn will inhibit AC and result in less cAMP production. The two most prominent PDE4 sub-families found in HEK293 cells are PDE4B and PDE4D, attached to the membrane or found in the cytosol, respectively (Terrin, Di Benedetto et al. 2006).

Studies from this thesis identify DHET as a new candidate for linking PDE4 activation/inhibition and regulation of cAMP levels in HEK293 cells. PKA-mediated activation of PDE4 is an important mechanism for shaping GPCR-mediated signals (Terrin, Di Benedetto et al. 2006; Willoughby, Wong et al. 2006; Rich, Xin et al. 2007). Forskolin-induced production of cAMP leads to phosphorylation of PKA, which in turn activates PDE and subsequent degradation of cAMP, thus, working as a negative feedback mechanism on cAMP production in cardiac myocytes (Rochais, Vandecasteele et al. 2004). The lack of effect of PKA inhibition in the current thesis suggests there was no involvement of PKA phosphorylation, and is consistent with a previous study, where H89 did not inhibit accumulation of prostaglandin-induced cAMP in HEK293 cells (Rich, Xin et al. 2007) However, we cannot rule out the role of PKA as we only tested one concentration of H89 (10 μM) and there is evidence suggesting that the inhibitory

effect of H89 is blocked in the presence of IBMX (Rochais, Vandecasteele et al. 2004). We have used IBMX in all our experiments in this study and this might explain why the blocking effect of H89 is not apparent.

Evidence for EET-mediated regulation of GPCRs has been demonstrated in various systems, yet the exact mechanism(s) remains unknown (Behm, Ogbonna et al. 2009). In the coronary circulation, 11,12-EET affects function through a cAMP-dependent process that activates vascular smooth muscle BKCa channels**.** EET-mediated BKCa activation can be blocked by the G protein inhibitor guanosine 5'-*O*-(2- thio) diphosphate, suggesting the role of G proteins (Li and Campbell 1997). Low concentrations of GDP and its stable analog guanosine 5'-O-(2-thio)diphosphate (GDP beta S) have been demonstrated to stimulate AC activity in canine cardiac sarcolemmal membranes (Piacentini, Mura et al. 1996). Gαs proteins activate AC, thereby producing cAMP and a direct interaction of Gαi with AC (following activation of the Gα subunit and dissociation from βγ) results in the inhibition of cAMP synthesis (Wong, Federman et al. 1991). Activation of receptors coupled to inhibitory G proteins (Gα i/o) has opposing consequences for cAMP accumulation (Watts and Neve 2005). For example, when cells have been pretreated with PTX it was found that PTX activates Gαi, resulting in cAMP accumulation. In a detailed study using FRET-based assays in intact HEK293 cells, data demonstrated key downstream signals involved in receptor-G protein interaction, G protein activation, and cAMP production (Mukhopadhyay and Ross 1999). Results suggested the three key events between receptor-G protein interaction and G protein activation are GDP release, GTP binding, and at least one conformational change. It is believed that GDP release from the G protein is a rate-limiting step. Consistent with this process, data presented in this thesis found that pretreatment of

HEK293 cells with GDP for 10 min before drug stimulation abolished the inhibitory effect of EET and DHET on cAMP production.

Gαi activation is typically associated with inhibition of Gαs-stimulated AC activity and thus reduced cAMP generation (Barnes 1998). Data presented in this thesis confirm that inhibition of Gαi subunit via pertussis toxin attenuates the EET-mediated effects. PTX treatment results in ADP-ribosylation of the Gαi subunit preventing Gαi coupling. The present results suggest that 11,12-EET-mediated events work to control intracellular cAMP levels partially via G proteins.

sEH is present in the human kidney in relatively high abundance and is preferentially expressed in the renal vasculature. Moreover, epoxide hydrolase activity has been detected in human and murine endothelial cells as well as in HEK293 cells (Barbosa-Sicard, Fromel et al. 2009), consistent with the current data demonstrating sEH expression. sEH functions predominantly to turnover lipid derived epoxides, which can act as signaling molecules with diverse functions in regulatory processes, such as control of blood pressure (Newman, Morisseau et al. 2005; Sporkova, Kopkan et al. 2011), inflammatory processes, and cell proliferation (Decker, Arand et al. 2009). An important observation in the thesis was EET-mediated inhibition of forskolin-induced cAMP production suggested it might potentially involve the metabolite DHET. Relative to EETs, DHETs are more hydrophilic and are more likely to be excreted (Yu, Davis et al. 2004). DHETs also have reduced intracellular protein binding and are incorporated to a lesser degree into the membrane phospholipids pool relative to the corresponding EETs (Yu, Davis et al. 2004). sEH converts EETs to DHETs, limiting many of the biological actions of EETs. *t*AUCB is an urea-based compound that works as a specific sEH enzyme inhibitor. By blocking sEH enzymatic activity there will be more EET formation and

less DHET formation. sEH inhibitors has been used experimentally to promote EET formation to examine its beneficial effects such as treatment of hypertension (Sporkova, Kopkan et al. 2011). In contrast, 11,12-DHET has been identified as the most potent coronary vasodilator (Oltman, Weintraub et al. 1998). However, the cellular ionic mechanisms through which DHETs induce vasorelaxation are unknown. The data from this thesis demonstrate involvement of the PDE in cAMP production. From our results we could suggest that EET might stimulate the formation of cAMP in time and concentration-dependent manner, whereas EET metabolite (DHET) has an inhibitory effect on the cAMP production. DHET also might work directly on G protein subunits or via interference with PDE activity. 11,12-DHET inhibits PDE activity in response to drug stimulation. This might explain the role of AA metabolites in controlling cAMP production in cells.

It is currently unclear exactly how EETs initiate their biological effects but several modes of EET-dependent signaling pathways have been proposed. For example, EETs may initiate their biological effects by interacting with a cell surface protein such as GPCRs (Pfister, Gauthier et al. 2010). Data presented in this thesis, as summarized in Fig. 3.1, suggest that EET-mediated events can be initiated at the G protein level, where they can enhance AC activity increasing cAMP production. This effect lasts for a short period until EETs are converted to their corresponding metabolites (DHETs) which further regulate cAMP production. It is hypothesized that DHETs work either directly by activating Gαi protein subunit leading to inhibition of cAMP production or activating PDE. Either mechanism results in an endogenous negative feedback mechanism, which works to regulate cAMP levels in HEK293 cells.

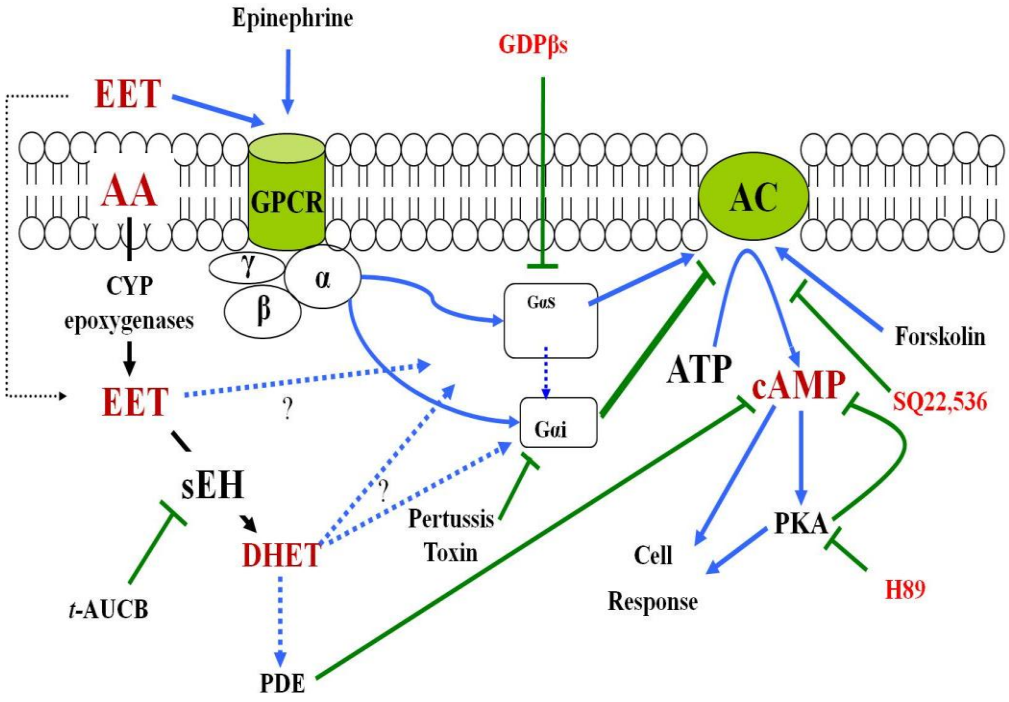


Figure 3-1 : Model of intracellular signaling pathways

When EETs administered exogenously and/or released from arachidonic acid at the cell membrane, it will be converted intracellularly to its metabolites (DHETs) via soluble epoxide hydrolase enzyme. EETs might work directly on Gαs protein which consequently activate adenylyl cyclase enzyme and lead to cAMP production. Alternatively, EETs might work through its metabolites (DHET) also through G αs and or Gαi subunits to control cAMP production. DHET might also work though activation of PDE as negative back mechanism for controlling cAMP.

3.2. Conclusion

Finally, arachidonic acid metabolites, eicosanoids, are key contributors to cardiovascular homeostasis and function. Dysregulation of eicosanoids can contribute to the progression of cardiovascular diseases (Sudhahar, Shaw et al. 2010). As such, it is important to obtain a better understanding of the underlying mechanism(s) of EET-mediated events. Data presented in this thesis have identified a novel signaling system in HEK293 cells, in which EETs and DHETs work as endogenous regulators of cAMP production. Elucidation of their specific site of action and their role in physiological and pathological conditions at the cellular and tissue level will be valuable in the development of new therapeutic agents for cardiovascular and renal diseases.

3.3. Future directions

1- To investigate the mechanistic role of EETs and DHET in regulating cAMP production under hypoxic-reoxygenation conditions in order to elucidate a novel protective response.
2- To further investigate the role of Gαi and PDE in the EET and DHET signaling mechanism.
3- To determine if this novel endogenous regulation system occurs in other cells and tissues, notably cardiomyocytes and the heart.
4- To investigate the role of EETs and DHETs in chronic disease conditions such as in *vivo* models of heart failure.

3.4. References

Adderley, S. P., R. S. Sprague, et al. (2010). "Regulation of cAMP by phosphodiesterases in erythrocytes." Pharmacol Rep **62**(3): 475-82.

Asanuma, H., M. Kitakaze, et al. (2001). "Nifedipine limits infarct size via NO-dependent mechanisms in dogs." Basic Res Cardiol **96**(5): 497-505.

Attwood, T. K. and J. B. Findlay (1994). "Fingerprinting G-protein-coupled receptors." Protein Eng **7**(2): 195-203.

Atwood, B. K., J. Lopez, et al. (2011). "Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis." BMC Genomics **12**: 14.

Barbosa-Sicard, E., T. Fromel, et al. (2009). "Inhibition of the soluble epoxide hydrolase by tyrosine nitration." J Biol Chem **284**(41): 28156-63.

Barki-Harrington, L., C. Perrino, et al. (2004). "Network integration of the adrenergic system in cardiac hypertrophy." Cardiovasc Res **63**(3): 391-402.

Barnes, P. J. (1998). "Pharmacology of airway smooth muscle." Am J Respir Crit Care Med **158**(5 Pt 3): S123-32.

Batchu, S. N., E. Law, et al. (2009). "Epoxyeicosatrienoic acid prevents postischemic electrocardiogram abnormalities in an isolated heart model." J Mol Cell Cardiol **46**(1): 67-74.

Batchu, S. N., S. B. Lee, et al. (2011). "Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischaemia reperfusion injury." Br J Pharmacol **162**(4): 897-907.

Beavo, J. A. and L. L. Brunton (2002). "Cyclic nucleotide research -- still expanding after half a century." Nat Rev Mol Cell Biol **3**(9): 710-8.

Behm, D. J., A. Ogbonna, et al. (2009). "Epoxyeicosatrienoic acids function as selective, endogenous antagonists of native thromboxane receptors: identification of a novel mechanism of vasodilation." J Pharmacol Exp Ther **328**(1): 231-9.

Billington, C. K. and R. B. Penn (2003). "Signaling and regulation of G protein-coupled receptors in airway smooth muscle." Respir Res **4**: 2.

Bjarnadottir, T. K., D. E. Gloriam, et al. (2006). "Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse." Genomics **88**(3): 263-73.

Bodiga, S., R. Zhang, et al. (2009). "Protective actions of epoxyeicosatrienoic acid: dual targeting of cardiovascular PI3K and KATP channels." J Mol Cell Cardiol **46**(6): 978-88.

Braden, G. A., H. R. Knapp, et al. (1991). "Suppression of eicosanoid biosynthesis during coronary angioplasty by fish oil and aspirin." Circulation **84**(2): 679-85.

Brash, A. R. (1999). "Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate." J Biol Chem **274**(34): 23679-82.

Burns, F., A. Z. Zhao, et al. (1996). "Cyclic nucleotide phosphodiesterases: gene complexity, regulation by phosphorylation, and physiological implications." Adv Pharmacol **36**: 29-48.

Buschmans, E., D. J. Hearse, et al. (1985). "Forskolin: effects on cyclic AMP and contractile function in the isolated rat and guinea pig heart." Can J Cardiol **1**(6): 385-94.

Campbell, W. B. and I. Fleming (2010). "Epoxyeicosatrienoic acids and endothelium-dependent responses." Pflugers Arch **459**(6): 881-95.

Campbell, W. B., D. Gebremedhin, et al. (1996). "Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors." Circ Res **78**(3): 415-23.

Capdevila, J., N. Chacos, et al. (1981). "Liver microsomal cytochrome P-450 and the oxidative metabolism of arachidonic acid." Proc Natl Acad Sci U S A **78**(9): 5362-6.

Caron, M. G. and R. J. Lefkowitz (1993). "Catecholamine receptors: structure, function, and regulation." Recent Prog Horm Res **48**: 277-90.

Carroll, M. A., A. B. Doumad, et al. (2006). "Adenosine2A receptor vasodilation of rat preglomerular microvessels is mediated by EETs that activate the cAMP/PKA pathway." Am J Physiol Renal Physiol **291**(1): F155-61.

Catella, F., J. A. Lawson, et al. (1990). "Endogenous biosynthesis of arachidonic acid epoxides in humans: increased formation in pregnancy-induced hypertension." Proc Natl Acad Sci U S A **87**(15): 5893-7.

Chakraborti, S., T. Chakraborti, et al. (2000). "beta-adrenergic mechanisms in cardiac diseases: a perspective." Cell Signal **12**(8): 499-513.

Chaudhary, K. R., M. Abukhashim, et al. (2010). "Inhibition of soluble epoxide hydrolase by trans-4- [4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid is protective against ischemia-reperfusion injury." J Cardiovasc Pharmacol **55**(1): 67-73.

Chaudhary, K. R., S. N. Batchu, et al. (2009). "Role of B-type natriuretic peptide in epoxyeicosatrienoic acid-mediated improved post-ischaemic recovery of heart contractile function." Cardiovasc Res **83**(2): 362-70.

Chehal, M. K. and D. J. Granville (2006). "Cytochrome p450 2C (CYP2C) in ischemic heart injury and vascular dysfunction." Can J Physiol Pharmacol **84**(1): 15-20.

Chen, W., W. Glasgow, et al. (1999). "Lipoxygenase metabolism of arachidonic acid in ischemic preconditioning and PKC-induced protection in heart." Am J Physiol **276**(6 Pt 2): H2094-101.

Chiamvimonvat, N., C. M. Ho, et al. (2007). "The soluble epoxide hydrolase as a pharmaceutical target for hypertension." J Cardiovasc Pharmacol **50**(3): 225-37.

Choi, S. H., S. Aid, et al. (2009). "The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research." Trends Pharmacol Sci **30**(4): 174-81.

Cooper, D. M., N. Mons, et al. (1995). "Adenylyl cyclases and the interaction between calcium and cAMP signalling." Nature **374**(6521): 421-4.

Corenblum, M. J., V. E. Wise, et al. (2008). "Altered soluble epoxide hydrolase gene expression and function and vascular disease risk in the stroke-prone spontaneously hypertensive rat." Hypertension **51**(2): 567-73.

Daaka, Y., L. M. Luttrell, et al. (1997). "Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A." Nature **390**(6655): 88-91.

Decker, M., M. Arand, et al. (2009). "Mammalian epoxide hydrolases in xenobiotic metabolism and signalling." Arch Toxicol **83**(4): 297-318.

Defer, N., M. Best-Belpomme, et al. (2000). "Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase." Am J Physiol Renal Physiol **279**(3): F400-16.

Dessauer, C. W. (2009). "Adenylyl cyclase--A-kinase anchoring protein complexes: the next dimension in cAMP signaling." Mol Pharmacol **76**(5): 935-41.

Devic, E., Y. Xiang, et al. (2001). "Beta-adrenergic receptor subtype-specific signaling in cardiac myocytes from beta(1) and beta(2) adrenoceptor knockout mice." Mol Pharmacol **60**(3): 577-83.

Dhanasekaran, A., S. K. Gruenloh, et al. (2008). "Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia." Am J Physiol Heart Circ Physiol **294**(2): H724-35.

Dorn, G. W., 2nd, N. Liel, et al. (1990). "Increased platelet thromboxane A2/prostaglandin H2 receptors in patients with acute myocardial infarction." Circulation **81**(1): 212-8.

England, P. J. and M. Shahid (1987). "Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart." Biochem J **246**(3): 687-95.

Falck, J. R., R. Kodela, et al. (2009). "14,15-Epoxyeicosa-5,8,11-trienoic acid (14,15-EET) surrogates containing epoxide bioisosteres: influence upon vascular relaxation and soluble epoxide hydrolase inhibition." J Med Chem **52**(16): 5069-75.

Falck, J. R., U. M. Krishna, et al. (2003). "Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation." Am J Physiol Heart Circ Physiol **284**(1): H337-49.

Falck, J. R., V. J. Schueler, et al. (1987). "Arachidonate epoxygenase: identification of epoxyeicosatrienoic acids in rabbit kidney." J Lipid Res **28**(7): 840-6.

Fang, X., T. L. Kaduce, et al. (2001). "Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition." J Biol Chem **276**(18): 14867-74.

Fang, X., N. L. Weintraub, et al. (2004). "Effect of soluble epoxide hydrolase inhibition on epoxyeicosatrienoic acid metabolism in human blood vessels." Am J Physiol Heart Circ Physiol **287**(6): H2412-20.

Fang, X., N. L. Weintraub, et al. (1999). "Epoxyeicosatrienoic acids increase intracellular calcium concentration in vascular smooth muscle cells." Hypertension **34**(6): 1242-6.

Ferdinandy, P., R. Schulz, et al. (2007). "Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning." Pharmacol Rev **59**(4): 418-58.

Ferreiro-Vera, C., J. M. Mata-Granados, et al. (2011). "Automated targeting analysis of eicosanoid inflammation biomarkers in human serum and in the exometabolome of stem cells by SPE-LC-MS/MS." Anal Bioanal Chem **399**(3): 1093-103.

Fichtlscherer, S., S. Dimmeler, et al. (2004). "Inhibition of cytochrome P450 2C9 improves endothelium-dependent, nitric oxide-mediated vasodilatation in patients with coronary artery disease." Circulation **109**(2): 178-83.

Fitzgerald, D. J. and G. A. Fitzgerald (1989). "Role of thrombin and thromboxane A2 in reocclusion following coronary thrombolysis with tissue-type plasminogen activator." Proc Natl Acad Sci U S A **86**(19): 7585-9.

Fitzgerald, D. J., L. Roy, et al. (1986). "Platelet activation in unstable coronary disease." N Engl J Med **315**(16): 983-9.

Fleming, I. (2001). "Cytochrome p450 and vascular homeostasis." Circ Res **89**(9): 753-62.

Foord, S. M., T. I. Bonner, et al. (2005). "International Union of Pharmacology. XLVI. G protein-coupled receptor list." Pharmacol Rev **57**(2): 279-88.

Fornage, M., E. Boerwinkle, et al. (2004). "Polymorphism of the soluble epoxide hydrolase is associated with coronary artery calcification in African-American subjects: The Coronary Artery Risk Development in Young Adults (CARDIA) study." Circulation **109**(3): 335-9.

Francis, S. H., M. A. Blount, et al. (2011). "Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions." Physiol Rev **91**(2): 651-90.

Fretland, A. J. and C. J. Omiecinski (2000). "Epoxide hydrolases: biochemistry and molecular biology." Chem Biol Interact **129**(1-2): 41-59.

Fukao, M., H. S. Mason, et al. (2001). "Regulation of BK(Ca) channels expressed in human embryonic kidney 293 cells by epoxyeicosatrienoic acid." Mol Pharmacol **59**(1): 16-23.

Gauthier, K. M., S. G. Jagadeesh, et al. (2003). "14,15-epoxyeicosa-5(Z)-enoic-mSI: a 14,15- and 5,6-EET antagonist in bovine coronary arteries." Hypertension **42**(4): 555-61.

Geng, W., Z. Wang, et al. (2005). "Cloning and characterization of the human soluble adenylyl cyclase." Am J Physiol Cell Physiol **288**(6): C1305-16.

Gilman, A. G. (1987). "G proteins: transducers of receptor-generated signals." Annu Rev Biochem **56**: 615-49.

Graham, F. L., J. Smiley, et al. (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." J Gen Virol **36**(1): 59-74.

Granneman, J. G. (2001). "The putative beta4-adrenergic receptor is a novel state of the beta1-adrenergic receptor." Am J Physiol Endocrinol Metab **280**(2): E199-202.

Gross, G. J., K. M. Gauthier, et al. (2008). "Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection produced by exogenous or endogenous EETs in the canine heart." Am J Physiol Heart Circ Physiol **294**(6): H2838-44.

Gschwendtner, A., S. Ripke, et al. (2008). "Genetic variation in soluble epoxide hydrolase (EPHX2) is associated with an increased risk of ischemic stroke in white Europeans." Stroke **39**(5): 1593-6.

Halpert, J., B. Naslund, et al. (1983). "Suicide inactivation of rat liver cytochrome P-450 by chloramphenicol in vivo and in vitro." Mol Pharmacol **23**(2): 445-52.

Hamm, H. E. (1998). "The many faces of G protein signaling." J Biol Chem **273**(2): 669-72.

Hanoune, J. and N. Defer (2001). "Regulation and role of adenylyl cyclase isoforms." Annu Rev Pharmacol Toxicol **41**: 145-74.

Harder, D. R., W. B. Campbell, et al. (1995). "Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone." J Vasc Res **32**(2): 79-92.

Harry, A., Y. Chen, et al. (1997). "Differential regulation of adenylyl cyclases by Galphas." J Biol Chem **272**(30): 19017-21.

Hearse, D. J., R. Zucchi, et al. (1986). "Forskolin and myocardial function in the normal, ischemic and reperfused rat heart." Can J Cardiol **2**(5): 303-12.

Henrich, W. L., J. R. Falck, et al. (1990). "Inhibition of renin release by 14,15-epoxyeicosatrienoic acid in renal cortical slices." Am J Physiol **258**(2 Pt 1): E269-74.

Hirt, D. L., J. Capdevila, et al. (1989). "Cytochrome P450 metabolites of arachidonic acid are potent inhibitors of vasopressin action on rabbit cortical collecting duct." J Clin Invest **84**(6): 1805-12.

Honetschlagerova, Z., A. Sporkova, et al. (2011). "Inhibition of soluble epoxide hydrolase improves the impaired pressure-natriuresis relationship and attenuates the development of hypertension and hypertension-associated end-organ damage in Cyp1a1-Ren-2 transgenic rats." J Hypertens **29**(8): 1590-1601.

Houslay, M. D. and D. R. Adams (2003). "PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization." Biochem J **370**(Pt 1): 1-18.

Houslay, M. D. and G. Milligan (1997). "Tailoring cAMP-signalling responses through isoform multiplicity." Trends Biochem Sci **22**(6): 217-24.

Hutchens, M. P., T. Nakano, et al. (2008). "Soluble epoxide hydrolase gene deletion reduces survival after cardiac arrest and cardiopulmonary resuscitation." Resuscitation **76**(1): 89-94.

Imig, J. D. (2005). "Epoxide hydrolase and epoxygenase metabolites as therapeutic targets for renal diseases." Am J Physiol Renal Physiol **289**(3): F496-503.

Insel, P. A. and R. S. Ostrom (2003). "Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling." Cell Mol Neurobiol **23**(3): 305-14.

Iyengar, R. (1993). "Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases." Faseb J **7**(9): 768-75.

Jiang, J. G., Y. G. Ning, et al. (2007). "Cytochrome p450 epoxygenase promotes human cancer metastasis." Cancer Res **67**(14): 6665-74.

Johnston, C. A. and V. J. Watts (2003). "Sensitization of adenylate cyclase: a general mechanism of neuroadaptation to persistent activation of Galpha(i/o)-coupled receptors?" Life Sci **73**(23): 2913-25.

Karara, A., S. Wei, et al. (1992). "Arachidonic acid epoxygenase: structural characterization and quantification of epoxyeicosatrienoates in plasma." Biochem Biophys Res Commun **182**(3): 1320-5.

Karmazyn, M. (1989). "Synthesis and relevance of cardiac eicosanoids with particular emphasis on ischemia and reperfusion." Can J Physiol Pharmacol **67**(8): 912-21.

Katragadda, D., S. N. Batchu, et al. (2009). "Epoxyeicosatrienoic acids limit damage to mitochondrial function following stress in cardiac cells." J Mol Cell Cardiol **46**(6): 867-75.

Keseru, B., E. Barbosa-Sicard, et al. (2008). "Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response." Faseb J **22**(12): 4306-15.

Kinsella, B. T. (2001). "Thromboxane A2 signalling in humans: a 'Tail' of two receptors." Biochem Soc Trans **29**(Pt 6): 641-54.

Kopf, P. G., K. M. Gauthier, et al. (2011). "Angiotensin II regulates adrenal vascular tone through zona glomerulosa cell-derived EETs and DHETs." Hypertension **57**(2): 323-9.

Kostenis, E. (2006). "G proteins in drug screening: from analysis of receptor-G protein specificity to manipulation of GPCR-mediated signalling pathways." Curr Pharm Des **12**(14): 1703-15.

Kreutner, W., R. W. Chapman, et al. (1985). "Bronchodilator and antiallergy activity of forskolin." Eur J Pharmacol **111**(1): 1-8.

Kroetz, D. L. and D. C. Zeldin (2002). "Cytochrome P450 pathways of arachidonic acid metabolism." Curr Opin Lipidol **13**(3): 273-83.

Kubler, W. and M. Haass (1996). "Cardioprotection: definition, classification, and fundamental principles." Heart **75**(4): 330-3.

Kuhn, H. and V. B. O'Donnell (2006). "Inflammation and immune regulation by 12/15-lipoxygenases." Prog Lipid Res **45**(4): 334-56.

Larsen, B. T., D. D. Gutterman, et al. (2006). "Emerging role of epoxyeicosatrienoic acids in coronary vascular function." Eur J Clin Invest **36**(5): 293-300.

Larsen, B. T., H. Miura, et al. (2006). "Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BK(Ca) channels: implications for soluble epoxide hydrolase inhibition." Am J Physiol Heart Circ Physiol **290**(2): H491-9.

Lattin, J., D. A. Zidar, et al. (2007). "G-protein-coupled receptor expression, function, and signaling in macrophages." J Leukoc Biol **82**(1): 16-32.

Lee, C. R., K. E. North, et al. (2007). "CYP2J2 and CYP2C8 polymorphisms and coronary heart disease risk: the Atherosclerosis Risk in Communities (ARIC) study." Pharmacogenet Genomics **17**(5): 349-58.

Lee, J., M. Dahl, et al. (2010). "Genetically reduced soluble epoxide hydrolase activity and risk of stroke and other cardiovascular disease." Stroke **41**(1): 27-33.

Li, P. L. and W. B. Campbell (1997). "Epoxyeicosatrienoic acids activate K+ channels in coronary smooth muscle through a guanine nucleotide binding protein." Circ Res **80**(6): 877-84.

Li, P. L., C. L. Chen, et al. (1999). "11,12-Epoxyeicosatrienoic acid stimulates endogenous mono-ADP-ribosylation in bovine coronary arterial smooth muscle." Circ Res **85**(4): 349-56.

Liu, J. Y., J. Yang, et al. (2010). "Inhibition of soluble epoxide hydrolase enhances the anti-inflammatory effects of aspirin and 5-lipoxygenase activation protein inhibitor in a murine model." Biochem Pharmacol **79**(6): 880-7.

Lu, T., P. V. Katakam, et al. (2001). "Dihydroxyeicosatrienoic acids are potent activators of Ca(2+)-activated K(+) channels in isolated rat coronary arterial myocytes." J Physiol **534**(Pt 3): 651-67.

Maier, K. G. and R. J. Roman (2001). "Cytochrome P450 metabolites of arachidonic acid in the control of renal function." Curr Opin Nephrol Hypertens **10**(1): 81-7.

Makita, K., J. R. Falck, et al. (1996). "Cytochrome P450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system." Faseb J **10**(13): 1456-63.

McGiff, J. C. and M. A. Carroll (1991). "Cytochrome P450-dependent arachidonate metabolites, renal function and blood pressure regulation." Adv Prostaglandin Thromboxane Leukot Res **21B**: 675-82.

McGiff, J. C., M. Steinberg, et al. (1996). "Missing links: Cytochrome P450 arachidonate products A new class of lipid mediators." Trends Cardiovasc Med **6**(1): 4-10.

Michel, F., J. S. Silvestre, et al. (2006). "Thromboxane A2/prostaglandin H2 receptor activation mediates angiotensin II-induced postischemic neovascularization." Arterioscler Thromb Vasc Biol **26**(3): 488-93.

Moncada, S. and J. R. Vane (1978). "Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin." Pharmacol Rev **30**(3): 293-331.

Mukhopadhyay, S. and E. M. Ross (1999). "Rapid GTP binding and hydrolysis by G(q) promoted by receptor and GTPase-activating proteins." Proc Natl Acad Sci U S A **96**(17): 9539-44.

Murray, C. J. and A. D. Lopez (1997). "Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study." Lancet **349**(9064): 1498-504.

Neer, E. J. (1994). "G proteins: critical control points for transmembrane signals." Protein Sci **3**(1): 3-14.

Neves, S. R., P. T. Ram, et al. (2002). "G protein pathways." Science **296**(5573): 1636-9.

Newman, J. W., C. Morisseau, et al. (2005). "Epoxide hydrolases: their roles and interactions with lipid metabolism." Prog Lipid Res **44**(1): 1-51.

Nithipatikom, K., M. P. Endsley, et al. (2006). "Effects of selective inhibition of cytochrome P-450 omega-hydroxylases and ischemic preconditioning in myocardial protection." Am J Physiol Heart Circ Physiol **290**(2): H500-5.

Nithipatikom, K., J. M. Moore, et al. (2006). "Epoxyeicosatrienoic acids in cardioprotection: ischemic versus reperfusion injury." Am J Physiol Heart Circ Physiol **291**(2): H537-42.

Node, K., Y. Huo, et al. (1999). "Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids." Science **285**(5431): 1276-9.

Node, K., X. L. Ruan, et al. (2001). "Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids." J Biol Chem **276**(19): 15983-9.

Nowak, J. Z. and J. B. Zawilska (1999). "[Adenylyl cyclase--isoforms, regulation and function]." Postepy Hig Med Dosw **53**(2): 147-72.

Ogletree, M. L. (1987). "Overview of physiological and pathophysiological effects of thromboxane A2." Fed Proc **46**(1): 133-8.

Oltman, C. L., N. L. Weintraub, et al. (1998). "Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation." Circ Res **83**(9): 932-9.

Pain T, Y. X., Critz SD, Yue Y, Nakano A, Liu GS, Heusch , Cohen MV, Downey JM (2000). "Opening of mitochondrial K(ATP) channels triggers the preconditioned state by genrating free radicals." Circ Res **87**: 460-6.

Patel, T. B., Z. Du, et al. (2001). "Molecular biological approaches to unravel adenylyl cyclase signaling and function." Gene **269**(1-2): 13-25.

Penela, P., C. Murga, et al. (2006). "Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease." Cardiovasc Res **69**(1): 46-56.

Penn, R. B., A. N. Pronin, et al. (2000). "Regulation of G protein-coupled receptor kinases." Trends Cardiovasc Med **10**(2): 81-9.

Pfister, S. L., K. M. Gauthier, et al. (2010). "Vascular pharmacology of epoxyeicosatrienoic acids." Adv Pharmacol **60**: 27-59.

Piacentini, L., R. Mura, et al. (1996). "Stable GDP analog-induced inactivation of G(i) proteins promotes cardiac adenylyl cyclase inhibition by guanosine 5'-(beta gamma-imino)triphosphate and muscarinic acetylcholine receptor." Biochim Biophys Acta **1282**(1): 11-6.

Przybyla-Zawislak, B. D., P. K. Srivastava, et al. (2003). "Polymorphisms in human soluble epoxide hydrolase." Mol Pharmacol **64**(2): 482-90.

Rich, T. C., W. Xin, et al. (2007). "Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells." Am J Physiol Cell Physiol **292**(1): C319-31.

Rochais, F., G. Vandecasteele, et al. (2004). "Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact

cardiac myocytes: an in vivo study using adenovirus-mediated expression of CNG channels." J Biol Chem **279**(50): 52095-105.

Rockman, H. A., W. J. Koch, et al. (2002). "Seven-transmembrane-spanning receptors and heart function." Nature **415**(6868): 206-12.

Rodbell, M. (1980). "The role of hormone receptors and GTP-regulatory proteins in membrane transduction." Nature **284**(5751): 17-22.

Rosolowsky, M. and W. B. Campbell (1993). "Role of PGI2 and epoxyeicosatrienoic acids in relaxation of bovine coronary arteries to arachidonic acid." Am J Physiol **264**(2 Pt 2): H327-35.

Rosolowsky, M. and W. B. Campbell (1996). "Synthesis of hydroxyeicosatetraenoic (HETEs) and epoxyeicosatrienoic acids (EETs) by cultured bovine coronary artery endothelial cells." Biochim Biophys Acta **1299**(2): 267-77.

Ruan, Y. C., W. Zhou, et al. (2011). "Regulation of smooth muscle contraction by the epithelium: role of prostaglandins." Physiology (Bethesda) **26**(3): 156-70.

Sable, C. L., N. Filippa, et al. (1997). "cAMP stimulates protein kinase B in a Wortmannin-insensitive manner." FEBS Lett **409**(2): 253-7.

Sacerdoti, D., A. Gatta, et al. (2003). "Role of cytochrome P450-dependent arachidonic acid metabolites in liver physiology and pathophysiology." Prostaglandins Other Lipid Mediat **72**(1-2): 51-71.

Sanada, S., M. Kitakaze, et al. (2001). "Cardioprotective effect afforded by transient exposure to phosphodiesterase III inhibitors: the role of protein kinase A and p38 mitogen-activated protein kinase." Circulation **104**(6): 705-10.

Sandberg, M., C. Hassett, et al. (2000). "Identification and functional characterization of human soluble epoxide hydrolase genetic polymorphisms." J Biol Chem **275**(37): 28873-81.

Schlondorff, D., E. Petty, et al. (1987). "Epoxygenase metabolites of arachidonic acid inhibit vasopressin response in toad bladder." Am J Physiol **253**(3 Pt 2): F464-70.

Scholich, K., C. Wittpoth, et al. (1997). "Identification of an intramolecular interaction between small regions in type V adenylyl cyclase that influences stimulation of enzyme activity by Gsalpha." Proc Natl Acad Sci U S A **94**(18): 9602-7.

Schwartzman, M., N. R. Ferreri, et al. (1985). "Renal cytochrome P450-related arachidonate metabolite inhibits (Na+ + K+)ATPase." Nature **314**(6012): 620-2.

Seibold, A., B. Williams, et al. (2000). "Localization of the sites mediating desensitization of the beta(2)-adrenergic receptor by the GRK pathway." Mol Pharmacol **58**(5): 1162-73.

Seki, K., A. Hirai, et al. (1992). "Epoxyeicosatrienoic acid stimulates ADP-ribosylation of a 52 kDa protein in rat liver cytosol." Biochem J **281 (Pt 1)**: 185-90.

Seubert, J., B. Yang, et al. (2004). "Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+ channels and p42/p44 MAPK pathway." Circ Res **95**(5): 506-14.

Seubert, J. M., C. J. Sinal, et al. (2006). "Role of soluble epoxide hydrolase in postischemic recovery of heart contractile function." Circ Res **99**(4): 442-50.

Seubert, J. M., D. C. Zeldin, et al. (2007). "Role of epoxyeicosatrienoic acids in protecting the myocardium following ischemia/reperfusion injury." Prostaglandins Other Lipid Mediat **82**(1-4): 50-9.

Sexton, A., M. McDonald, et al. (2007). "12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/reperfusion injury via activation of neuronal TRPV1." Faseb J **21**(11): 2695-703.

Skeberdis, V. A. (2004). "Structure and function of beta3-adrenergic receptors." Medicina (Kaunas) **40**(5): 407-13.

Smith, W. L., E. A. Meade, et al. (1994). "Pharmacology of prostaglandin endoperoxide synthase isozymes-1 and -2." Ann N Y Acad Sci **714**: 136-42.

Spector, A. A. and A. W. Norris (2007). "Action of epoxyeicosatrienoic acids on cellular function." Am J Physiol Cell Physiol **292**(3): C996-1012.

Spiecker, M. and J. Liao (2006). "Cytochrome P450 epoxygenase CYP2J2 and the risk of coronary artery disease." Trends Cardiovasc Med **16**(6): 204-8.

Spiecker, M. and J. K. Liao (2005). "Vascular protective effects of cytochrome p450 epoxygenase-derived eicosanoids." Arch Biochem Biophys **433**(2): 413-20.

Sporkova, A., L. Kopkan, et al. (2011). "Role of cytochrome P-450 metabolites in the regulation of renal function and blood pressure in 2-kidney 1-clip hypertensive rats." Am J Physiol Regul Integr Comp Physiol **300**(6): R1468-75.

Stevens, T., J. Creighton, et al. (1999). "Control of cAMP in lung endothelial cell phenotypes. Implications for control of barrier function." Am J Physiol **277**(1 Pt 1): L119-26.

Sudhahar, V., S. Shaw, et al. (2010). "Epoxyeicosatrienoic acid analogs and vascular function." Curr Med Chem **17**(12): 1181-90.

Sunahara, R. K., C. W. Dessauer, et al. (1996). "Complexity and diversity of mammalian adenylyl cyclases." Annu Rev Pharmacol Toxicol **36**: 461-80.

Sunahara, R. K., C. W. Dessauer, et al. (1997). "Interaction of Gsalpha with the cytosolic domains of mammalian adenylyl cyclase." J Biol Chem **272**(35): 22265-71.

Tang, C. M. and P. A. Insel (2004). "GPCR expression in the heart; "new" receptors in myocytes and fibroblasts." Trends Cardiovasc Med **14**(3): 94-9.

Taussig, R. and A. G. Gilman (1995). "Mammalian membrane-bound adenylyl cyclases." J Biol Chem **270**(1): 1-4.

Terrin, A., G. Di Benedetto, et al. (2006). "PGE(1) stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases." J Cell Biol **175**(3): 441-51.

Thomas, P. and T. G. Smart (2005). "HEK293 cell line: a vehicle for the expression of recombinant proteins." J Pharmacol Toxicol Methods **51**(3): 187-200.

Tirapelli, C. R., S. R. Ambrosio, et al. (2010). "Hypotensive action of naturally occurring diterpenes: a therapeutic promise for the treatment of hypertension." Fitoterapia **81**(7): 690-702.

Tobise, K., Y. Ishikawa, et al. (1994). "Changes in type VI adenylyl cyclase isoform expression correlate with a decreased capacity for cAMP generation in the aging ventricle." Circ Res **74**(4): 596-603.

VanRollins, M., T. L. Kaduce, et al. (1996). "Arachidonic acid diols produced by cytochrome P-450 monooxygenases are incorporated into phospholipids of vascular endothelial cells." J Biol Chem **271**(24): 14001-9.

Violin, J. D., L. M. DiPilato, et al. (2008). "beta2-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics." J Biol Chem **283**(5): 2949-61.

Wang, Y., X. Wei, et al. (2005). "Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways." J Pharmacol Exp Ther **314**(2): 522-32.

Watts, V. J. and K. A. Neve (2005). "Sensitization of adenylate cyclase by Galpha i/o-coupled receptors." Pharmacol Ther **106**(3): 405-21.

Willoughby, D., G. S. Baillie, et al. (2007). "Dynamic regulation, desensitization, and cross-talk in discrete subcellular microdomains during beta2-adrenoceptor and prostanoid receptor cAMP signaling." J Biol Chem **282**(47): 34235-49.

Willoughby, D., W. Wong, et al. (2006). "An anchored PKA and PDE4 complex regulates subplasmalemmal cAMP dynamics." Embo J **25**(10): 2051-61.

Wittpoth, C., K. Scholich, et al. (1999). "Regions on adenylyl cyclase that are necessary for inhibition of activity by beta gamma and G(ialpha) subunits of heterotrimeric G proteins." Proc Natl Acad Sci U S A **96**(17): 9551-6.

Wong, P. Y., P. S. Lai, et al. (2000). "Mechanism and signal transduction of 14 (R), 15 (S)-epoxyeicosatrienoic acid (14,15-EET) binding in guinea pig monocytes." Prostaglandins Other Lipid Mediat **62**(4): 321-33.

Wong, P. Y., P. S. Lai, et al. (1997). "Post-receptor signal transduction and regulation of 14(R),15(S)-epoxyeicosatrienoic acid (14,15-EET) binding in U-937 cells." J Lipid Mediat Cell Signal **16**(3): 155-69.

Wong, P. Y., K. T. Lin, et al. (1993). "14(R),15(S)-epoxyeicosatrienoic acid (14(R),15(S)-EET) receptor in guinea pig mononuclear cell membranes." J Lipid Mediat **6**(1-3): 199-208.

Wong, Y. H., A. Federman, et al. (1991). "Mutant alpha subunits of Gi2 inhibit cyclic AMP accumulation." Nature **351**(6321): 63-5.

Worth, C. L., A. Kreuchwig, et al. (2011). "GPCR-SSFE: A comprehensive database of G-protein-coupled receptor template predictions and homology models." BMC Bioinformatics **12**: 185.

Wu, S., W. Chen, et al. (1997). "Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes." J Biol Chem **272**(19): 12551-9.

Xiao, Y. F. (2007). "Cyclic AMP-dependent modulation of cardiac L-type Ca2+ and transient outward K+ channel activities by epoxyeicosatrienoic acids." Prostaglandins Other Lipid Mediat **82**(1-4): 11-8.

Xiao, Y. F., L. Huang, et al. (1998). "Cytochrome P450: a novel system modulating Ca2+ channels and contraction in mammalian heart cells." J Physiol **508 (Pt 3)**: 777-92.

Xin, W., T. M. Tran, et al. (2008). "Roles of GRK and PDE4 activities in the regulation of beta2 adrenergic signaling." J Gen Physiol **131**(4): 349-64.

Yan, S. Z., Z. H. Huang, et al. (1998). "Conversion of forskolin-insensitive to forskolin-sensitive (mouse-type IX) adenylyl cyclase." Mol Pharmacol **53**(2): 182-7.

Yang, B., L. Graham, et al. (2001). "Overexpression of cytochrome P450 CYP2J2 protects against hypoxia-reoxygenation injury in cultured bovine aortic endothelial cells." Mol Pharmacol **60**(2): 310-20.

Yoshimoto, T. and Y. Takahashi (2002). "Arachidonate 12-lipoxygenases." Prostaglandins Other Lipid Mediat **68-69**: 245-62.

Yu, Z., B. B. Davis, et al. (2004). "Vascular localization of soluble epoxide hydrolase in the human kidney." Am J Physiol Renal Physiol **286**(4): F720-6.

Yu, Z., F. Xu, et al. (2000). "Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids." Circ Res **87**(11): 992-8.

Zaccolo, M. (2009). "cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies." Br J Pharmacol **158**(1): 50-60.

Zeldin, D. C. (2001). "Epoxygenase pathways of arachidonic acid metabolism." J Biol Chem **276**(39): 36059-62.

Zhang, W., T. Otsuka, et al. (2008). "Soluble epoxide hydrolase gene deletion is protective against experimental cerebral ischemia." Stroke **39**(7): 2073-8.

Zhang, Y., H. El-Sikhry, et al. (2009). "Overexpression of CYP2J2 provides protection against doxorubicin-induced cardiotoxicity." Am J Physiol Heart Circ Physiol **297**(1): H37-46.

Zhao, X., T. Yamamoto, et al. (2004). "Soluble epoxide hydrolase inhibition protects the kidney from hypertension-induced damage." J Am Soc Nephrol **15**(5): 1244-53.

Zou, A. P., J. T. Fleming, et al. (1996). "Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K(+)-channel activity." Am J Physiol **270**(5 Pt 2): F822-32.