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Cytochrome P450 Eicosanoids Regulate Vascular Function Via Peroxisome Proliferator-Activated Receptor α

by

Yong-Hsin Valerie Ng

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CEHMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Cytochrome P450 Eicosanoids Regulate Vascular Function Via Peroxisome Proliferator-Activated Receptor α

Yong-Hsin Valerie Ng

Eicosanoids generated from the metabolism of arachidonic acid by cytochrome P450 (CYP) metabolism are mediators of diverse biological functions including the regulation of vascular tone, cellular proliferation, and inflammation. The major products of CYP-catalyzed arachidonic acid metabolism are 20-hydroxyeicosatetraenoic acid and the regio- and stereospecific epoxyeicosatrienoic acids, which can be further metabolized by soluble epoxide hydrolase (sEH) into their corresponding dihydroxyeicosatrienoic acids. Regulation of CYP eicosanoid levels is determined by numerous factors including the expression and function of the CYP enzymes responsible for their formation. Induction and repression of CYP and sEH can be mediated by the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). Ligands for PPARα include fatty acids, eicosanoids, and fibrate drugs. Given that eicosanoids are PPARα agonists, and that PPARα can regulate CYP eicosanoid levels, the overall hypothesis tested in this dissertation is that components of the CYP-catalyzed pathway of arachidonic acid metabolism affect and/or mediate biological effects via PPARα-associated transcriptional signaling. CYP eicosanoids and a class of urea-based inhibitors of sEH are identified as novel PPARα ligands that alter the transcription of PPARα-responsive genes. The latter group of compounds consists of urea-based alkanoic acids which potently attenuate smooth muscle cell proliferation, an action that requires PPARα

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expression. In smooth muscle cells, CYP eicosanoids also induced the expression of cyclooxygenase-2 (COX-2) via increased NF-κB signaling and in a PPARα-dependent manner. Upregulation of COX-2 resulted in the surprising attenuation of inflammatory prostaglandin release, showing that cross-talk between the cyclooxygenase and CYP-catalyzed pathways of arachidonic acid metabolism may have anti-inflammatory effects. In vivo, CYP and sEH expression, especially in small diameter vessels and the heart, are altered after treatment with a PPARα agonist, demonstrating that these enzymes are responsive to PPARα activation physiologically. In summary, these results suggest that CYP eicosanoids and urea-based inhibitors of sEH favorably affect vascular function and mediate their effects through PPARα. Further investigations into the actions of these molecules may be helpful in the design of pharmacological modulators for the treatment of inflammation and vasculoproliferative diseases.

Deanna L. Kroetz

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Chapter 1

Introduction

1.1 Arachidonic Acid Metabolism by Cytochrome P450 Enzymes

1.1.1 Source of Arachidonic Acid

Unsaturated fatty acids are important physiological mediators of diverse biological processes such as inflammation, allergic reactions, pain, sleep, and hypertension. Arachidonic acid (AA or $\omega 6$, 20:4, $\Delta^{5,8,11,14}$) is one such biologically significant fatty acid. Mainly synthesized from the essential fatty acid linoleic acid by desaturase and elongase enzyme systems, AA is a long-chain polyunsaturated fatty acid with four *cis* double bonds (Mayes and Botham 2003). The cellular uptake of AA is controversial and various lines of evidence suggest that proteins, receptors, transporters, or simple diffusion mediate AA transfer into the cell (Brash 2001). The concentration of free AA is usually low in non-stimulated cells due to its esterification and incorporation into the phospholipid pools of cellular membranes. Depending on the cell type, esterified arachidonate is present in the micromolar to millimolar range. This intracellular store can be rapidly released and could generate high local concentrations, resulting in the efficient regulation of downstream signaling cascades (Chilton et al. 1996; Neufeld and Majerus 1983; Ramanadham et al. 1992).

In the presence of agonists such as thrombin, bradykinin, angiotensin II, or epinephrine, the activation of phospholipase A_2 (PLA_2) releases esterified AA from phospholipid pools (Roman et al. 2000). This mechanism is a critical step in regulating the levels of free AA for the production of eicosanoids. The group IV cytosolic PLA_2

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($cPLA_2$) is the main enzyme responsible for the release of AA from the sn-2 position of phospholipids, although the Ca^{2+}-dependent secretory PLA_2 and the Ca^{2+}-independent intracellular PLA_2 have also been implicated (Chakraborti 2003). The activity of $cPLA_2$ itself is tightly regulated and in most cell types, its full activation requires both phosphorylation by mitogen-activated protein kinases and elevated intracellular calcium levels (Leslie 2004).

Once released from the phospholipid pool, AA is susceptible to metabolism by three distinct sets of enzymes: cyclooxygenases, lipoxygenases, and the cytochrome P450 (CYP) monooxygenases. Metabolites of AA are collectively known as eicosanoids which are biologically active in many tissues including the brain, liver, kidney, immune, and cardiovascular systems. Prostaglandins, prostacyclins, thromboxanes, and leukotrienes are products of the cyclooxygenase and lipoxygenase metabolism of AA (Funk 2001). The major products of CYP-mediated AA metabolism are 20-hydroxyeicosatetraenoic acid (20-HETE) and regio- and stereospecific epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs, Figure 1.1) (Kroetz and Zeldin 2002).

1.1.2 CYP ω-hydroxylases

20-HETE-, and to a lesser extent, 19-HETE production from AA are catalyzed by the CYP4 family of enzymes. Hydroxylation of other medium and long-chain fatty acids is also catalyzed by the CYP4 enzymes. Evolutionarily, the CYP4 enzymes are one of the oldest members of the CYP superfamily. CYP4A and CYP4F isoforms with AA ω/ω-1 activities have been discovered in rats (4A1, 4A2, 4A3, 4F1,4F4), humans (4A11,

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4F2, 4F3, 4F12), rabbits (4A4, 4A6, 4A7), and mice (4A10, 4A12) (Hashizume et al. 2001; Honeck et al. 2000; Kawashima and Strobel 1995; Kikuta et al. 1993; Kikuta et al. 1999; Lasker et al. 2000; Nguyen et al. 1999; Powell et al. 1998; Roman et al. 1993; Xu et al. 2004a).

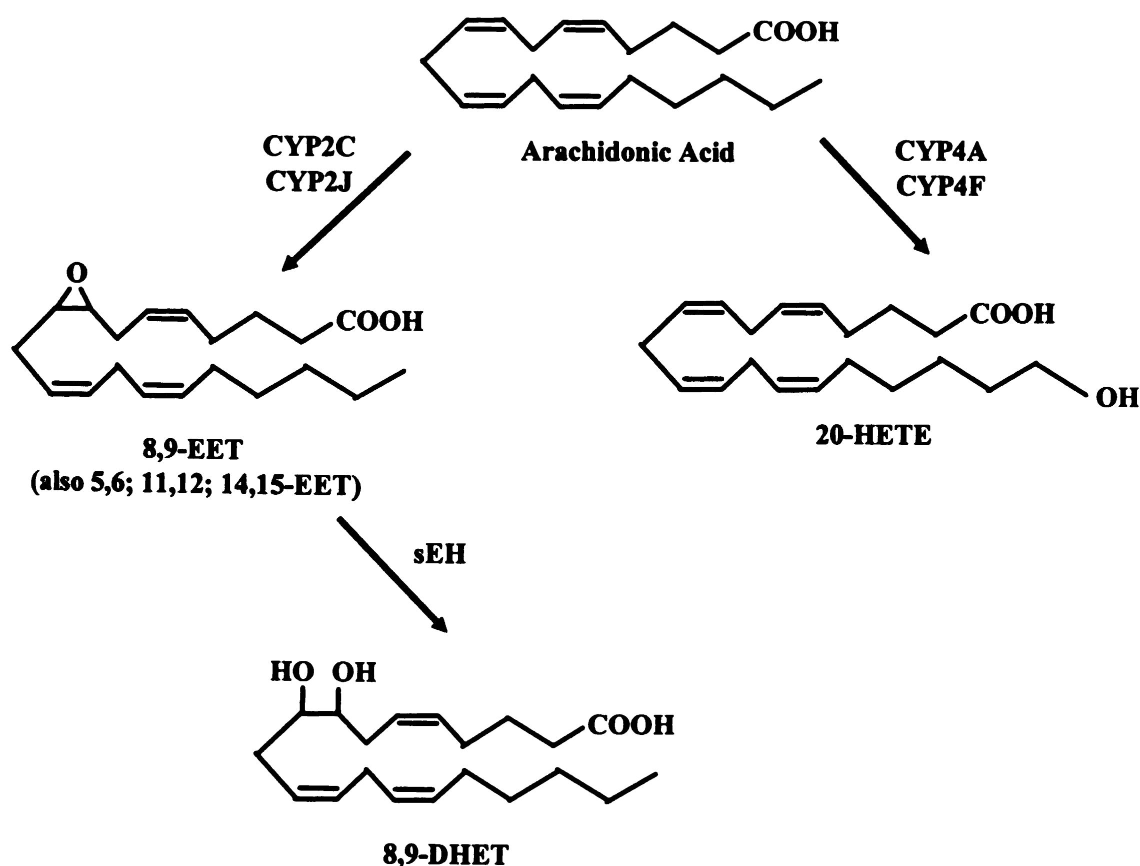

Figure 1.1 Metabolism of arachidonic acid by cytochrome P450 enzymes. Epoxidation by CYP2C and CYP2J epoxygenases leads to the formation of four regioisomeric EETs, which can be further hydrolyzed by the soluble epoxide hydrolase to form the corresponding DHETs. Oxidation at the ω position by CYP4A and CYP4F hydroxylases results in the formation of 20-HETE.

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In the rat, CYP4A expression is the highest in the kidney, and is also detected in the liver, lung, brain, skeletal muscle, and small arterioles within these tissues (Ito et al. 1998; Kimura et al. 1989; Marji et al. 2002; Stromstedt et al. 1994; Wang et al. 2004a; Zhu et al. 2002). In the vasculature, CYP4A protein levels are highest in vessels less than 100 μm and decrease with increasing vessel diameter (Marji et al. 2002). Recombinant CYP4A1 displays a 10-fold greater AA catalytic activity compared with CYP4A2 and 4A3, although CYP4A2 appears to be the isoform that is constitutively expressed in most tissues (Ito et al. 1998; Kroetz et al. 1997; Nguyen et al. 1999). Regulation of CYP4A expression is associated with activation of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). Hypolipidemic agents such as fibrates and synthetic compounds that induce peroxisome proliferation (known as peroxisome proliferators) upregulate CYP4A expression in the rodent liver and kidney (Aldridge et al. 1995; Muerhoff et al. 1992; Okita et al. 1993). PPARα knockout mice are refractory to the induction of CYP4A, demonstrating the indispensable role of this receptor in the regulation of CYP4A expression (Honkakoski and Negishi 2000; Lee et al. 1995).

Similar to CYP4A, CYP4F proteins are also highly expressed in the rat kidney and also detected in the liver and brain (Kalsotra et al. 2002). Studies of CYP4Fs were initially focused on the hydroxylation of leukotriene B_4, but CYP4F1 and 4F4 have recently been shown to ω-hydroxylate AA with k_{cat} values comparable to that of CYP4A1 (Xu et al. 2004a). In addition, CYP4Fs display hydroxylase activity towards prostaglandins, prostaglandin endoperoxides, lipoxins, several HETEs, fatty acid epoxides, and tocopherols (Kikuta et al. 1993; Le Quere et al. 2004; Parker et al. 2004).

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Xenobiotics such as erythromycin, benzphetamine, ethylmorphine, chlorpromazine, and imipramine are also CYP4F substrates (Kalsotra et al. 2004).

In humans, CYP4A and CYP4F enzymes are widely distributed. CYP4A11 is mainly expressed in the liver, and its expression has also been detected in the kidney, heart, skeletal muscle, keratinocytes, human mononuclear cells and saphenous veins (Asghar et al. 2002; Bertrand-Thiebault et al. 2004; Cui et al. 2000; Gonzalez et al. 2001). CYP4F2 enzymes are highly expressed in the kidney and liver (Kikuta et al. 1994; Lasker et al. 2000) with the expression of CYP4F3 in human polymorhonuclear leukocytes and CYP4F12 in the small intestine (Hashizume et al. 2001; Kikuta et al. 1998). Unlike in the rat, vascular expression of human AA ω-hydroxylases has not been as extensively characterized. The major CYP4A to exhibit AA-specific ω-hydroxylase activity is CYP4A11, although its efficiency is much lower compared to the rat CYP4A enzymes (Hoch et al. 2000). CYP4F2 appears to be the enzyme that is mainly responsible for the formation of 20-HETE, since immunoinhibition of CYP4F2 results in a 67% reduction in 20-HETE production in human kidney microsomes, whereas a CYP4A11 antibody only reduced 20-HETE formation by 32% (Powell et al. 1998).

1.1.3 CYP Epoxygenases

Unlike the ω-hydroxylation of AA, epoxidation is catalyzed by a variety of CYP enzymes in the CYP1 and CYP2 families (Daikh et al. 1994; Imaoka et al. 1993; Oleksiak et al. 2000; Rifkind et al. 1995; Wu et al. 1996). CYP2C and CYP2J-catalyzed EET formation has been the most extensively characterized. CYP2C11 is the major epoxygenase in the rodent liver, whereas CYP2C23 is believed to be the principal

epoxygenase in rat kidney (Capdevila et al. 1992; Holla et al. 1999). CYP2J3 is also expressed in the proximal tubule and collecting duct in rat kidney (Ma et al. 1999). In the rat brain, CYP2C11 is highly expressed in astrocytes (Alkayed et al. 1996). CYP2J3 is expressed in the heart, pancreas, liver, lung, and kidney of rats (Wu et al. 1997).

In humans, CYP2C8, CYP2C9, CYP2C19, and CYP2J2 are expressed in the liver and liver microsomes produced EETs when incubated with AA (Bylund et al. 1998; Daikh et al. 1994; Rifkind et al. 1995; Wu et al. 1996; Zeldin et al. 1996). Using the CYP2C9 inhibitor sulfaphenazole, it was shown that CYP2C9 was responsible for the formation of 50% of the epoxygenase activity in human liver microsomes (Rifkind et al. 1995). Another study however showed that immunoprecipitation of CYP2C8 in human liver microsomes reduced EET formation by 85% (Zeldin et al. 1996). In the heart and lung, CYP2J2 is emerging as a major enzyme responsible for the production of EETs; in the heart, it is highly expressed in cardiac myocytes and serves as the primary catalyst of AA to EETs (Wu et al. 1996). CYP2C8, CYP2C9, and CYP2J2 are the principal AA epoxygenases in human endothelial cells and support the role of EETs as putative endothelial-derived hyperpolarizing factors (EDHFs) (Fisslthaler et al. 1999; Node et al. 1999; Yang et al. 2001).

1.1.4 Metabolic Fate of EETs

EETs can be further converted into a variety of metabolites by different pathways. Soluble epoxide hydrolase (sEH), and to a lesser extent, microsomal epoxide hydrolase (mEH), hydrates EETs into their corresponding dihydroxyeicosatrienoic acids (DHETs) (Zeldin et al. 1993). DHETs are generally considered to have less biological activity

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compared to the EETs, and EET hydration is perceived as a means to limit the actions of EETs. However, the biological function of DHETs has not been extensively characterized. sEH expression is widely distributed and its activity has been detected in the liver, kidney, lungs, heart, brain, and vascular tissues (Enayetallah et al. 2004; Johansson et al. 1995; Pinot et al. 1995; Sellers et al. 2005; Sevanian et al. 1980; Yu et al. 2004b). sEH metabolism of EETs is highly regioselective with 14,15-EET being the preferred substrate while 11,12-EET and 8,9-EET are hydrated at significantly lower rates (Yu et al. 2000; Zeldin et al. 1993).

EETs, as well as 20-HETE, can also be further metabolized by β-oxidation or cyclooxygenases, and can be esterified and reincorporated into membrane phospholipid pools (Roman 2002). The latter mechanism represents an important method for storing large pools of eicosanoids which can be rapidly released in response to stimuli. High concentrations of EETs (up to micromolar) have been extracted from kidney, platelets, and endothelial cells (Karara et al. 1991; VanRollins et al. 1993; Zhu et al. 1995b). All four EET regioisomers can also be converted to their corresponding ω/ω-1 hydroxy metabolites by CYP ω-hydroxylases. Interestingly, 20-hydroxy metabolites of 11,12- and 14,15-EET bind to and transactivate PPARα, suggesting that these eicosanoids may be involved in the regulation of fatty acid metabolism (Cowart et al. 2002).

1.2 Biological Significance of CYP Eicosanoids

1.2.1 Vascular Tone

20-HETE potently constricts small arteries and arterioles by blocking large conductance calcium activated potassium channels (K_{Ca}), resulting in depolarization of

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smooth muscle cells and increased calcium entry via the L-type calcium channel (Harder et al. 1994; Zou et al. 1996) . The vasoconstrictive effects of 20-HETE have been documented in the renal, cerebral, mesenteric, and skeletal microvasculature, with little or no effect on larger arteries or the aorta (Alonso-Galicia et al. 1999a; Gebremedhin et al. 2000; Harder et al. 1996; Marji et al. 2002; Wang et al. 2001). Deviations from the established vasoconstrictive properties of 20-HETE have been reported in coronary and pulmonary vessels where 20-HETE was vasodilatory (Birks et al. 1997; Pratt et al. 1998). In addition, 20-HETE interacts with intracellular proteins that activates the protein kinase C signaling pathway in smooth muscle cells isolated from cerebral arteries, thereby increasing cellular calcium sensitivity and altering vascular tone (Lange et al. 1997).

EETs are generally regarded as vasodilators and have been shown to dilate renal, mesenteric, cerebral, pulmonary, and coronary arteries (Campbell et al. 2001; Medhora et al. 2001; Miller et al. 2001; Pomposiello et al. 2001; Yaghi et al. 2001). EETs are produced in endothelial cells and through an unknown mechanism, are transported to smooth muscle cells where they reduce vascular tone. This is accomplished by the hyperpolarization of smooth muscle cells via increasing the open-state probability of K_{Ca} channels. In light of their actions and mechanism, EETs have been proposed to be EDHF (Campbell et al. 1996; Fisslthaler et al. 1999; Node et al. 1999). This hypothesis is supported by findings from many groups including the observation that inhibition of CYP2C enzymes diminishes EDHF-mediated responses (Campbell et al. 1996; Fisslthaler et al. 1999; Lischke et al. 1995).

Depending on the species and vascular bed examined, specific regioisomers may or may not be equipotent in their vasoactive properties. For example, 11,12-EET exhibits

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greater vasodilatory actions in cat cerebral arteries whereas in canine coronary arteries, all the regioisomers elicit the same vasodilatory response (Gebremedhin et al. 1992; Oltman et al. 1998). Some evidence of vasoconstrictive effects of EETs have also been reported (Carroll et al. 1992; Zhu et al. 2000). The effects of DHETS have not been thoroughly investigated, and studies have shown that DHETs are more, less, and as potent as EETs in their vasoactive functions (Gebremedhin et al. 1992; Imig et al. 1996; Lu et al. 2001; Oltman et al. 1998).

1.2.2 Inflammation

Distinct from their role in vascular tone regulation, EETs were shown to exert anti-inflammatory effects by inhibiting cytokine-induced expression of adhesion molecules in endothelial cells and preventing leukocyte adhesion to the vascular wall (Node et al. 1999). Overexpression of CYP2J2 or exogenous application of nanomolar amounts of 11,12-EET, but not 14,15-EET, attenuated the increased expression of vascular cell adhesion molecule-1(VCAM-1) and E-selectin induced by tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and lipopolysaccharide (LPS) (Node et al. 1999). The mechanism for the anti-inflammatory properties of EETs is related to the inhibition of NF-κB signaling. By interfering with the nuclear translocation of an NF-κB subunit required for transcription initiation, 11,12-EET potently reduced the expression of pro-inflammatory genes. These effects were independent of membrane hyperpolarization as selective K_{Ca} channel blockers did not attenuate their anti-inflammatory actions. In other studies, EETs decreased the attachment of polymorphonuclear leukocytes to endothelial cells and attenuated LPS-induced PGE_2 synthesis by monocytes (Kozak et al. 2003; Pratt

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et al. 2002). Interestingly in a different report, although 11,12-EET inhibited NF-κB activation and expression of adhesion molecules in endothelial cells, overexpression or induction of CYP2C9 enhanced NF-κB activity (Fleming et al. 2001). EETs also activate other signaling pathways such as the tyrosine and mitogen-activated protein (MAP) kinases in both endothelial and smooth muscle cells, which may be other mechanisms involved in the anti-inflammatory effects of EETs (Mombouli et al. 1999).

1.2.3 Vascular Growth

20-HETE and EETs contribute to vascular growth by acting as mitogenic mediators. 20-HETE increases the incorporation of thymidine in proximal tubular cells and activates the MAP kinase system in response to norepinephrine and angiotensin II (Lin et al. 1995; Muthalif et al. 2000b; Uddin et al. 1998). EETs have been shown to promote cell proliferation in renal epithelial cells and vascular endothelial cells (Chen et al. 1998; Munzenmaier and Harder 2000). These effects are associated with an increase in intracellular Ca^{2+} concentration and activation of the tyrosine kinase and MAP kinase signaling cascades. These results suggest that 20-HETE and EETs may promote angiogenesis in the vascular system. Administration of HET0016, a 20-HETE inhibitor, suppressed angiogenic responses in the rat cornea, and in cerebral microvascular and umbilical endothelial cells, EETs resulted in increased formation of endothelial tubes (Chen et al. 2005; Michaelis et al. 2005; Munzenmaier and Harder 2000). The effects of EETs were significantly inhibited by the cytochrome P450 inhibitor 17-ODYA, further confirming the angiogenic effect of EETs. EETs, particularly 11,12-EET, also inhibit smooth muscle cell migration, whose effects were attenuated by inhibitors of MAP

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kinase and phosphatidylinositol 3-kinase/Akt pathways (Sun et al. 2002; Wang et al. 2005).

1.2.4 Ischemia

There is increasing evidence that EETs are protective against injury after ischemia-reperfusion. In an isolated-perfused rat heart model, incubation with 11,12-EET significantly improved myocardial contractility after ischemia-reperfusion (Wu et al. 1997). Endogenous generation of EETs by CYP2J2 in coronary and aortic endothelial cells reduces hypoxia-reoxygenation injury and reactive oxygen species production, effects that can be mimicked by exogenous application of 11,12-EET, and to a lesser extent, 11,12-DHET and 14,15-EET (Yang et al. 2001). Furthermore, cardiomyocyte-specific transgenic expression of CYP2J2 in mice resulted in significant improvement of cardiac function post ischemic injury compared to wild type hearts. These protective effects were proposed to be mediated via the activation of p42/p44 MAP kinase signaling and the mitochondrial ATP-sensitive K^+ channels (Seubert et al. 2004).

1.3 Clinical Relevance of CYP Eicosanoids

1.3.1 Experimental Models of Hypertension

Rats treated with deoxycorticosterone (DOCA) and a high-salt diet result in a hypertensive phenotype. The elevation of 20-HETE formation has been implicated in this disease model and 20-HETE inhibitors have been used to examine the effects of 20-HETE on blood pressure regulation. Administration of 1-aminobenzotriazole (ABT), a specific inhibitor of 20-HETE and EET synthesis, prevented the development of

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hypertension in DOCA-salt-treated animals by inhibiting renal ω-hydroxylase activity and decreasing MAP kinase signaling in smooth muscle cells (Muthalif et al. 2000a; Oyekan et al. 1999). In contrast, decreased ω-hydroxylase activity has also been reported in DOCA salt-treated animals and induction of 20-HETE production by fibrates resulted in an anti-hypertensive phenotype associated with increased renal Cyp4a14 expression (Honeck et al. 2000). Angiotensin (ANG) II treatment results in increased formation of 20-HETE in the rat renal circulation (Croft et al. 2000). Chronic inhibition of CYP eicosanoid production using ABT significantly reduced blood pressure in ANG II-infused rats in vivo and blockade of 20-HETE in isolated renal arterioles attenuated the vasocontrictive effects of ANG II (Alonso-Galicia et al. 1999b; Muthalif et al. 2000b; Xu et al. 2004a)

In the spontaneously hypertensive rat (SHR), 20-HETE formation is elevated in the kidney and mesenteric artery (Kroetz et al. 1997; Sacerdoti et al. 1988; Zhang et al. 2001). These increases are consistent with increased renal arachidonic acid ω-hydroxylase activity and expression (Kroetz et al. 1997; Stec et al. 1996) . Using anti-sense oligonucleotides against CYP4A, vascular reactivity was reduced in SHR renal microvessles (Wang et al. 2001). In vivo, treatment with the 20-HETE-specific inhibitor sodium 10-undecynyl sulfate resulted in the acute reduction of blood pressure in SHRs (Xu et al. 2002).

Deletion of murine Cyp4a14 provided additional evidence that 20-HETE is involved in blood pressure regulation, although hypertension was evident only in male mice (Holla et al. 2001). Interestingly, animals lacking Cyp4a14 exhibited increased ω-hydroxylase activity and higher renal levels of 20-HETE due to a compensatory induction of

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Cyp4a12. The hypertension however is androgen-sensitive and castration normalized blood pressure in these animals.

EETs are also important regulators of blood pressure. The targeted disruption of the sEH gene resulted in decreased EET and DHET formation in the kidney of both male and female mice, and in male mice, decreased systolic blood pressure was apparent (Sinal et al. 2000). In SHRs, sEH expression, urinary DHET excretion, and blood pressure are greatly increased relative to normotensive Wistar-Kyoto rats, and inhibition of sEH with a tight-binding sEH inhibitor reversed the hypertensive phenotype, implicating the antihypertensive properties of EETs (Yu et al. 2000). Similarly, inhibition of sEH using another urea-based inhibitor in the ANG-II and DOCA-salt models of hypertension decreased blood pressure (Imig et al. 2002; Loch et al. 2004). The activity of CYP epoxygenases is induced with excess dietary salt intake as an adaptive response to limit salt retention, and inhibition of CYP epoxygenase activity by clotrimazole leads to a salt-dependent hypertension phenotype (Holla et al. 1999). The inability of Dahl salt-sensitive rats to induce renal CYP epoxygenase activity upon excess dietary salt intake indicates that abnormalities in epoxygenase activity may be related to their hypertensive phenotype.

1.3.2 Humans

Despite extensive characterization of CYP eicosanoids in animal models of hypertension, relatively little is known about the role of 20-HETE and EETs in humans. In obese patients with essential hypertension, urinary 20-HETE excretion was negatively correlated with insulin levels, suggesting that ω-hydroxylase function may be inhibited

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by circulating insulin (Laffer et al. 2004). 20-HETE excretion is also regulated by salt intake in hypertensive subjects, where salt-sensitive patients have a disrupted relationship between sodium excretion and 20-HETE compared with salt-resistant patients (Laffer et al. 2003). In pregnancy-induced hypertensive patients, increased urinary exretion of 11,12- and 14,15-DHET is evident compared with healthy pregnant patients, suggesting that EETs may be involved in the pathophysiology of pregnancy-induced hypertension (Catella et al. 1990).

Genetic polymorphisms have been identified in human ω-hydroxylase and CYP epoxygenase genes that could potentially result in physiological changes. A variant in the human CYP4A11 gene (T590C) demonstrated a significantly reduced ω-hydroxylase activity and is associated with a hypertensive phenotype (Gainer et al. 2005). CYP2C8, the major epoxygenase in human liver and kidney, has three reported non-synonymous single-nucleotide polymorphisms (SNPs). CYP2C8*3 (Arg139Lys and Lys399Arg)-mediated AA activity is roughly one-third that of the reference enzyme, however there is no significant association between the CYP2C8*3 genotype and hypertension in Caucasian males (Dai et al. 2001; King et al. 2005). In a study carried out in healthy and hypertensive Chinese subjects, the CYP2C9*3 genetoype was associated with a protective phenotype, especially in females (Yu et al. 2004a).

Similarly, four CYP2J2 variants also exhibited reduced AA metabolism (King et al. 2002). A polymorphism located at -50 site in the proximal promoter of CYP2J2 (CYP2J2*7) results in the loss of Sp1 transcription factor binding to the promoter, decreased CYP2J2 promoter activity, and lower plasma levels of EET metabolites. Importantly, this polymorphism is associated with increased risk of coronary artery

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disease (Spiecker et al. 2004). In contrast, another recent study showed an association between the CYP2J2*7 genotype with a lower frequency of hypertension in Caucasian individuals, suggesting the protective effect of this variant (King et al. 2005).

Polymorphism in the sEH gene has also been described in the human population. Alterations in sEH activity may lead to an imbalance between intracellular levels of epoxides and dihydrodiols. Two sEH variants, the Arg287Gln and the Arg287Gln/Arg103Cys haplotype show decreased 14,15-EET enzymatic activity, whereas elevated activity was evident in the Lys55Arg and Cys154Tyr variants (Przybyla-Zawislak et al. 2003). In an intrafamilial hypercholesterolemia association study, among the carriers of a LDL receptor mutation, a Arg287Gln allele in sEH resulted in a significant elevation in plasma cholesterol and triglyceride levels (Sato et al. 2004). In a separate study, African-American subjects with the Arg287Gln allele had an approximately 2-fold increase in the risk of having coronary artery calcification (Fornage et al. 2004). Investigations into the association of CYP/sEH SNPs and their phenotypes will be important in further understanding the relationship between CYP eicosanoids and their physiological function in humans.

1.4 Overview of the Peroxisome Proliferator-Activated Receptor Family

1.4.1 Structure and Expression of PPAR Isoforms

Peroxisome proliferator-activated receptors (PPARs, NR1C) belong to one of the 48 members of the nuclear receptor superfamily presently identified in the human genome. There are three known isoforms within the PPAR subfamily, α, β, and γ (Issemann and Green 1990; Kliewer et al. 1994). Despite variations in ligand sensitivity,

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the structural organization of these nuclear receptors is highly similar (Chawla et al. 2001). They contain an NH_2-terminal region containing a ligand-independent transcriptional activation function (AF-1), a DNA-binding domain containing two highly conserved zinc finger motifs that allows the receptor to bind to specific DNA response elements, a hinge region permitting protein flexibility that allows for simultaneous receptor dimerization and DNA binding, and a large COOH-terminus that includes the ligand-binding domain, the dimerization interface, and a ligand-dependent activation function (AF-2, Figure 1.2). AF-2 is an important region as it allows for the modulation of transcriptional activity through corepressor or coactivator associations (McKenna et al. 1999).

Figure 1.2 Schematic diagram of the PPAR secondary structure.

PPARs are widely expressed in a variety of tissues. PPARα is found predominantly in the liver, heart, kidney, and intestines, and PPARγ is expressed in the adipose tissue, large intestine, spleen, and heart (Braissant et al. 1996; Issemann and Green 1990; Kliewer et al. 1994; Mukherjee et al. 1997). In addition, PPARγ exists in three distinct isoforms derived from alternative promoter usage, PPARγ1, PPARγ2, and PPARγ3 with distinct tissue distribution; PPARγ1 is ubiquitously expressed but primarily in the adipose tissue and macrophages, PPARγ2 is expressed exclusively in the adipose tissue, and PPARγ3 is found in the large intestine (Braissant et al. 1996; Zhu et al. 1995a). The expression of PPARδ is more ubiquitous (Kliewer et al. 1994). In the last

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few years, PPARα and PPARγ has been detected in macrophages, vascular endothelial and smooth muscle cells, and increasing interest has been placed on their role in the cardiovascular system (Chinetti et al. 1998; Marx et al. 1999a; Marx et al. 1998; Marx et al. 1999b; Ricote et al. 1998; Staels et al. 1998).

1.4.2 PPAR Ligands

Ligands for the PPARs are biologically and structurally diverse. Exogenous ligands for PPARα include a group of chemicals known as peroxisome proliferators, including hypolipidemic drugs such as fibrates, gemfibrozil, Wy 14,643, pathalate esters, plasticizers, and herbicides (Table 1.1). There is some correlation between the activity of a peroxisome proliferator towards PPARα and its potency as an inducer of hepatocarcinogenesis in rodents, supporting the hypothesis that hepatic PPARα is a target of hepatocarcinogenic peroxisome proliferators (Bayly et al. 1994; Lee et al. 1995; Marsman et al. 1988; Reddy et al. 1982). The antidiabetic thaizolidinediones (TZDs) such as troglitazone, rosiglitazone, and ciglitazone are xenobiotic ligands of PPARγ (Willson et al. 1996). Several nonsteroidal anti-inflammatory drugs such as indomethacin and ibuprofen activate both PPARα and PPARγ (Lehmann et al. 1997).

Endogenous PPAR ligands include oxidized low-density lipoproteins, fatty acids such as arachidonic acid and various eicosanoids, which bind to the three PPAR isoforms to varying degrees (Forman et al. 1997; Kliewer et al. 1995; Krey et al. 1997). LTB_4 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-Δ-PGJ_2) were one of the first endogenous ligands described for PPARα and PPARγ, respectively (Devchand et al. 1996; Forman et al. 1995). To date, 8(S)-HETE is the most potent endogenous PPARα agonist (Forman et al. 1997). In

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general, fatty acids with chain lengths under 16 and over 22 carbons weakly activate the PPARs. PPARα appears to be the most promiscuous of the three isoforms, exhibiting strong binding affinity for both saturated and unsaturated fatty acids. Similar to PPARα, PPARδ binds to a diverse array of fatty acids, albeit with lower affinity. PPARγ appears to be the most selective as it binds mainly to polyunsaturated fatty acids (Xu et al. 1999). Compared with other nuclear receptors, the ligand binding cavity of PPARs is much larger, 1300 $Å^3$ compared to about 450 $Å^3$ for the estrogen receptor, and this difference has been used to explain the promiscuity of the receptor (Moras and Gronemeyer 1998; Nolte et al. 1998; Tanenbaum et al. 1998; Xu et al. 2001).

Compounds	α	γ	β/δ
Hypolipidemic drugs			
Wy 14,643	+++	+	++
Clofibrate	++	–	+
Ciprofibrate	++	–	+
Gemfibrozil	++	+++	–
Phthalate esters	+++	+	++
Thiazolidinediones			
BRL-49653	–	+++	–
Pioglitazone	–	++	–
Ciglitazone	–	++	–
Rosiglitazone	–	++	–
NSAIDs			
Indomethacin	+	++	–
Ibuprofen	+	+	–
Fenoprofen	++	+	–
Saturated fatty acids			
Palmitic (16:0)	+++	–	++
Stearic (18:0)	+++	–	++
Monounsaturated fatty acids			
Palmitoleic (16:1)	+++	+	++
Oleic (18:1)	+++	++	++

Polyunsaturated fatty acids			
Linoleic (18:2)	+++	+	+
Arachidonic (20:4)	+++	+++	++
Eicosapentanoic (22:5)	+++	++	++
Docosahexaenoic (22:6)	+++	+	++
Eicosanoids			
PGA_1	+	+	++
PGA_2	+	+	+
PGD_1	++	++	+
PGD_2	++	++	+
PGJ_2	+	+++	+/–
PGI_2	++	++	+
15-Δ-PGJ_2	+	+++	+/–
8(S)-HETE	+++	–	–
12-HETE	+	ND	ND
15-HETE	ND	ND	+
LTB_4	+/–	ND	–
Oxidized LDL	ND	++	ND
Oxidized linoleic acid products			
9-HODE	ND	++	ND
13-HODE	ND	++	ND

Table 1.1 Exogenous and endogenous activators of PPARs (adapted from Corton et al., 2000 and Bishop-Bailey, 2001). +/–, conflicting reports in the literature regarding activity towards PPAR; ND, not determined for this compound.

1.4.3 Molecular Mechanism

PPARs are required to heterodimerize with the retinoid X receptor (RXR) in order to bind to and activate PPAR-responsive genes (Kliewer et al. 1992). Binding of cognate ligands to the nuclear receptors induces a conformational change that enables binding to DNA sequences known as the peroxisome proliferator response elements (PPREs). Through analysis of several promoters of PPAR target genes, the PPRE sequence is

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defined as two direct repeats of AGGTCA separated by a single nucleotide (DR1) (Bishop-Bailey 2000). In some rare cases, the PPAR/RXR heterodimer can bind to and activate DR2 sequences such as the human REV-ERBα gene and the estrogen response elements (Gervois et al. 1999; Nunez et al. 1997). The sequences flanking the PPREs are also important for receptor stabilization. In the CYP4A6 PPRE, six nucleotides adjacent to the DR1 element are critical for optimal receptor binding and receptor gene activation (Palmer et al. 1995).

Like other nuclear receptors, PPARs are additionally regulated by coactivators and corepressors that can act as bridging proteins between the nuclear receptor and the transcriptional machinery, resulting in enhanced or decreased transactivation, respectively. In the absence of ligand, PPARs can associate with corepressors containing histone deacetylase activity such as nuclear co-repressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Chen and Evans 1995; Horlein et al. 1995). Coactivators, some with histone acetylase activity, are recruited upon ligand binding and are required for the activation of PPAR-responsive genes. These include the steroid receptor co-activator-1 (SRC-1), p300/CBP, and PPARγ-coactivator-1 (PGC-1) (Lonard and O'Malley 2005). The spatial and temporal expression of such proteins provides an additional regulatory control of PPAR activity.

1.4.4 Cross Talk

The fact that RXR is a common heterodimerization partner of the PPARs and other nuclear receptors indicates that competition for available RXR will provide a level of cross talk. Thyroid hormone inhibits the expression of PPARα-regulated genes in vivo

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due to limiting amounts of RXR. Similarly, overexpression of PPARs limited the formation of TR/RXR heterodimers, resulting in the inhibition of TR-responsive genes (Chu et al. 1995; Jow and Mukherjee 1995; Juge-Aubry et al. 1995). PPARα and PPARγ reduce binding of the liver X receptor (LXR) and RXR to the LXR response element, resulting in reduced promoter activity of the sterol regulatory element-binding protein 1c, an LXR-activated gene. Addition of excess RXR attenuated the inhibitory effects of PPARs, indicating that competition for RXR is a plausible mechanism (Ide et al. 2003).

PPARs and other nuclear receptors can also compete for DNA binding at a common response element. TRα homodimers can bind to PPREs and result in the induction of PPARα-responsive genes (Chu et al. 1995; Hunter et al. 1996). PPAR/RXR also competes with the estrogen receptor (ER) homodimer for binding and transactivation of estrogen response elements; conversely, ER lowers both basal and stimulated PPAR-mediated reporter activity (Nunez et al. 1997; Wang and Kilgore 2002). The hepatocyte nuclear factor-4 (HNF) homodimer competes with the PPAR/RXR heterodimer for binding to DR1 elements, blocking the expression of various liver-specific genes (Hertz et al. 1995). Interestingly, both PPARα and HNF-4 bind to a degenerate DR1 sequence upstream of the human PPARα promoter, resulting in induction of PPARα expression (Pineda Torra et al. 2002). Other nuclear receptors such as the retinoid acid receptor, and the chicken ovalbumin upstream promoter transcription factor also bind to PPRE sequences (Jansen et al. 1995; Marcus et al. 1996; Miyata et al. 1993).

Besides interacting with nuclear receptors, PPARs also engage in cross talk with other signaling cascades. Several lines of evidence implicate the response of p38 MAP kinase to peroxisome proliferators. The peroxisome proliferator nafenopin activates

MAP kinase in hepatocytes, and inhibition of MAP kinase blocks the mitogenic response, but not peroxisome proliferation, caused by nafenopin (Cosulich et al. 2000). Docosahexaenoic acid induces apoptosis in smooth muscle cells via a p38 MAP kinase-dependent pathway that regulates PPARα, and selective p38 inhibitors decreased apoptosis and PPARα expression (Diep et al. 2000). PPARα and PPARγ themselves are phosphoproteins and studies show that phosphorylation by MAP kinase leads to enhanced PPAR ligand-dependent transactivation (Barger et al. 2001; Camp and Tafuri 1997; Hu et al. 1996; Shalev et al. 1996). Other kinases such as the Janus kinase-signal transducer and activator of transcription (JAK/STAT), MAP kinase kinase (MEK), and extracellular signal-related kinases (ERK) also engage in cellular cross talk with PPARs (Cosulich et al. 2000; Mounho and Thrall 1999; Shipley and Waxman 2004).

PPARs can also be involved in the trans-repression of signaling mediators such as NF-κB and activation protein-1 (AP-1) (Delerive et al. 1999a). In the vasculature, PPAR ligands inhibits many inflammatory processes such as smooth muscle migration, cytokine and prostaglandin production, adhesion molecule and endothelin-1 expression (Delerive et al. 1999b; Goetze et al. 1999; Marx et al. 1999b; Staels et al. 1998). These PPAR functions do not occur via the binding of PPAR to regulatory elements of these genes, but rather by inhibiting the activation of other transcription factors. NF-κB activation, for example, requires the degradation of its inhibitor IκBα, whose level is subject to regulation by PPARα and PPARγ (Delerive et al. 2000; Mishra et al. 2004; Zingarelli et al. 2003). PPARα and PPARγ also decrease AP-1 activity by inhibiting DNA binding and transactivation of the AP-1 transcription factors (Irukayama-Tomobe et al. 2004; Ryoo et al. 2004; Wang et al. 2002; Zingarelli et al. 2003).

1.5 Biological Roles of PPARs

1.5.1 Energy Homeostasis

The actions of PPARα have been mostly defined at the hepatic level with regards to lipid metabolism. PPARα is the main PPAR subtype expressed in the liver and is a major regulator of fatty acid metabolism, synthesis, and catabolism. It is also involved in HDL synthesis and reverse cholesterol transport (Berthou et al. 1995; Staels et al. 1995; Vu-Dac et al. 1998; Vu-Dac et al. 1995). The binding of fatty acids, eicosanoids, and fibrate drugs can lead to PPARα activation and induction of numerous genes involved in the β-oxidation and cellular uptake of fatty acids, such as acyl-CoA oxidase (ACOX), very-long-chain and medium-chain acyl-CoA dehydrogenase (VLCAD), 3-keto-acyl-CoA thiolase (ACT) , carnitine palmitoyl transferase type I (CPT I), and acyl-CoA synthetase (ACS) (Gulick et al. 1994; Lee et al. 1995; Mascaro et al. 1998; Osumi et al. 1991; Tugwood et al. 1992). PPARα activation also leads to increased cellular uptake of fatty acids by upregulating the expression of fatty acid transport protein and fatty acid translocase (FAT) (Motojima et al. 1998). PPARα agonists enhance components of the HDL synthesis pathway and promote reverse cholesterol transport. In humans, activation of PPARα leads to increased levels of apolipoprotein (Apo) A-I and ApoA-II (Vu-Dac et al. 1998; Vu-Dac et al. 1995). PPARα can also regulate the expression of the ATP-binding cassette transporter A1 (ABCA1), whose activity is involved in the delivery of phospholipids and cholesterol to ApoA-I (Knight et al. 2003). In addition, PPARα activation decreases triglyceride levels by inducing the expression of lipoprotein lipase, and inhibiting ApoC-III in the liver (Schoonjans et al. 1996; Staels et al. 1995). Taken

together, it is clear that PPARα plays a critical role in the maintenance of lipid homeostasis.

The role of PPARγ in adipocyte differentiation has been extensively investigated. This isoform is present at high levels in adipocytes and its activation alone is sufficient for adipocyte differentiation in vitro (Tontonoz et al. 1994). The activation of PPARγ also promotes apoptosis in mature lipid-filled adipocytes, causing an increased formation of insulin-sensitive adipocytes (Okuno et al. 1998). Much interest has also been paid to the insulin-sensitizing actions of PPARγ ligands. PPARγ agonists enhance adipocyte insulin signaling by regulating the synthesis of secreted adipocyte proteins such as adiponectin, thereby affecting insulin signaling in the liver and skeletal muscles (Bajaj et al. 2004; Combs et al. 2002). Increasing evidence suggests that PPARγ agonists improve insulin secretion in patients with type 2 diabetes (Bays et al. 2004).

The actions of PPARδ agonists on the regulation of lipid metabolism have also been described. In skeletal muscles, activation of PPARδ induces the expression of genes involved in lipid utilization, β-oxidation, cholesterol efflux, and energy uncoupling such as ApoA-I, lipoprotein lipase, uncoupling protein-3 and fatty acid binding protein (Dressel et al. 2003). In type 2 diabetic animal models, activation of PPARδ with a selective agonist resulted in significant decreases in blood insulin and glucose levels (Tanaka et al. 2003).

1.5.2 Cardiovascular System

1.5.2.1 Vasculature

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Atherosclerosis is an inflammatory disease marked by the recruitment and activation of a variety of cell types including monocytes/macrophages, endothelial, and smooth muscle cells (Ross 1999). In macrophages, PPARα and PPARγ agonists can inhibit foam cell formation, reduce the uptake of low-density lipoprotein (LDL) by downregulating the apoB48 receptor and upregulating lipoprotein lipase, and stimulate cholesterol efflux via the induction of ABCA1 and scavenger receptor-B1 (Chinetti et al. 2000; Chinetti et al. 2001; Gbaguidi et al. 2002; Haraguchi et al. 2003; Li et al. 2004). In contrast, other results show that PPARγ activation can also promote foam cell formation by the induction of the oxidized LDL receptor CD36, thus creating a positive feedback loop of monocyte activation and foam cell formation (Tontonoz et al. 1998). Similarly, the effect of PPARδ activation on macrophage lipid homeostasis is not clear. PPARδ activators increase ABCA1 expression and cholesterol efflux in macrophages, but also induce the scavenger receptors CD36 and scavenger receptor A, resulting in cholesterol loading and storage (Oliver et al. 2001b; Vosper et al. 2001).

In vascular endothelial cells, activation of PPARα and PPARγ interfere with chemoattraction and cell adhesion of lymphocytes. PPARα agonists inhibit TNFα-induced expression of VCAM-1 in part by inhibiting NF-κB signaling (Marx et al. 1999b). Conflicting studies showed both the induction and lack of effect of PPARα activation by different agonists on the expression and release of monocyte chemotactic protein-1 (MCP-1) and IL-8 (Lee et al. 2000; Marx et al. 2000). Limited evidence also shows that activation of PPARδ reduced TNFα-induced VCAM-1 and MCP-1 expression (Rival et al. 2002). Both PPARα and PPARγ ligands repress thrombin-induced expression of endothelin-1 (ET-1), a potent vasoconstrictor and inducer of smooth muscle

proliferation, by inhibiting AP-1 signaling (Delerive et al. 1999b). Notably, in the mesenteric vasculature of DOCA-salt-induced hypertensive rats, activators of both PPAR isoforms abrogated the increase in prepro-ET-1 mRNA content (Iglarz et al. 2003). Endothelial nitric oxide synthase expression and nitric oxide release are also enhanced in the presence of eicosapentaenoic acid and fenofibrate, suggesting the vasoprotective effect of PPARα (Goya et al. 2004; Omura et al. 2001).

In vascular smooth muscle cells, PPARα activators inhibit IL-1-induced levels of IL-6 and prostaglandins by negatively affecting the NF-κB pathway (Delerive et al. 2000; Staels et al. 1998). PPARγ agonists inhibit vascular smooth muscle migration, the release of matrix-degrading enzymes, and expression of the ANG II type 1 receptor (Diep et al. 2002; Marx et al. 1998). These effects can potentially interfere with fatty streak formation. Surprisingly, in SHRs, PPARα and PPARγ expression were upregulated in the vasculature and in cultured smooth muscle cells, presumably playing a compensatory role in remodeling of blood vessels (Diep and Schiffrin 2001). After treatment with a PPARα/γ dual agonist, hypercholesterolemic rabbits showed significant reduction of intimal thickening and reduced macrophage and smooth muscle recruitment, showing that PPARα and PPARγ activation can lead to reduction of intimal hyperplasia (Seki et al. 2005). In addition, TZDs inhibit smooth muscle cell growth and proliferation by increasing cyclin-dependent kinase inhibitor expression, leading to cell cycle arrest (Bruemmer et al. 2003; de Dios et al. 2003).

1.5.2.2 Heart

Effects of PPAR activity in the heart are beginning to be recognized. PPARα regulates cardiac metabolism and function by inducing genes that are implicated in lipid metabolism and mitochondrial fatty acid β-oxidation, the latter an important source of fuel for the heart. These include genes involved in fatty acid uptake such as FAT, FATP, mitochondrial transport such as CPT I, and mitochondrial β-oxdiation such as medium-chain acyl CoA dehydrogenase (Huss and Kelly 2004). PPARα activators reduced the expression of LPS-induced cardiac TNFα production and NF-κB activation, which could result in the improvement of congestive heart failure (Takano et al. 2000). In animals with cardiac hypertrophy due to pressure-overload, fenofibrate decreased transcript levels of ET-1 and attenuated interstitial and perivascular fibrosis, presumably via the suppression of AP-1 activation (Ogata et al. 2002). In addition, fenofibrate decreased inflammation and collagen deposition in the heart of ANG II-infused animals by decreasing VCAM-1, a platelet endothelial cell adhesion molecule (Diep et al. 2004).

The role of PPARγ in the heart is less clearly defined. Its expression is low compared to PPARα and PPARδ (Gilde et al. 2003). Conflicting results demonstrate both beneficial and deleterious effects of PPARγ on cardiac function. TZDs attenuate cardiomyocyte hypertrophy triggered by ANG II in vitro, but may result in an aggravation of congestive heart failure in humans (Asakawa et al. 2002; Wang et al. 2004b). Recently, PPARδ was shown to be necessary in the maintenance of energy balance and normal cardiac function. By specific deletion of cardiomyocyte PPARδ, it was demonstrated that these animals have cardiac dysfunction, progressive myocardial lipid accumulation, cardiac hypertrophy, and congestive heart failure (Cheng et al. 2004).

PPARδ also inhibits LPS-induced NF-κB activation and decreases MCP-1 production (Planavila et al. 2005).

1.5.3 Summary and Significance

A summary of the biological actions of PPARα, γ, and δ is shown in Figure 1.3. In the liver, PPARα regulates the expression of genes involved in the oxidation of fatty acid and HDL metabolism. In the heart, activation of PPARα increases fatty acid oxidation and decreases the expression of inflammatory cytokines. Activators of PPARα, PPARγ, and PPARδ inhibit inflammatory marker expression and induce macrophage cholesterol efflux in the vascular wall. PPARγ exerts its actions in the adipose tissue where insulin sensitivity is increased. In the skeletal muscle, PPARγ and PPARδ activity increases insulin sensitivity and fatty acid oxidation.

By acting as general lipid and glucose sensors, PPAR activation in distinct target tissues results in the coordinate regulation of metabolic pathways that maintain energy homeostasis. Furthermore, in the cardiovascular system, PPARs limit processes implicated in the development of atherosclerosis. Overall, activation of the PPARs results in improvement in the cardiovascular risk profile.

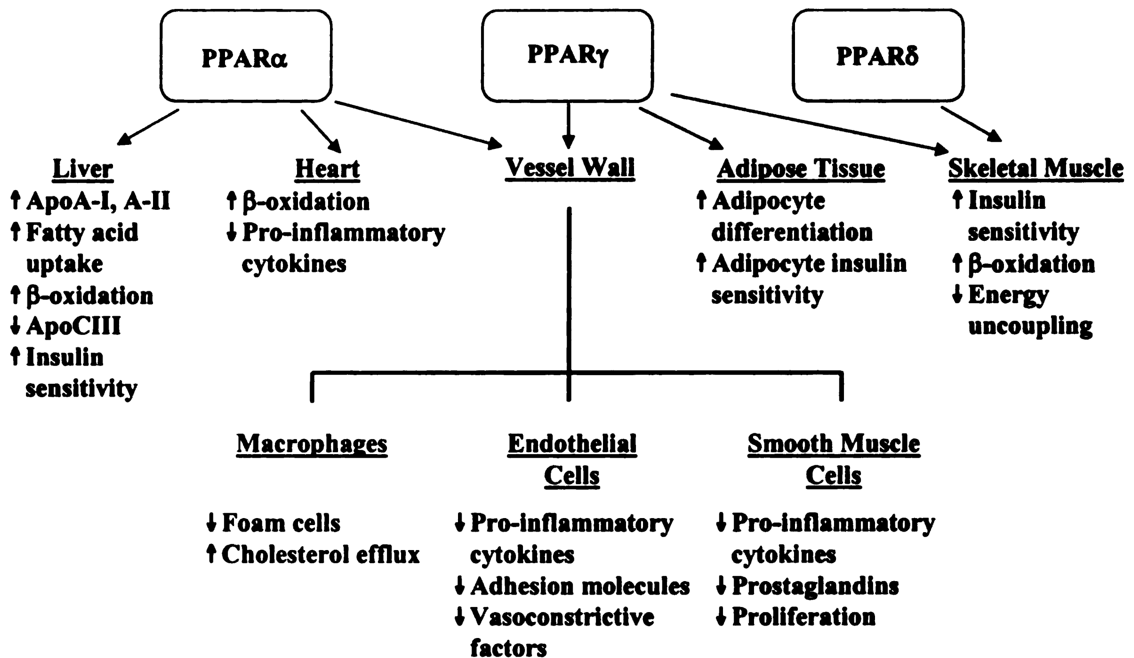

Figure 1.3 Summary of the metabolic and vascular effects of PPARα, PPARγ, and PPARδ activation.

1.6 PPARs and Clinical Relevance

1.6.1 Clinical Studies

In several different clinical trials, fibrates reduce the progression of coronary atherosclerosis (Ericsson et al. 1997; Frick et al. 1997; Steiner and Diabetes Athersclerosis Intervention Study Investigators 2001). In patients with type 2 diabetes, treatment with fibrates resulted in improved insulin sensitivity and endothelial function, and similar improvements in endothelial function were evident in non-diabetic patients with dyslipidemia (Capell et al. 2003; Evans et al. 2000; Playford et al. 2002). In addition, studies have shown an association between PPARα genetic polymorphisms and the risk of coronary atherosclerosis and ischemic heart disease (Flavell et al. 2002). The PPARα L162V missense mutation results in decreased PPARα activity and is associated

with higher high-density lipoprotein cholesterols and triglyceride levels, and alterations in triglyceride-rich lipoprotein metabolism (Robitaille et al. 2004; Sapone et al. 2000; Tai et al. 2002). Conversely, this genetic polymorphism is linked to a lower body mass index in patients with non-insulin-dependent diabetes mellitus and a better response to gemfibrozil with respect to their high-density lipoprotein cholesterol profile (Bosse et al. 2002; Evans et al. 2001).

Vasoprotective effects of TZDs have been demonstrated in different patient populations. In subjects with type 2 diabetes, TZDs significantly reduced C-reactive protein (CRP), matrix metalloproteinase-9 levels (MMP-9), and white blood cell counts, which are surrogates for the prediction of cardiovascular events (Haffner et al. 2002). Furthermore, in patients with coronary artery disease and type 2 diabetes, rosiglitazone significantly decreased MMP-9, TNFα levels and serum amyloid A levels, all of which are risk factors for cardiovascular events (Marx et al. 2003). The Pro12Ala polymorphism in PPARγ-2 results in a missense mutation that is present at a high frequency. It is also the most widely studied PPARγ genetic polymorphism. Although an initial study reported a decrease in risk for diabetes in subjects carrying the Ala allele, multiple subsequent studies showed both beneficial, detrimental, and the lack of association with this polymorphism (Clement et al. 2000; Deeb et al. 1998; Doney et al. 2004a; Ghoussaini et al. 2005; Horiki et al. 2004; Mancini et al. 1999; Ringel et al. 1999; Snitker et al. 2004). Some evidence also shows that the Pro12Ala genotype is linked to increased insulin sensitivity in normal and diabetic subjects, as well as in childhood obesity (Buzzetti et al. 2005; Chuang et al. 2001; Ek et al. 2001; Ghoussaini et al. 2005). Other studies show that the Ala allele is protective against colorectal adenoma and

myocardial infarction (Doney et al. 2004b; Gong et al. 2005). A number of other genetic variants have also been identified, including a gain-of-function mutation (Pro115Gln) associated with obesity but not insulin sensitivity, loss of function mutations (Val290Met and Pro467Leu) associated with severe insulin resistence, and the C1431T polymorphism that is linked to decreased risk of diabetes (Barroso et al. 1999; Ristow et al. 1998; Tai et al. 2004). All of these associations require confirmation in large study populations.

To date, data from clinical studies using PPARδ activators are lacking although results from animal studies show much promise in their potential as therapeutic targets. In obese rhesus monkeys, a PPARδ agonist decreased elevated triglyceride levels and increased high density lipoprotein cholesterol (HDL-C), suggesting that activation of PPARδ may impart beneficial effects on dyslipidemic subjects (Oliver et al. 2001a). The role of PPARδ in the attenuation of colon carcinogenesis was also demonstrated using PPARδ-deficient mice (Harman et al. 2004). A genetic polymorphism identified in the 5′ promoter region of PPARδ (+294T/C) displays increased transcriptional activity and is associated with higher plasma levels of low-density cholesterol (Skogsberg et al. 2003).

1.6.2 Therapeutic Modulators

Given that modulation of PPAR activity can result in the positive outcome of conditions associated with diabetes and cardiovascular function, active research in the past decade has focused on pharmacological agents that target PPARs without producing undesirable side effects. Thiazolidinediones are PPARγ agonists which are routinely used for their anti-diabetic activities. However, their use is associated with increased

adverse events such as increased adiposity and weight gain (Tiikkainen et al. 2004). A new generation of non-thiazolidinedione compounds that are selective PPARγ modulators improves hyperglycemia and insulin resistance profiles in diabetic animals without the excessive weight gain and increased adipose-depot size (Berger et al. 2003). Since PPARα and PPARγ produce favorable effects on lipid metabolism and vascular disease, dual PPARα/γ agonists have been developed recently (Berger et al. 2005). For example, insulin resistance and hyperglycemia is attenuated by the hydrocinnamic acid LY51029 in diabetic rodents, and raises HDL-C in human ApoA-I transgenic mice (Xu et al. 2004b). Tesaglitazar, a potent dual agonist of PPARα and PPARγ, has shown desirable efficacy in rats with dietary-induced insulin resistance (Hegarty et al. 2004). In phase II clinical trials in hypertriglyceridemic type 2 diabetic subjects, improved plasma glucose and lipid profiles were noted following treatment with ragaglitazar (Saad et al. 2004). Altogether, these data support the beneficial role of PPARα/γ dual agonists in improving lipid and glycemic profiles in diabetic patients.

1.7 Focus of Dissertation

Based on the large body of research supporting central roles of CYP eicosanoids and PPARs in positively modulating cardiovascular disease, the overall hypothesis tested in this dissertation is that components of the CYP-catalyzed pathway of AA metabolism affect and/or mediate biological effects via PPAR-associated transcriptional signaling. Since most evidence indicates that expression of cytochrome P450 levels is regulated by PPARα, this isoform will be the focus of these studies. The overall goal of these studies is two-fold. First, to identify novel endogenous and exogenous PPAR activators that are

also constituents of the CYP metabolism cascade. Second, to determine the biological effects of these novel ligands in the regulation of fatty acid metabolism and vascular function. The objectives of the dissertation are as follows:

1. Determine whether CYP eicosanoids are endogenous activators of PPARs and if so whether they regulate PPAR-responsive genes (Chapter 2). Transactivation and gel shift assays were established to examine the effects of CYP eicosanoids on PPARs. Expression of genes involved in fatty acid and CYP metabolism in response to CYP eicosanoid treatment were investigated.
2. Identify novel exogenous activators of PPAR and determine their effects on PPAR-mediated biological activities (Chapter 3). Inhibitors of sEH were tested for their ability to act as ligands of PPARα and PPARγ, and their effects on fatty acid metabolism and smooth muscle cell proliferation were explored.
3. Investigate the role of PPAR agonists on the expression of cyclooxygenase-2 in smooth muscle cells (Chapter 4). The molecular mechanism underlying the effects of CYP eicosanoids on interleukin-induced cyclooxygenase-2 expression was demonstrated.
4. Examine the in vivo effects of a PPARα agonist on the cardiovascular expression of CYPs and sEH (Chapter 5). CYP and sEH expression in multiple vascular beds and the heart were surveyed from rats treated with clofibrate.

In summary, these studies contribute towards understanding the interaction between the CYP-mediated pathway of AA metabolism and PPARα signaling. Results suggest that CYP eicosanoids and sEH inhibitors favorably affect vascular function and mediate their effects at least partially through PPARα. Further investigations of the molecular

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Chapter 2

Cytochrome P450 Eicosanoids are Endogenous Activators of Peroxisome Proliferator-Activated Receptor α

2.1 Introduction

Eicosanoids generated from arachidonic acid metabolism by cytochrome P450 (CYP) enzymes are important autocrine and paracrine factors that have diverse biological functions. CYP eicosanoids are involved in the regulation of vascular tone, renal tubular transport, cardiac contractility, cellular proliferation, and inflammation (Roman 2002). The major products of CYP-catalyzed arachidonic acid metabolism are 19- and 20-hydroxyeicosatetraenoic acid (19- and 20-HETE), and the regio- and stereoisomeric epoxyeicosatrienoic acids (EETs, Figure 1.1) (Kroetz and Zeldin 2002; Roman 2002). 20-HETE formation is catalyzed by the CYP4A (Nguyen et al. 1999; Wang et al. 1999) and CYP4F (Powell et al. 1998; Xu et al. 2004) family of enzymes whereas the EETs are products of arachidonic acid metabolism by the CYP2C and CYP2J enzymes (Karara et al. 1993; Wu et al. 1997; Wu et al. 1996; Zeldin et al. 1996). EETs are subsequently metabolized by the soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs) (Yu et al. 2000; Zeldin et al. 1993).

Regulation of CYP eicosanoid levels is determined by many factors, which includes the induction or repression of the CYP enzymes responsible for their formation. Large numbers of studies have focused on the induction of CYP4A protein and mRNA levels by anti-hyperlipidemic agents such as clofibrate (Gibson et al. 1990; Kimura et al. 1989; Muerhoff et al. 1992; Sabzevari et al. 1995; Sundseth and Waxman 1992). In

addition, starvation and fasting also induce CYP4A expression in both rat liver and kidney (Imaoka et al. 1990; Kroetz et al. 1998; Qu et al. 1998). Recently, renal CYP2C23 has also been shown to be inducible by fibrates (Muller et al. 2004) whereas hepatic CYP2C is repressed (Corton et al. 1998). Hepatic and renal sEH expression are also induced after treatment with clofibrate (Pinot et al. 1995).

Fibrate drugs such as clofibrate are part of a diverse group of compounds known as peroxisome proliferators, which also include herbicides and phthalate ester plasticizers (Badr 1992; Butler et al. 1988; Watanabe et al. 1985). Peroxisome proliferators act via the peroxisome proliferator-activated receptor alpha (PPARα) (Issemann and Green 1990). This receptor is a member of the PPAR nuclear receptor family that also consists of the PPARγ and PPARδ isoforms. PPARα is expressed in the heart, liver, kidney, skeletal muscle, and in vascular smooth muscle and endothelial cells (Bishop-Bailey 2000). Its biological role as a lipid sensor has been well established. In the human liver, PPARα activation results in the upregulation of apolipoprotein (Apo) A-I and ApoA-II and increased expression of β-oxidation enzymes, fatty acid transport proteins, fatty acid binding proteins, and other genes involved in the control of triglyceride and fatty acid metabolism (Fruchart et al. 2001). More recent studies have focused on the role of PPARα in the cardiovascular system. In both smooth muscle and endothelial cells, PPARα inhibits the inflammatory response by repressing NF-κB signaling. Expression of genes involved in inflammation such as interleukin-6, cyclooxygenase 2, and vascular adhesion molecule are all inhibited after PPARα activation (Marx et al. 1999; Staels et al. 1998).

Mechanistically, PPARs are ligand-activated transcription factors which, upon ligand binding, will heterodimerize with the retinoid X receptor and bind to its response element, the peroxisome proliferator response element (PPRE). Ligands for PPARα include fatty acids, eicosanoids and fibrate drugs (Forman et al. 1997). One of the more potent endogenous activators has been identified as the eicosanoid 8(S)-HETE (Forman et al. 1997). 8(S)-HETE is generated from the lipoxygenase pathway of arachidonic acid metabolism and activates and binds to PPARα at nanomolar concentrations. Other related eicosanoids such as prostacyclin, some prostaglandins, and leukotriene B_4 also activate PPARα (Devchand et al. 1996). A recent report also showed that hydroxylated EETs are high affinity ligands of PPARα (Cowart et al. 2002).

Given that related eicosanoids bind to and activate PPARα, and that CYPs and sEH are regulated by peroxisome proliferators, we hypothesize that CYP eicosanoids are also agonists of PPARα. To test whether CYP eicosanoids can functionally activate PPARα, transactivation assays were carried out and the results demonstrated that 11,12-EET and 14,15-DHET are potent activators of PPARα and PPARγ. By using gel shift assays, CYP eicosanoids were shown to induce the binding of PPARα to a PPRE. Furthermore, we show that 11,12-EET and 14,15-DHET behave like peroxisome proliferators in that they were able to alter ApoA-I and ApoA-II mRNA expression in primary rat hepatocytes. Since ApoA-I and ApoA-II are involved in the transport of HDL, these findings suggest that CYP eicosanoids may play a role in the regulation of triglyceride levels via PPARα. In addition, we show that CYP and sEH mRNA levels were increased in primary rat hepatocytes after treatment with these eicosanoids. These

results suggest that CYP eicosanoids may regulate their own levels through a complex autoregulatory mechanism.

2.2 Materials and Methods

2.2.1 Materials

Wy 14,643 and ciglitazone were obtained from Biomol (Plymouth Meeting, PA) and eicosanoids were purchased from Cayman Chemical (Ann Arbor, MI). The EET agonist 11,12-epoxyeicosa-8(Z)-enoic acid (11,12-EEZE) was synthesized by Dr. John Falck as described previously (Falck et al. 2003). 3,3′,5-triiodo-L-thyronine (T_3) was purchased from Sigma Chemical Company (St. Louis, MO). CYP4A1 and CYP2C11 primary antibodies were purchased from Gentest (Woburn, MA). The sEH antibody was a kind gift from Dr. Bruce Hammock (University of California, Davis), and the CYP2C23 antibody was a kind gift from Dr. Jorge Capdevila (Vanderbilt University). Gal4 expression and reporter plasmids were provided by Dr. Thomas Scanlan (University of California, San Francisco) and pCMX-mPPARα and pRS-hRXRα plasmids were a generous gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA). The CYP2J2 antibody and the expression plasmids pcDNA/CYP2J2 and pcDNA/GFP were a kind gift from Dr. Darryl Zeldin (National Institute of Environmental Health Sciences).

2.2.2 Cell Culture

CV-1, HepG2, and H4IIE cells were obtained from American Type Cell Culture and maintained in Modified Eagle's Medium with Earle's Balanced Salt Solution and contained 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino

acids, and penicillin-streptomycin. Sprague Dawley primary hepatocytes were isolated by the UCSF Liver Center Core Facility, were cultured on Collagen Type I plates (BD Biosciences, Bedford, MA) and overlaid with 0.25 mg/ml Matrigel (BD Biosciences, Bedford, MA) in HCM™ medium (Cambrex, Walkersville, MD). Primary hepatocytes were treated on the third day after isolation. All cells were cultured at 37°C in 5% CO_2.

2.2.3 Transactivation Assays

HepG2 cells were plated in 24-well plates at 8×10^4 cells per well and transfected the next day using Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) with 0.1-1 ng of Gal4-hPPARα or Gal4-hPPARγ, 100 ng UAS_4-LUC, and 35 ng of pCMV-βgal. After 24 hours, cells were dosed with 50 μM Wy 14,643, 10 μM ciglitazone, or 10 μM CYP eicosanoids in serum-free medium containing up to 0.1% DMSO for 6 h. Cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI) and subjected to one freeze-thaw cycle. Transfection of CV-1 cells were also carried out using 50 ng pcDNA/CYP2J2 or pcDNA/GFP, 1 ng Gal4-hPPARα, 100 ng UAS_4-LUC, and 20 ng pCMV-βgal. At 24 h after transfection, 50 μM Wy or DMSO was added and incubated for an additional 24 h before cell lysis. Primary hepatocytes were plated on 6-well Collagen I-coated plates at 6.25×10^5 cells per well and transfected with 4 μg $PPRE_3$-tk-LUC and 1 μg pCMV-βgal using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 24 h before addition of Wy or CYP eicosanoids. Cells were lysed 24 h or 48 h later. A single-tube format luminometer (MGM Instruments, Hamden, CT) was used for obtaining luciferase values using the Luciferase Assay Reagent as substrate (Promega, Madison, WI). To determine β-galactosidase activity, cell lysates were

incubated with the substrate *o*-nitrophenyl-β-D-galactopyranoside and absorbance was measured at 420 nm. Luciferase activity is expressed relative to β-galactosidase activity, and fold activation is calculated relative to DMSO control. Each experiment was carried out in quadruplicate and repeated three times.

2.2.4 Gel Shift Assays

pCMX-mPPARα (1 μg) and pRS-hRXRα (1 μg) were translated using the TNT reticulocyte in vitro translation system (Promega, Madison, WI). The binding reaction contained 1 μl of translated PPARα, 0.3 μl of RXRα in 10 mM Tris, 150 mM KCl, 6% glycerol, 0.05% Igepal, 1 mM DTT, 2 μg poly (dI-dC), and CYP eicosanoids with or without 1 μl PPARα or RXRα antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The ratio of translated PPARα and RXRα is dependent on the efficiency of each in vitro translation reaction and optimization is generally required when a new batch of translated protein is used. The reactions were incubated for 10 min on ice before 250,000 cpm [γ-^{32}P]-labeled PPRE was added. The sequence for the consensus PPRE oligonucleotide is 5′-CAA AAC TAG GTC AAA GGT CA-3′, the sequence for the mutant oligonucleotide is 5′-CAA AAG TAG CAC AAA GCA CA-3′, and for the CYP4A6-RE is 5′-CGC GGA TCC GCG AGG GCA AAG TTC AGG AA-3′. The oligonucleotides were end-labeled with [γ-^{32}P] using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA). Following incubation for 30 min at room temperature, the reaction was separated on a 5% pre-run polyacrylamide gel at 4°C. The gel was dried and radioactive bands were visualized using a phosphorimager and ImageQuant software (Amersham Biosciences, Piscataway, NJ).

2.2.5 Real-Time Quantitative PCR

H4IIE cells or primary hepatocytes were treated with peroxisome proliferators or eicosanoids for 24 to 48 h. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription is carried out using M-MLV reverse transcriptase (Promega, Madison, WI). Primers and probe sets were designed using Primer Express with the help of Dr. David Ginzinger (UCSF Cancer Center) and are listed in Table 2.1. Probes were labeled with the reporter dye, 6-carboxy-fluorescein phosphamidite (FAM) at the 5′-end and the dye quencher, Black Hole Quencher at the 3′-end. The ApoAI, ApoAII, and CPT1A primer and probe sets were Assays-on-Demand purchased from Applied Biosystems (Foster City, CA). Reactions were run on an ABI Prism 7700 and cycling conditions were: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of specific transcripts was calculated by the following formula: Relative expression = $2^{-\Delta\Delta Ct}$ where Ct = $(\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{treated} - (\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{control}$.

2.2.6 Western Immunoblotting

Hepatocytes were lysed with buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Igepal and supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). Protein concentration was quantitated using the BCA method (Pierce, Rockford, IL). Cell lysates (20 μg) were prepared and electrophoresed through NuPage™ 10% Bis-Tris gels and transferred using a wet-transfer method onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Western blots were incubated with

a 1:1000 dilution of primary antibodies followed by incubation with alkaline phosphatase- (CYP4A1) or horseradish peroxidase-conjugated (CYP2C11, CYP2C23, and sEH) secondary antibodies. Immunoreactive proteins were visualized using an alkaline phosphatase kit (Biorad, Hercules, CA) or an ECL detection kit (Amersham Biosciences, Piscataway, NJ).

Table 2.1 Primers and probes used in quantitative RT-PCR. Sequences were designed using Primer Express.

Rat cyclophilin	
Forward primer	5′-CGA TGA CGA GCC CTT GG-3′
Reverse primer	5′-TCT GCT GTC TTT GGA ACT TTG TC-3′
Probe	6FAM-CGC GTC TGC TTC GAG CTG TTT GCA-BHQ
Rat CYP4A1	
Forward primer	5′-TCA CCT CCC TTC CAC TGG TT-3′
Reverse primer	5′-TCC ACA CAT GTC ATA ATT TGC T-3′
Probe	6FAM-TCA CCT TGA AAC TGC TTG TGC CCA-BHQ
Rat sEH	
Forward primer	5′-CTC TAA ACT GGT ATC GAA ACA CAG AAA G-3′
Reverse primer	5′-ATG TCC TTC TCA GCT GTG ACC AT-3′
Probe	6FAM-CGT TGG GAA GGA AGA TCT TGG TCC CT-BHQ
Rat CYP2C11	
Forward primer	5′-GCC TTG TGG AGG AAC TGA GG-3′
Reverse primer	5′-AGC ACA GCC CAG GAT AAA GGT-3′
Probe	6FAM-AGC AAA GGT GCC CCT TTT GAT CCC-TAMRA
Rat CYP2C23	
Forward primer	5′-TTC GGG CTC CTG CTC CTT A-3′
Reverse primer	5′-CGT CCA ATC ACA CGG TCA AG-5′
Probe	6FAM-AGA GGT GCA AGC CAA AGT TCA TGA GGA-BHQ

2.2.7 Lipid Extraction and Liquid Chromatography Tandem Mass Spectrometry

A confluent culture of HepG2 cells in a T75 flask was incubated with 30 nmol CYP eicosanoids for 6 h. Extraction of lipids from culture medium and cells have been described previously (Fang et al. 2001). Briefly, lipids from cell culture medium were extracted twice with water-saturated ice-cold ethyl acetate in a ratio of 1:4 (v/v). For the extraction of intracellular lipids, cells were first trypsinized and washed with PBS, then homogenized by hand with a Dounce homogenizer. Ice-cold chloroform/methanol (2:1, v/v) was then added and the organic phase was removed. In some cases, hydrolysis of intracellular lipids was carried out by adding methanol containing 5% NaOH and 10% H_2O to the organic phase and incubating the reaction for 1 h at 50°C. Lipids were then extracted with ethyl acetate as described above. The extracted layer was evaporated under nitrogen gas and stored at -80°C. On the day of analysis, lipids were reconstituted in acetonitrile (100 μl). Quantitation of eicosanoids were carried by out Dr. Zhigang Yu as previously described (Yu 2004). Briefly, analysis of lipids by high-performance liquid chromatography (HPLC) was performed using a 4.6 x 150 mm 5μm Luna C18(2) column (Phenomenex, Torrance, CA) and lipids were eluted from the reverse-phase HPLC column. The effluent was then injected into a Quattro Ultima tandem-quadrupole mass spectrometer (Micromass, Manchester, UK) and subjected to negative mode electrospray ionization (ESI). Multi-reaction moitoring (MRM) was employed to detect the eicosanoids simultaneously, and data were analyzed using the MassLynx 3.5 software (Milford, MA).

2.2.8 Statistics

Statistical significance of differences between values was evaluated by an unpaired Student's t test. Significance was set at a p value of <0.05.

2.3 Results

2.3.1 Optimization of PPARα transactivation by Wy and CYP eicosanoids.

It was first of interest to determine the effects of serum on the transactivation of PPARα by CYP eicosanoids (Figure 2.1). Initial transactivation studies were carried out in CV-1 monkey kidney cells although further experiments were performed in HepG2 human hepatoma cells. The Gal4 plasmid containing the ligand binding domain of human PPARα and a luciferase reporter plasmid containing 4 repeats of the Gal4 response element (UAS_4-LUC) were transfected into CV-1 cells followed by incubation with putative activators. The optimal exposure of the PPARα agonist Wy 14,643 to transfected CV-1 cells in serum-deficient medium was 6 h as demonstrated by the almost 3-fold increase in corrected luciferase activity compared with the activity at 3, 9, 12 or 24 h (Figure 2.1A). In cells that were not transfected with PPARα, minimal basal activation was observed. Cells that were transfected with PPARα and treated with the vehicle control DMSO resulted in a marginal increase in activation. In the presence of Wy 14,643, 8,9-, 11,12-, or 14,15-DHET, significant PPARα activation was apparent only when CYP eicosanoids were administered in medium devoid of serum (Figure 2.1B). Wy 14,643 and 14,15-DHET transactivated PPARα 23-fold and 20-fold, respectively in serum-free medium, and both activated PPARα only 4-fold in serum-containing medium. In the absence of serum, no cytotoxicity was observed for the duration of the experiment (personal observations).

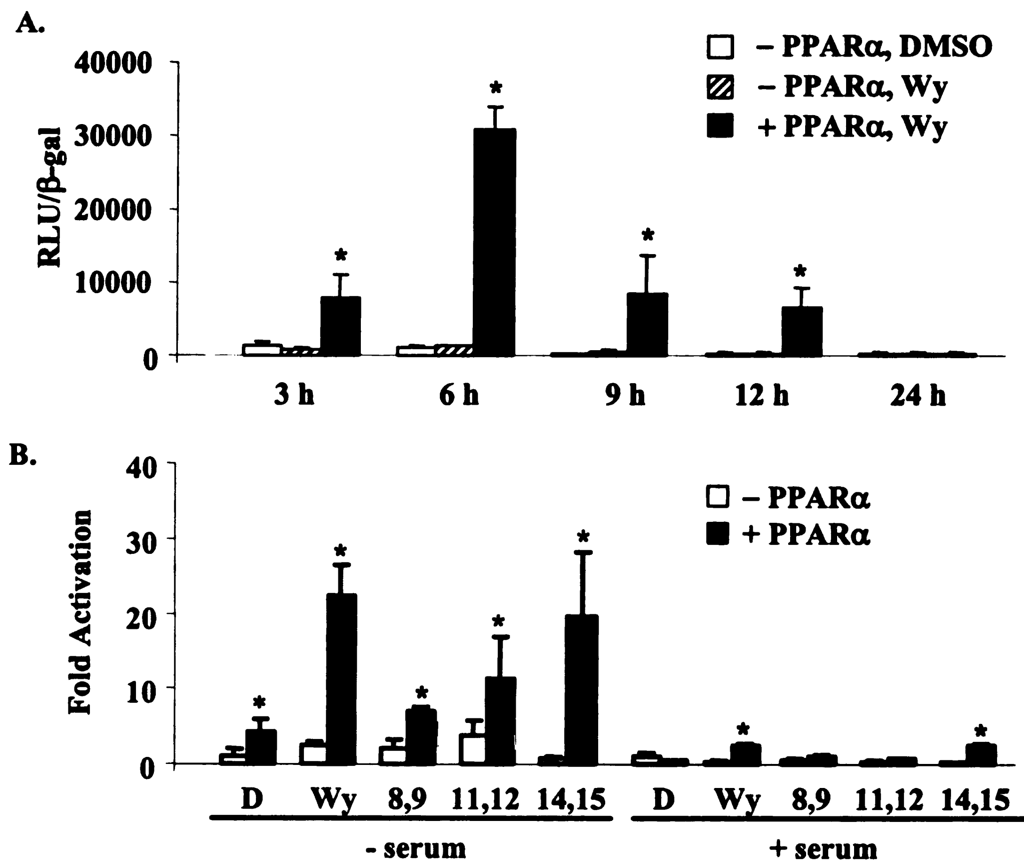


Figure 2.1 Optimization of PPARα transactivation by Wy and CYP eicosanoids. Transactivation assays were performed in CV-1 cells that were transfected with Gal4-hPPARα and the reporter UAS_4-LUC. A) Wy 14,643 (Wy, 50 μM) was added to cells in serum-free medium 24 h post transfection and cells were lysed 3, 6, 9, 12, or 24 h later. Luciferase activity was normalized to *β*-galactosidase values. B) Wy 14,643 (50 *μ*M) or DHETs (10 μM) were added to cells in serum-free or serum-containing medium 24 h post transfection and cells were lysed 6 h later. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over the DMSO (D) control. The values shown are the mean ± SD of a representative experiment performed in quadruplicate. Similar results were obtained in additional experiments. * Significant difference compared to DMSO not transfected with PPARα within each treatment group (p<0.05).

2.3.2 CYP eicosanoids transactivate PPARδ in CV-1 cells.

Using conditions determined in the previous experiment (Figure 2.1), transactivation of Gal4-PPARδ by CYP eicosanoids was determined in CV-1 cells (Figure 2.2A). Carbaprostacyclin I_2, a stable analog of prostaglandin I_2 was used as a positive control. With the exception of 8,9-EET, PPARδ was strongly activated by all the CYP eicosanoids tested, and the EETs in general were better activators than the DHETs. In HepG2 cells, transfection with PPARδ did not result in increased transactivation with any of the CYP eicosanoids, and in some cases decreased PPARδ activity was observed (Figure 2.2B and C).

2.3.3 CYP eicosanoids transactivate PPARα and PPARγ in HepG2 cells.

Transactivation assays were next carried out to determine whether EETs or DHETs were able to functionally activate PPARα and PPARγ in HepG2 cells (Figure 2.3). In cells not transfected with PPARα or PPARγ, only minimal basal activation was observed. When PPARα-transfected cells were treated with the PPARα-specific activator Wy 14,643, PPARα was activated greater than 30-fold. Similarly, PPARγ was transactivated by ciglitazone, a PPARγ ligand. The highest activation was observed when PPAR-transfected cells were dosed with 11,12-EEZE, a monounsaturated 11,12-EET analog (Figure 2.3A). Significant activation was also evident with 10 μM 11,12-EET, 11,12-EEZE, or 14,15-EET treatment (Figure 2.3B). 8,9-EET did not significantly activate either PPARα or PPARγ.

The effects of DHETs on PPAR activation were also examined (Figure 2.3B).

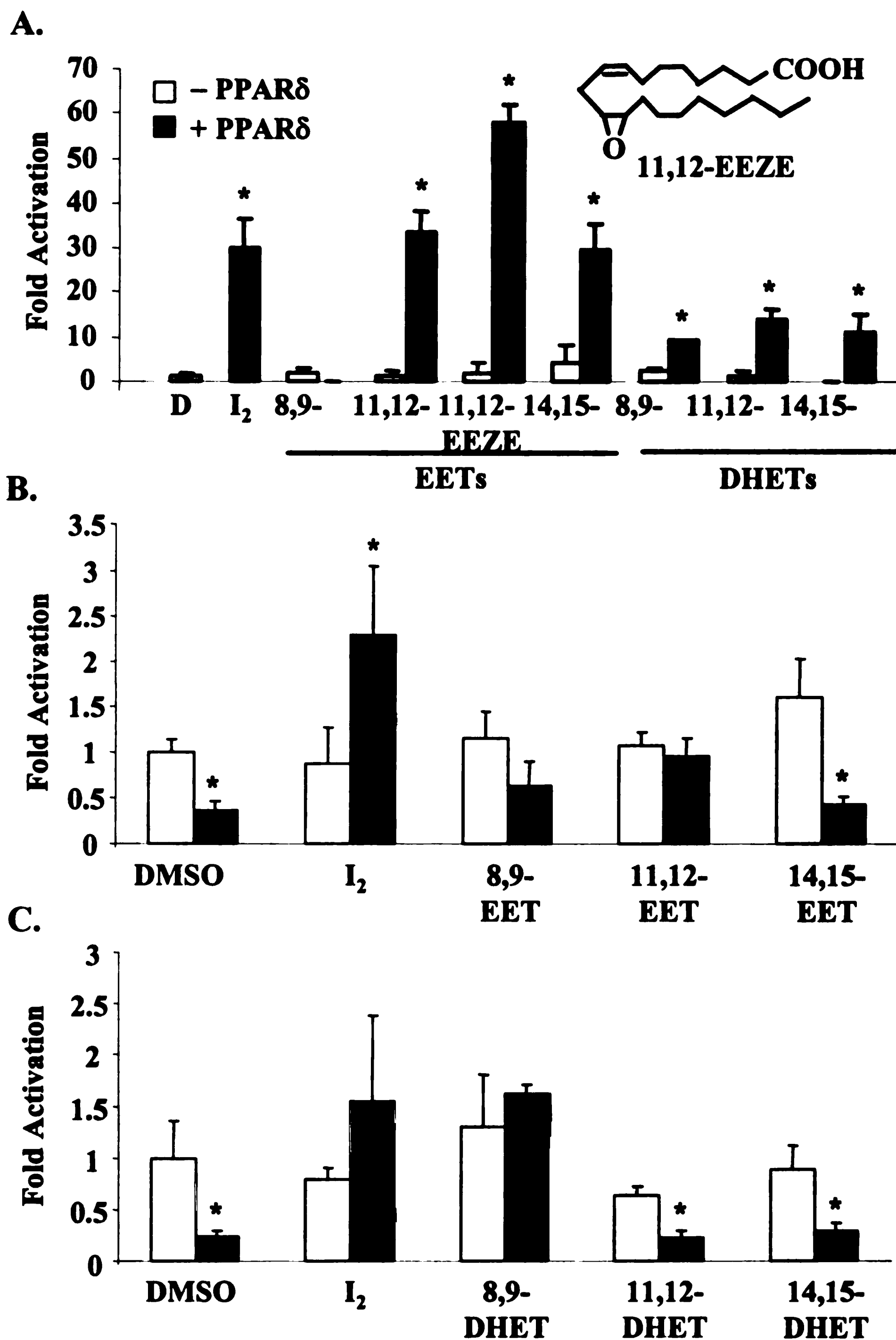


Figure 2.2 CYP eicosanoids transactivate PPARδ in CV-1 cells. Transactivation assays were carried out in CV-1 (A) or HepG2 (B and C) cells as described in Figure 2.1, except that cells were transfected with Gal4-hPPARδ. Cells were treated for 6 h with $cPGI_2$ (I_2, 10 μM) as a positive control for PPARδ, EETs, 11,12-EEZE (10 μM; A,B), or DHETs (10 μM; A,C). The values shown are mean ± SD of a representative experiment performed in quadruplicate. * Significant difference compared to DMSO (p<0.05).

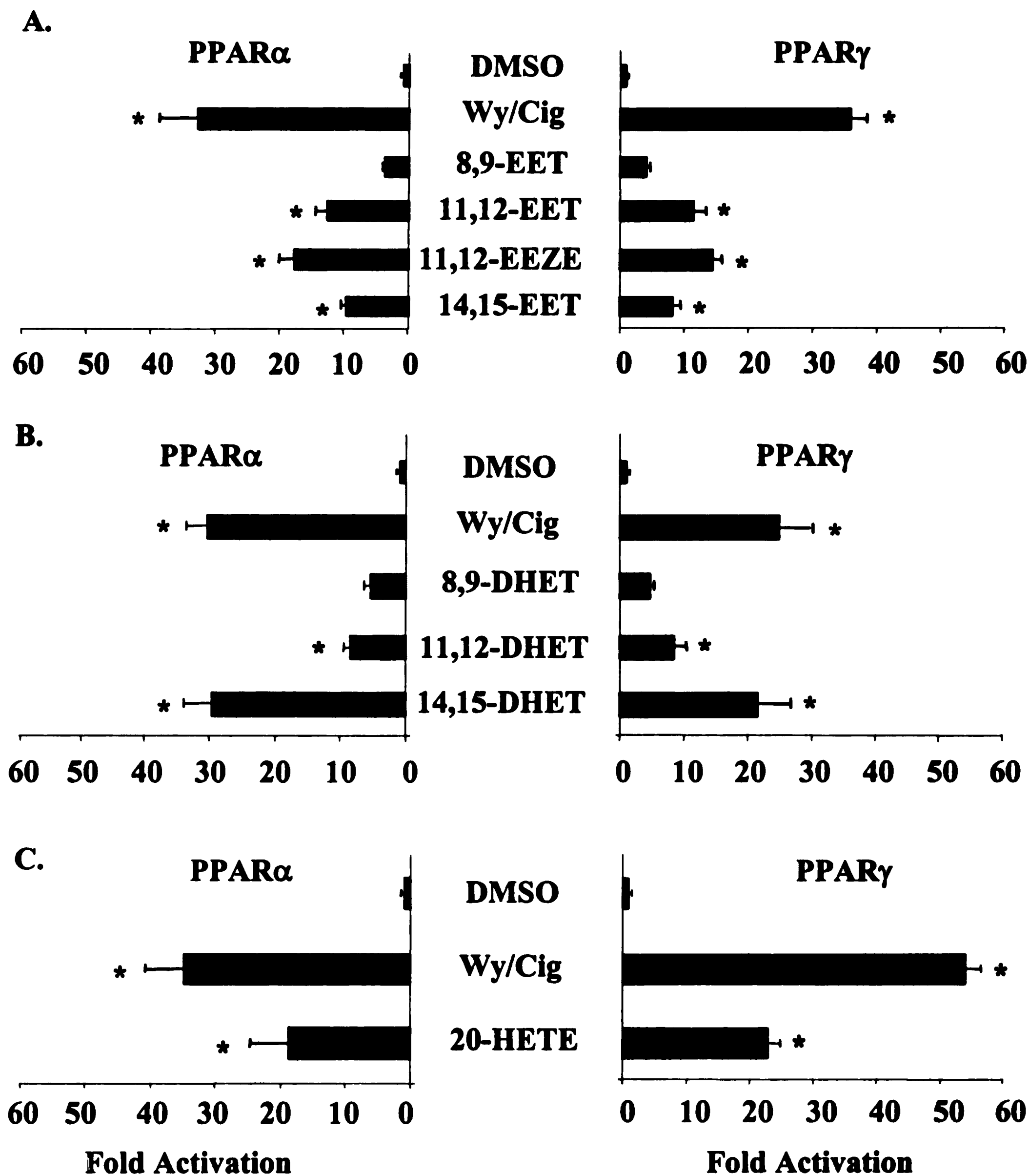


Figure 2.3 CYP eicosanoids transactivate PPARα and PPARγ in HepG2 cells. Transactivation assays were performed in HepG2 cells that were transfected with Gal4-hPPARα or Gal4-hPPARγ and the reporter UAS$_4$-LUC. Wy 14,643 (Wy, 50 μM), ciglitazone (Cig, 10 μM), or A) EETs (10 μM), B) DHETs (10 μM), or C) 20-HETE (10 μM) were added to cells in serum-free medium 24 h post transfection and cells were lysed 6 h later. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over the DMSO control (transfected only with UAS$_4$-LUC). The values shown are the mean ± SD of a representative experiment performed in quadruplicate. Similar results were obtained in additional experiments. Significant difference compared to DMSO (p<0.05).

PPAR-transfected HepG2 cells were treated with 10 μM 8,9-DHET, 11,12-DHET, or 14,15-DHET. 14,15-DHET was the most potent activator and was as effective as Wy 14,643 and ciglitazone at transactivating the respective PPARs. PPARα was activated 30-fold and PPARγ 21-fold by 14,15-DHET. Activation of PPARα and PPARγ by 11,12-DHET was 8- to 9-fold and 8,9-DHET resulted in a 5-fold activation of both PPARα and PPARγ. 20-HETE activated PPARα and PPARγ 18-23-fold (Figure 2.3C).

To exclude the possibility that the activation observed with CYP eicosanoids was non-specific for nuclear receptors, we tested their ability to transactivate an unrelated receptor, the thyroid hormone receptor beta (TRβ) (Figure 2.4). The endogenous ligand of TRβ, triiodo-L-thyronine (T_3) strongly activated this receptor whereas 11,12-EET and 14,15-DHET did not have any effect on TRβ activation.

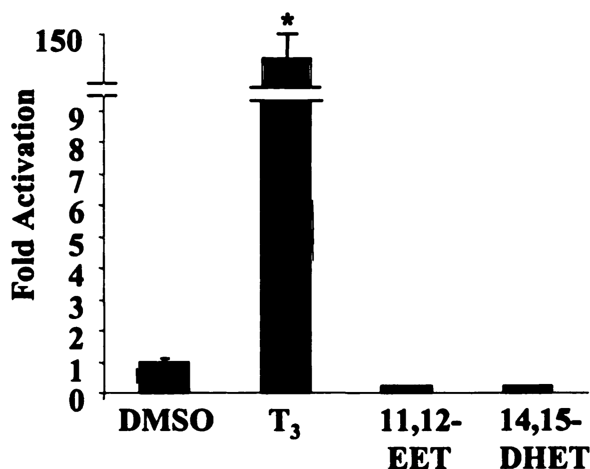


Figure 2.4 TRβ is not activated by CYP eicosanoids. HepG2 cells were transfected with Gal4-TRβ and UAS_4-LUC and treated with 10 μM 11,12-EET and 10 μM 14,15-DHET as described in Figure 2.3. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over the DMSO control (transfected only with UAS_4-LUC). The values shown are the mean ± SD of a representative experiment performed in quadruplicate. Similar results were obtained in additional experiments. * Significant difference compared to DMSO (p<0.05).

14,15-DHET was further tested for its ability to transactivate PPARα and PPARγ in a dose-dependent and saturable manner (Figure 2.5). Cells were transfected as described above and treated with 0.1 μM to 100 μM 14,15-DHET. A steep dose-dependence was observed between 1 μM and 10 μM of 14,15-DHET. Maximal activation was observed at 50 μM for both receptors with EC_{50} values of 1.5 μM and 2.5 μM for PPARα and PPARγ, respectively.

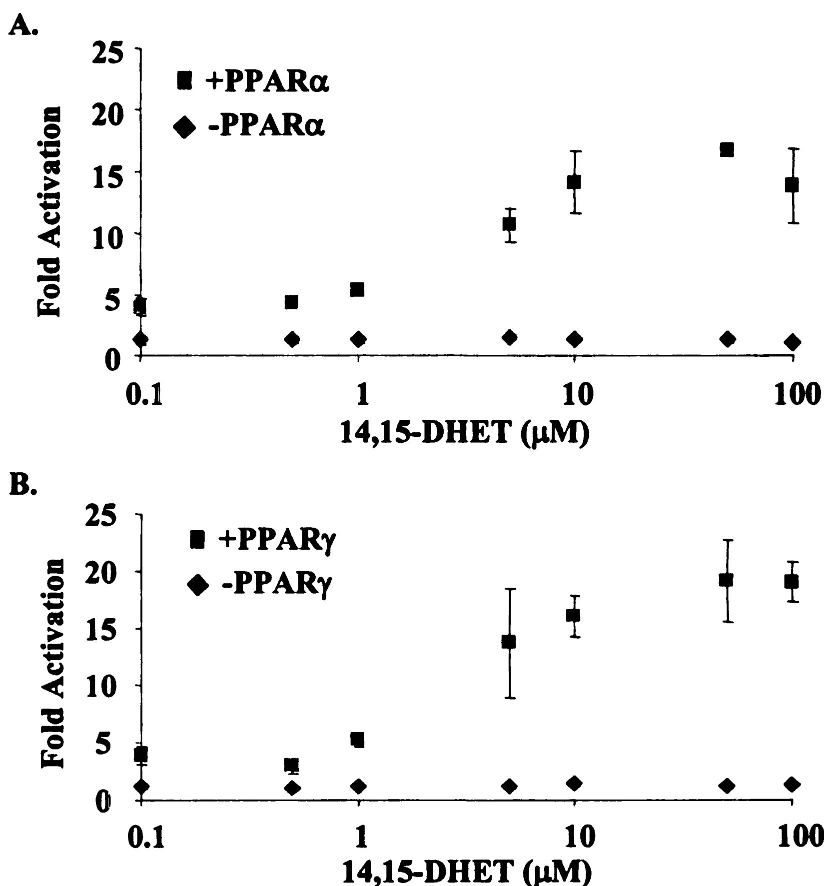
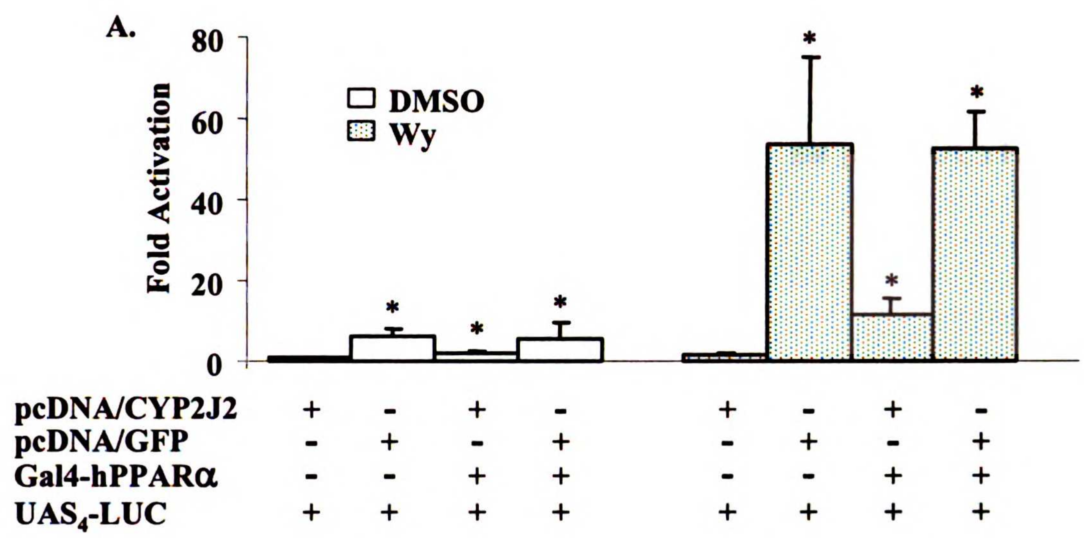


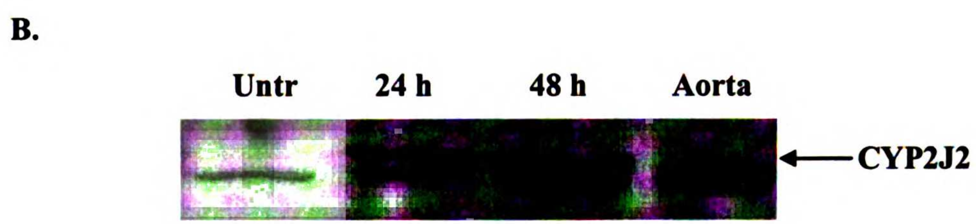
Figure 2.5 Dose dependent transactivation of PPARα and PPARγ by 14,15-DHET. Transactivation assays were performed as described in Figure 1. Transactivation of A) PPARα and B) PPARγ by 0.1-100 μM 14,15-DHET is shown in the absence and presence of PPARα or PPARγ. The values shown are mean ± SD of a representative experiment performed in quadruplicate. EC_{50} values of 1.5 μM for PPARα and 2.5 μM for PPARγ were estimated using GraphPad Prism.

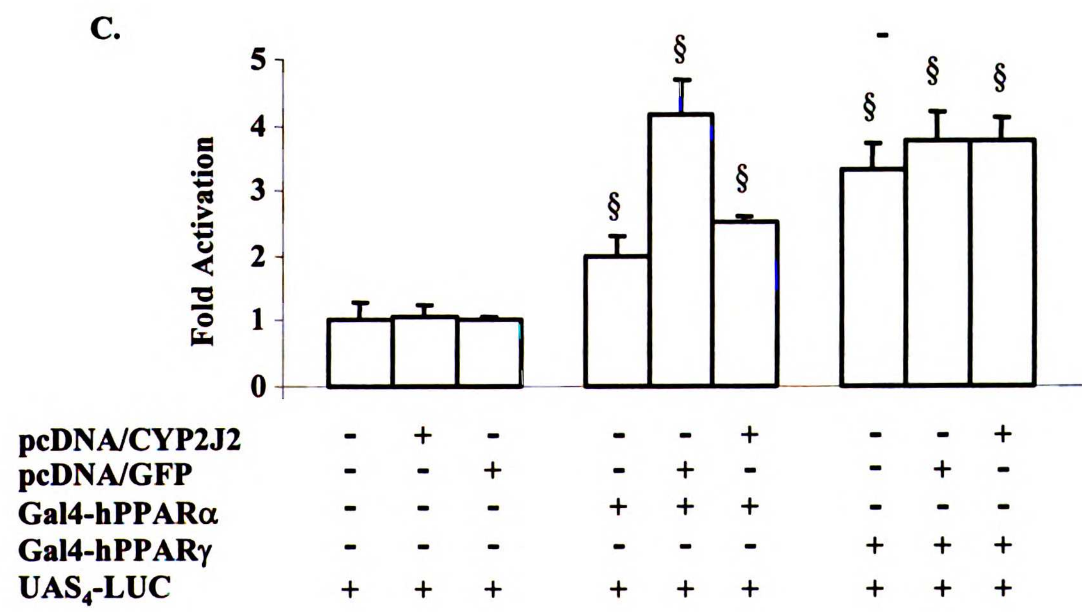
2.3.4 CYP2J2 transfection does not increase PPARα activation.

The effect of endogenously-generated CYP eicosanoids on PPARα was ascertained by transfecting CV-1 cells with the CYP2J2 expression plasmid or the corresponding GFP control (Figure 2.6A top panel). Transfection with CYP2J2 did not result in increased transactivation of PPARα compared with the GFP control plasmid. Cells transfected with the control vector displayed the same level of luciferase activity in the absence or presence of transfected PPARα. Wy 14,643 was used as a positive control to insure functionality of the system. Although Wy 14,643 activated PPARα, the presence of the GFP control plasmid resulted in a marked increase in luciferase activity, even in the absence of PPARα. Expression of CYP2J2 was confirmed by Western immunoblotting 24 h and 48 h after transfection (Figure 2.6B). In untransfected cells, a CYP2J2 immunoreactive protein was detected which was assumed to be non-specific. After transfection with CYP2J2, an immunoreactive band with a slightly higher molecular weight was detected that was not present in untransfected cells, and the higher molecular weight protein was assumed to be the CYP2J2 protein. Based on this assumption, CYP2J2 expression was detected at 24 h and further increased at 48 h.

Similar experiments were performed in HepG2 cells and transfection with CYP2J2 and either PPARα or PPARγ expression plasmids did not result in significant activation over GFP-transfected cells (Figure 2.6C). Further experiments were performed in the same manner using a luciferase reporter containing the PPAR consensus response element and with the addition of a phospholipase A_2 activating peptide (PLAP) to increase the pool of free arachidonic acid, however significant changes in PPARα activation were not observed (Figure 2.6D)







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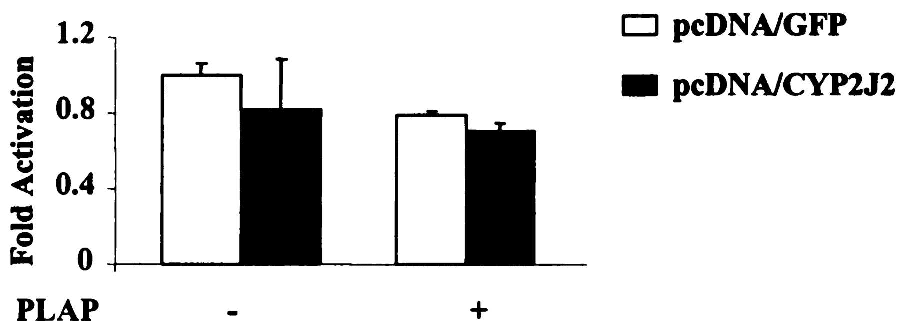

Figure 2.6 CYP2J2 transfection does not increase PPARα activation. A) Transactivation assays were carried out in CV-1 cells that were transfected with Gal4-hPPARα, UAS_4-LUC, and pcDNA/CYP2J2 or pcDNA/GFP control. B) Detection of CYP2J2 expression by Western immunoblotting of CV-1 cells 24 h or 48 h after transfection with pcDNA/CYP2J2. Untransfected CV-1 cells (Untr) and human aorta were used as negative and positive controls, respectively. C) Transactivation assays were performed in HepG2 cells as described in (A). Cells were also transfected with Gal4-hPPARγ. D) HepG2 cells were transfected with pcDNA/CYP2J2 or pcDNA/GFP and a reporter plasmid containing the consensus PPAR response element, $PPRE_3$-tk-LUC, with or without the addition of 10 μg/ml of PLAP. The values shown are the mean ± SD of a representative experiment performed in triplicate. * Significant difference compared to DMSO; § significant difference compared to cells not transfected with PPARα or PPARγ ($p<0.05$)

2.3.5 Characterization of the distribution of 14,15-EET and 14,15-DHET in HepG2 cells.

In order to further characterize the availability of eicosanoids in cell culture conditions, the distribution of exogenously administered CYP eicosanoids was examined using liquid chromatography tandem mass spectrometry (Figure 2.7). In cells treated with 30 nmols of 14,15-EET, approximately 6 nmols were incorporated into phospholipid pools, whereas unbound intracellular EETs were not detected (Figure 2.7A). A small

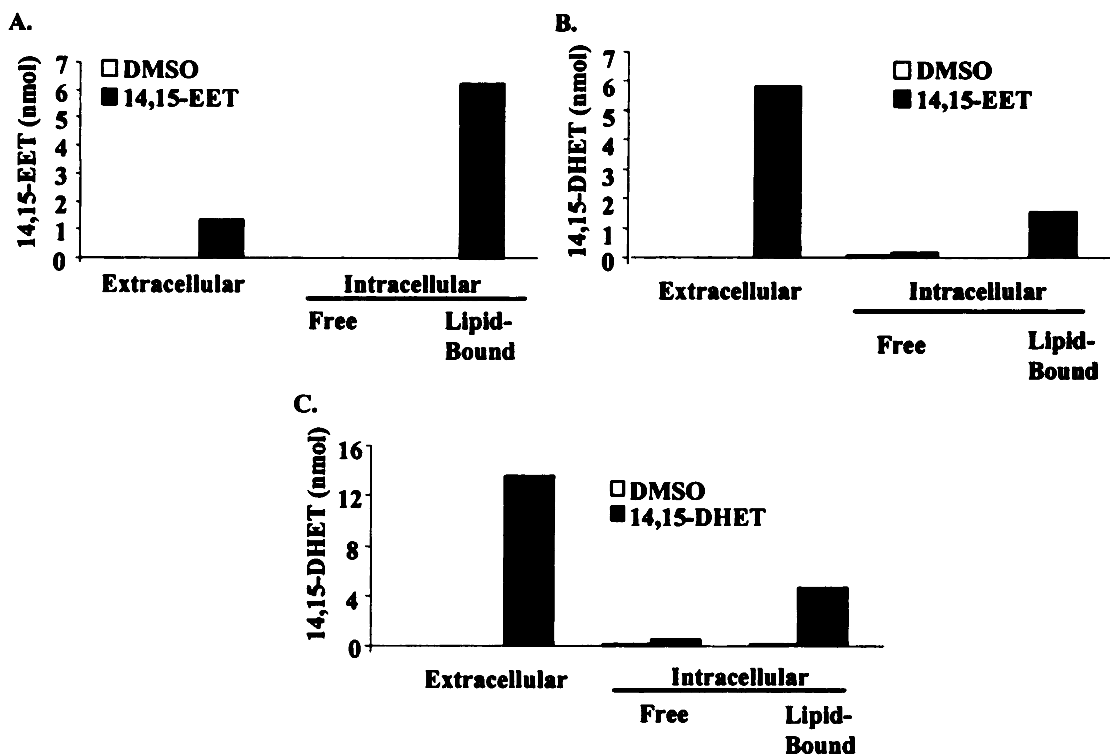

Figure 2.7 Characterization of the distribution of 14,15-EET and DHET in HepG2 cells. HepG2 cells were treated with 30 nmol 14,15-EET (A and B), 14,15-DHET (C), or DMSO for 6 h. Cells and culture medium were collected and lipid extraction was carried out as described in *Materials and Methods.* Amount of A) 14,15-EET, B) 14,15-DHET formed from 14,15-EET, and C) 14,15-DHET present in the extracellular medium or intracellularly were determined using liquid chromatography tandem mass spectrometry. No EETs and DHETs were detected in cells treated with DMSO (A-C). Unbound 14,15-EETs was not detected intracellulary in cells dosed with 14,15-EET (A). The values shown are single determinations.

amount was also detected in the extracellular medium. Approximately 6 nmols of 14,15-EET were hydrolyzed to 14,15-DHET. DHETs were found mostly in the medium, with only 1.5 nmols detected in the phospholipid pool (Figure 2.7B). Similarly, exogenously administered 14,15-DHET was largely found in the extracellular medium, with a small percentage bound within lipid pools (Figure 2.7C).

2.3.6 EETs and DHETs induce PPAR/RXR binding to a PPRE

Gel shift assays were carried out to determine whether CYP eicosanoids can induce a conformational change in PPARα resulting in subsequent binding of the PPARα/RXRα heterodimer to a PPRE. EETs, DHETs, and 20-HETE induced heterodimer binding which was not observed with a mutant PPRE or unprogrammed reticulocytes (Figure 2.8).

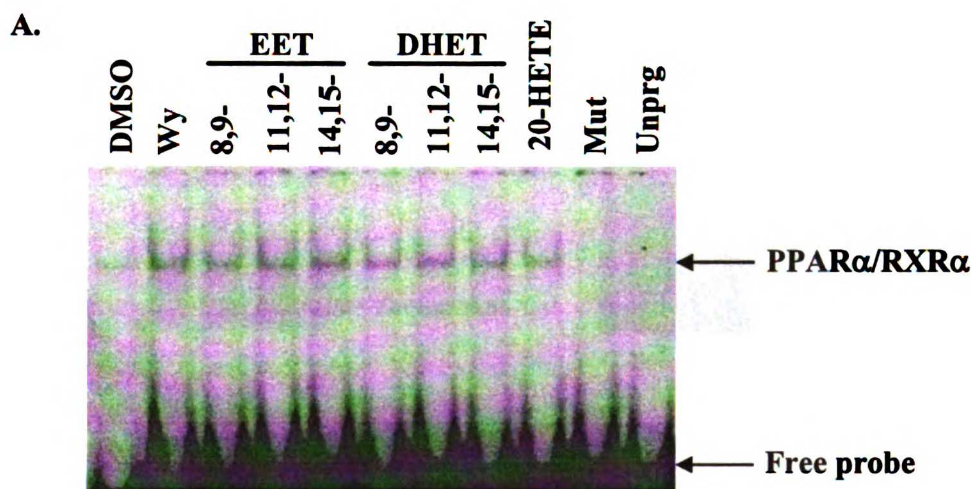

Figure 2.8 CYP eicosanoids induce PPARα/RXRα-PPRE binding. EMSAs were performed by incubating in vitro translated PPARα and RXRα with DMSO, 5 μM Wy 14,643, or 1 μM CYP eicosanoids and ^{32}P-labeled PPRE. The complex was resolved through a 5% non-denaturing polyacrylamide gel and analyzed using a PhosphorImager. Mutant PPRE (Mut) and unprogrammed reticulocyte (Unpgr) were used as controls.

Supershifts with PPARα and RXRα specific antibodies demonstrated that heterodimers consisted of PPARα and RXRα (Figures 2.9 and 2.10). To further validate binding specificity, increasing amounts of cold PPRE were incubated with the complex, which resulted in a dose-dependent decrease in PPARα/RXRα bound to radiolabeled PPRE (Figure 2.11). Likewise, 20-HETE induced PPARα/RXRα-specific

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heterodimerization and subsequent binding to the PPRE (Figure 2.12). In addition, CYP eicosanoids induce the binding of PPARα/RXR to the natural promoter sequence of the CYP4A6 gene (Figure 2.13).

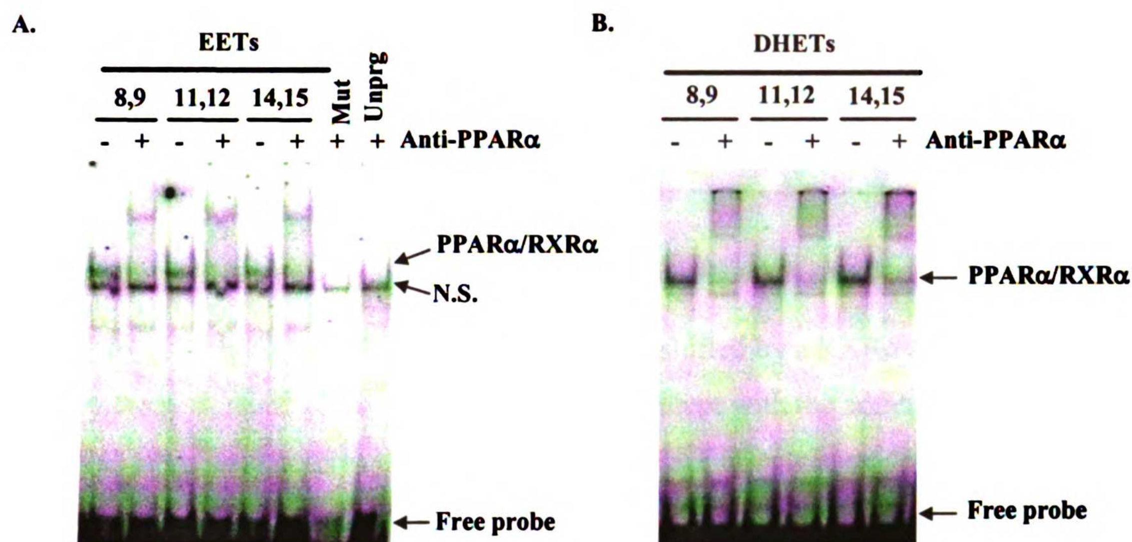

Figure 2.9 PPARα/RXRα-PPRE complex is supershifted by a PPARα antibody. EMSAs were performed as described in Figure 2.8 except with the addition of a PPARα antibody (1μl). Specificity of the A) EET and B) DHET effects were determined by supershifts with a PPARα antibody. N.S. denotes non-specific binding. Mutant PPRE (Mut) and unprogrammed reticulocyte (Unpgr) were used as controls.

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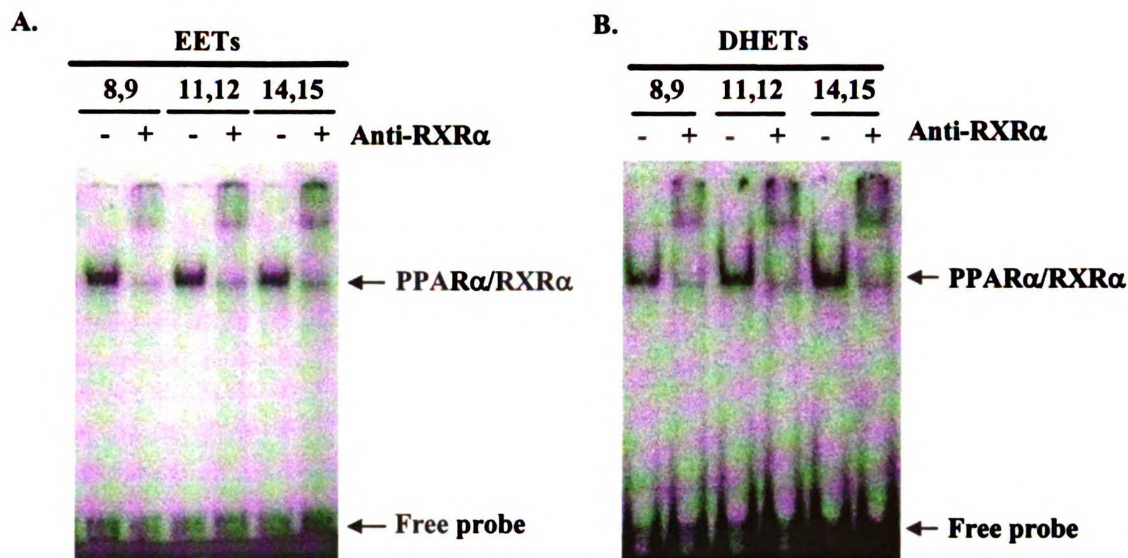

Figure 2.10 PPARα/RXRα-PPRE complex is supershifted by a RXRα antibody. EMSAs were performed as described in Figure 2.8 except with the addition of a RXRα antibody (1 μl). Specificity of the A) EET and B) DHET effects were determined by supershifts with a RXRα antibody.

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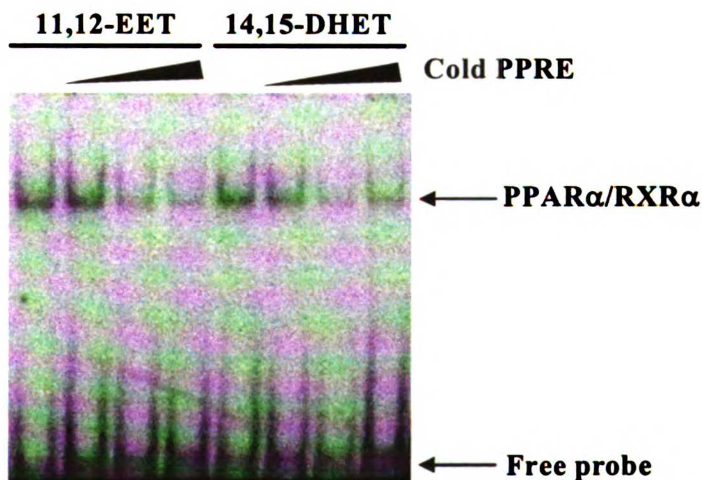

Figure 2.11 Cold PPRE can bind PPARα/RXRα. EMSAs were performed as described in Figure 2.8 with the addition of 10-fold increasing amounts of cold PPRE to compete with labeled PPRE for PPARα/RXRα binding.

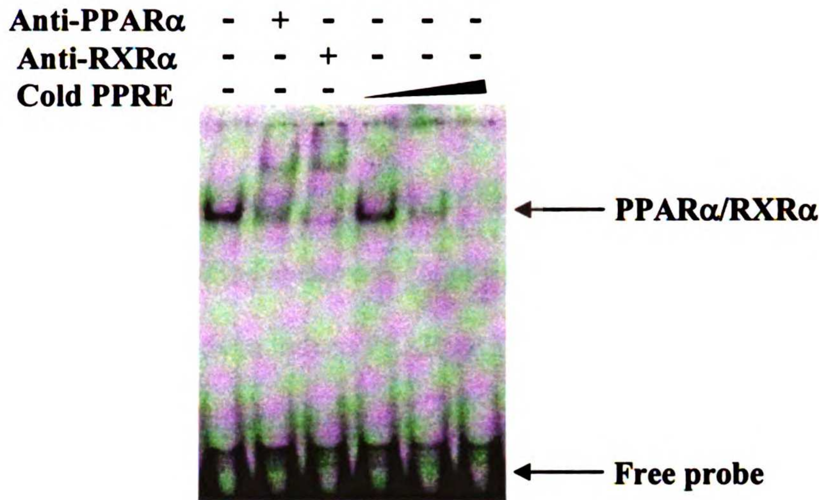

Figure 2.12 20-HETE induces PPARα/RXRα-specific binding to a PPRE. EMSAs were performed by incubating in vitro translated PPARα and RXRα with 20-HETE and ^{32}P-labeled PPRE in the presence of a PPARα or RXRα-specific antibody (1 μl), or increasing amounts of cold PPRE.

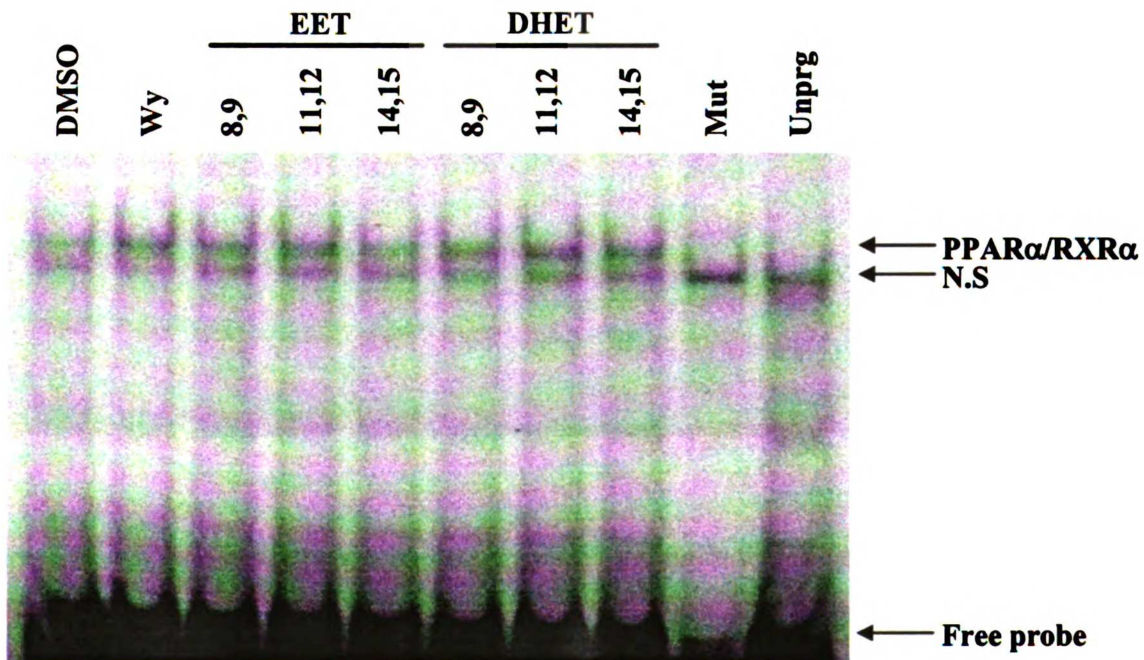

Figure 2.13 CYP eicosanoids induce PPARα/RXRα-CYP4A6-RE binding. EMSAs were performed as described in Figure 2.8 except a ^{32}P-labeled CYP4A6-RE was used.

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It was also of interest to investigate whether CYP eicosanoids can result in the binding of PPARγ/RXR to the PPRE. Attempts have been carried out using both wheat germ and reticulocyte-translated receptors, however, it was not possible to distinguish between heterodimer binding under vehicle or ligand-treated conditions. Therefore the ability of CYP eicosanoids to induce PPARγ/RXR heterodimer binding to the PPRE could not be examined (Figure 2.14).

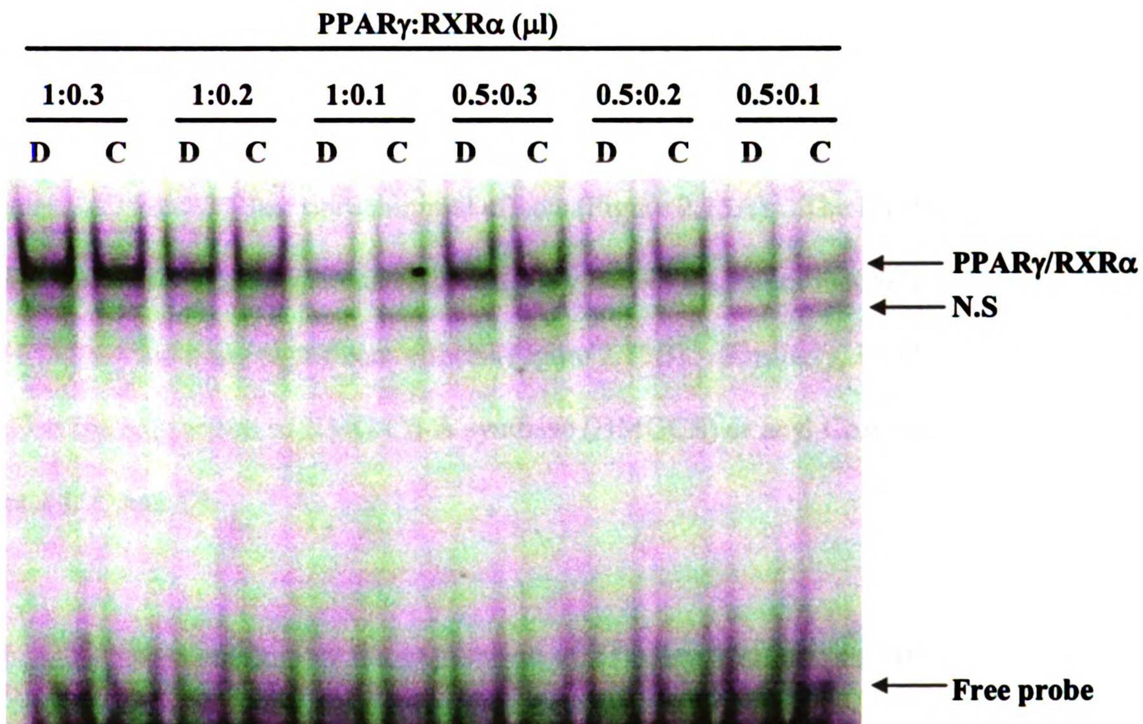

Figure 2.14 PPARγ/RXRα induces strong PPRE binding in the absence of ligand. EMSAs were performed as described in Figure 2.8, except with in vitro translated PPARγ. Different ratios of PPARγ and RXRα were incubated with DMSO control (D) or 10 μM ciglitazone (C) to optimize the assay.

2.3.7 CYP eicosanoids have minimal effects on PPARα-responsive genes in H4IIE

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rat hepatoma cells.

After demonstration of PPARα activation by CYP eicosanoids, it was of interest to investigate whether CYP eicosanoids were able to mimic the actions of peroxisome proliferators and alter the RNA levels of known PPARα-responsive genes involved in the metabolism and transport of triglycerides. Since the inherent expression of PPARα is low in human cell lines (Palmer et al. 1998), the rat hepatoma cell line H4IIE was utilized. The responsiveness of carnitine palmitoyl transferase 1A (CPT1A) and ApoA-II to Wy 14,643 and 14,15-DHET was determined from 24 h to 96 h (Figure 2.15). The expression of CPT1A was induced in the presence of Wy 14,643 at all times examined, however, 14,15-DHET had only minimal effects (Figure 2.15A). The level of ApoA-II mRNA was repressed approximately 20% by Wy 14,643 48 h after incubation, but 14,15-DHET did not have any significant effects (Figure 2.15B). Similarly, 14,15-DHET did not affect the expression of HMG Co-A synthase (HMGCS) or acyl CoA synthase (ACS, Figure 2.15C and D).

2.3.8 CYP eicosanoids alter the expression of PPARα-responsive genes in primary rat hepatocytes

Since primary cells represent a more physiologically relevant system, the expression of PPARα-responsive genes was determined in primary rat hepatocytes. Primary hepatocytes also provide the added ability to examine cytochrome P450 levels since CYP expression is not maintained in cell lines. Primary rat hepatocytes were treated with 10 μM 11,12-EET, 14,15-DHET, or 20-HETE and RNA levels of ApoA-I, ApoA-II, and CPT1A were determined by real time quantitative PCR (Figure

2.16). Wy 14,643 and 14,15-DHET slightly decreased ApoA-I expression. Interestingly,

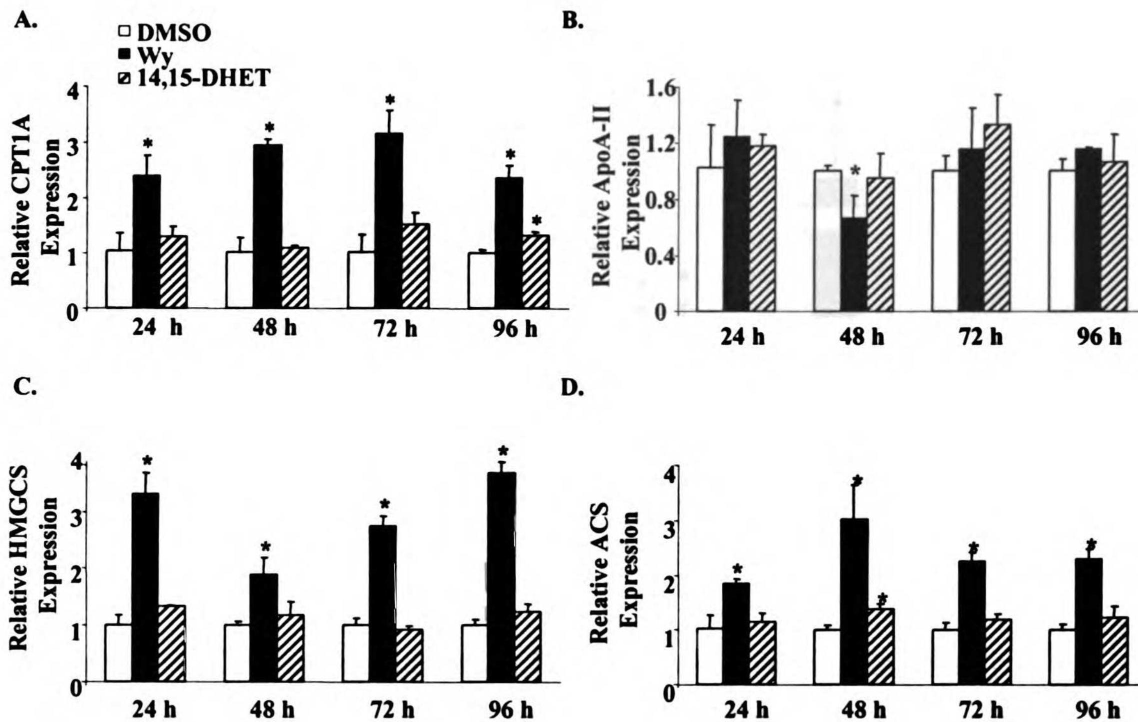

Figure 2.15 CYP eicosanoids have minimal effects on PPARα-responsive genes in H4IIE rat hepatoma cells. H4IIE cells were treated for 24-96 h with 50 μM Wy or 10 μM 14,15-DHET. Total RNA was isolated and RNA expression was determined by real-time quantitative PCR. Expression of A) CPT1A, B) ApoA-II, C) HMGCS, and D) ACS are normalized to cyclophilin levels and expressed relative to DMSO control. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO (p<0.05).

20-HETE resulted in a 70% decrease in ApoA-I expression. In contrast ApoAII expression was increased after treatment with PPARα activators. Treatment with Wy 14,643 resulted in a 2-fold increase in ApoA-II expression and induction was more than 3-fold with 11,12-EET, 14,15-DHET, and 20-HETE. The expression of CPT1A was

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only minimally affected by CYP eicosanoids, in contrast to potent activation by Wy 14,643.

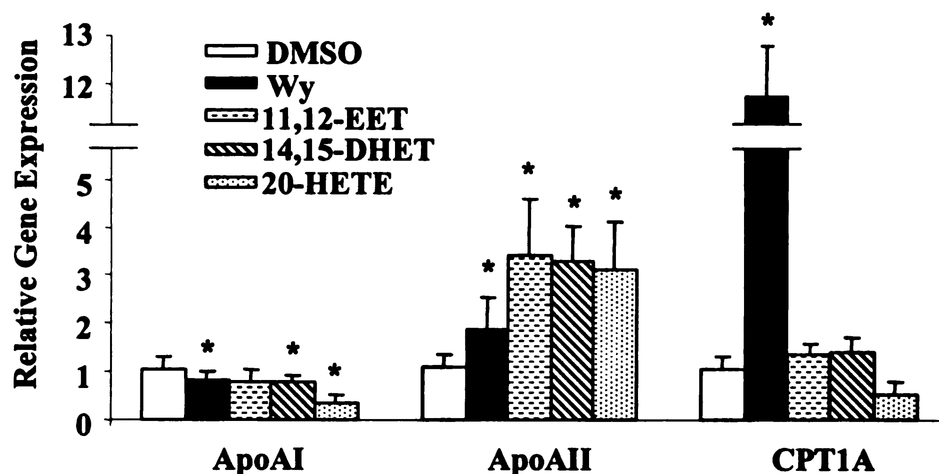


Figure 2.16 CYP eicosanoids induce PPARα-responsive genes in primary rat hepatocytes. Primary hepatocytes from Sprague Dawley rats were treated for 24 h with DMSO, 50 μM Wy, or 10 μM 11,12-EET, 14,15-DHET, or 20-HETE. Total RNA was isolated and RNA expression was determined by real-time quantitative PCR. Expression of ApoA-I, ApoA-II and CPT1A are normalized to cyclophilin levels and expressed as increase over DMSO. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO ($p<0.05$).

Since CYP and sEH levels can be modulated by peroxisome proliferators, we investigated whether CYP eicosanoids had the ability to modify CYP and sEH levels, thereby regulating their own expression (Figure 2.17). As expected, Wy 14,643 resulted in a marked increase in CYP4A1 and sEH expression. In contrast, CYP4A1 and sEH mRNA levels were increased only 2- to 3-fold by 10 μM 11,12-EET, 14,15-DHET and 20-HETE. Interestingly, CYP eicosanoids were more potent than Wy 14,643 in inducing CYP2C11 expression. 20-HETE was the most effective inducer of CYP2C11, resulting

in an 8-fold increase of CYP2C11 mRNA. CYP2C23 mRNA was increased only 1.5-fold after treatment with Wy 14,643 and 11,12-EET.

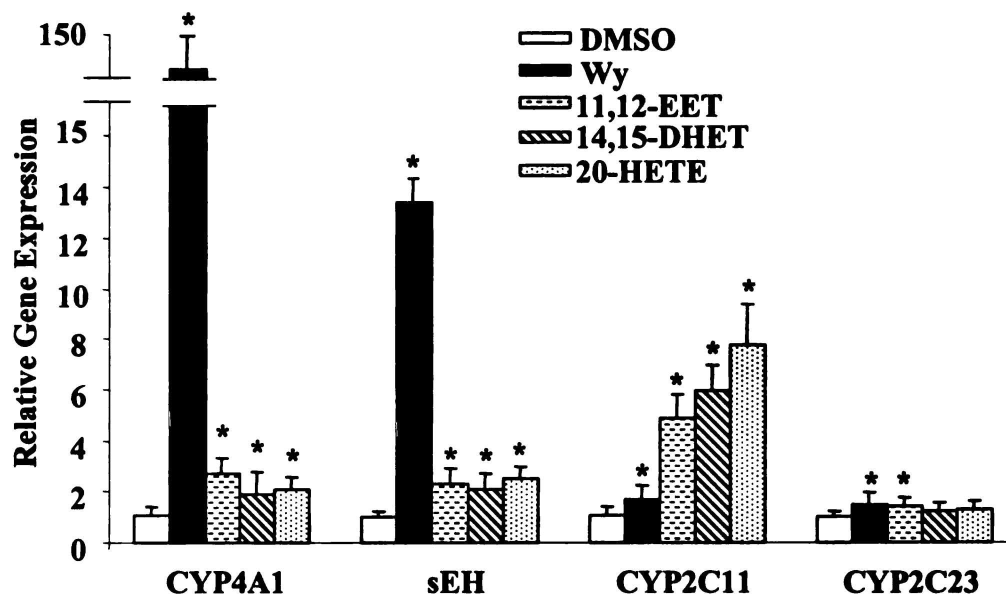


Figure 2.17 CYP eicosanoids induce CYP and sEH expression in primary rat hepatocytes. Primary hepatocytes were treated as described in Figure 2.16. The expression of CYP4A1, sEH, CYP2C11, and CYP2C23 RNA was determined by real-time quantitative PCR and normalized to cyclophilin levels and expressed as increase over DMSO. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO ($p<0.05$).

Given that RNA levels of CYPs and sEH can be modulated by CYP eicosanoids, we further tested their ability to change corresponding protein levels in primary rat hepatocytes (Figure 2.18). Marked induction of CYP4A1 (Figure 2.13A) and sEH (Figure 2.18B) were evident with Wy 14,643 treatment, and marginal increases in CYP2C11 (Figure 2.18C) and CYP2C23 (Figure 2.18D) protein levels were observed.

However, 10 μM 20-HETE, 11,12-EET, and 14,15-DHET did not significantly affect CYP4A1, sEH, CYP2C11, and CYP2C23 immunoreactive protein levels.

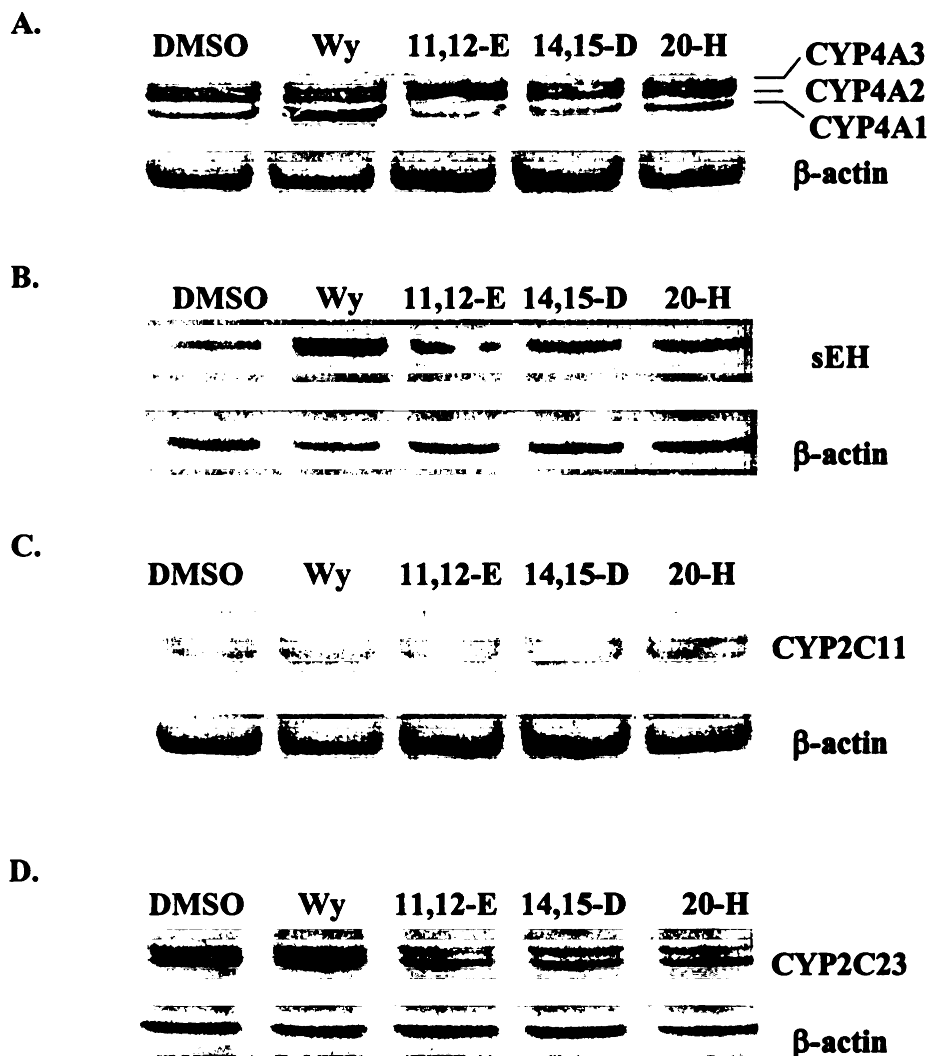
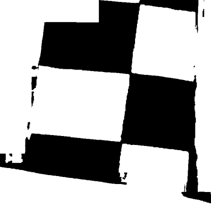


Figure 2.18 The effect of CYP eicosanoids on CYP4A1, sEH, CYP2C11, and CYP2C23 protein expression. Primary hepatocytes from Sprague Dawley rats were treated with 50 μM Wy, 10 μM CYP eicosanoids, or DMSO. A) CYP4A1, B) sEH, C) CYP2C11, and D) CYP2C23 immunoreactive proteins were detected by Western blot as described in the *Materials and Methods*. 11,12-EET, 14,15-DHET, and 20-HETE are denoted as *11,12-E*, *14,15-D*, and *20-H*, respectively. β-Actin was used as a loading control. The CYP4A1 antibody detects CYP4A1, CYP4A2, and CYP4A3 proteins.



2.3.9 CYP eicosanoids do not transactivate a PPRE in primary rat hepatocytes.

Although CYP eicosanoids have been identified as potent activators of PPARα in HepG2 cells, their effects on PPARα-responsive genes in primary rat hepatocytes were not as significant as expected. In an attempt to understand this discrepancy, transactivation of PPARα in primary rat hepatocytes was examined. Hepatocytes were transfected with a luciferase reporter containing the consensus PPRE so that the activation of endogenous PPARα can be determined (Figure 2.19). Only Wy 14,643 resulted in PPARα transactivation, with a transactivation of 2-fold over the vehicle control. 11,12-EET, 14,15-DHET, and 20-HETE did not significantly transactivate PPARα.

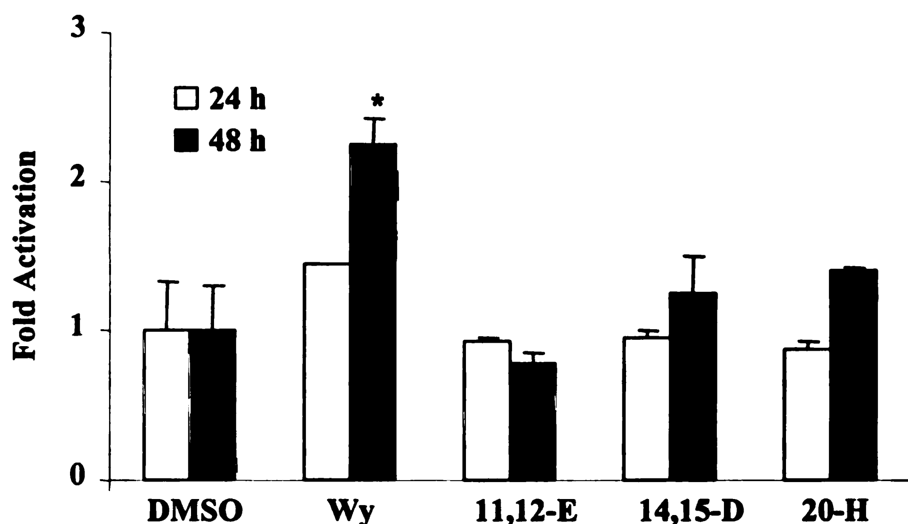


Figure 2.19 CYP eicosanoids do not activate a PPRE in primary rat hepatocytes. Primary hepatocytes from Sprague Dawley rats were transfected with $PPRE_3$-tk-LUC for 24 h and treated with 50 μM Wy, 10 μM CYP eicosanoids, or DMSO for 24 h or 48 h. Luciferase activity and fold activation were determined as described in Figure 2.1. 11,12-EET, 14,15-DHET, and 20-HETE are denoted as *11,12-E*, *14,15-D*, and *20-H*, respectively. The values shown are the mean ± SD of a representative experiment performed in triplicate. * Significant difference compared to DMSO ($p<0.05$).

2.4 Discussion

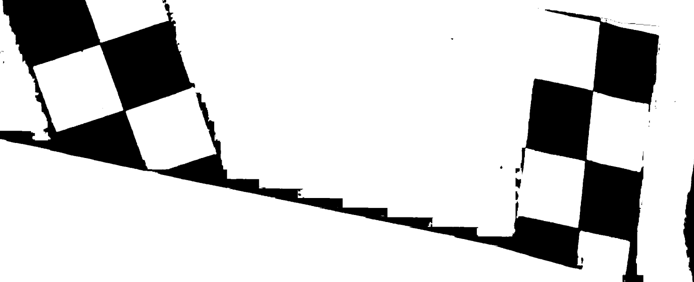
CYP-derived eicosanoids have been identified as novel activators of PPARα and PPARγ. Of the eicosanoids tested, 11,12-EET, 14,15-DHET, and 20-HETE functionally activate the nuclear receptors at low micromolar concentrations and are the most potent activators of both PPARα and PPARγ. It is recognized that the exogenous concentrations added to cell culture media are likely to be much higher than intracellular eicosanoid levels. A significant attenuation of PPAR activation by CYP eicosanoids when dosed in medium containing serum is consistent with binding to cellular proteins. Preliminary analysis of intracellular EET and DHET levels by liquid chromatography tandem mass spectrometry indicates that they are in the nanomolar range, consistent with endogenous EET levels in rodent plasma (Yu and Kroetz, unpublished results) and even lower than the levels in the human liver (Karara et al. 1991). It is thus plausible that CYP eicosanoids can activate PPARα and PPARγ in vivo. Although transfection with CYP2J2 did not result in any increase in the transactivation of PPARα, this result does not necessarily imply that endogenous levels of CYP eicosanoids do not activate PPARα. It is likely that CYP2J2 cannot generate sufficient amounts of eicosanoids to reflect in vivo concentration due to its relatively low CYP epoxygenase activity (Barbosa-Sicard et al. 2005; Wu et al. 1996). It will be important to carry out these assays by expressing CYP2C9, since this epoxygenase is also utilized for the purpose of producing endogenous EETs (Michaelis et al. 2005; Michaelis et al. 2003; Potente et al. 2003).

Regioisomeric CYP eicosanoids may also be preferentially metabolized in the cell, thus influencing PPAR activation. Differences in PPARα activation by regioisomeric EET/DHET pairs suggest that the DHET alone cannot account for all of the activity associated with EET treatment. To address the relative contributions of EETs

and DHETs it would be of interest to measure EET transactivation in a cell system devoid of sEH activity. Unfortunately, inhibition of sEH by urea-based inhibitors was not possible in the present studies since these inhibitors also activated PPARs (Chapter 3).

11,12-EET and 14,15-DHET transactivated PPARα and PPARγ to a similar extent, and PPARδ was strongly transactivated by 11,12- and 14,15-EET. Many reports have shown the dual activation of PPARs by polyunsaturated fatty acids. Linoleic, arachidonic, and eicosapentaenoic acids activate PPARα and PPARδ (Forman et al. 1997), and palmitic, oleic, linolenic, and arachidonic acids are agonists for PPAα and PPARγ (Kliewer et al. 1997). Recently, there have been increased efforts to synthesize compounds that act as PPARα/PPARγ dual agonists. Compounds such as ragaglitazar, AX242, and MK-0767 show beneficial effects on insulin resistance and display antihyperglycemic and hypolipidemic activities (Brand et al. 2003; Doebber et al. 2004; Ljung et al. 2002). Since CYP eicosanoids are endogenous compounds that are released into the intracellular environment in response to various stimuli, it is of interest to explore whether CYP eicosanoids play a role in improving insulin sensitivity and maintaining lipid homeostasis.

It was recently shown that 10-20 μM ω-hydroxylated EETs (HEETs) transactivated Gal4-mPPARα about 2- to 3- fold over control (Cowart et al. 2002). In the present study, 10 μM 11,12-EET and 14,15-EET activated Gal4-hPPARα more than 10-fold, suggesting that the non-hydroxylated EETs may be more potent activators of PPARα. It is important however, to take into account the differences in cell type and the species of PPARα that were employed in the assays. 20,14,15-HEET displaced *cis*-

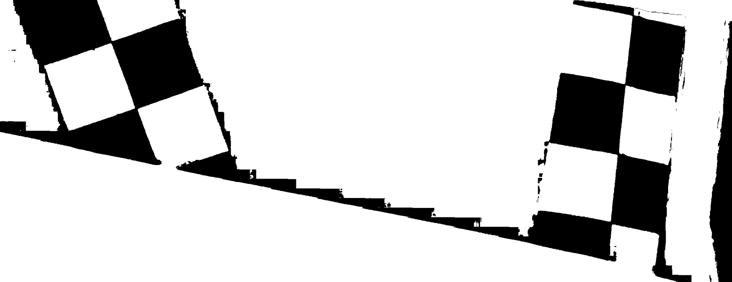


parinaric acid from the ligand binding domain of PPARα with high affinity (K_i = 3 nM). The corresponding K_is for EETs are about 10-fold higher and is 26-fold higher for Wy 14,643. Based on ligand displacement assays, EETs and Wy 14,643 are expected to have lower affinity for PPARα than the hydroxylated EETs. However, Wy 14,643 was functionally as potent at transactivating full length mPPARα and hPPARα as 20,14,15-HEET and in assays using Gal4-mPPARα chimeras, Wy 14,643 was 4 times more potent than 20,14,15-HEET. The lack of correlation between *cis*-parinaric acid displacement constants and transactivation potential makes it difficult to assign relative potencies for PPARα activation to the CYP eicosanoids. The possibility exists that in vivo, EETs are converted to their hydroxylated products in the presence of the CYP4A enzymes. However CYP4A expression was not detected in HepG2 cells where the present transactivation studies were performed, consistent with a direct effect of EETs on PPARα in this system.

8(S)-HETE, 15-deoxy-$\Delta^{12,14}$-PGJ_2, and hydroxylated EETs are naturally occurring eicosanoid ligands for PPARα and PPARγ (Cowart et al. 2002; Forman et al. 1997; Kliewer et al. 1997). Given the similarity in structure between these eicosanoids and CYP eicosanoids, and the promiscuous and large ligand binding pocket of PPARs, it is not entirely surprising that CYP eicosanoids can also activate these nuclear receptors. A major determinant of substrate specificity between PPARα and PPARγ is the Tyr314 residue. It will be interesting to model whether the dual agonist CYP eicosanoids can efficiently hydrogen bond with Tyr314 and its equivalent in PPARγ. Preliminary docking efforts were inconclusive and hindered by the large number of configurations that these eicosanoids can assume. Structural information will provide additional insight

as to why certain CYP eicosanoids are better agonists than others despite their high degree of structural similarity.

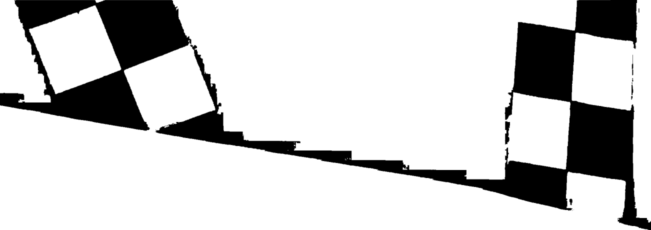
Most of the interest in PPARα activation has focused on its role in regulating lipid metabolism and transport. Examination of genes involved in maintaining lipid homeostasis in the H4IIE rat hepatocyte cell line after treatment with 14,15-DHET did not reveal significant changes in their expression. In primary Sprague Dawley hepatocytes however, alterations in the levels of ApoA-I and ApoA-II were evident in the presence of CYP eicosanoids. CYP eicosanoids decreased ApoA-I mRNA levels in primary rat hepatocytes to a similar or greater degree as the well-characterized PPARα agonist Wy 14,643. The regulation of the ApoA-I gene by PPARα is species and ligand-specific (Vu-Dac et al. 1998). In previous rat hepatocyte studies, fenofibrate transcriptionally decreased ApoA-I mRNA while benzafibrate, gemfibrozil, and Wy 14,643, had no effect (Staels et al. 1992). The difference in Wy 14,643 effects between these studies could be due to differences in the rat strain and the mode of activator exposure. Since CYP eicosanoids cannot be administered *in vivo*, hepatocytes were dosed after isolation. In contrast, for the fibrate studies, hepatocytes were isolated after in vivo administration of the activators (Staels et al. 1992). Remarkably, 20-HETE was significantly more potent and resulted in a 70% decrease in ApoA-I mRNA. It may be possible that the effect of 20-HETE is not due solely to its activation of PPARα. The RXR homodimer has been implicated in ApoA-I regulation (Nagasaki et al. 1994) and saturated fatty acids have been proposed to alter hepatic ApoA-I levels via post-transcriptional mechanisms (Srivastava 1994). Moreover, studies on the human ApoA-I promoter have shown that other transcription factors such as Rev-erbα (Vu-Dac et al.



1998) and HNF-4 (Chan et al. 1993) may be involved in ApoA-I transcription. It is important to note however, that in contrast to rodents, PPARα activation in human hepatocytes leads to an increase in ApoA-I expression (Berthou et al. 1996), which is regulated by more complex mechanisms. The effect of CYP eicosanoids on ApoA-I expression in humans requires further study.

ApoA-II expression in rat hepatocytes was significantly induced after treatment with Wy 14,643 and CYP eicosanoids, with EETs, DHETs, and 20-HETE causing an almost 4-fold increase in ApoA-II mRNA. In earlier studies, fenofibrate decreased ApoA-II mRNA in the rat liver (Staels et al. 1992) and increased hepatic production of ApoA-II in humans (Vu-Dac et al. 1995). The conflicting effects of PPARα agonists on ApoA-II expression may be related to strain-specific regulation of this gene. As is the case with ApoA-I, we cannot rule out the possibility that CYP eicosanoids mediate their effects via other mechanisms in addition to acting as PPARα ligands.

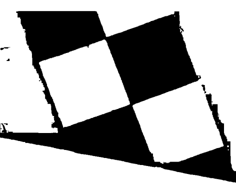
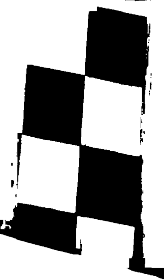
The ability of CYP eicosanoids to regulate their own levels via PPARα was also examined by looking at their effects on PPARα-responsive genes in the arachidonic acid metabolism cascade. CYP4A and sEH mRNA, protein, and activity are highly responsive to treatment with fibrates and Wy 14,643 (Kimura et al. 1989; Pinot et al. 1995; Tollet et al. 1994). In this study, moderate differences in CYP4A1 RNA expression were observed with CYP eicosanoid treatment, however changes in protein levels were not detected. This result could have several explanations. First, the change in CYP4A1 mRNA was not translated to increased protein production in these hepatocytes. Second, compared with the high sensitivity of quantitative PCR, Western blotting may not be sensitive enough to detect changes in protein levels. CYP4A1

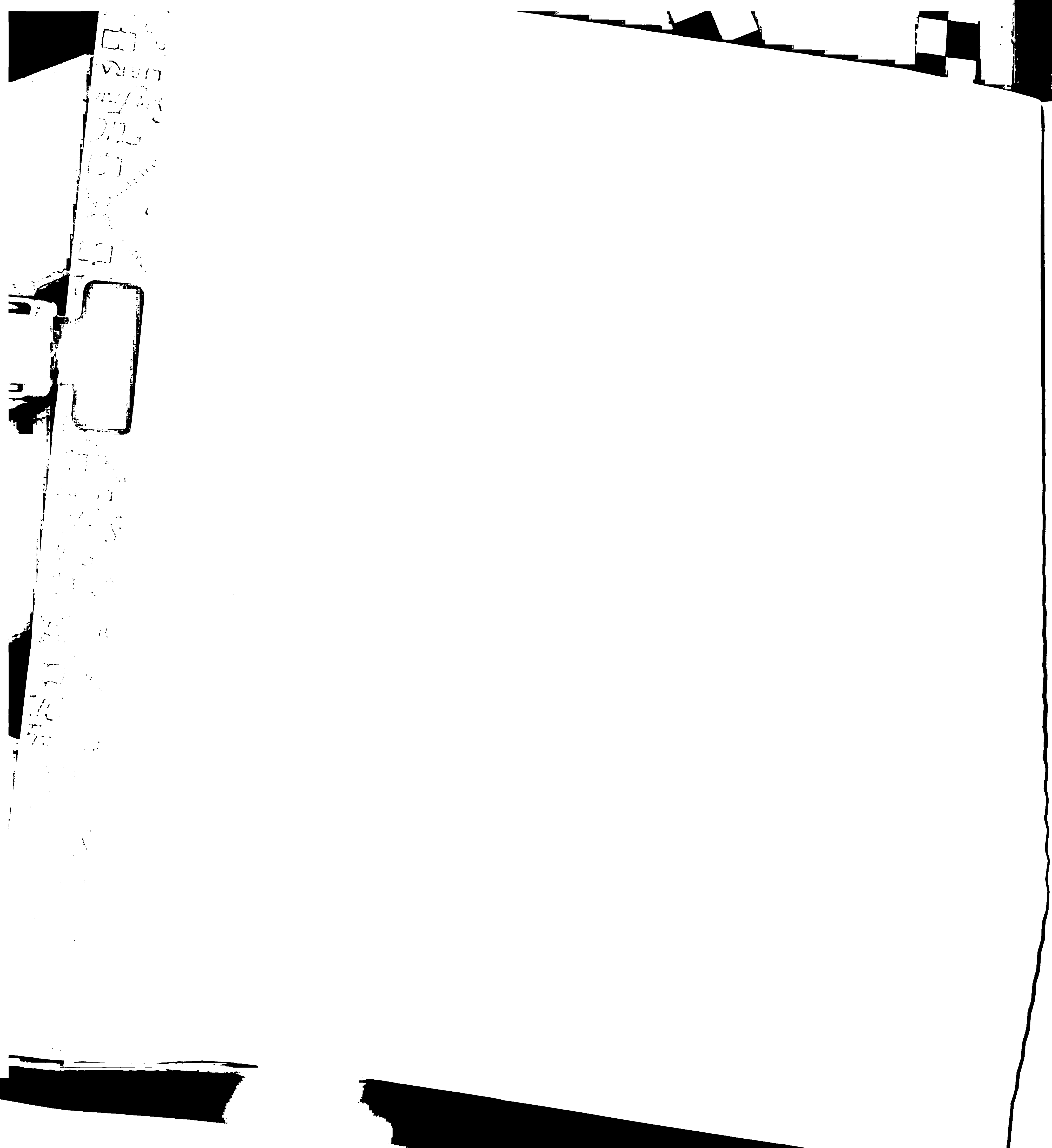


protein induction by Wy 14,643 was roughly 2-fold by densitometric analysis, however the induction corresponded to an almost 150-fold increase in its mRNA level. Therefore, it can be expected that an increase of 2- to 3-fold in mRNA may not be detectable at the protein level. It has previously been shown that PPARα-mediated induction of CYP4A1 mRNA does not correspond to the same extent of protein induction (Kroetz et al. 1998; Ram and Waxman 1994; Sharma et al. 1989).

One of the best characterized roles of PPARγ is the induction of adipocyte differentiation (Forman et al. 1995; Kliewer et al. 1995). Activation of PPARγ can lead to an increase in specialized proteins involved in lipid storage and metabolism during adipogenesis, such as aP2 and PEPCK (Tontonoz et al. 1995; Tontonoz et al. 1994). Although, we have shown that EETs, DHETs, and 20-HETE activate PPARγ, the expression of CYP4A and CYP2C enzymes responsible for their formation have not been described in adipocytes. Therefore, the biological effects of CYP eicosanoids in adipocytes are not clear.

PPARα is well established as a mediator of lipid homeostasis in the liver. Although many studies have demonstrated the responsiveness of hepatic CYPs to fibrate treatment, the biological consequences of increased CYPs in the liver have not been elucidated. Through activation of PPARα, CYP metabolites of arachidonic acid alter ApoA-I and ApoA-II expression in hepatocytes and may play a role in the regulation of triglyceride transport. It will be interesting to examine the overall effect of CYP eicosanoids on fatty acid transport and metabolism by analyzing a panel of genes involved in these processes. CYP levels are often altered in experimental models of hypertension and diabetes. Since CYP eicosanoids may also be moderately auto-



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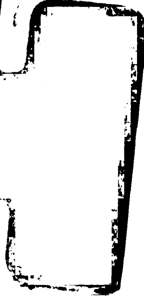
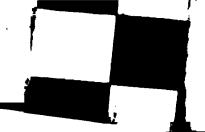

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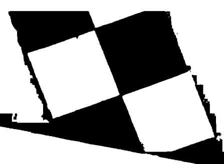
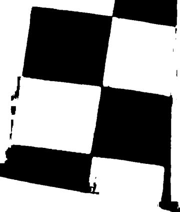
Chapter 3

Inhibition of smooth muscle cell proliferation by urea-based alkanoic acids via peroxisome proliferator-activated receptor α-dependent repression of cyclin D1

3.1 Introduction

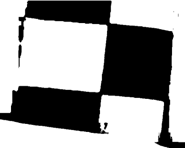
Smooth muscle cell (SMC) proliferation is a critical event in atherosclerosis (Ross 1986) and in restenosis following interventional procedures (Virmani and Farb 1999). In atherosclerotic animal models, lesions are marked by the accumulation of alternating layers of smooth muscle cells and lipid-laden macrophages (Ross 1993) and in human atherosclerotic plaques, SMCs constitute a prominent portion of the lesion (Haust et al. 1960). After injury to the vasculature caused by either mechanical or pathological interventions, a vasculoproliferative cascade is initiated that includes the recruitment and proliferation of SMCs (Clowes et al. 1983). This proliferative response can eventually lead to occlusive lesions that result in myocardial ischemia (Sriram and Patterson 2001). Much effort has been made to inhibit SMC proliferation using pharmacological and genetic approaches that interfere with cell cycle regulators such as cyclins and cyclin-dependent kinases (CDKs) (Brooks et al. 1997; Morishita et al. 1994; Ruef et al. 1999; Zhu et al. 1997).

Cyclins and CDKs are part of the regulatory machinery controlling smooth muscle cell progression through the cell cycle. Binding of cyclin to its cognate CDK serves to activate the complex which promotes cell-cycle progression by phosphorylation of specific target proteins. The D and E cyclins and their associated kinases are viewed

as essential for entry into and progression through the G1-phase of a cell cycle (Sherr and Roberts 2004). When cells are arrested in G0, the presence of mitogens such as platelet-derived growth factor (PDGF) will stimulate them to re-enter the cell cycle, during which cyclins D1, D2, and D3 and their kinases are activated rapidly by multiple processes involving transcription, translation, and stabilization of the cyclin-CDK complex (Jones and Kazlauskas 2000). Overexpression of the D cyclins can shorten G1 implying that they are critical for cell cycle progression through this phase (Resnitzky et al. 1994).

In leukemic and breast cancer cells, xenobiotics such as clofibrate and troglitazone inhibited cyclin D expression, resulting in cell cycle arrest (Laurora et al. 2003; Yin et al. 2001). These compounds are ligands for the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors. PPARs are ligand-activated nuclear receptors of which there are three isoforms (α, γ, and δ). Activators of PPARα include polyunsaturated fatty acids and fibrate drugs (Forman et al. 1997; Staels et al. 1998). PPARγ ligands include the prostaglandin D_2 derivative 15-deoxy-$\Delta^{12,14}$-prostaglindin J_2, forms of oxidized linoleic acid, and the antidiabetic thiazolidinediones such as troglitazone, rosiglitazone, and pioglitazone (Kliewer et al. 1995; Lehmann et al. 1995; Nagy et al. 1998). All three PPAR isoforms are expressed in vascular smooth muscle and endothelial cells and recent studies have elucidated the importance of these receptors, especially PPARα and PPARγ, in atherogenesis (Marx et al. 2004). Upon ligand activation, PPAR heterodimerizes with the retinoid X receptor (RXR) and they subsequently bind to the peroxisome proliferator response element (PPRE). By recruiting large complexes of coactivators, target gene transcription is then initiated. Besides binding to PPRE, PPARs can also repress gene expression by interfering with other



signaling pathways such as the NF-κB and AP-1 pathways (Irukayama-Tomobe et al. 2004; Marx et al. 1999).

Eicosanoids derived from the cytochrome P450-catalyzed metabolism of arachidonic acid include the regio-and sterioisomeric epoxyeicosatrienoic acids (EETs) which have potent vasoactive as well as anti-inflammatory effects in smooth muscle and endothelial cells (Node et al. 1999; Roman 2002). Soluble epoxide hydrolase (sEH) plays a major role in the CYP-mediated arachidonic acid cascade by hydrating EETs into their corresponding dihydroxyeicosatrienoic acids (DHETs), and is thus critical in controlling the biological effects of EETs. Interestingly, sEH is strongly induced by PPARα ligands such as clofibrate (Hammock and Ota 1983). In a recent study, 1-cyclohexyl-3-dodecyl urea (CDU), a urea-based sEH inhibitor, decreased PDGF induced-SMC proliferation by inhibiting cyclin D1 expression (Davis et al. 2002). This study suggested an increase in intracellular EET concentration caused by inhibition of sEH may be responsible for the decrease in SMC proliferation. However other studies have indicated that EETs are mitogenic in SMCs (Fang et al. 1998). Thus the ability of CDU to inhibit SMC proliferation may be independent of its effects on sEH.

In this chapter, we show that the sEH inhibitors, urea-based alkanoic acids, activate PPARα and in turn attenuate PDGF-induced SMC proliferation. In addition, cyclin D1 expression is repressed by alkanoic ureas, implying SMCs are arrested in the G1 phase of the cell cycle. To unambiguously determine whether the decrease in cyclin D1 expression is via alkanoic acid urea-mediated activation of PPARα, the endogenous expression of PPARα in SMCs was knocked-down using siRNA. Results indicate that

PPARα is at least partially responsible for the observed attenuation of SMC proliferation by urea-based alkanoic acids.

3.2 Materials and Methods

3.2.1 Materials

Cyclin D1 (C-20), PPARα (N-19), RXRα (D-20), and β-Actin (I-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The synthesis of all sEH inhibitors has been described in detail elsewhere (Morisseau et al. 2002). The inhibitors used in this study were cyclohexyl butanoic acid urea (CUBA), cyclohexyl heptanoic acid urea (CUHpA), cyclohexyl octanoic acid urea (CUOA), cyclohexyl undecanoic acid urea (CUUA), cyclohexyl dodecanoic acid urea (CUDA), and adamantyl dodecanoic acid urea (AUDA). Wy 14,643 was purchased from Biomol (Plymouth Meeting, PA). Via-Probe™ (7-AAD) was purchased from BD Biosciences (San Diego, CA). Human recombinant platelet-derived growth factor-BB (PDGF-BB) was obtained from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]-ATP and [^{3}H]-thymidine were obtained from Perkin Elmer (Boston, MA). All other reagents were from Sigma Chemical Company (St. Louis, MO).

3.2.2 Cell Culture

HepG2 cells were obtained from American Type Cell Culture and maintained in Modified Eagle's Medium with Earle's Balanced Salt Solution and contained 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and penicillin-streptomycin. Human aortic and coronary artery smooth muscle cells were purchased

from Cambrex (Walkersville, MD) at passage 3 and maintained in SmGM®-2 medium. All experiments using smooth muscle cells were carried out at passages 4-9. All cells were cultured at 37°C under 5% CO_2.

3.2.3 HepG2 transfection and Gel Shift Assays

Transactivation assays carried out in HepG2 cells have been described in Chapter 2. For the analysis of PPARα-responsive genes, 8 x 10^4 cells per well were transfected with 2 ng pCMX-PPARα (plasmid kindly provided by Dr. Ronald Evans) using the Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA). Gel shift assays are carried out exactly as described in Chapter 2, except that the binding reaction included the indicated urea-based alkanoic acids.

3.2.4 Proliferation Assays

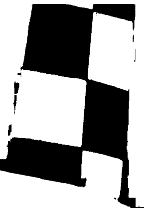
Smooth muscle cells were grown to 80-90% confluence in 24-well plates then incubated in quiescence medium containing SmBm basal medium, GA-1000 (Cambrex, Walkersville, MD), 5 mg/ml transferrin, and 0.5 mg/ml BSA. After 24 h, cell medium was changed to growth medium containing SmBm basal medium, GA-1000, 5 mg/ml insulin, and 30 ng/ml PDGF-BB with or without the test compounds. For quantitation of cell proliferation, [^{3}H]-thymidine was added after 6-8 h and incubated for another 14-16 h. Medium was then aspirated and DNA was precipitated for 30 min at 4°C with 1 ml 15% ice cold trichloroacetic acid. Cells were washed extensively with H_2O and 0.5 ml 1N NaOH was added. After 20 min, the mixture was neutralized with 0.5 ml 1N HCl and transferred into scintillation vials containing 5 ml scintillation fluid for counting. For

flow cytometry, 24 h after addition of growth medium containing alkanoic acids or PPAR activators, cells were trypsinized and resuspended in cold PBS. For detection of viable cells, 7-AAD (20 μl) was added to each sample and incubated for 10 min. Cells were detected with a BD FACSCalibur™ (San Jose, CA) and the data were analyzed using the FlowJo software from Treestar (Palo Alto, CA). Events positive for 7-AAD represent dead cells.

3.2.5 3T3-L1 Differentiation and Lipid Quantitation

Two days after reaching confluence, 3T3-L1 cells were incubated in culture medium containing 10 μg/ml insulin, 0.5 mM 1-methyl-3-isobutylxanthine, and 1 μM dexamethasone for 2 days. Medium is replaced with culture medium containing 10 μg/ml insulin with or without ciglitazone or alkanoic acids and replaced every 48 h until 90% of the cells have differentiated into adipocytes. The quantitation of intracytoplasmic lipids has been previously described (Ramirez-Zacarias et al. 1992). Briefly, cells were fixed for at least 1 h with 10% formalin in isotonic phosphate buffer. After washing with H_2O, cells were immersed for at least 2 h in a working solution of Oil Red O and rinsed exhaustively with H_2O. Excess water was evaporated by placing the stained cultures at 32°C. Isopropyl alcohol is added to each well and the extracted dye was immediately removed by gentle pipeting. Absorbance was monitored spectrophotometrically at 510 nm.

3.2.6 siRNA Transfection



Aortic smooth muscle cells were grown to 50-60% confluence and transfected with chemically synthesized PPARα (ID# 5439) or negative control siRNA #1 using siPORT™ *Amine* (all from Ambion, Austin, TX) according to the manufacturer's instructions. Fresh culture medium was added 4 h after transfection to minimize cytotoxicity. For detection of cyclin D1 expression, transfected cells were incubated in quiescence medium 24 h after transfection for 1 day. Cells were then exposed to growth medium with or without the alkanoic acids or Wy 14,643 for the indicated times. Cell lysates were prepared and used in Western immunoblotting as described below.

3.2.7 Real-Time Quantitative PCR and Western immunoblotting

Reverse transcription and real-time quantitative PCR were carried out as described in Chapter 2. The 18S primers and probe set was designed using Primer Express from Applied Biosystems (Foster City, CA). Primer sequences are as follows: forward primer 5′-CGG CTA CCA CAT CCA AGG AA-3′, reverse primer 5′-GCT GGA ATT ACC GCG GCT-3′. The probe sequence is 5′-TCG TGG CAC CAG ACT TGC CT C-3′. All other primers and probes were Assays-on-Demand purchased from Applied Biosystems. Western immunoblotting techniques have been described (Chapter 2).

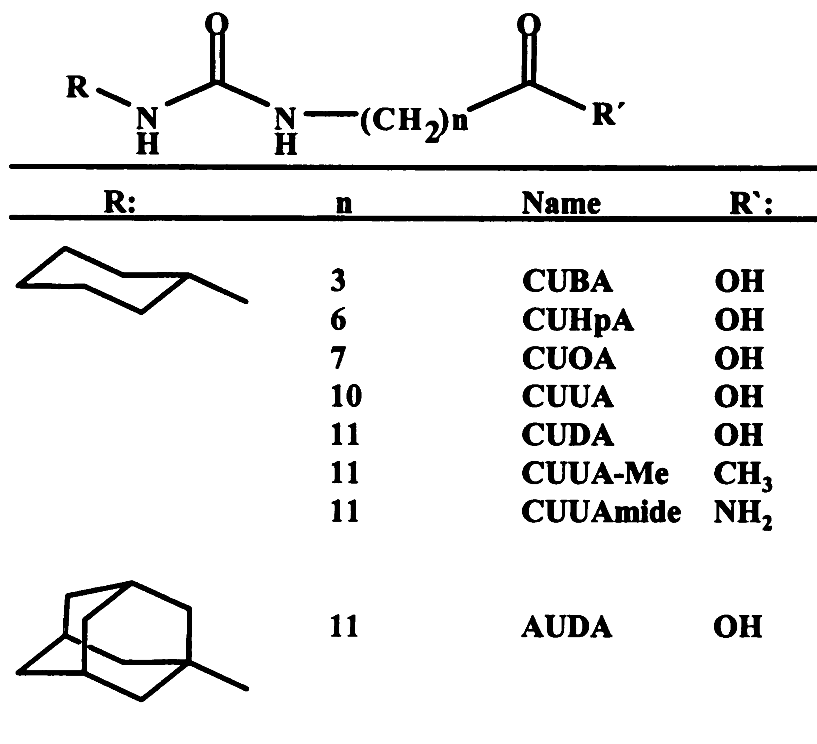
3.2.8 Statistics

Statistical significance of differences between values was evaluated by an unpaired Student's t test. Significance was set at a p value of <0.05.

3.3 Results

3.3.1 Urea-based alkanoic acids activate PPARα.

An adamantyl and a series of cyclohexyl urea-based alkanoic acids were tested for their ability to activate PPARα. The structures of these acids are shown in Figure 3.1.



R:	n	Name	R`:
	3	CUBA	OH
	6	CUHpA	OH
	7	CUOA	OH
	10	CUUA	OH
	11	CUDA	OH
	11	CUUA-Me	CH_3
	11	CUUAmide	NH_2
	11	AUDA	OH

Figure 3.1 Structures of urea-based compounds used in transactivation assays.

In transactivation assays, HepG2 cells were transfected with Gal4-hPPARα or Gal4-hPPARγ and tested for its ability, in the presence of alkanoic acids, Wy 14,643, or ciglitazone, to transactivate the Gal4 response element UAS tagged to a luciferase reporter gene (UAS_4-LUC). All of the inhibitors significantly activated PPARα and PPARγ compared to the DMSO vehicle control (Figure 3.2). Receptor activation increased as the carbon chain length in the cyclohexyl-based acids was lengthened. A

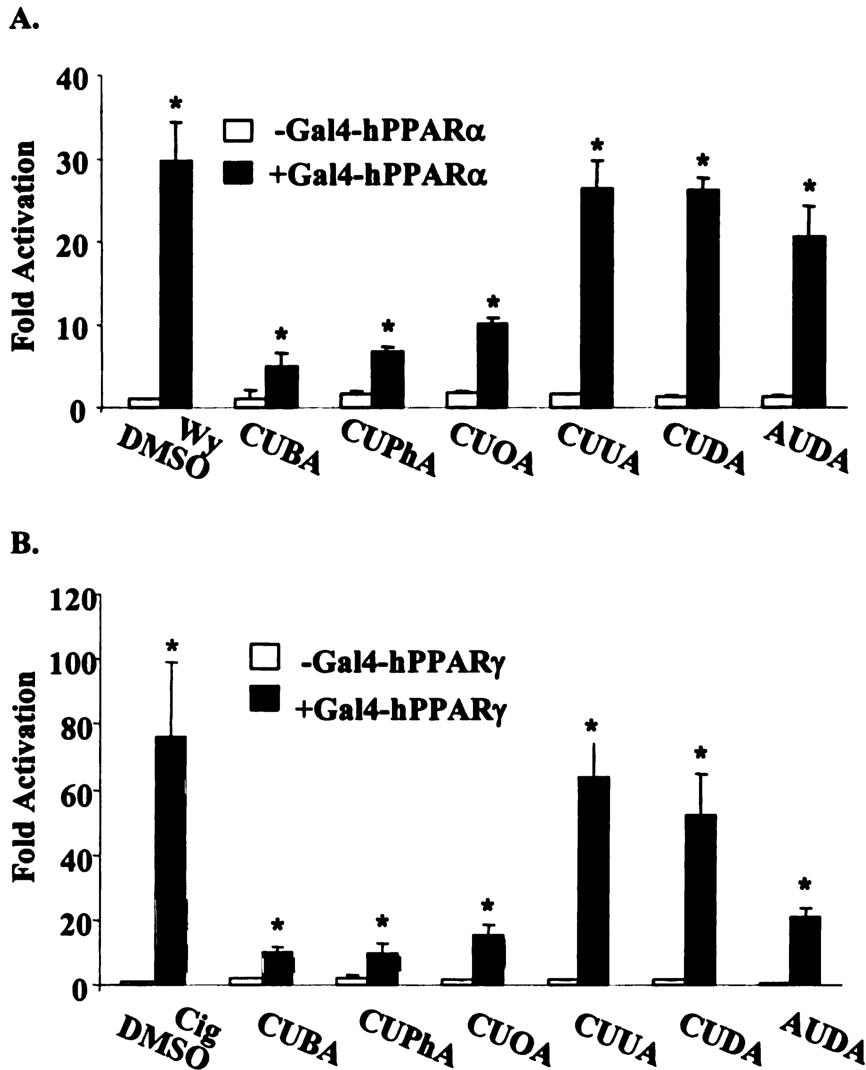


Figure 3.2 Long chain urea-based alkanoic acids transactivate PPARα and PPARγ in HepG2 cells. Transactivation assays were performed in HepG2 cells which were transfected with the UAS_4-LUC reporter with or without the A) Gal4-hPPAR*α* or B) Gal4-hPPARγ expression plasmids. HepG2 cells were treated with 50 μM of alkanoic acids, 50 *μ*M Wy 14,643 (Wy), or 10 *μ*M ciglitazone (Cig) and transactivation was measured 24 h later. Luciferase values were normalized to β-galactosidase and fold activation was calculated as increase over the DMSO solvent control. The values shown are the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained from additional experiments. * Significant difference compared to DMSO ($p<0.05$).

maximum activation was observed with CUUA and CUDA. The adamantyl urea-based acid, AUDA, also strongly activated PPARα and PPARγ more than 20-fold over control. As expected, activation required PPARα or PPARγ transfection.

A steep dose-dependence for PPARα activation was observed between 10 and 100 μM CUUA and AUDA with saturation evident at 50 μM (Figure 3.3). Due to the limited solubility of CUUA-Me and CUUA-Amide, it was only possible to access their ability to transactivate PPARα at 5 μM. CUUA and CUUA-Amide transactivated PPARα to an equal extent, whereas CUUA-Me was slightly more potent (Figure 3.4).

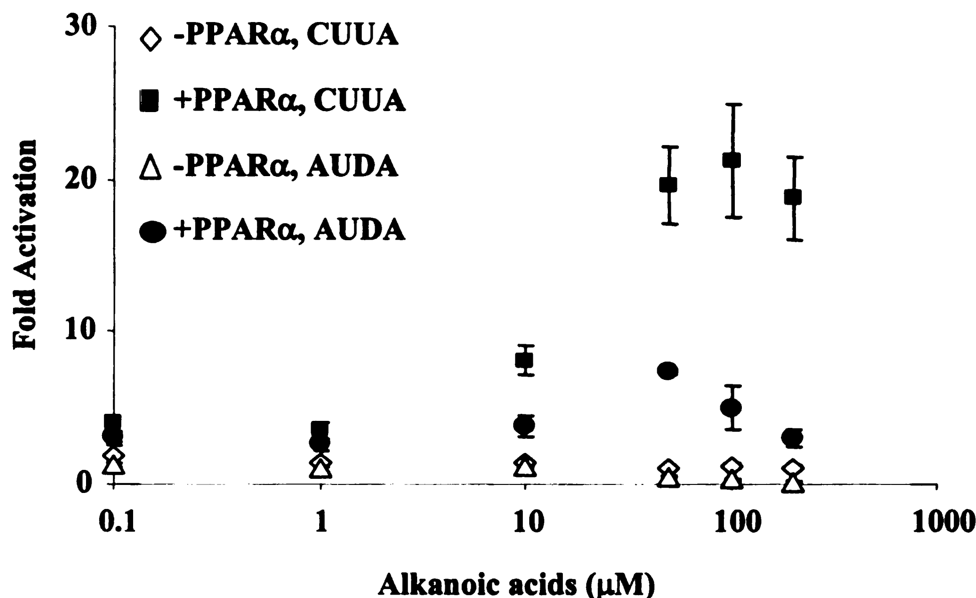


Figure 3.3 CUUA and AUDA dose-dependently transactivate PPARα in HepG2 cells. Transactivation assays were performed in HepG2 cells which were transfected with the UAS_4-LUC reporter with or without the Gal4-hPPARα expression plasmid. HepG2 cells were treated with 0.1-200 μM CUUA or AUDA and transactivation was measured 24 h later. Luciferase values were normalized to β-galactosidase and fold activation was calculated as increase over the DMSO solvent control. The values shown are the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained from additional experiments. * Significant difference compared to DMSO (p<0.05).

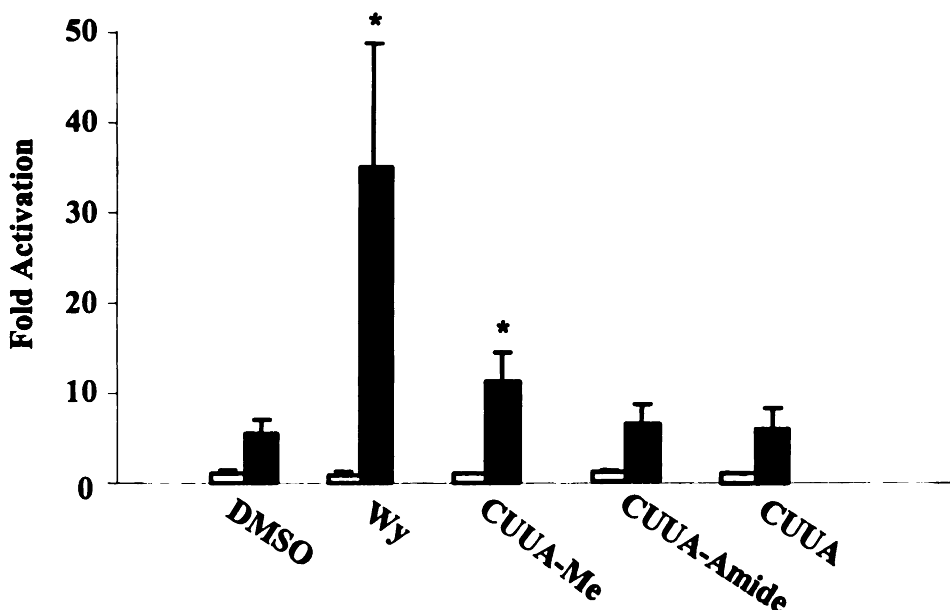


Figure 3.4 Amide and ester forms of CUUA transactivate PPARα in HepG2 cells. Transactivation assays were performed in HepG2 cells which were transfected with the UAS_4-LUC reporter with or without the Gal4-hPPARα expression plasmid. HepG2 cells were treated with 5 μM CUUA-Amide, CUUA-Me, or CUUA, or 50 μM Wy 14,643 and transactivation was measured 24 h later. Luciferase values were normalized to β-galactosidase and fold activation was calculated as increase over the DMSO solvent control. The values shown are the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained from additional experiments. * Significant difference compared to DMSO ($p<0.05$).

As a specificity control, Gal4-TRβ and UAS_4-LUC were transfected into HepG2s cells which were treated with 50 μM CUUA, AUDA, or the TRβ positive control, 3,3′,5-triiodo-L-thyronine (T_3) (Figure 3.5). T_3 strongly activated TRβ whereas CUUA and AUDA had no effect, showing that the activation of nuclear receptors is specific for PPARα.

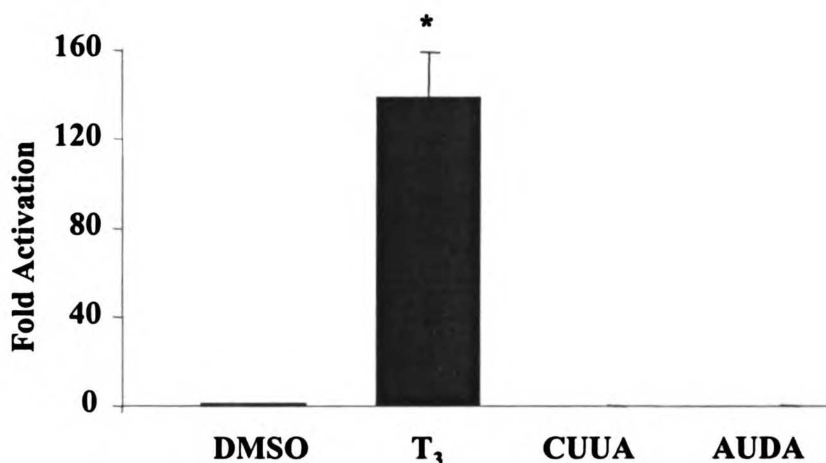


Figure 3.5 TRβ is not activated by CUUA or AUDA. Transactivation assays were performed in HepG2 cells which were transfected with the UAS_4-LUC reporter with or without the Gal4-hTRβ expression plasmid. HepG2 cells were treated with 50 μM CUUA or AUDA, or 1 μM T_3 and transactivation was measured 24 h later. Luciferase values were normalized to β-galactosidase and fold activation was calculated as increase over the DMSO solvent control. The values shown are the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained from additional experiments. * Significant difference compared to DMSO ($p<0.05$).

3.3.2 Urea-based alkanoic acids induce formation of a PPARα-PPRE complex.

Using electrophoretic-mobility shift assays (EMSA), it was next determined whether these alkanoic acids could induce the formation of a PPARα-PPRE complex. In vitro-translated PPARα and RXRα were tested for their ability to heterodimerize in the presence of Wy 14,643 or the alkanoic acids and bind to the PPRE. The addition of Wy 14,643 and the alkanoic acids examined resulted in the detection of a PPRE-protein complex (Figure 3.6). PPARα and RXRα were also able to heterodimerize and bind the PPRE in the absence of ligand; however the extent of this basal binding was significantly less than the binding observed with Wy 14,643 or the alkanoic acids, except CUBA and

CUHpA. The negative controls, a mutated response element and unprogrammed lysates did not result in heterodimer formation and DNA binding.

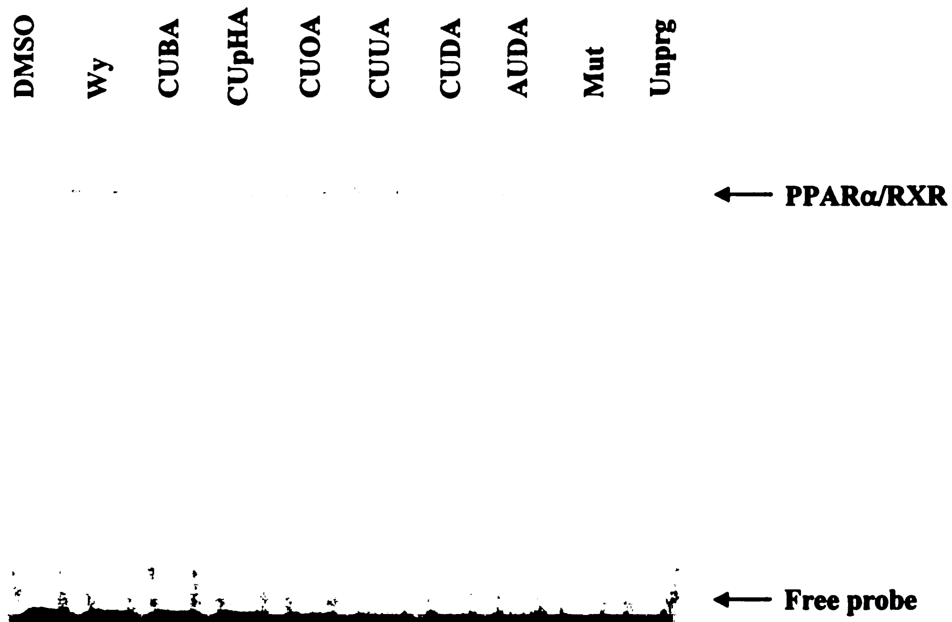
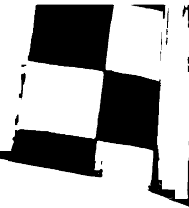


Figure 3.6 CUUA and AUDA induce PPARα/RXRα-specific binding to PPRE. EMSAs were performed by incubating in vitro translated PPARα and RXRα with DMSO, 50 μM Wy 14,643, CUUA, or AUDA and ^{32}P-labeled PPRE. The complex was resolved through a 5% non-denaturing polyacrylamide gel and visualized using a PhosphorImager. A mutant PPRE oligonucleotide (mut) and unprogrammed reticulocyte (unprg) were used as controls.

The DNA-protein complex was supershifted by PPARα- and RXRα-specific antibodies and unlabeled PPRE competed for PPARα/RXRα binding to the PPRE (Figure 3.7). In addition, CUUA dose-dependently increased formation of the PPARα/RXRα-PPRE complex (Figure 3.8).



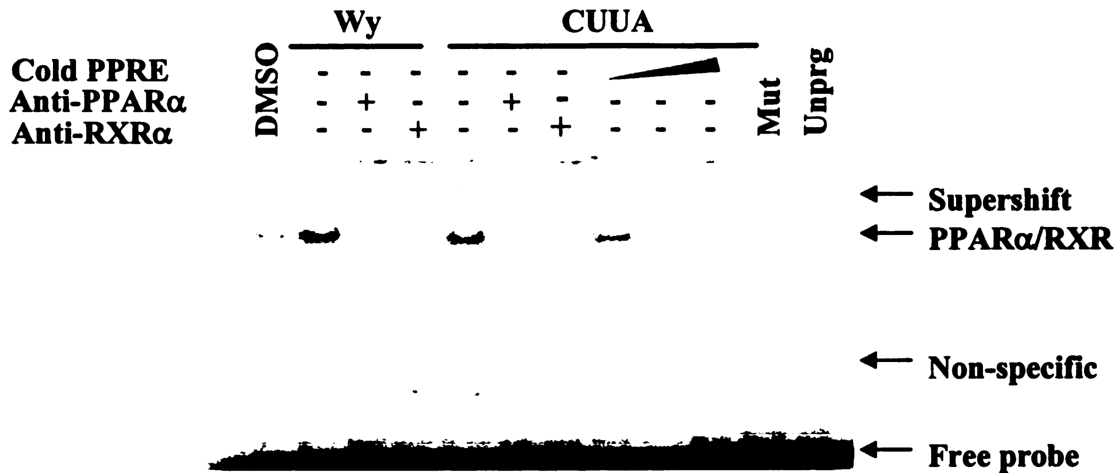


Figure 3.7 PPARα and RXRα antibodies supershift the PPAR/RXR-PPRE complex induced by CUUA. EMSAs were performed by incubating in vitro translated PPARα and RXRα with DMSO, 50 μM Wy 14,643 or CUUA and ^{32}P-labeled PPRE in the absence or presence of PPARα- or RXRα-specific antibodies. The complex was resolved through a 5% non-denaturing polyacrylamide gel and visualized using a PhosphorImager. A mutant PPRE oligonucleotide (mut) and unprogrammed reticulocyte (unprg) were used as controls.

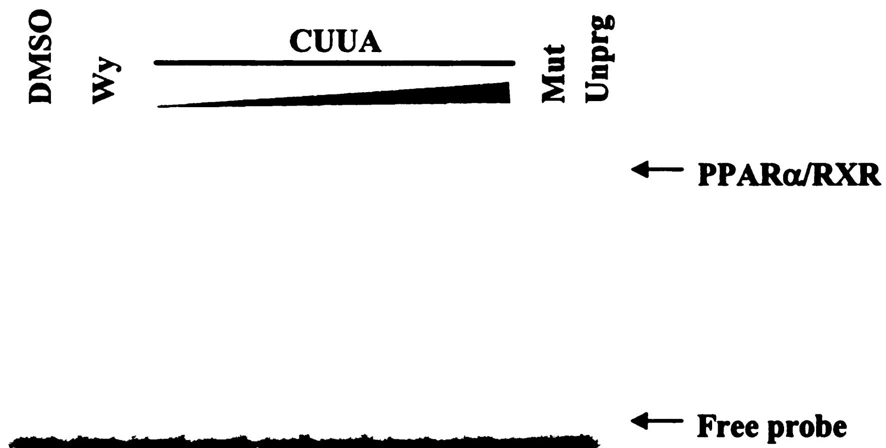


Figure 3.8 CUUA dose-dependently increases PPARα/RXRα binding to PPRE. EMSAs were performed by incubating in vitro translated PPARα and RXRα with DMSO, 50 μM Wy 14,643 or 0.1-100 μM CUUA and ^{32}P-labeled PPRE. The complex was resolved through a 5% non-denaturing polyacrylamide gel and visualized using a PhosphorImager. A mutant PPRE oligonucleotide (mut) and unprogrammed reticulocyte (unprg) were used as controls.

3.3.3 Urea-based alkanoic acids induce expression of PPARα-responsive genes.

To determine whether these PPARα activators could affect known PPARα-responsive genes involved in the regulation of fatty acid metabolism, HepG2 cells were transfected with PPARα and treated with 50 μM Wy 14,643, CUUA, or AUDA. mRNA expression of three well-established PPARα downstream genes, acyl-CoA synthetase (ACS), carnitine palmitoyl transferase 1A (CPT1A) and acyl-CoA oxidase (ACOX) were determined using Taqman quantitative PCR (Figure 3.9). Treatment with Wy 14,643

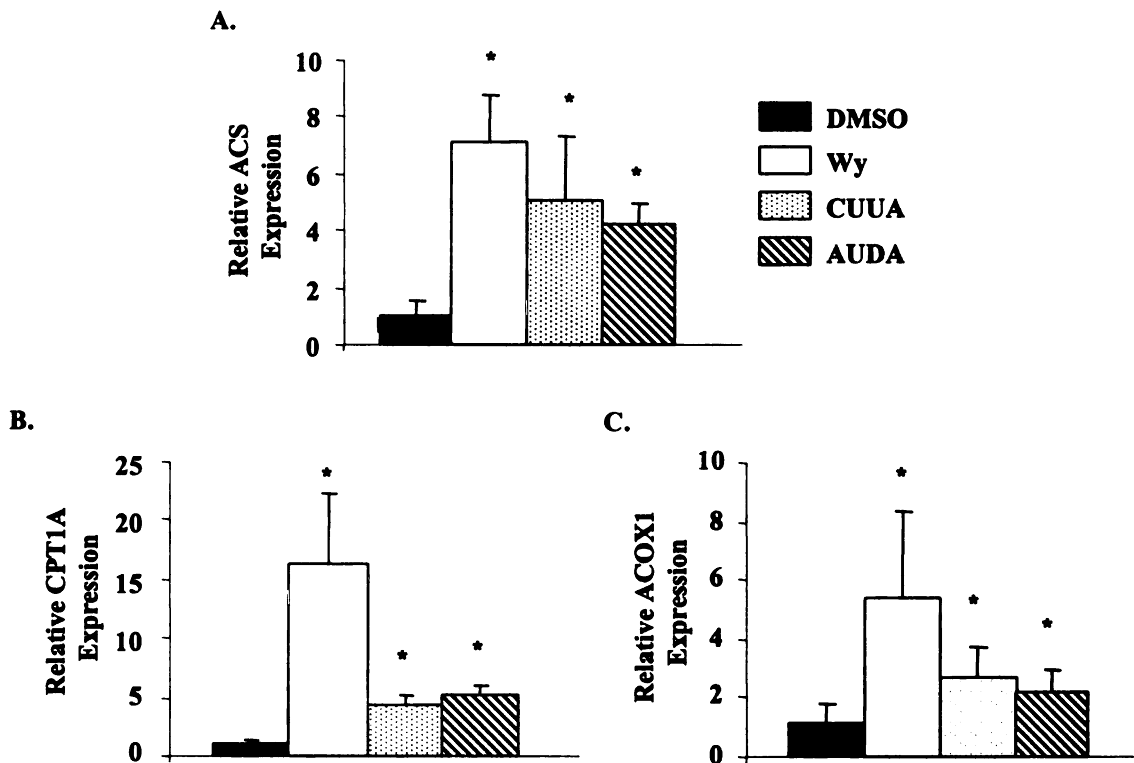


Figure 3.9 CUUA and AUDA induce PPARα-responsive genes. HepG2 cells were transfected with the full-length PPARα expression vector and 24 h later cells were dosed with 50 μM Wy 14,643, CUUA, or AUDA for an additional 24 h. RNA was isolated and A) ACS, B) CPT1A, and C) ACOX expression were determined using Taqman quantitative PCR. Specific gene expression was normalized to 18S expression. The values shown are the mean ± SD of a representative experiment performed in triplicate.
* Significant difference in expression between control and treated cells (p<0.05)

resulted in the expected induction of all three genes. ACS expression was up-regulated 7-fold, CPT1A 16–fold, and ACOX1 5-fold by Wy 14,643. CUUA and AUDA increased ACS expression 4- to 5-fold. Similarly, CPT1A and ACOX expression were induced 2- to 5-fold by CUUA and AUDA.

In primary Sprague Dawley hepatocytes, CUUA and AUDA had minimal effects on the PPARα downstream genes (Figures 3.10). Wy 14,643 repressed apolipoprotein (Apo) A-I expression by 20%, and induced CPT1A expression 6-fold. The expression of ApoA-II and CPT1A were only moderately induced by CUUA, while AUDA had no effect on any of the genes examined.

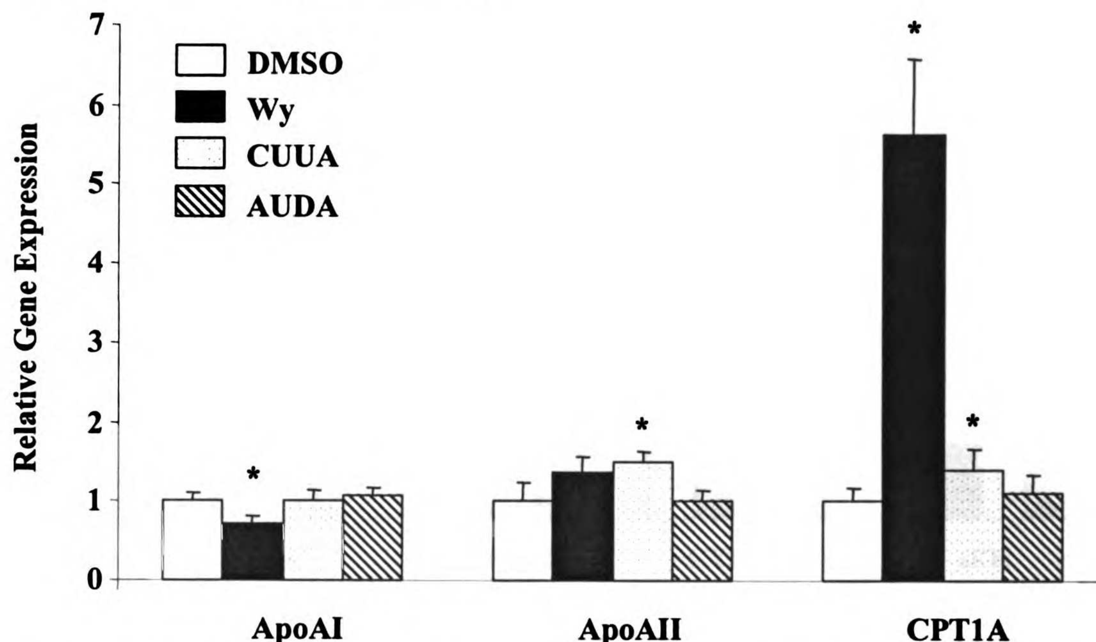


Figure 3.10 CUUA and AUDA have minimal effects on PPARα-responsive genes in primary hepatocytes. Primary hepatocytes from Sprague Dawley rats were treated for 48 h with DMSO, 50 μM Wy, 30 μM CUUA, or 30 μM AUDA. Total RNA was isolated with TRIzol reagent and RNA expression was determined by real-time quantitative PCR. Expression of ApoA-I, ApoA-II, and CPT1A are normalized to cyclophilin levels and expressed as increase over DMSO control. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO (p<0.05).

The expression of cytochrome P450 enzymes and sEH in the presence of urea-based alkanoic acids was also explored (Figure 3.11). Wy 14,643 potently induced CYP4A1 and sEH expression, whereas CYP2C11 expression was repressed. CUUA induced CYP2C23 2-fold, and CYP2C11 and sEH only 1.5-fold. CYP4A1, CYP2C23, and CYP2C11 expression were all induced by AUDA by about 2-fold (Figure 3.11). Protein levels of the CYP enzymes and sEH were not altered after treatment with CUUA or AUDA (Figure 3.12).

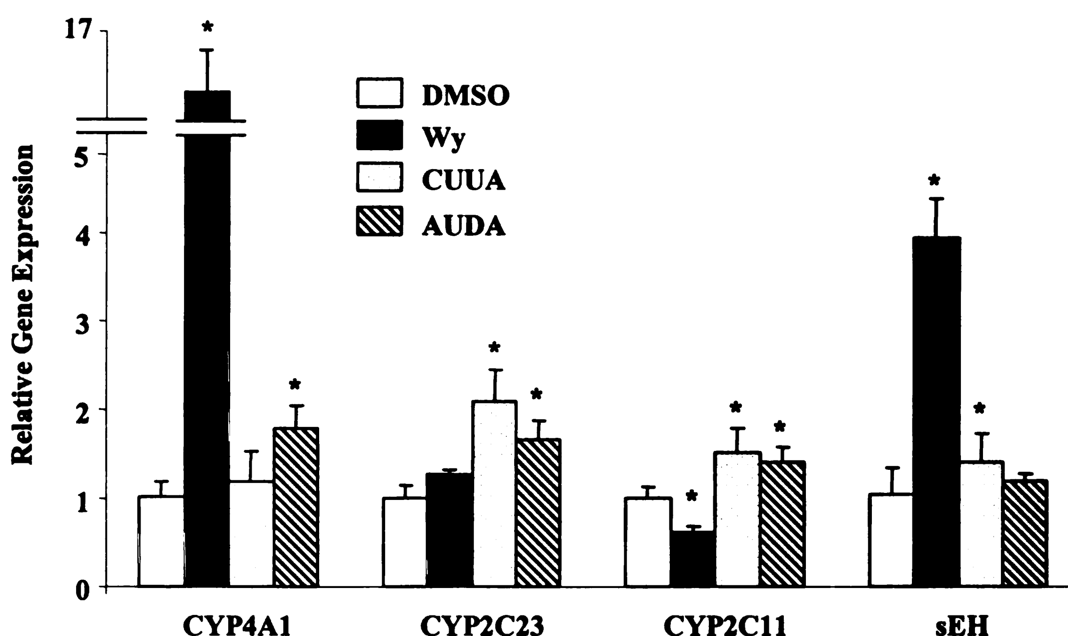


Figure 3.11 CUUA and AUDA have minimal effects on CYP RNA expression in primary hepatocytes. Primary hepatocytes from Sprague Dawley rats were treated for 48 h with DMSO, 50 μM Wy, 30 μM CUUA, or 30 μM AUDA. Total RNA was isolated with TRIzol reagent and RNA expression was determined by real-time quantitative PCR. Expression of CYP4A1, sEH, CYP2C11, and CYP2C23 are normalized to cyclophilin levels and expressed as increase over DMSO control. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO ($p<0.05$).

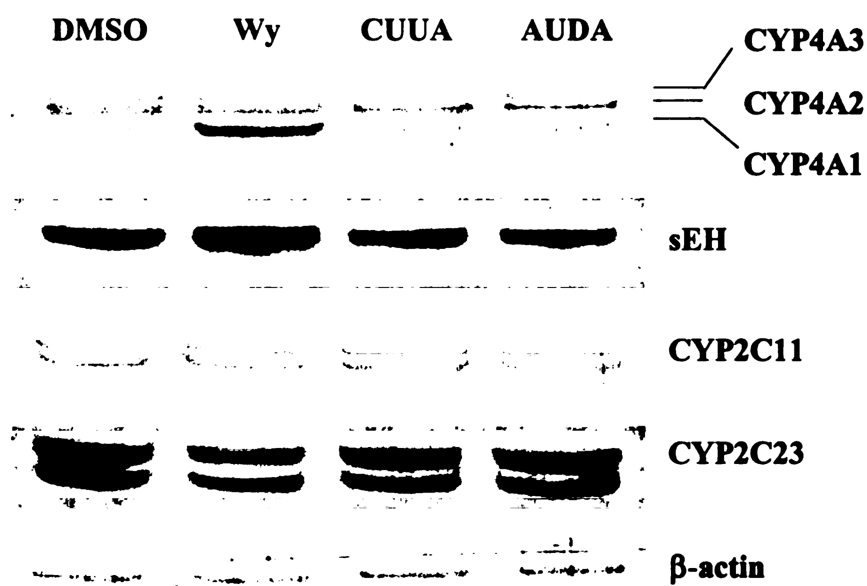


Figure 3.12 CUUA and AUDA have minimal effects on CYP and sEH protein expression in primary hepatocytes. Primary hepatocytes from Sprague Dawley rats were treated for 48 h with DMSO, 50 μM Wy, 30 μM CUUA, or 30 μM AUDA. CYP4A1, sEH, CYP2C11, and CYP2C23 immunoreactive proteins were detected by Western immunoblotting as described in the *Materials and Methods*. β-Actin was used as a loading control. The CYP4A1 antibody detects CYP4A1, CYP4A2, and CYP4A3 proteins.

3.3.4 Urea-based alkanoic acids have moderate effects on PPARγ-responsive genes.

The expression of the well-characterized PPARγ gene aP2 was investigated in 3T3-L1 pre-adipocytes (Figure 3.13). CUUA or AUDA (30 μM) was incubated with 3T3-L1 cells during differentiation and aP2 expression was determined by real-time PCR.

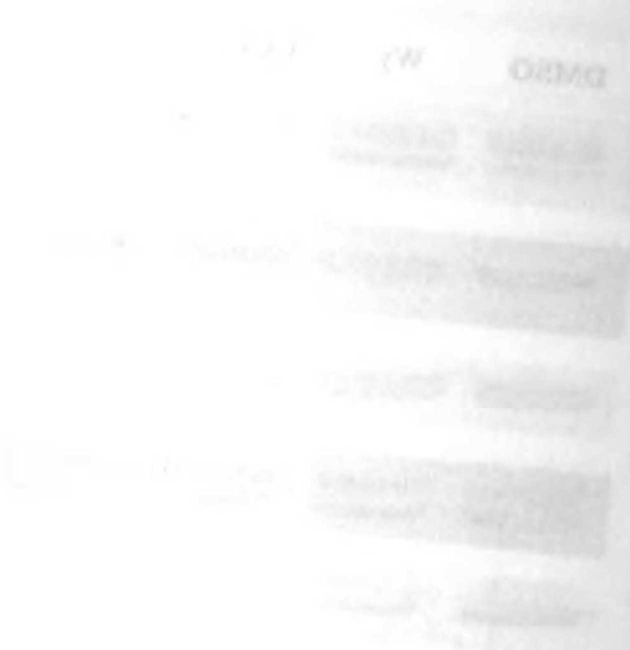


Figure 3.12 CUUA and AUDA ... expression in primary hepatocytes ... treated for 48 h with DMSO, ... 48h, CYP2C11, and CYP3A23 ... immunoblotting as described ... loading control. The CYP4A1 ... proteins.

3.3.4 Urea-based alkanoic acids ... genes.

The expression of the well-characterized ... investigated in 3T3-L1 pre-adipocytes (Figure 3.13). ... treated with ... 3T3-L1 cells during differentiation and ... quantified by real-time PCR.

Ciglitazone, the PPARγ activator, strongly induced the level of aP2 by 18-fold. CUUA and AUDA however only induced aP2 expression about 3-fold.

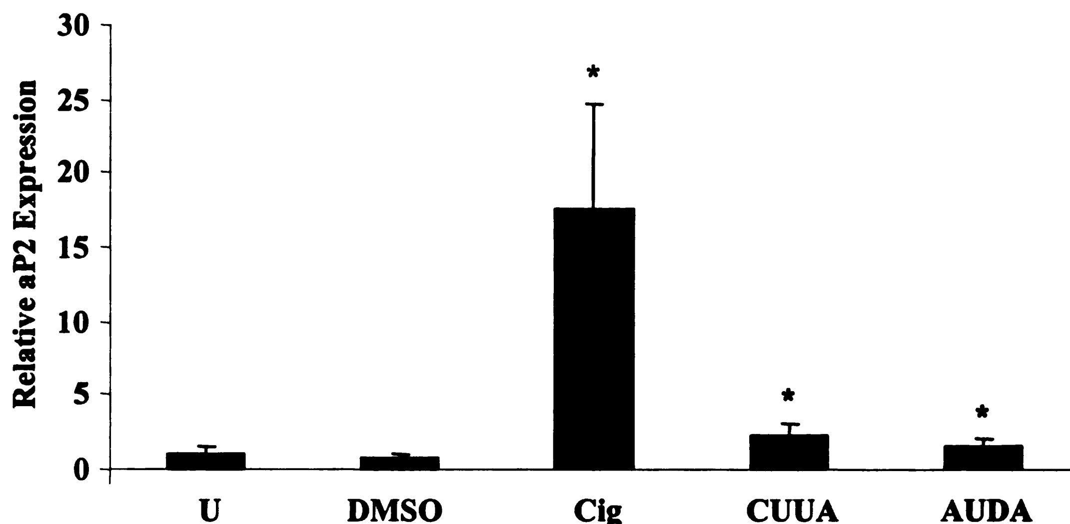


Figure 3.13 CUUA and AUDA induce aP2 expression. 3T3-L1 cells were incubated in maintenance (U) or differentiation medium for 7 days in the presence of 10 μM ciglitazone (Cig), or 30 μM CUUA or AUDA. Expression of aP2 was determined by real-time quantitative PCR and normalized to GAPDH levels and expressed as increase over DMSO control. The values shown are mean ± SD of triplicate determinations.
* Significant difference compared to DMSO (p<0.05).

Since activation of PPARγ leads to increased adipocyte differentiation, the effects of CUUA and AUDA on intracytoplasmic lipid accumulation was explored (Figure 3.14). Incubation of 3T3-L1 pre-adipocytes with ciglitazone resulted in an approximate 2-fold increase of lipid accumulation compared with cells treated only with the vehicle control. CUUA moderately increased the amount of intracellular lipids. Due to the high variability that was present between each replicate, it was not possible to definitively

determine whether AUDA had any effects on adipocyte differentiation, although there is an upward trend of lipid accumulation in the presence of AUDA.

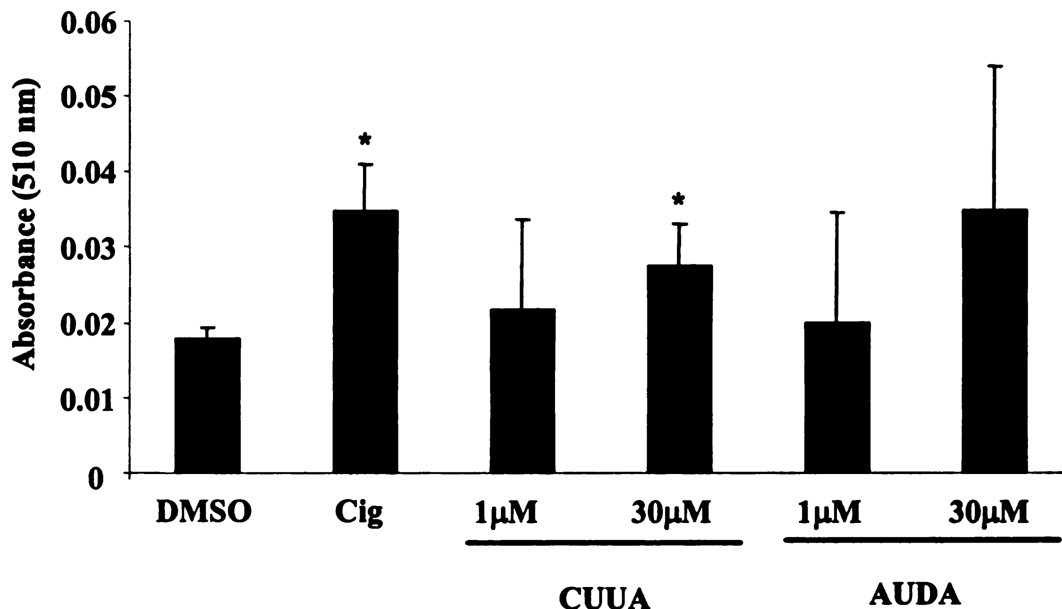


Figure 3.14 CUUA and AUDA have minimal effects on inducing adipocyte differentiation. 3T3-L1 cells were incubated for 10 days in differentiation medium with 10 μM ciglitazone (Cig), or 1 μM or 30 μM CUUA or AUDA. Intracytoplasmic lipids were quantitated as described in *Materials and Methods*. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO (p<0.05).

3.3.5 Urea-based alkanoic acids inhibit human smooth muscle cell proliferation.

Human aortic smooth muscle cells were quiesced for 24 h then incubated in medium containing PDGF-BB with or without the PPARα activators Wy 14,643, CUUA, or AUDA. As expected, PDGF resulted in a marked increase in SMC proliferation (Figure 3.15). Wy 14,643, CUUA, and AUDA significantly attenuated PDGF-induced proliferation. Compared to PDGF alone, proliferation was decreased 72%, 53%, and

90% by Wy 14,643, CUUA and AUDA, respectively. Most remarkable was the effect of AUDA, which almost completely abolished SMC proliferation.

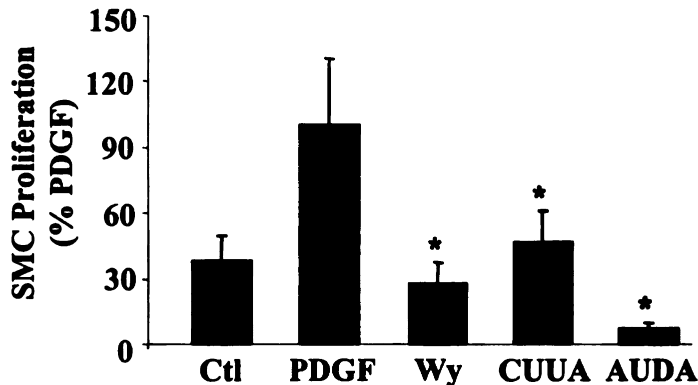
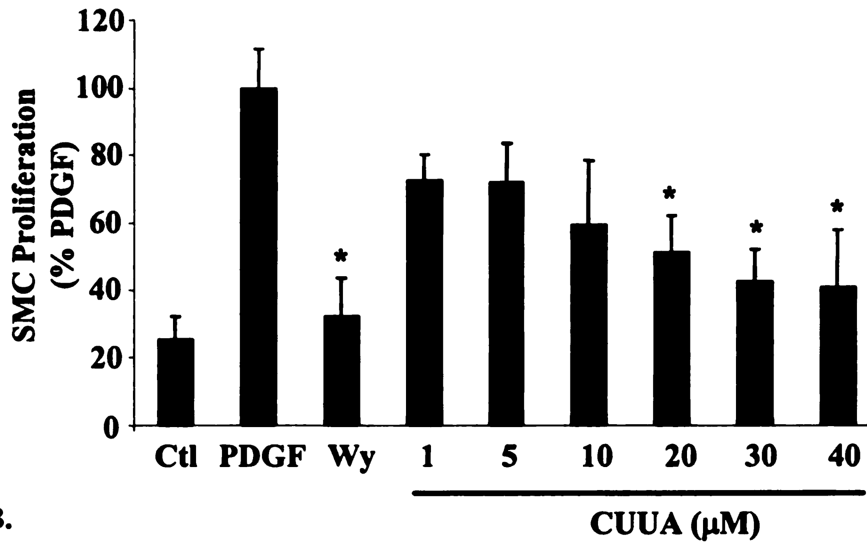


Figure 3.15 CUUA and AUDA decrease proliferation in human aortic SMCs. Human aortic SMCs were grown to 85-90% confluence and growth-arrested in quiescence medium (Ctl). PDGF medium was added and [^{3}H]-thymidine incorporation was assessed by scintillation counting after 24 h. SMC proliferation was measured in the presence of 50 μM Wy 14,643, CUUA or AUDA. The values shown are the mean ± SD of a representative experiment performed in triplicate. Each experiment was repeated three times. * Significant difference over PDGF positive control (p<0.05).

In dose-dependence studies, a significant decrease in proliferation was evident starting at 20 μM CUUA, and the decrease continued gradually at 30 μM and 40 μM (Figure 3.16A). AUDA had significant anti-proliferative effects at 1 μM (Figure 3.16B). Interestingly, a sharp decrease in proliferation was observed when human aortic SMCs were treated with 20 μM AUDA. To determine whether this was a phenomenon was cell-type specific, the same studies were repeated in human coronary SMC. A similar sharp decrease was observed when coronary artery smooth muscle cells were treated with 20 μM AUDA (Figure 3.17).

A.



B.

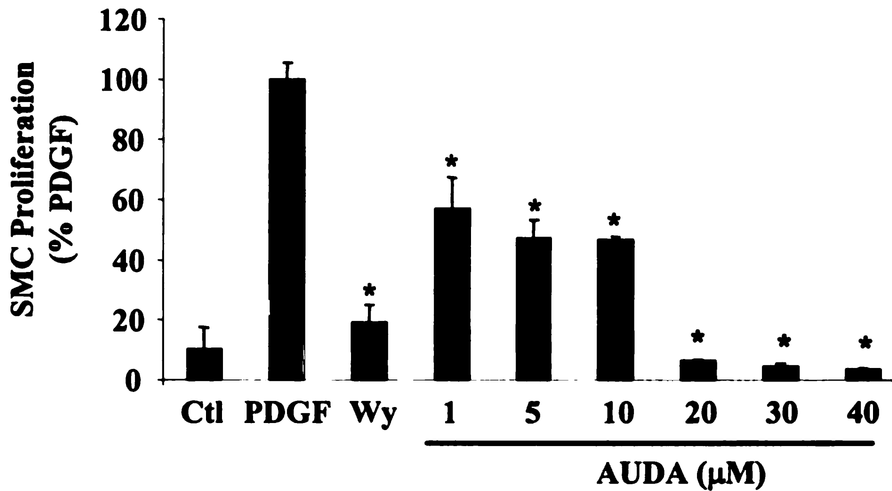


Figure 3.16 CUUA and AUDA decrease human aortic SMC proliferation in a dose-dependent manner. Human aortic SMCs were grown to 85-90% confluence and growth-arrested in quiescence medium (Ctl). PDGF medium was added and [^{3}H]-thymidine incorporation was assessed by scintillation counting after 24 h. SMC proliferation was measured in the presence of 50 μM Wy 14,643, A) 1-40 μM CUUA, or B) 1-40 μM AUDA. The values shown are the mean ± SD of a representative experiment performed in triplicate. Each experiment was repeated three times. * Significant difference over PDGF positive control (p<0.05).

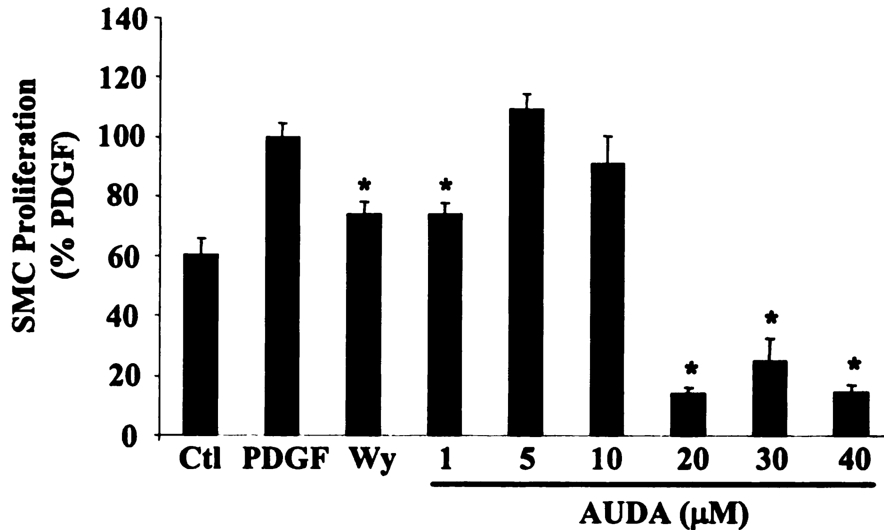


Figure 3.17 AUDA decreases human coronary SMC proliferation in a dose-dependent manner. Human coronary SMCs were grown to 85-90% confluence and growth-arrested in quiescence medium (Ctl). PDGF medium was added and [^{3}H]-thymidine incorporation was assessed by scintillation counting after 24 h. SMC proliferation was measured in the presence of 50 μM Wy 14,643, or 1-40 μM AUDA. The values shown are the mean ± SD of a representative experiment performed in triplicate. Each experiment was repeated three times. * Significant difference over PDGF positive control ($p<0.05$).

3.3.6 Decreased SMC proliferation is not due to cell death.

To show that decreased SMC proliferation is not attributed to increased cellular toxicity, SMCs were subjected to proliferation conditions and fluorescently labeled with a viability stain, 7-AAD (Figure 3.18). Based on the dose dependence shown in Figure 3.16, if cell death were responsible for the observed decrease in proliferation with increasing concentrations of the alkanoic acids, higher 7-AAD fluorescence would be expected. However, no differences in 7-AAD incorporation were observed between 10

μM and 40 μM CUUA or AUDA. The attenuation of SMC proliferation by CUUA and AUDA is therefore not accounted for by cell toxicity.

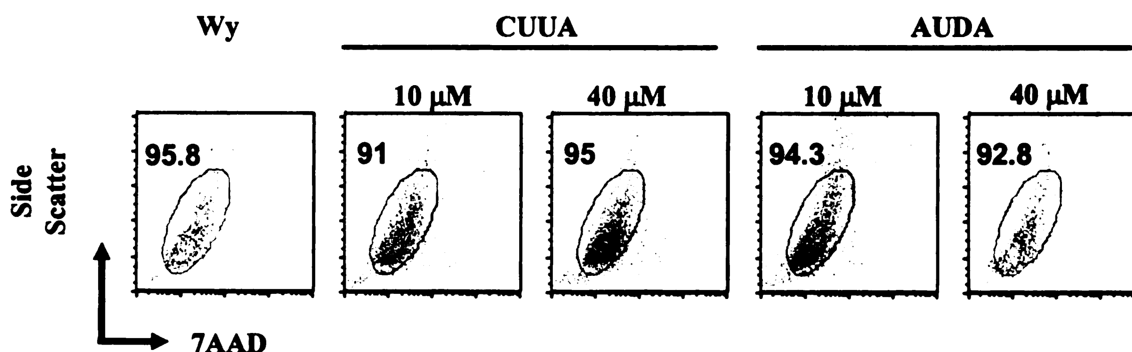


Figure 3.18 Attenuation of aortic SMC proliferation by CUUA and AUDA is not due to increased cell death. Human aortic SMCs were growth-arrested and incubated in PDGF medium containing 50 μM Wy 14,643, 10 or 40 μM CUUA, or 10 or 40 μM AUDA. After a 24 h incubation in PDGF medium, cells were stained for viability using ViaProbe™ (7-AAD) and fluorescence was detected by flow cytometry. The numbers represent the percentage of events that are negative for 7-AAD.

3.3.7 Urea-based alkanoic acids decrease cyclin D1 expression.

In order to understand the mechanism whereby CUUA and AUDA induce cell cycle arrest, cyclin RNA and protein levels were examined. Cyclin D1 RNA expression was induced 12-24 h after incubation in PDGF medium (Figure 3.19). With the addition of 50 μM Wy 14,643, cyclin D1 levels are significantly decreased at 24 h compared to PDGF treatment alone. Cyclin D1 expression is also repressed at 12 and 24 h by 30 μM AUDA. In contrast, treatment with 30 μM CUUA increased cyclin D1 RNA expression at 12 h compared to PDGF alone.

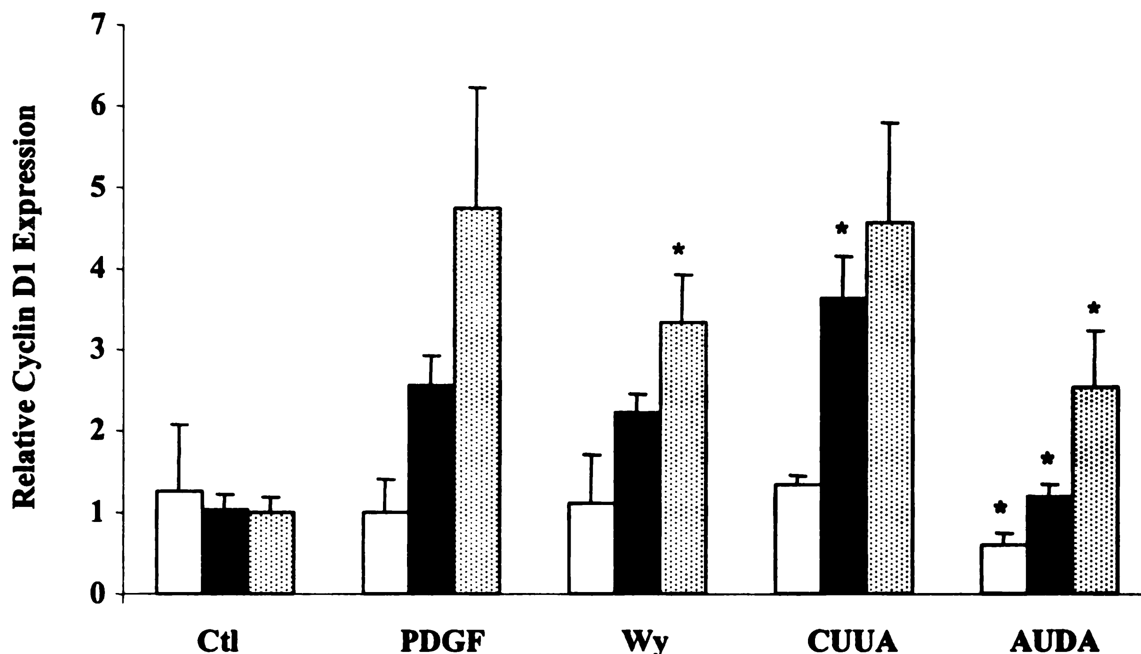


Figure 3.19 Cyclin D1 mRNA expression is attenuated by CUUA and AUDA in aortic SMCs. Human aortic SMCs were growth-arrested for 24 h and incubated in PDGF medium containing 50 μM Wy 14,643, 30 μM CUUA or 30 μM AUDA for 6, 12, and 24 h. Cyclin D1 mRNA expression was quantified by real-time quantitative PCR and normalized to 18S. The values shown are the mean ± SD of a representative experiment performed in triplicate. * Significant difference over PDGF positive control at a given time point (p<0.05).

Increased cyclin D1 protein levels are detected already at 6 h following PDGF treatment, consistent with its early expression in the cell cycle. Addition of Wy 14,643 attenuated PDGF-induced cyclin D1 expression from 6-24 h (Figure 3.20). When compared to PDGF treatment alone, CUUA effectively reduced cyclin D1 levels at 6 and 12 h. Remarkably, cyclin D1 protein expression was ablated by AUDA 6-24 h after PDGF treatment.

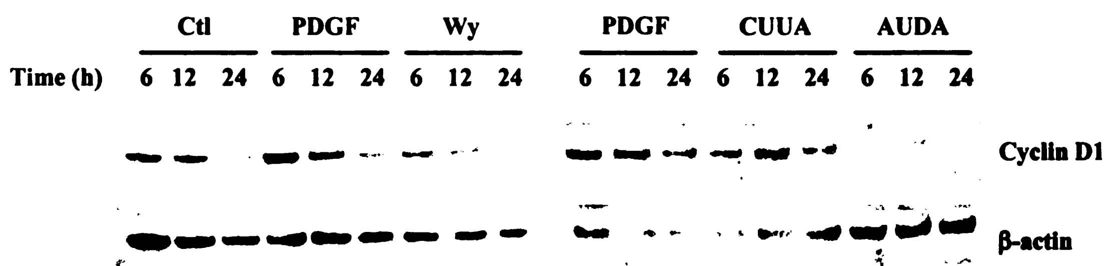


Figure 3.20 Cyclin D1 protein expression is attenuated by CUUA and AUDA in aortic SMCs. Human aortic SMCs were growth-arrested for 24 h and incubated in PDGF medium containing 50 μM Wy 14,643, 30 μM CUUA or 30 μM AUDA for 6, 12, and 24 h. Cyclin D1 protein expression was determined by Western immunoblotting using β-actin as a loading control. The blot is representative of additional experiments.

3.3.8 Repression of cyclin D1 by urea-based alkanoic acids is partially mediated by PPARα.

In order to determine if inhibition of cyclin D1 is attributed to CUUA/AUDA activation of PPARα, PPARα expression was silenced using siRNA in SMCs. SMCs were transfected with a negative control or PPARα-specific siRNA and PPARα RNA and protein expression were determined. Within 24 h of transfection, PPARα mRNA was significantly decreased by 70% with only a minimal additional decrease by 48 h (Figure 3.21A). The knockdown in RNA level was maintained even at 72 h post-transfection. Similar to RNA levels, there was a significant decrease in PPARα protein levels from 24-72 h following PPARα siRNA transfection (Figure 3.21B). Transfection with the negative control siRNA had no effect on PPARα transcript or protein expression.

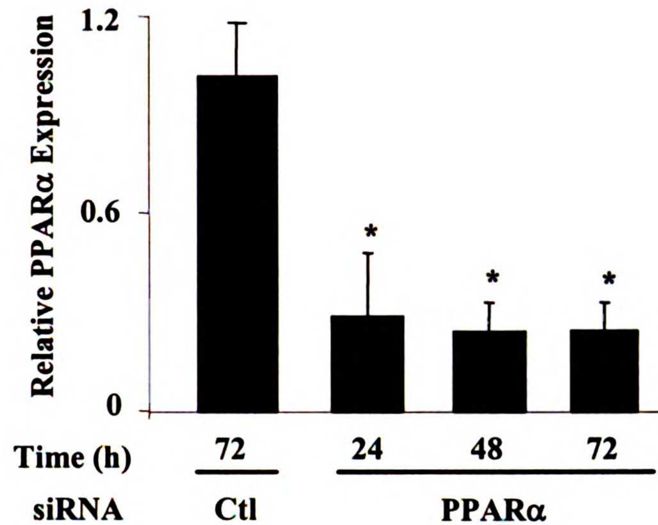
Time (h)	6	12	24	6	12	24	6	12	[illegible]
	CH			PDGF			W[illegible]		

Figure 3.20 Cyclin D1 protein [illegible] **SMCs. Human aortic SMCs** [illegible] **medium containing 50 μM WY1**[illegible] **h. Cyclin D1 protein expression** [illegible] **actin as a loading control. The** [illegible] **by**

3.1.8 Repression of cyclin D1 by [illegible] PPARα

In order to determine if inhibi[illegible] activation of PPARα, PPARα expre[illegible] of PPARα RNA were transfected with a negative contro[illegible] PPARα mRNA and protein expression were determined [illegible] to 48 h was significantly decreased by 70% [illegible] h post- (Figure 3.21A). The knockdown in RNA [illegible] PPARα protein transfection. Similar to RNA levels, [illegible] (Figure 3.21B). Transfection levels from 24-72 h following PPARα siR[illegible] with the negative control siRNA had no [illegible] PPARα transcript or protein expression.

A.



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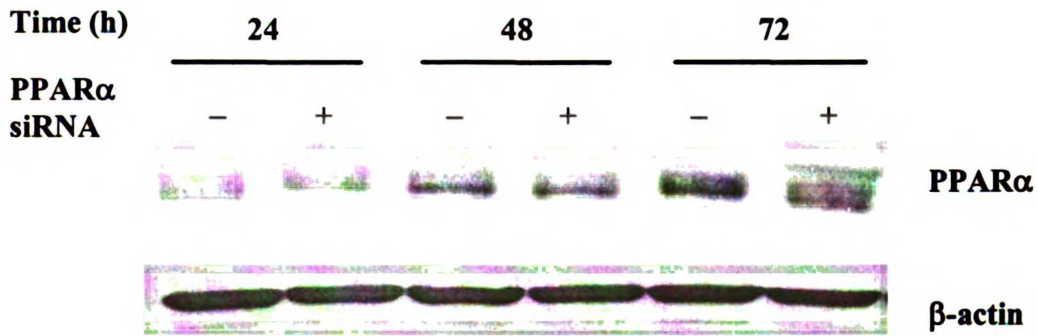

Figure 3.21 Silencing of PPARα by siRNA. Human aortic SMCs were transfected with PPARα-specific siRNA or negative control siRNA using siPORT Amine™ for 24-72 h. A) Total RNA was isolated and PPARα expression was determined by real-time quantitative PCR and normalized to 18S expression. The values shown are the mean ± SD of a representative experiment performed in triplicate. * Significant difference over negative control siRNA-transfected cells ($p<0.05$). B) Cells were lysed and PPARα protein expression was determined using Western immunoblotting. β-actin was used as a loading control.

In SMCs transfected with negative control siRNA, cyclin D1 protein expression is induced with the addition of PDGF medium, and attenuated in the presence of Wy 14,643, CUUA, and AUDA (Figure 3.22). These results indicate that transfection of SMCs with the control siRNA does not interfere with the normal response of cyclin D1 to PPARα activators. Interesting results were obtained when PPARα-specific siRNA was used to transfect SMCs. PDGF failed to induce cyclin D1, whose level was comparable to that in growth-arrested cells. Since Wy 14,643 is a known agonist of PPARα, silencing of PPARα should dampen the attenuation of cyclin D1 expression that was observed in control-transfected cells. Consistent with this reasoning, compared with PDGF treatment alone, addition of Wy 14,643 resulted in increased cyclin D1 expression. Furthermore, the repressive effects of CUUA and AUDA were partially mitigated by knocking down PPARα. In CUUA-treated cells, cyclin D1 expression increased dramatically whereas a slight increase is seen with AUDA treatment. These results show that PPARα plays a role in mediating the inhibitory effects of CUUA and AUDA on cyclin D1 expression.

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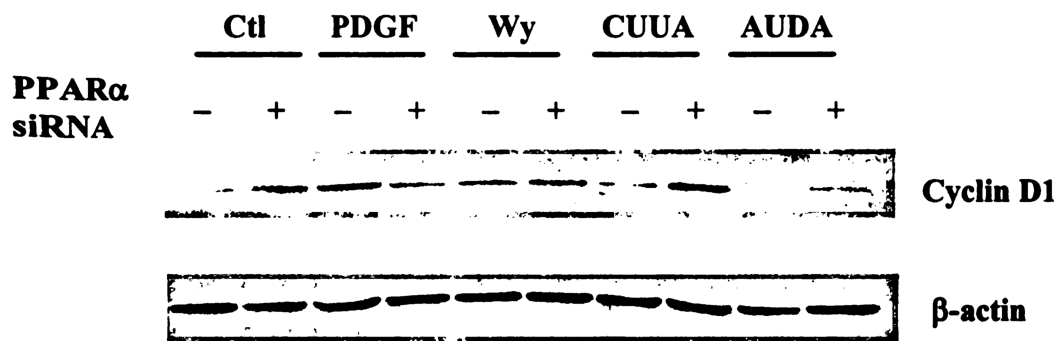

Figure 3.22 Silencing of PPARα by siRNA decreased effects of CUUA and AUDA on cyclin D1 expression. Human aortic SMCs were transfected with PPARα-specific siRNA or negative control siRNA using siPORT Amine™ for 24-72 h. Cyclin D1 expression was detected 6 h after treatment of aortic SMCs in PDGF medium with or without 50 μM Wy 14,643, 30 μM CUUA, or 30 μM AUDA. β-actin was used as a loading control.

3.4 Discussion

The 1,3-distubstituted urea-based alkanoic acids are potent and tight-binding inhibitors of sEH with nanomolar K_i values (Morisseau et al. 1999; Morisseau et al. 2002). The studies described within have demonstrated that urea-based alkanoic acids, particularly CUUA and AUDA, functionally activated PPARα and induced the expression of ACS, CPT1A, and ACOX, PPARα-responsive genes involved in the metabolism of fatty acids. Since the concentration required for PPARα activation is in the micromolar range, the effects of sEH inhibition are distinct from that of PPARα activation. CUUA and AUDA also attenuated PDGF-induced SMC proliferation and repressed cyclin D1 expression, an action that is mediated in part by PPARα. Compounds like CUUA and AUDA may represent a novel structural lead for the synthesis of more potent PPARα ligands for therapeutic use.

In this study, 1-cyclohexyl-3-n-alkyl and 1-adamantyl-3-dodecanoic acid ureas were tested as putative PPARα activators. Functional assays showed that increasing activity was observed with increasing alkyl chain length, with a maximum effect observed with the 11 and 12 carbon analogs CUUA and CUDA. The presence of a hydrophilic group followed by a hydrophobic central group are shared features of most known PPAR agonists and such features are present in these disubstituted ureas. The crystal structure of AZ 242 bound to the ligand-binding domain of human PPARα shows extensive hydrophobic interactions between its central ring systems and the receptor (Cronet et al. 2001). The fact that shorter chain 1,3-disubstituted ureas are less effective as PPARα activators compared with longer chain compounds may be due to decreased hydrophobic interactions that are essential for forming a more stable complex with the

receptor. CUDA and AUDA differ only in the substitution of the cyclohexyl for an adamantyl head group and AUDA is a slightly weaker PPARα activator. The bulkiness of the adamantyl moiety might limit accessibility to the ligand binding site, however this effect is not limiting since AUDA potently transactivates PPARα. Maximum PPARα activation by CUUA and AUDA was apparent at 50-100 μM, after which a decline in activation was observed that prevented the accurate calculation of the EC_{50} for these compounds. The decreased activation could be due to the aggregation of these alkanoic acids at high concentrations (Brian Feng, Valerie Ng, Deanna Kroetz, and Brian Shoichet, unpublished observations) which would decrease their effective availability. Interestingly, a lower concentration of AUDA is required for aggregation compared with CUUA, consistent with the decline in PPARα activation at a lower AUDA concentration. Surprisingly, AUDA is two times more soluble in water than CUDA, which is only one carbon longer than CUUA, therefore the aggregatory properties of these compounds may not be related to their solubility (Morisseau et al. 2002).

Recently, a similar study was published which also reported the activation of PPARα by the substituted ureas CUDA and AUDA (Fang et al. 2005). Reminiscent of the findings presented here, the authors showed that chain-shortened products of CUDA and AUDA were progressively less potent activators of PPARα. In addition, the PPARα agonist GW7647 inhibited sEH activity and reduced DHET production by 30-50%, suggesting that PPARα agonists and sEH inhibitors can be rationally optimized to perform both biological functions.

Fibrates and other peroxisome proliferators are known to induce the expression of numerous genes involved in fatty acid metabolism and transport, including ACS, CPT1A,

and ACOX1, via PPARα-mediated mechanisms (Mascaro et al. 1998; Schoonjans et al. 1995; Varanasi et al. 1996). Acyl-CoA synthetases are critical enzymes facilitating the uptake of fatty acids into the mitochondria for metabolism. They act by catalyzing the conversion of a free fatty acid to an "active" fatty acid by conjugating an acyl-CoA group, thus enabling its uptake into the mitochondria by carnitine palmitoyl transferases. CPT1A is found on the outer mitochondrial membrane, and plays a critical role in the entry of fatty acids into the β-oxidation pathway (Mayes and Botham 2003b). ACOX1 is the first and rate-limiting enzyme of the β -oxidation pathway (Reddy et al. 1986). The novel PPARα activators, CUUA and AUDA, are also inducers of ACS, CPT1A, and ACOX1, indicating that CUUA and AUDA can upregulate PPARα-responsive genes. In general CUUA was slightly more effective than AUDA, which is consistent with its higher activity towards PPARα. The inherent low level of PPARα expression in HepG2 cells made it necessary to transfect them with a PPARα expression plasmid in order to detect changes in the levels of PPARα-responsive genes. In primary hepatocytes, CUUA and AUDA did not have profound effects on the expression of genes involved in maintaining fatty acid homeostasis. Their effects on cytochrome P450 and sEH expression were slightly more apparent, although no changes in protein level were evident. The consequences of CUUA and AUDA on the RNA and protein expression of these genes may be cell-type and species-dependent and warrants further characterization.

aP2 is a well-recognized PPARγ-responsive gene that serves as an adipocyte-specific marker that transports long-chain fatty acids (Mayes and Botham 2003a). During adipogenesis, aP2 expression is upregulated and its expression has been

associated with increased lipolysis and insulin resistance (Boord et al. 2002). The expression of aP2 is regulated by thiazolidinediones and 15-deoxy-$\Delta^{12,14}$-PGJ_2 in adipocytes (Forman et al. 1995; Tontonoz et al. 1994). In these studies, CUUA and AUDA, although potent activators of PPARγ, only had moderate effects on aP2 expression when compared to ciglitazone. Since the latter is a stronger activator of PPARγ compared with CUUA and AUDA, this difference may account for the disparate effects on aP2 induction. Availability of CUUA and AUDA may also be an experimental issue, given that these compounds are quite hydrophobic. Although premature, it is exciting to speculate on the potential use of urea-based alkanoic acids as PPARγ activators that do not have the undesired effects on adipocyte-specific gene expression.

The effect of CUUA and AUDA on adipocyte differentiation was also explored by measuring the accumulation of intracellular lipids using Oil Red O. This method of quantitation may not have been sensitive enough to detect differences between the various treatments. Exposure of adipocytes to ciglitazone, which has well-characterized effects on adipocyte differentiation, only resulted in a two-fold increase in absorbance, thus any subtle changes in adipocyte differentiation will not be apparent. Further experimental optimization or selection of an alternative technique is required to more conclusively demonstrate the effect of CUUA and AUDA on adipocyte differentiation.

In addition to the regulation of fatty acid metabolism, CUUA and AUDA attenuated PDGF-induced SMC proliferation. Another 1,3-disubstituted urea, CDU, was previously shown to inhibit SMC proliferation (Davis et al. 2002). The authors postulated that the inhibition of sEH led to an increase in intracellular EET concentration and that EETs were responsible for inhibiting SMC proliferation. However, sEH

transcript or protein was not detected in the human aortic SMCs used in the current study. In addition, 1,3-disubstituted urea-based sEH inhibitors inhibit the enzyme at nanomolar concentrations, whereas their anti-proliferative effect could only be observed using micromolar concentrations, indicating that the inhibitors could be signaling via a different pathway to decrease SMC proliferation. CDU resulted in an approximate 70% reduction of SMC proliferation at 5 μM (Davis et al. 2002), whereas 5 μM AUDA decreased proliferation by 50%. At the same concentration however, CUUA only moderately inhibited SMC proliferation. The fact that CUUA and AUDA have relatively comparable effects on PPARα activation, yet CUUA is not as potent at inhibiting SMC proliferation as AUDA suggests that the inhibition may not be solely due to PPARα activation. A steep decrease in proliferation was observed between 10 μM and 20 μM AUDA in two different types of SMCs and toxicity could not account for the sudden proliferative decrease. Possibly the phenomenon is associated with AUDA's aforementioned ability to form aggregates. Small molecules may inhibit enzymes non-specifically by the formation of aggregates at micromolar concentrations (Seidler et al. 2003). At 20 μM AUDA, aggregates form readily whereas minimal aggregation was observed with the same concentration of CUUA. It may be possible that AUDA, by forming aggregates, indirectly inhibits cyclin D1 expression. Unfortunately it was not possible to test the aggregation hypothesis since aggregates require disruption using Triton-X, and even at low concentration, Triton-X is detrimental to cell integrity. Alternatively, the steep decrease is reminiscent of cooperative binding in enzyme kinetics.

Inhibition of cyclin D1 RNA and protein was observed when SMCs stimulated with PDGF were incubated with AUDA. AUDA completely abolished cyclin D1 protein

expression, indicating SMCs were growth-arrested in G1. Despite the complete repression of cyclin D1 immunoreactive protein, its transcripts were readily detectable. The discrepancy could be due to the high sensitivity of Taqman real time PCR, or that expression of cyclin D1 is regulated by both transcriptional and non-transcriptional mechanisms. Interestingly, CUUA inhibited cyclin D1 protein expression at 6 h yet had minimal effects on cyclin D1 RNA. These results would suggest that CUUA does not regulate cyclin D1 at the transcriptional level. Non-transcriptional mechanisms for nuclear receptor signaling have been reported. Estrogen receptor alpha (ERα) regulates NO release by physically interacting with the regulatory subunit of the lipid kinase PI3K (Simoncini et al. 2000). ER also mediates estrogen-dependent transport of ribonucleoprotein from the nucleus to the cytoplasm (Govind and Thampan 2001). It is therefore plausible that CUUA and AUDA exert their effects on cyclin D1 via non-transcriptional mechanisms. An alternative explanation would suggest that CUUA and AUDA inhibit SMC proliferation in a PPARα-independent manner, however, by silencing PPARα expression, the role of PPARα was evident in this study.

PPARα RNA and protein expression were significantly attenuated by PPARα siRNA up to 72 h post-transfection. In the absence of normal levels of PPARα, the repression of cyclin D1 by sEH inhibitors was greatly reduced. Interestingly, when PPARα expression is reduced, PDGF failed to induce expression of cyclin D1, indicating that PPARα may be involved as a downstream member of the PDGF-induced signaling cascade.

PPAR ligands have been implicated in the progression of the cell cycle. In breast cancer carcinoma cells, troglitazone and 15-deoxy-$\Delta^{12,14}$-PGJ_2 inhibit cyclin D1

expression (Yin et al. 2001) whereas the inhibition is not observed in PPARγ-deficient cells (Wang et al. 2001). Similarly, in rat intestinal epithelial cells, troligtazone reduced cyclin D1 expression and induced G1 cell cycle arrest (Kitamura et al. 2001). In rat aortic smooth muscle cells, a non-thaizolidinedione partial PPARγ agonist attenuated the induction of G1 cyclins D1, A, and E by PDGF (Bruemmer et al. 2003). Although most studies have been carried out using PPARγ agonists, some evidence also implicates PPARα and PPARδ in the regulation of cell cycle proteins. One report showed that PPARδ in SMCs promotes the proliferation of post-confluent cells by increasing cyclin A and CDK2 expression (Zhang et al. 2002). In human leukemic cells, clofibrate arrested cells in G0/G1 by decreasing cyclin D2 and c-myc expression (Laurora et al. 2003). Some studies however, show that PPAR ligands regulate cell division through PPAR-independent mechanisms. For example, in both PPARγ$^{+/+}$ and PPARγ$^{-/-}$ mouse embryonic stem cells, thiazolidinediones were able to cause G1 cell cycle arrest to the same extent. The mechanism is thought to be mediated by the inhibition of translation initiation (Palakurthi et al. 2001).

Urea-based inhibitors have been widely used to block the conversion of EETs into DHETs and to examine the role of EETs in numerous biological functions. To date, effects associated with sEH inhibition has been attributed to the increased levels of EETs, including the reduction of blood pressure and protection against kidney failure in hypertensive rats (Imig et al. 2002; Yu et al. 2000; Zhao et al. 2004). Results from the present study suggest that some of the effects derived from urea-based sEH inhibitors may also be due to the activation of PPARα. PPARα activation and sEH inhibition/EET overexpression display overlapping biological effects such as their anti-inflammatory

properties in vascular cells (Marx et al. 1999; Node et al. 1999), and protective effects during hypoxic and ischemic conditions (Narravula and Colgan 2001; Seubert et al. 2004; Yang et al. 2001; Yue et al. 2003). Re-examination of PPARα functions in the studies that involved the use of sEH inhibitors or overexpression of cytochrome P450 epoxygenases will provide additional insight into its role in these disease models.

In summary, the present study identified a novel class of PPARα activators, urea-based alkanoic acids, that are also inhibitors of sEH. CUUA and AUDA can induce fatty acid metabolism as well as inhibit SMC proliferation. Since excess smooth muscle accumulation and proliferation is a hallmark of atherosclerosis, the use of these compounds for treatment of atherosclerosis is an intriguing possibility. In addition to possessing qualities of other PPARα agonists, CUUA and AUDA have the added ability to inhibit sEH, thereby increasing vascular protective EET levels. The combined biological effects of these urea-based alkanoic acids may prove to be well-suited for the treatment of vascular diseases such as hypertension and inflammation.

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Chapter 4

Peroxisome Proliferator-Activated Receptor α Activators Potentiate Interleukin 1β-induced Expression of Cyclooxygenase-2

4.1 Introduction

Prostaglandin H synthases-1 and 2 (PGHS-1 and 2), also called cyclooxygenase-1 and 2 (COX-1 and COX-2) are the targets of nonsteroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, and celecoxib. COX-1 and COX-2 are implicated in a variety of diseases such as thrombosis, inflammation, pain, fever, and various cancers (Smith et al., 2000). COX-1 is constitutively expressed in most cells and is generally considered to perform housekeeping functions. Conversely, COX-2 expression is barely detectable in most cells but it can be dramatically induced by various stimuli; this distinction however is not absolute (Funk, 2001).

Arachidonic acid is released from membrane phospholipid pools in response to diverse physiological and pathological stimuli and can be metabolized by COX into prostanoids, including the prostaglandins (PGs) and thromboxanes (Txs). Prostanoids can also be derived from other ω3 and ω6 polyunsaturated fatty acids, although arachidonic acid is the major precursor. The biosynthesis reaction results in the formation of the unstable prostaglandin endoperoxide H_2 (PGH_2) which is then metabolized to the more biologically active end products such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2 by specific isomerases and synthases (Smith et al., 2000). Though hydrophobic, these prostanoids require the prostaglandin transporter to exit the cell (Chan

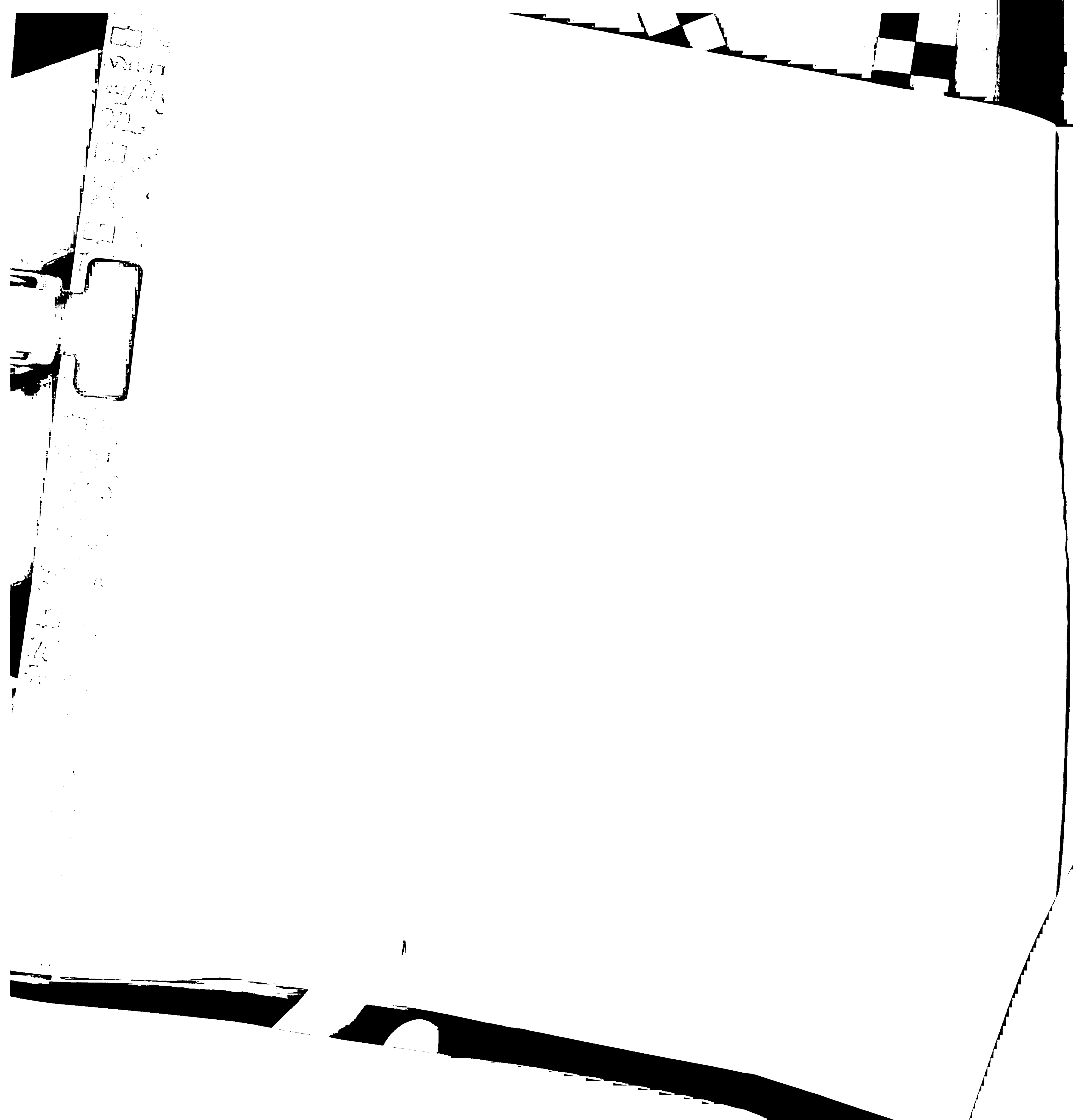
et al., 1998). Due to their short half lives, these prostanoids act as paracrine or autocrine factors (Funk, 2001; Hamberg and Samuelsson, 1971).

Most prostanoids exert their actions by activating membrane receptors on the surface of target cells, thus the biological consequences of prostaglandins and thromboxanes depend on the specific prostaglandin receptors present in different cell types and the enzymes available to further metabolize the parent prostanoids. There have been eight types of membrane receptors associated with prostanoids, all of which are G protein-coupled receptors with seven transmembrane spanning domains. Four of the receptors bind PGE_2, and are termed EP_1-EP_4; FP, IP, and TP bind $PGF_{2\alpha}$, PGI_2, and TxA_2, respectively (Narumiya et al., 1999).

Prostanoids mediate diverse biological actions in a large variety of cell types in the bone, lungs, heart, nervous, immune and vascular systems. In bone resorption, PGE_2 mediates inflammatory osteoclastogenesis and impaired osteoclast formation occurs in cells deficient in the EP_4 or EP_2 receptor (Li et al., 2000; Miyaura et al., 2000). In the immune system, PGE_2 inhibits T and B cell functions such as T cell activation, proliferation and antibody production (Chouaib et al., 1985; Pene et al., 1988; Roper et al., 1994). In animals deficient in the IP receptor, the consequences of acute inflammation such as vasodilation, swelling, and pain are completely absent, indicating that PGI_2 mediates these inflammatory responses (Murata et al., 1997). In the vascular smooth muscle, PGI_2 and PGE_2 have vasodilatory properties (Walch et al., 1999). PGE_2 is also involved in the regulation of systemic blood pressure and counteracts the hypertensive effects caused by a high-salt diet (Kennedy et al., 1999).

Increase in COX-2 expression is a source of rapid upregulation of prostanoid abundance. COX-2 induction is linked to signaling pathways leading to increased transcription and enhanced mRNA stability (Chun and Surh, 2004). Inflammatory cytokines such as TNFα, IL-1β, and LPS can upregulate COX-2 expression by activating the NF-κB, C/EBP, and mitogen-activated protein kinase signaling cascades. These cascades have been shown to contribute to the increase in COX-2 expression either in concert or individually (Smith et al., 2000). Examination of the human COX-2 promoter region shows the presence of a canonical TATA box and various transcriptional regulatory elements including NF-IL6, C/EBP, CRE, SP-1, and two NF-κB binding sites (Yamamoto et al., 1995).

NF-κB is a transcription factor composed of dimers of various combinations of the REL family of polypeptides consisting of RELA/p65, c-REL, RELB, p105, p50, p100, and p52. Normally, NF-κB is sequestered in the cytoplasm by its inhibitor protein IκB, which includes IκBα, IκBβ, IκBγ, IκBε, and BCL3. These inhibitors block the nuclear localization sequences of NF-κB, preventing its entry into the nucleus. In the classic NF-κB activation pathway, the prototypical NF-κB complex consists of the heterodimers p50 and RELA/p65 and is mainly sequestered in the cytoplasm through their association with IκBα, the most studied member of the IκB family (Figure 4.1). Following activation by a stimulus such as IL-1β, IκBα is phosphorylated at serines 32 and 36 by the signalsome, which is a macromolecular complex containing the IκB kinases (IKKs). The phosphorylation of IκBα stimulates its rapid ubiquitination and degradation by the 26S proteosome complex. Simultaneously, release of the NF-κB heterodimer reveals its nuclear localization sequences, thereby promoting its rapid



translocation into the nucleus. Once in the nucleus, NF-κB can bind to its cognate response element (κBRE) and recruit co-activators necessary for target gene transcription (Chen and Greene, 2004). One of the genes that it regulates is its own inhibitor IκBα, resulting in de novo synthesis of the protein. The newly synthesized IκBα enters the nucleus and shuttles NF-κB back to the cytoplasm, turning off NF-κB-responsive gene transcription (Beg et al., 1993; Brown et al., 1993; Sun et al., 1993). Thus NF-κB is involved in an autoregulatory mechanism. Other target genes of the RELA/p65 and p50 heterodimer include inflammatory cytokines such as IL-6, IL-8, and TNFα (Kunsch and Rosen, 1993; Libermann and Baltimore, 1990; Shakhov et al., 1990).

An increasing number of reports have demonstrated cross-talk between the NF-κB and PPAR signaling pathways (Bishop-Bailey, 2000). One study showed that Wy inhibited IL-1-induced IL-6 expression in aortic smooth muscle cells where the physical interaction of PPARα with p65 was proposed to interfere with IL-6 gene transcription (Delerive et al., 1999). PPARα activators also induce the expression of IκBα as a means of limiting the actions of NF-κB (Delerive et al., 2000). Agonists of PPARγ such as troglitazone abolish p65 transactivation activity and PPARγ itself binds directly to p65 and p50 to mediate the decrease in LPS-induced IL-12 production (Chung et al., 2000; Ruan et al., 2003).

Recently, a PPRE has been described in the distal 5'-regulatory region of human COX-2. This region is in part responsible for the regulation of COX-2 expression by fatty acids, prostaglandins, and NSAIDS in colonic and mammary epithelial cell lines (Meade et al., 1999). It has also been shown that LPS-induced COX-2 is regulated by a negative feedback loop mediated via PPARγ in the macrophage-like differentiated

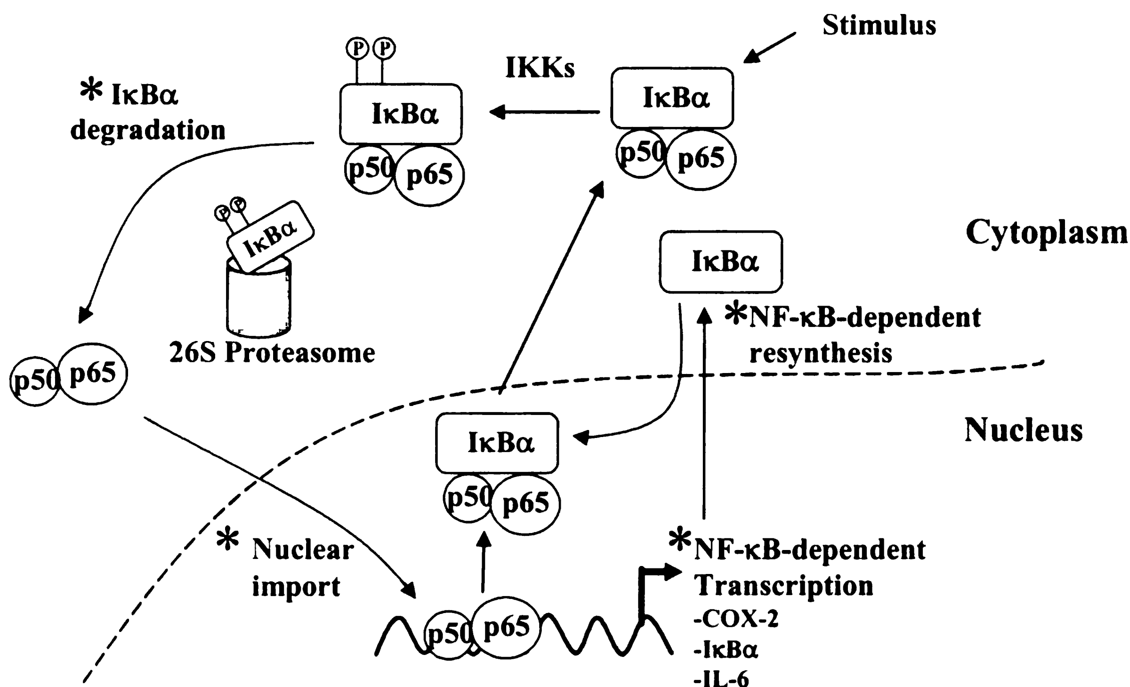


Figure 4.1 Activation of the NF-*κ*B pathway. In this pathway, the p50/p65 heterodimers are mainly sequestered in the cytoplasm via the association with I*κ*B*α*. After stimulation by factors such as IL-1*β*, I*κ*B*α* is phosphorylated by IKKs and degraded by the 26S proteasome. Degradation of I*κ*B*α* releases the NF-*κ*B heterodimer (p50/p65), promoting the rapid translocation of the NF-*κ*B complex into the nucleus and binding to its response element resulting in target gene transcription (adapted from Chen and Greene, 2004). * Components of pathway that are tested in this chapter.

U937 cells, but not in endothelial cells (Inoue et al., 2000). In human airway smooth muscle cells, PPARγ, but not PPARα, activators enhanced IL-1β-induced COX 2 expression in a PPARγ-dependent and NF-κB-independent manner (Pang et al., 2003). In contrast, in human aortic smooth muscle cells, IL-1β-induced COX-2 expression is suppressed by PPARα and not PPARγ activation. The inhibition was a result of cross-talk between the PPARα and NF-κB pathways (Staels et al., 1998). Angiotensin II-induced increase in COX-2 protein was attenuated by both PPARα and PPARγ

activators, although the involvement of NF-κB with PPAR signaling was not explored. In general, the effect of PPAR activators on COX-2 expression seems to depend on the context of COX-2 activation and the cell type studied.

The studies described below tested the hypothesis that previously identified PPARα agonists, 11,12-EET and 14,15-DHET, potentiate the actions of IL-1β on COX-2 expression in human aortic smooth muscle cells in a NF-κB- and PPARα-dependent manner. Examination of specific members in the NF-κB signaling cascade were examined to determine the effects of 11,12-EET and 14,15-DHET (Figure 4.1). In addition, by using small interfering RNA against PPARα, the direct consequence of PPARα activation by CYP eicosanoids on COX-2 expression was investigated.

4.2 Materials and Methods

4.2.1 Materials

COX-2 (N-20), IκBα (C-15), β-Actin (I-19), β-tubulin (H-235), p65 (C-20), and HDAC1 (H-51) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-IκBα (9246) antibody was obtained from Cell Signaling Technology (Beverly, MA). Wy 14,643 and MG-132 were obtained from Biomol (Plymouth Meeting, PA). All eicosanoids were from Cayman Chemical (Ann Arbor, MI). IL-1β was purchased from R&D Systems (Minneapolis, MN). The [γ-^{32}P]-ATP was obtained from Perkin Elmer (Boston, MA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

4.2.2 Cell Culture

HepG2 cells were obtained from American Type Cell Culture and maintained in Modified Eagle's Medium with Earle's Balanced Salt Solution containing 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and penicillin-streptomycin. Human aortic smooth muscle cells were obtained from Cambrex (Walkersville, MD) at passage 3 and maintained in SmGM-2 medium. Cells were cultured at 37°C under 5% CO_2 and all experiments were carried out at passages 4-9.

4.2.3 Western Immunoblotting

Except where indicated, cells were lysed with buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Igepal and supplemented with Complete Protease Inhibitor Cocktail Tablets from Roche Applied Science (Indianapolis, IN). Protein concentration was quantitated using the BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL). Protein electrophoresis was carried out on NuPage™ 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) or Criterion Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto nitrocellulose membranes using a wet transfer method. Except for the phospho-IκBα antibody which was diluted 1:1000, all Western blots were incubated with a 1:200 dilution of primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) for detection of low amounts of antigen.

4.2.4 Nuclear Extract Preparation

Smooth muscle cells were harvested by trypsinization and centrifuged for 2 min at 400 x *g*. Cell pellets were washed once in PBS, and suspended in 50 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% Igepal, 1 mM DTT, 1 mM PMSF) and incubated on ice for 15 min. Cells were then centrifuged at 5000 x *g* for 30 s and the supernatant was extracted as the cytosolic fraction. The nuclear pellet was washed once with buffer A, then resuspended in 20 μl buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) and shaken on ice for 5 min. The suspension was then centrifuged for 1 min at 5000 x *g*, and the supernatant collected as the nuclear fraction.

4.2.5 Real-Time Quantitative PCR

Total RNA was isolated from smooth muscle cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcription was carried out using M-MLV reverse transcriptase (Promega, Madison, WI). The 18S primers and probe set was designed using Primer Express from Applied Biosystems (Foster City, CA). All other primers and probes were Assays-on-Demand purchased from Applied Biosystems. Reactions were run on an ABI Prism 7700 and cycling conditions were: 95°C for 10 min, 95°C for 15 s followed by 60°C for 1 min for 45 cycles. Relative expression of specific transcripts was calculated by the following formula: Relative expression = $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (\Delta Ct_{target} - \Delta Ct_{18S})_{treated} - (\Delta Ct_{target} - \Delta Ct_{18S})_{control}$

4.2.6 Gel Shift Assays

Nuclear extract (1 μg) was combined with binding buffer consisting of 20 mM HEPES (pH 7.9), 5% Glycerol, 0.1 M KCl, 0.2 mM EDTA (pH 8.0), 0.2 mM EGTA (pH 8.0), 1 mM DTT, and poly (dI-dC) (1 μg) in a total volume of 20 μl and incubated on ice for 10 min. [γ-^{32}P]-labeled κBRE or PPRE (250 000 cpm) were then added and incubated at room temperature for 20 minutes. The consensus sequence for the κBRE oligonucleotide is 5′-AGT TGA GGG GAC TTT CCC AGG C-3′. The sequence for the consensus PPRE oligonucleotide is 5′-CAA AAC TAG GTC AAA GGT CA-3′. The reaction was run on a 5% pre-run polyacrylamide gel for 2 h at 200 V in 4°C. The gel was dried and the image visualized using a phosphorimager and ImageQuant software from Amersham Biosciences (Piscataway, NJ).

4.2.7 EIA and ELISA Assays

Prostaglandin EIA kits were purchased from Cayman Chemical (Ann Arbor, MI) and the hIL-6 ELISA kit was obtained from R&D Systems (Minneapolis, MN). At the end of the experimental period, cell supernatants were harvested and stored at -80°C until time of assay. Dilutions were carried out using SmBm basal medium when required. The sensitivity for PGE_2 is about 15 pg/ml and there is negligible cross-reactivity with most prostanoids except PGE_2 ethanoloamide (100%), PGE_3 (43%), 8-*iso*-PGE_2 (37.4%), and PGE_1 (18.7%). The sensitivity for the 6-keto$PGF_{1\alpha}$ EIA kit is about 15 pg/ml and it cross reacts with 6-keto PGE_1 (151%), and $PGF_{1\alpha}$ (11%); all other cross-specificities were under 10%. The minimum detectable dose of IL-6 was 0.70 pg/ml with no cross-reactivity or interference with other factors tested. Determination of prostaglandin and IL-6 levels was carried out as indicated by the manufacturer. Briefly, prostagladin levels

were assayed by adding cell supernatant, $PGE_2/PGF_{1\alpha}$ AchE tracer, and specific monoclonal antibody to each well of the EIA plate and incubated for at least 18 h at 4°C. The plate was washed extensively, developed with reconstituted Ellman's Reagent, and incubated in the dark at room temperature for 60-90 min. Absorbance at 410 nm was measured to quantitate the optical density of each well. IL-6 levels were determined by incubating cell supernatant and provided Assay Diluent for 2 h at room temperature. Wells were washed and IL-6 conjugate was added and incubated for another 2 h. After extensive washing, the Substrate Solution was added to each well and incubated for 20 min. The Stop Solution was then added and the plate was read at 450 nm and 550 nm. Subtraction of the values obtained from the latter wavelength from the former wavelength corrects for optical imperfections in the plate.

4.2.8 Transactivation Assays

HepG2 cells were plated in antibiotic-free medium in 24-well plates at 8×10^4 cells per well and transfected the next day with 150 ng of either $PPRE_3$-tk-LUC (a gift from Dr. Ronald Evans, Salk Institute, La Jolla, CA) or κBRE_5-tk-LUC (a kind gift from Dr. Warner Greene, Gladstone Institute of Virology and Immunology, San Francisco, CA) and 35 ng of pCMV-βgal as an internal control using Lipofectamine PLUS reagents from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were pretreated for 90 min with 50 μM Wy, or 10 μM eicosanoids in serum-free medium, IL-1β (1 ng/ml) was then added and the cells were incubated for another 24 h (total DMSO<0.1%). Cells were lysed using Glo Lysis Buffer and assayed using Bright-Glo™ Luciferase Assay system from Promega (Madison, WI).

Luciferase activity was normalized to β-galactosidase values. The fold activation was calculated relative to DMSO control.

4.2.9 siRNA Transfection

Smooth muscle cells were grown to 50-70% confluence and transfected with chemically synthesized PPARα (ID# 5439) or negative control siRNA #1 using siPORT™ *Amine* (all from Ambion, Austin, TX) according to the manufacturer's instructions. Fresh culture medium was added 4 h after transfection to minimize cytotoxicity. Twenty-four hours after transfection, cells were treated as indicated and lysed for Western immunoblotting.

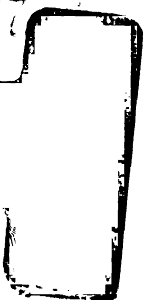
4.2.10 Statistics

Statistical significance of differences between values was evaluated by an unpaired Student's t test. Significance was set at a p value of <0.05.

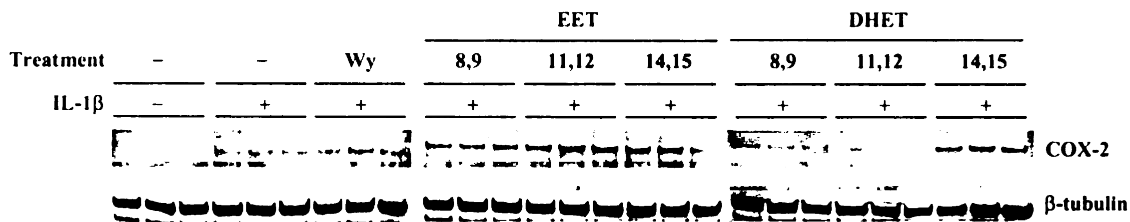
4.3 Results

4.3.1 IL-1β-induced COX-2 expression is potentiated by CYP eicosanoids

To determine the effect of CYP eicosanoids on COX-2 expression in the presence of IL-1β, primary human aortic smooth muscle cells (SMCs) were pre-incubated with 10 μM of 8,9-, 11,12-, or 14,15-EET or the corresponding DHETs before the addition of 1 ng/ml IL-1β in serum-free medium (Figure 4.2A). COX-2 expression was determined by Western immunoblotting 24 h later. As expected, COX-2 expression was clearly induced in the presence of IL-1β compared with solvent-treated cells. Co-incubation of IL-1β



with Wy or CYP eicosanoids further potentiated COX-2 expression. EETs, in particular 11,12-EET, generally elicited a stronger response than DHETs, except for 14,15-DHET which strongly potentiated COX-2 expression. 8,9-DHET and 11,12-DHET, unlike 14,15-DHET, did not further upregulate the level of COX-2. In cells treated only with Wy or eicosanoids, COX-2 expression was undetectable (Figure 4.2B).



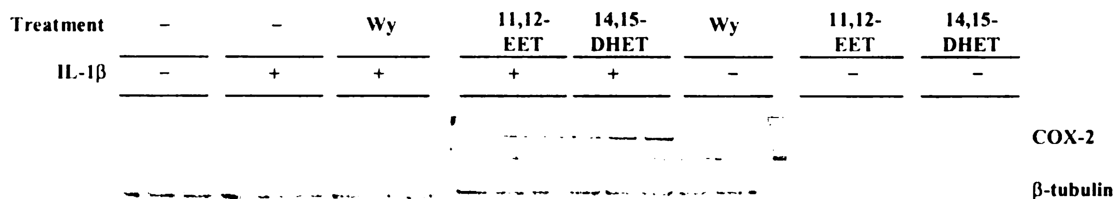


Figure 4.2 Wy and CYP eicosanoids potentiate IL-1β-induced COX-2 protein expression. SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids. After 90 min, 1 ng/ml IL-1β was added and incubated for 24 h. Cells lysates were resolved on a 10% Tris-HCl gel and transferred onto a nitrocellulose membrane. Western immunoblotting was carried out as indicated in *Material and Methods*. β-tubulin was used as a loading control. The blots presented are representative results obtained in three independent experiments.

COX-2 expression was also potentiated in a time-dependent manner (Figure 4.3). With IL-1β treatment alone, COX-2 expression peaked at 4 h and slightly decreased at 8 h and 14 h, although the decrease was not back to baseline level. Similarly, treatment with IL-1β and Wy, 11,12-EET, or 14,15-DHET also resulted in maximal COX-2 expression at 4 h post-IL-1β treatment. It is worthy to note here that the pattern of COX-2 expression, with a 4 h peak followed by a decline at 8 h and plateau at 14 h, is reminiscent of the cyclical pattern observed with other NF-κB responsive genes (Connelly et al., 2001; Towne et al., 2004). As mentioned in the introduction, NF-κB activation results in the upregulation of its inhibitor IκB, thereby resulting in the cyclical expression of its target genes.

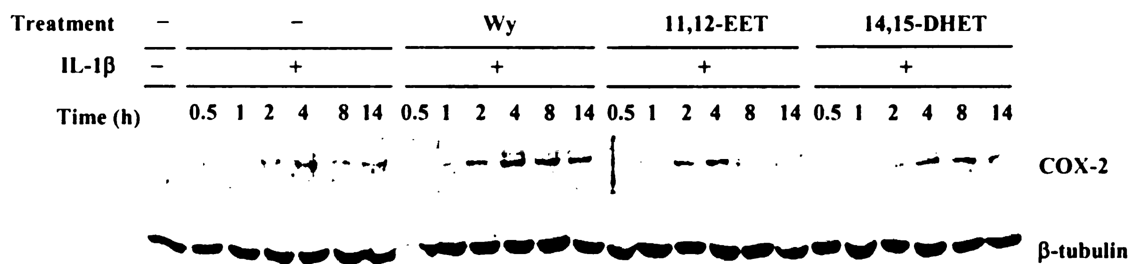
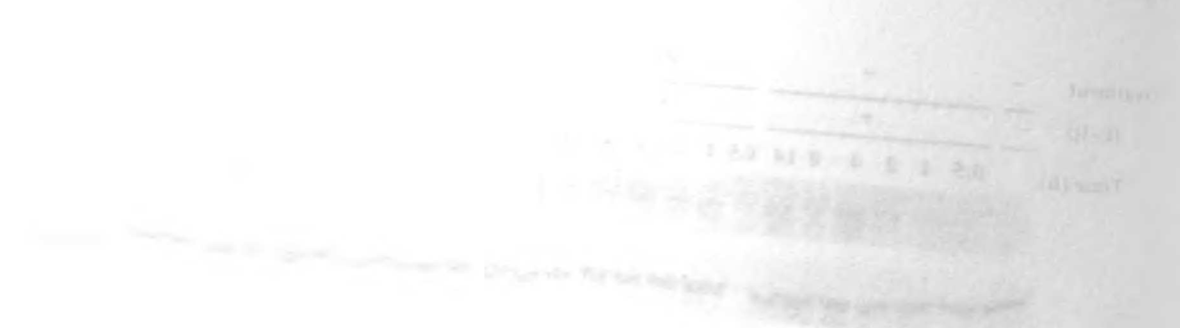


Figure 4.3 Wy and CYP eicosanoids potentiate IL-1β-induced COX-2 protein in a time-dependent manner. SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids. After 90 min, 1 ng/ml IL-1β was added and incubated for the indicated times. Cells were harvested and lysates were resolved on a 10% Tris-HCl gel and transferred onto a nitrocellulose membrane. Western immunoblotting was carried out as indicated in *Materials and Methods*. β-tubulin was used as a loading control. The blots presented are representative of additional experiments.



In agreement with the protein results, RNA expression of COX-2 was also potentiated by Wy and CYP eicosanoids in a time-dependent manner (Figure 4.4). Two hours after IL-1β treatment, COX-2 transcript increased about 30-fold over solvent-treated control, and at 6 h, transcript levels were 85-fold greater than control. With the addition of 50 μM Wy, synergistic effects on COX-2 over IL-1β alone are detected at 6 h with a greater than 100-fold increase observed; at 2 h, no significant difference was found. 11,12-EET (10 μM) was able to potentiate COX-2 levels as early as 2 h after IL-1β treatment, with 110-fold increase over baseline COX-2 expression, however no

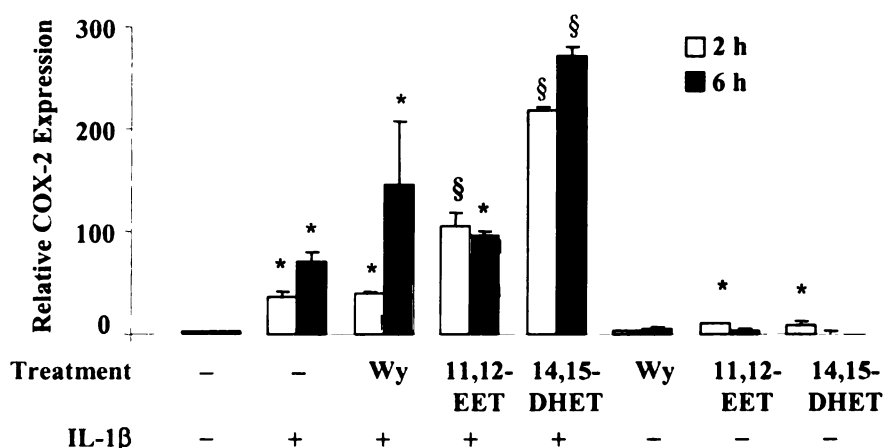


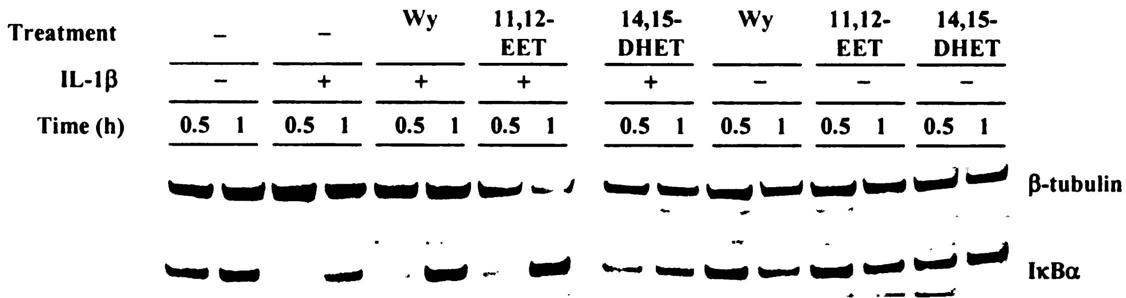
Figure 4.4 Wy and CYP eicosanoids potentiate IL-1β-induced COX-2 RNA expression in a time-dependent manner. SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids. After 90 min, 1 ng/ml IL-1β was added and incubated for the indicated times. Total RNA was isolated with the TRIzol reagent. COX-2 mRNA expression was determined by real-time quantitative PCR and normalized to 18S. * Difference over control and § difference over IL-1*β* treatment alone at the same time point ($p<0.05$). Each experiment was carried out in triplicate and repeated at least three times with similar results. Data are presented as mean ± SD. Results are representative of three separate experiments.

further changes were detected at 6 h. Strikingly, COX-2 transcript levels were greatly potentiated by 10 μM 14,15-DHET, with increases of 230- and almost 300-fold at 2 h and 6 h, respectively. In the absence of IL-1β, Wy, 11,12-EET and 14,15- DHET had minimal effects on COX-2 expression, although a significant increase of about 5-fold was observed with 11,12-EET and 14,15-DHET.

4.3.2 Wy, 11,12-EET, and 14,15-DHET affect IκBα and phospho-IκBα expression

To further understand the mechanism responsible for the synergistic effects of IL-1β and Wy/CYP eicosanoids, components of the NF-κB signaling pathway were examined. The expression of IκBα and its phosphorylated form were analyzed since the degradation of IκBα marks an early event in NF-κB signaling (Figure 4.1). Treatment with IL-1β alone resulted in IκBα degradation within 30 min and immediate upregulation within 1 h (Figure 4.5A). This type of rapid response is expected and typical of IL-1β-induced NF-κB signaling (Arenzana-Seisdedos et al., 1995). In the presence of preincubated Wy and 11,12-EET, the same pattern of rapid degradation and reconstitution similar to IL-1β alone was evident. Pretreatment with 14,15-DHET also brought about rapid IκBα degradation as seen with the other compounds, however its recovery at 1 h did not reach baseline levels. Slower recovery of IκBα expression implies increased translocation of NF-κB. In the absence of IL-1β, Wy and 11,12-EET appeared to slightly decrease IκBα expression, whereas 14,15-DHET had no effect. No further changes were manifested at later times (Figure 4.5B).

A.



B.

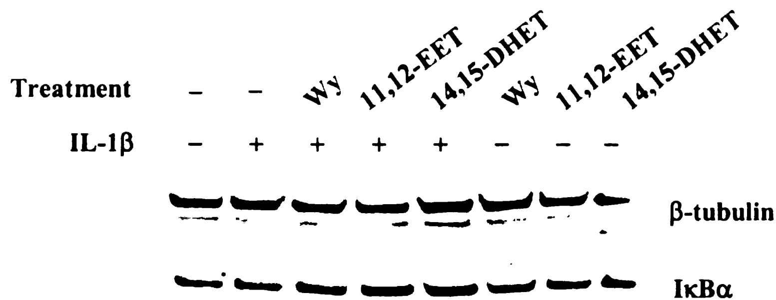


Figure 4.5 Wy, 11,12-EET, and 14,15-DHET affect IκBα expression. SMCs were pre-treated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids. After 90 min, 1 ng/ml IL-1β was added and incubated for A) indicated times or B) 2h. Whole cell lysates were resolved on 10% Bis-Tris gels followed by Western immunoblotting. β-tubulin was used as a loading control. The blots presented are representative of results obtained in three separate experiments.

The expression of the phosphorylated species of IκBα was also examined (Figure 4.6). As expected, rapid and considerable accumulation of phospho-IκBα occurred within 5 min of IL-1β treatment. Pre-incubation with Wy, 11,12-EET, and 14,15-DHET did not cause significant changes in phospho-IκBα expression up to 10 min after IL-1β treatment. At 20 min, phospho-IκBα levels returned to baseline in SMCs treated only

with IL-1β. In contrast, pre-treatment with Wy or CYP eicosanoids resulted in the sustained generation of phospho-IκBα, with the effects of the CYP eicosanoids being more potent than that of Wy. Interestingly, Wy and 11,12-EET alone caused increased phospho-IκBα expression in the absence of IL-1β, consistent with the published effect of Wy on IκBα (Delerive et al., 2000).

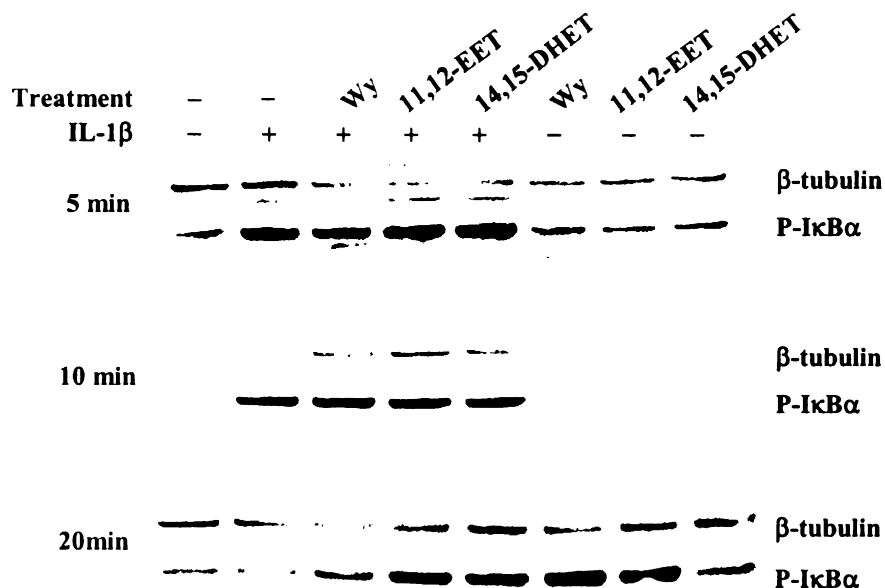


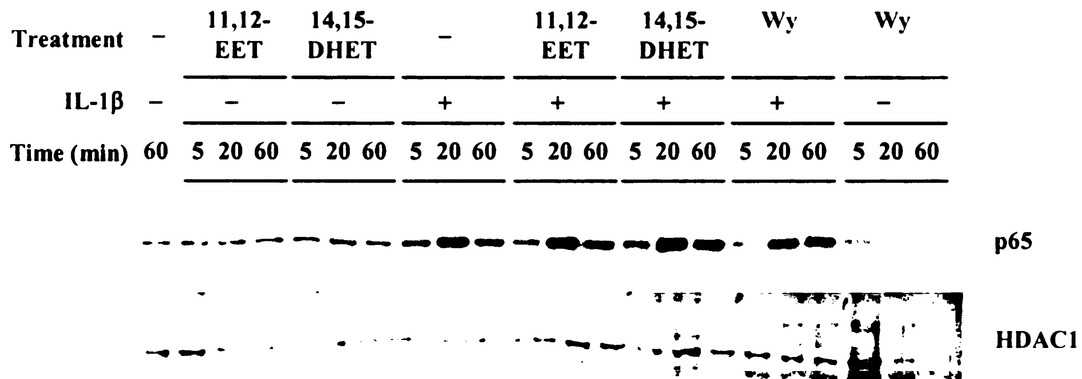
Figure 4.6 Wy, 11,12-EET, and 14,15-DHET affect phospho- IκBα expression. Wy, 11,12-EET, and 14,15-DHET affect IκBα and phospho-IκBα expression. SMCs were pre-treated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, and 30 μM MG-132 for 30 min to inhibit proteasomal degradation of phospho-IκBα (P-IκBα). After 90 min, 1 ng/ml IL-1β was added and incubated for the indicated times. Whole cell lysates were resolved on 10% Bis-Tris gels followed by Western immunoblotting. β-tubulin was used as a loading control. The blots presented are representative of results obtained in three separate experiments.

4.3.3 Wy, 11,12-EET, and 14,15-DHET increase p65 translocation and NF-κB binding

The expression of translocated p65, a subunit of NF-κB, was interrogated as an indication of NF-κB accumulation in the nucleus (Figure 4.7A). As in the previous experiments, SMCs were pre-incubated with Wy, 11,12-EET, or 14,15-DHET before addition of IL-1β, after which cells were disrupted and nuclear extracts obtained at various time points. IL-1β caused the expected translocation of p65 into the nucleus within 5 min of treatment; p65 levels peaked at 20 min and then decreased at 60 min, although not to baseline levels. Pre-incubation with Wy resulted in enhanced and prolonged accumulation of p65, with higher p65 expression detected at 60 min compared with IL-1β treatment alone. At all time points examined, SMCs that were pre-treated with 11,12-EET and 14,15-DHET showed a marked increase of p65 over cells incubated only with IL-1β. Basal levels of p65 in non-IL-1β-stimulated SMCs were readily detectable, although no changes were evident in the presence of Wy or eicosanoids. Since histone deacetylases (HDACs) are expressed solely in the nucleus, HDAC1 expression was probed as a loading control and as a confirmation of nuclear lysate quality. In other experiments, β-tubulin was used as a marker of cytosolic contamination in the nuclear extract preparation and its expression was not detected (Figure 4.7B).

In addition, we examined the ability of nuclear NF-κB to bind to its cognate response element in gel shift assays (Figure 4.8). In the absence of IL-1β, no NF-κB/DNA complex formation was evident when cells were treated with Wy, 11,12-EET, and 14,15-DHET. Twenty min post-IL-1β incubation, the presence of nuclear factors resulted in the gel retardation of κBRE, which diminished at 60 min. This observation is

A.



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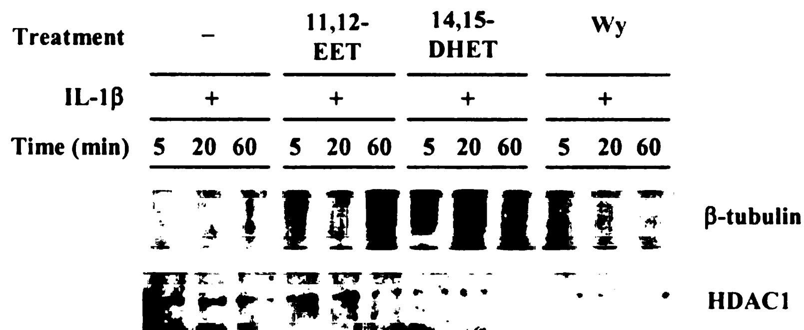


Figure 4.7 Wy, 11,12-EET, and 14,15-DHET increase p65 translocation. SMCs were pre-treated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, then 1 ng/ml IL-1β was added. Nuclear lysates were extracted as described in *Materials and Methods*. Time points were taken after IL-1β addition. A) p65 expression and B) β-tubulin expression were detected by Western immunoblotting. HDAC1 was used as a loading control. The blots are representative of results obtained from three independent experiments.

consistent with the increased nuclear accumulation of p65 at 20 min and its decrease at 60 min (Figure 4.7A). Addition of Wy prior to IL-1β treatment clearly resulted in increased complex formation at all time points, consistent with an increased duration of

binding. 11,12-EET and 14,15-DHET pre-treatment both increased NF-κB binding to its response element with more intense complex formation evident at 5 min and 20 min after IL-1β addition; increased DNA binding however was not observed 60 min following IL-1β treatment.

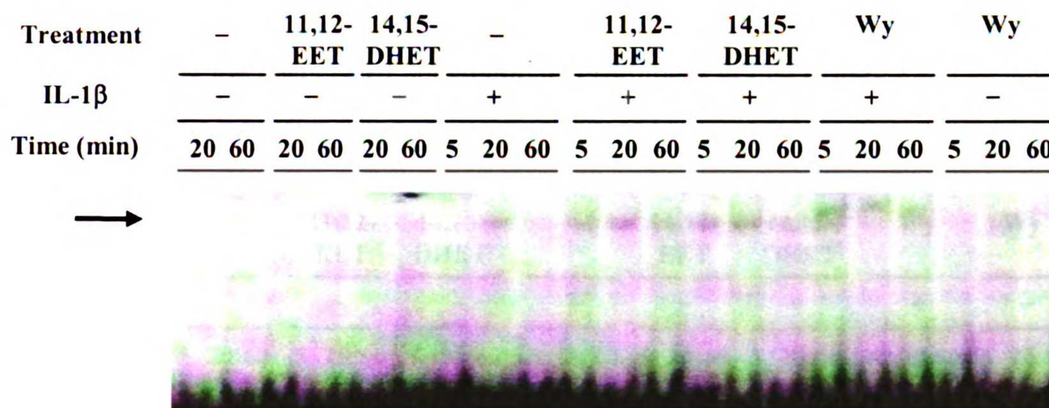


Figure 4.8 Wy, 11,12-EET, and 14,15-DHET increase NF-κB binding to κBRE. SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, then 1 ng/ml IL-1β was added. Nuclear lysates were extracted as described in *Materials and Methods.* Time points were taken after IL-1β addition. Gel shifts were performed by incubating nuclear extracts with ^{32}P-labeled κBRE and resolved through a 5% non-denaturing polyacrylamide gel followed by visualization. The arrow indicates the presence of the NF-κB/κBRE complex. The blot is representative of results obtained from three independent experiments.

Since Wy, 11,12-EET and 14,15-DHET are PPARα agonists, it was next determined whether these same nuclear lysates would also result in PPAR/RXR heterodimer formation with its response element, the PPRE (Figure 4.9). In gel shift assays, no heterodimer formation was apparent using nuclear lysates from any of the SMC treatments. These data conflict with results presented in Chapter 2 where Wy,

11,12-EET, and 14,15-DHET were shown to induce PPARα/RXRα heterodimer formation. It is important to note however, that in the current studies, gel shift assays were conducted using nuclear extracts whereas those described in Chapter 2 were performed with in vitro-translated PPARα and RXRα. From protein and RNA analysis the expression of PPARα in SMCs was difficult to detect (Chapter 3), and its scarcity may account for the absence of detectable heterodimers in this instance.

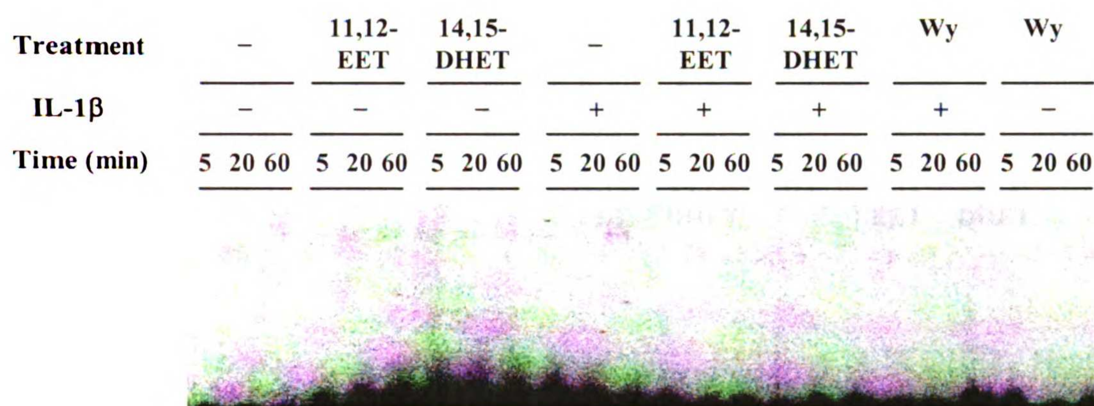


Figure 4.9 Wy, 11,12-EET, and 14,15-DHET do not induce PPAR/RXR binding to PPRE. SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, then 1 ng/ml IL-1β was added. Nuclear lysates were extracted as described in *Materials and Methods*. Time points were taken after IL-1β addition. Gel shifts were performed by incubating nuclear extracts with ^{32}P-labeled PPRE and resolved through a 5% non-denaturing polyacrylamide gel followed by visualization. The blot is representative of results obtained from three independent experiments.

4.3.4 Transactivation of κBRE and PPRE by Wy, 11,12-EET, and 14,15-DHET

To determine the functional consequences of increased p65 translocation and binding to the κBRE, transactivation assays were performed in HepG2 cells using a

luciferase reporter assay. NF-κB promoter activity increased 100% after IL-1β treatment (Figure 4.10). Pre-incubation with Wy, 11,12-EET, or 14,15-DHET did not result in further changes in NF-κB activity. In the absence of IL-1β, treatment with Wy or eicosanoids showed minimal activity towards NF-κB.

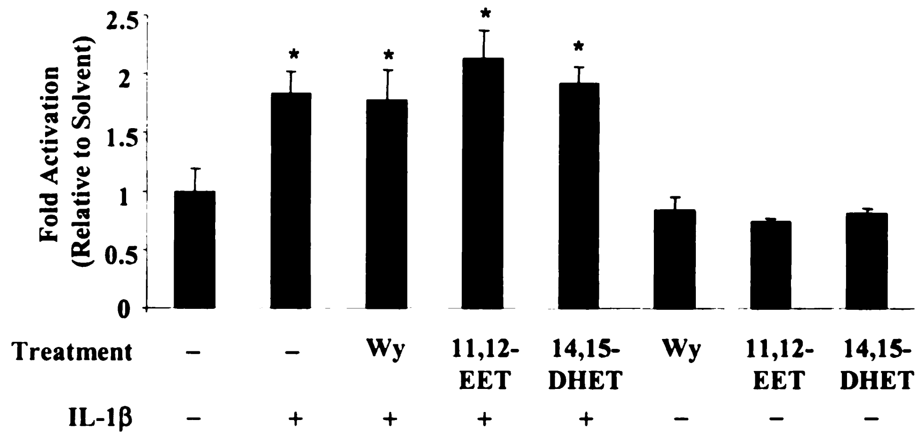


Figure 4.10 Transactivation of κBRE by Wy, 11,12-EET, and 14,15-DHET. HepG2 cells were transfected with the κBRE_5-tk-LUC reporter plasmid. After 24 h, transfected SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, then 1 ng/ml IL-1β was added. Cell lysates were assayed for luciferase and β-galactosidase activity 24 h later. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over solvent control. Results are typical of three separate experiments. Each experiment was performed at least in triplicate and repeated three separate times. Data are presented as mean ± SD. * Significant difference from the solvent control ($p<0.05$).

PPAR promoter activity was also measured with luciferase assays (Figure 4.11). IL-1β resulted in a 30% decrease in PPAR activation which was abolished by pretreatment with Wy and 14,15-DHET. In contrast, 11,12-EET had similar effects as IL-1β alone. PPAR promoter activity was upregulated after treatment with Wy, 11,12-

EET, or 14,15-DHET in the absence of IL-1β since these compounds are PPARα ligands. The potency of Wy and the eicosanoids were however moderate when compared with the reported values in Chapter 2. The most likely explanation is the low level of endogenous PPARα in HepG2 cells. Moreover, the transactivation assays in Chapter 2 were performed using the Gal4-PPAR/UAS luciferase system, which provided a higher sensitivity for the detection of PPAR activity.

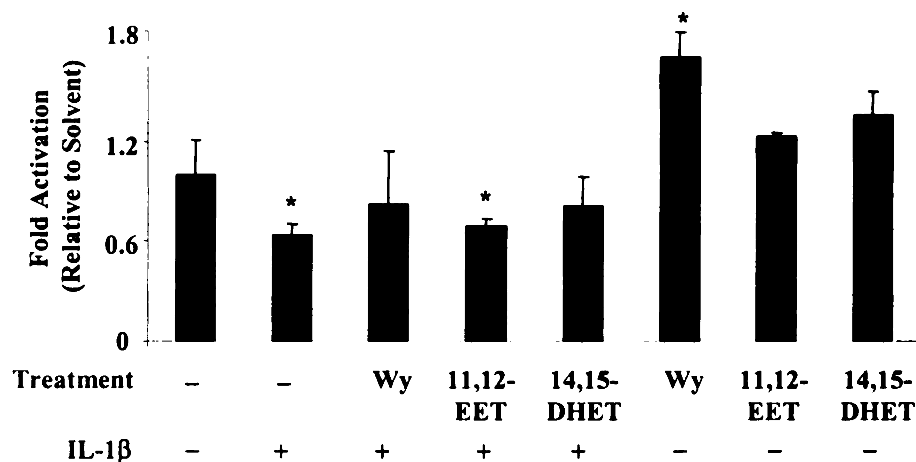
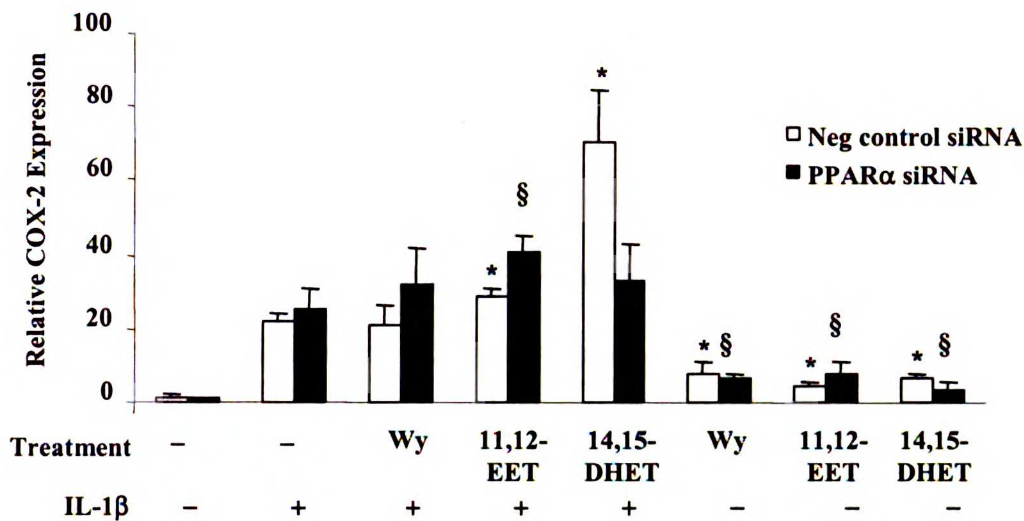


Figure 4.11 Transactivation of PPRE by Wy, 11,12-EET, and 14,15-DHET. HepG2 cells were transfected with the $PPRE_3$-tk-LUC reporter plasmid. After 24 h, transfected SMCs were pretreated for 90 min in SmBm basal medium containing 50 μM Wy or 10 μM eicosanoids, then 1 ng/ml IL-1β was added. Cell lysates were assayed for luciferase and β-galactosidase activity 24 h later. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over solvent control. Results are typical of three separate experiments. Each experiment was performed at least in triplicate and repeated three separate times. Data are presented as mean ± SD. * Significant difference from the solvent control (p<0.05).

4.3.5 PPARα expression is required for IL-1β-induced COX-2 potentiation by Wy and 14,15-DHET

In order to unambiguously determine the role of PPARα in COX-2 potentiation, PPARα expression in SMCs was silenced by siRNA. As reported in Chapter 3, PPARα RNA was reduced to approximately 30% of control values and its protein was moderately reduced by siRNA silencing. In negative control siRNA-transfected SMCs, COX-2 induction and potentiation was consistent with our earlier results. IL-1β resulted in a 22-fold upregulation of COX-2 transcript, and pre-treatment with 11,12-EET and 14,15-DHET caused a further increase to 30- and 70-fold, respectively, over solvent treated cells (Figure 4.12A). Curiously, Wy pre-treatment did not cause the expected potentiation of COX-2 expression, presumably an effect of transfection with the control siRNA. When SMCs were transfected with PPARα siRNA and treated with IL-1β or pre-treated with Wy, the extent of COX-2 induction was indistinguishable from COX-2 levels in control-siRNA transfected cells. Decreasing PPARα dampened but did not eliminate the synergy between 11,12-EET and IL-1β, where COX-2 was increased 1.5-fold over IL-1β treatment alone whereas previously a 3-fold increase was observed (Figure 4.4). PPARα silencing eliminated COX-2 potentiation by 14,15-DHET, with COX-2 expression maintained at the level of IL-1β alone. Interestingly, treatment with Wy, 11,12-EET, and 14,15-DHET without IL-1β also resulted in significant induction of COX-2 expression over solvent control, although those effects were minimal compared with IL-1β-treated cells and consistent with our results presented earlier (Figure 4.4).

A.



B.

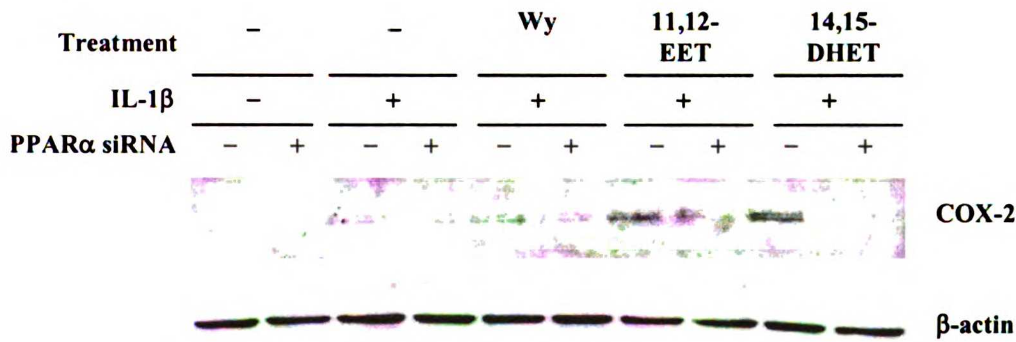


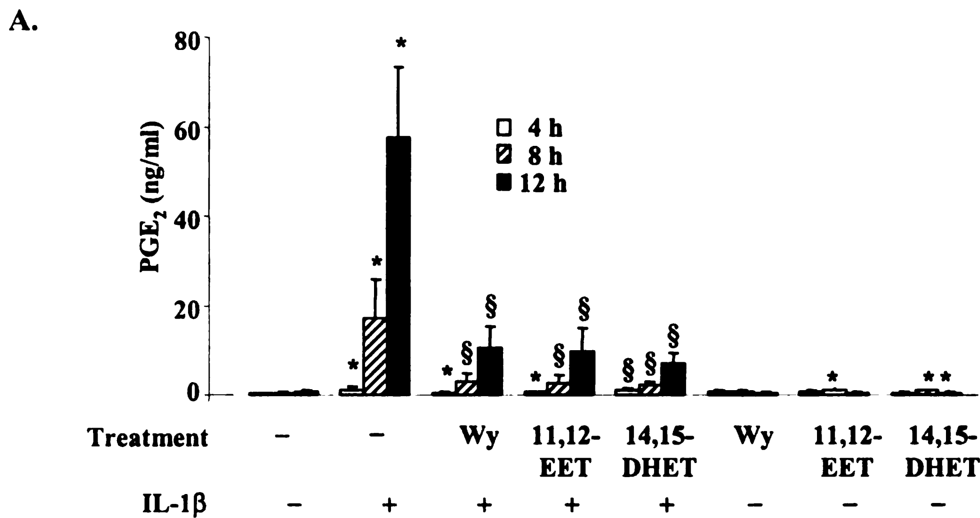
Figure 4.12 PPARα expression is required for IL-1β-induced COX-2 potentiation by Wy, 11,12-EET, and 14,15-DHET. SMCs were transfected with either a negative control or PPARα-specific siRNA. Twenty-four hours after transfection, cells were pre-incubated with Wy or eicosanoids for 90 min and then dosed with 1 ng/ml IL-1β. Cells were harvested after A) 6 h and B) 24 h for the determination RNA and protein expression, respectively. RNA data are presented as mean ± SD and each experiment was performed in triplicate. Whole cell lysates were resolved on 10% Tris-HCl gels followed by Western immunoblotting. β-actin was used as a loading control.
* Significance over negative control siRNA-transfected, IL-1β-treated expression and § significance over PPARα siRNA-transfected, IL-1β-treated expression. Results are representative of three separate experiments.

Analysis of COX-2 protein expression was generally consistent with the RNA data (Figure 4.12B). Identical to results obtained in untransfected SMCs (Figure 4.2A), transfection with control siRNA still resulted in COX-2 induction by IL-1β which was further potentiated by Wy, 11,12-EET, and 14,15-DHET. The observed potentiation was greatly diminished by knocking down PPARα. In PPARα-siRNA transfected cells, expression of the COX-2 immunoreactive protein was comparable to the level in cells treated solely with IL-1β, and no synergistic effects of CYP eicosanoids were observed. These data indicate that CYP eicosanoid-mediated COX-2 potentiation requires the actions of PPARα.

4.3.6 Wy, 11,12-EET, and 14,15-DHET have opposite effects on IL-1β-induced PGE_2 and IL-6 levels

To determine the biological consequences of IL-1β induced COX-2 potentiation caused by Wy, 11,12-EET, and 14,15-DHET, secreted PGE_2, $PGF_{1\alpha}$, and IL-6 levels were determined using enzyme-linked immunoassays (Figures 4.13-4.15). PGE_2 and $PGF_{1\alpha}$ were chosen as they are commonly used as indices of COX-2 activity. IL-1β caused the expected increase in PGE_2 production in a time-dependent manner, however pre-incubation with Wy, 11,12-EET, or 14,15-DHET resulted in the unexpected attenuation of secreted PGE_2 levels (Figure 4.13A). COX-2 protein expression was confirmed in these samples by Western blotting and results were identical to those in Figure 4.2 where Wy and CYP eicosanoids potentiated COX-2 expression relative to IL-1β-treated cells (Figure 4.13B). Wy, 11,12-EET, and 14,15-DHET alone had minimal effects on PGE_2 production.

A.



B.

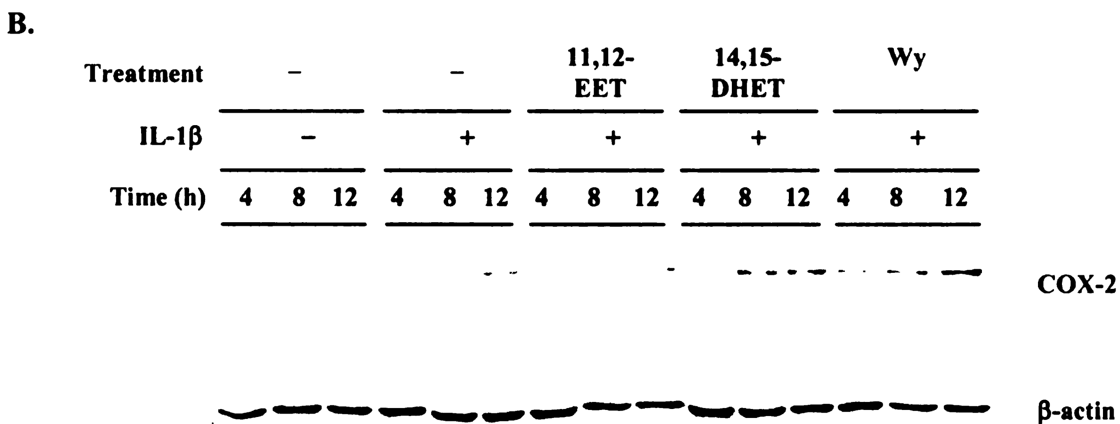


Figure 4.13 Wy, 11,12-EET, and 14,15-DHET decrease IL-1β-induced PGE_2 secretion. SMCs were preincubated for 90 min with 50 μM Wy or 10 μM eicosanoids then dosed with 1 ng/ml IL-1β. Cell supernatants were collected to determine PGE_2 secretion by EIA. A) PGE_2 levels were determined at 4 h, 8 h, and 12 h after IL-1β. B) A Western blot of lysates prepared from cells used to detect PGE_2 secretion shows expected effects on COX-2 expression. Data are presented as mean ± SD and each experiment was performed in quadruplicate. * Significant difference from solvent treatment at the same time point and § significant difference from both solvent and IL-1β treatments.

Generation of $PGF_{1\alpha}$ was significantly increased after dosing with IL-1β and eicosanoid pre-treatment failed to induce further changes in $PGF_{1\alpha}$ levels (Figure 4.14). Pre-incubation with Wy resulted in a 25% decrease in $PGF_{1\alpha}$ production. Again, in the absence of IL-1β, no effects on $PGF_{1\alpha}$ were detected with Wy or eicosanoid treatment alone. In contrast to prostaglandin production, IL-6 secretion was potentiated about 2-fold by pre-incubating SMCs with Wy, 11,12-EET, and 14,15-DHET compared to IL-1β alone (Figure 4.15).

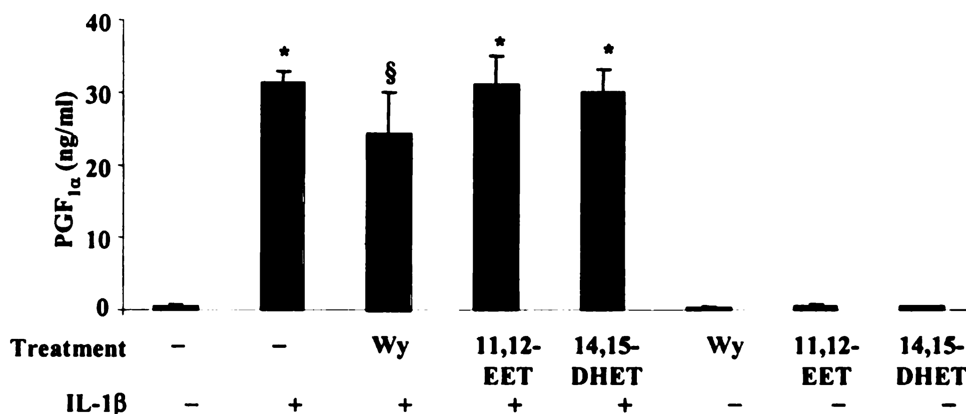


Figure 4.14 Wy, but not 11,12-EET and 14,15-DHET, decrease IL-1β-induced $PGF_{1\alpha}$ secretion. SMCs were preincubated for 90 min with 50 μM Wy or 10 μM eicosanoids then dosed with 1 ng/ml IL-1β. Cell supernatants were collected to determine $PGF_{1\alpha}$ secretion by EIA. The same supernatant collected at 12 h from Figure 4.13 was used to assay $PGF_{1\alpha}$ secretion. Data are presented as mean ± SD and each experiment was performed in quadruplicate. * Significant difference from solvent treatment at the same time point and § significant difference from both solvent and IL-1β treatments.

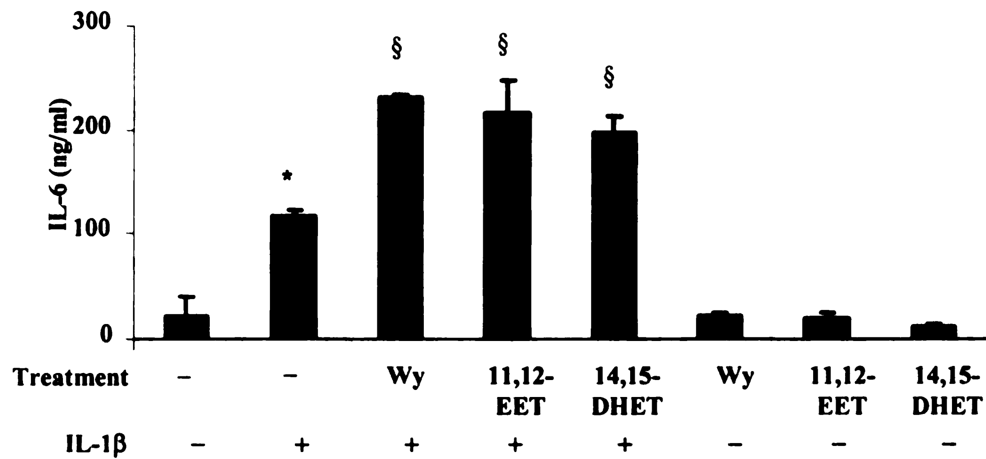


Figure 4.15 Wy, 11,12-EET, and 14,15-DHET potentiate IL-1β-induced IL-6 secretion. SMCs were preincubated for 90 min with 50 μM Wy or 10 μM eicosanoids then dosed with 1 ng/ml IL-1β. Cell supernatants were collected after 24 h to determine IL-6 secretion by ELISA. Data are presented as mean ± SD and each experiment was performed in quadruplicate. * Significant difference from solvent treatment at the same time point and § significant difference from both solvent and IL-1β treatments.

4.4 Discussion

In this study, we show that the PPARα activators Wy, 11,12-EET, and 14,15-DHET potentiate the effects of IL-1β on COX-2 protein and mRNA expression in human aortic smooth muscle cells. The effects of PPARα agonists on IL-1β-induced COX-2 expression in human aortic smooth muscle cells have been described (Staels et al., 1998). Surprisingly, our results were in direct contrast to these earlier data that showed that PPARα activators inhibited IL-1β-induced COX-2, IL-6, and prostaglandin production. As a direct comparison, in our experiments, pre-treatment with 50 μM Wy clearly and reproducibly resulted in increased COX-2 expression, whereas at this concentration, no effect was evident in the earlier study (Staels et al., 1998). When 250 μM Wy was used as a pre-treatment however, Staels and colleagues detected significant attenuation of COX-2 protein and mRNA expression. Repeated attempts were made to reproduce the previous report by optimizing the length of agonist incubation and agonist concentration on multiple lots of primary smooth muscle cells, however the results obtained remained consistent with the data presented here. The reasons underlying the discrepancy remain to be determined. However, the fact that both COX-2 protein and mRNA data are in agreement validate our findings. In other cell lines, reports of COX-2 induction by Wy and CYP eicosanoids have been reported. For example, COX-2 expression stimulated by TNFα is additionally induced in the presence of Wy, and more recently, CYP2C9-derived eicosanoids such as 11,12-EET can upregulate COX-2 expression in the absence of other stimuli (Ikawa et al., 2001; Michaelis et al., 2005).

NF-κB has been widely implicated in the regulation of COX-2 expression (Smith et al., 2000), thus we decided to further investigate the mechanism whereby this receptor

may potentiate IL-1β-induced COX-2 expression. A summary of these findings are schematically depicted in Figure 4.16. Compared with IL-1β treatment alone or pre-treatment with Wy or 11,12-EET, only 14,15-DHET resulted in slower recovery of the inhibitor protein IκBα. It is possible that 14,15-DHET, but not Wy or 11,12-EET, retards de novo synthesis of IκBα. Conversely, Wy and 11,12-EET may be more efficient at stimulating IκBα transcription. Although NF-κB has typically been identified as the primary regulator of IκBα transcription, PPARα activation by GW9578 dramatically increases p65-dependent IκBα promoter activity (Delerive et al., 2002). A recent report showed that coactivators, including p300/CBP, p/CAF, and p160 dynamically regulate IκBα expression following NF-κB activation (Gao et al., 2005). It is therefore possible that the expression of these coactivators may be differentially affected by Wy/11,12-EET and 14,15-DHET.

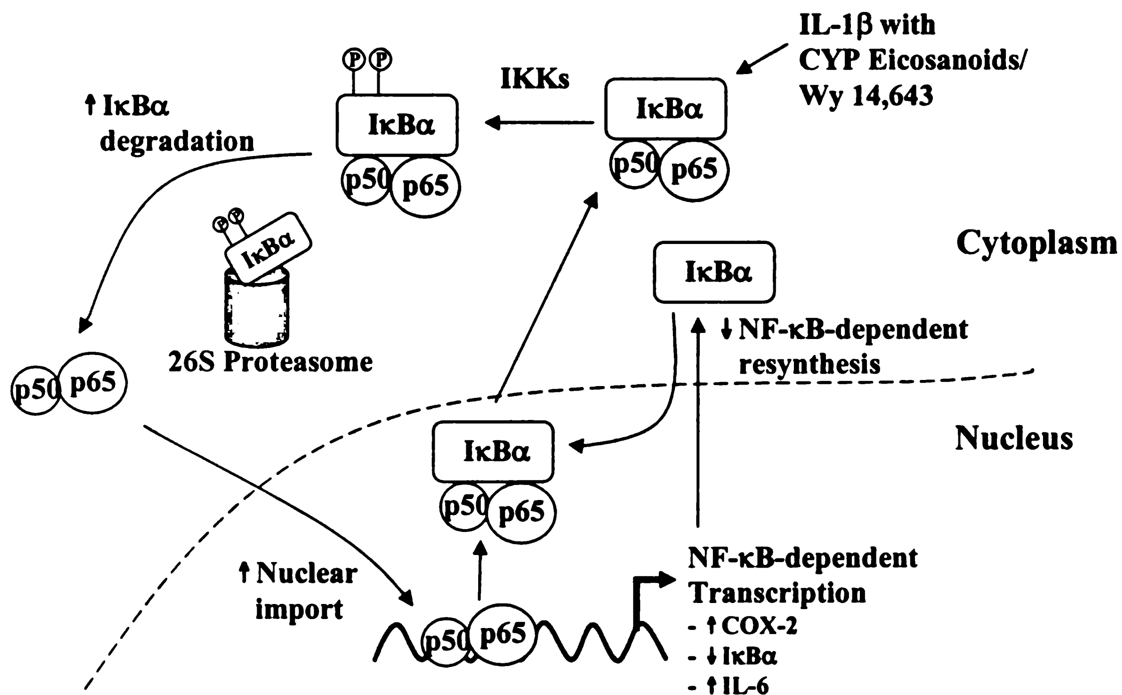


Figure 4.16 Summary of the effects of IL-1β in the presence of CYP eicosanoids or Wy 14,643 on components of the NF-κB signaling pathway.

Examination of the phosphorylated form of IκBα showed that Wy, 11,12-EET and 14,15-DHET sustained its expression to a greater extent than IL-1β alone, where 11,12-EET and 14,15-DHET had greater effects compared to Wy. This result suggests that Wy and CYP eicosanoids exert different effects on the upstream events leading to IκBα phosphorylation. In a study conducted in human aortic smooth muscle cells, Wy failed to affect IKK activity induced by IL-1β (Delerive et al., 2000). In another report, 11,12-EET decreased TNFα-induced IKK phosphorylation of IκBα in bovine aortic endothelial cells (Node et al., 1999). Other eicosanoids such as cyclopentone prostaglandins of the A- and J-type have also been shown to inhibit TNFα-stimulated IKK activity (Rossi et al., 2000). A direct comparison of the effects of Wy and CYP eicosanoids on IL-1β-induced IKK activity may help explain our observations. Nevertheless, sustained phospho-IκBα expression caused by Wy, 11,12-EET, and 14,15-DHET pre-treatment supports the fact that COX-2 potentiation is due to increased NF-κB signaling. Moreover, in cells pre-incubated with Wy or CYP eicosanoids, p65 translocation into the nuclear compartment was prolonged, and increased binding to a κBRE was detected. These data all point to NF-κB as a mechanism for the COX-2 potentiation that was observed in this study. However, in transactivation assays, additional activation of the κBRE by IL-1β after pre-incubation with Wy, 11,12-EET, or 14,15-DHET was not apparent. Since these assays were conducted in a different cell line (HepG2), it may not be accurate to correlate these results to the observations in SMCs. Technical difficulties were encountered with the transfection of primary SMCs, however future efforts will concentrate on introducing plasmids into SMCs to elucidate the effects of Wy and CYP eicosanoids on IL-1β-induced NF-κB transactivation. In addition, it will

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be important to examine their effects on a natural COX-2 promoter containing NF-κB response elements.

Many reports have described the regulation of COX-2 by a variety of stimuli in many cell types and via different signaling cascades. Studies in microvascular endothelial cells, rat vascular smooth muscle cells, and airway smooth muscle cells reported that NF-κB activation alone is insufficient to induce COX-2 expression by IL-1β, and that p38 mitogen-activated protein (MAP) kinase activity is necessary to regulate the IL-1β response (Chen et al., 1999; Laporte et al., 2000; Said et al., 2002). In cardiac myocytes and tracheal smooth muscle cells, COX-2 induction by IL-1β depends on both p42/p44 and p38 MAP kinase activation (LaPointe and Isenovic, 1999; Lin et al., 2004). Most recently, is was reported that CYP2C9-derived EETs were able to induce the expression of COX-2 in endothelial cells in a cAMP/PKA-dependent manner (Michaelis et al., 2005). Interestingly, an EDHF, suggested to be 11,12-EET, has been reported to activate p38 MAPK and Erk1/2 signaling in endothelial and smooth muscle cells (Fleming et al., 2001). It will be of interest to further investigate whether altered MAP kinase signaling in the presence of Wy, 11,12-EET, or 14,15-DHET affects the IL-1β potentiation of COX-2.

A feedback loop that regulates COX-2 expression via PPAR has been proposed (Davidge, 2001). Among the CYP eicosanoids tested, 11,12-EET and 14,15-DHET had the largest effect on COX-2 potentiation and markedly increased IL-1-induced COX-2 mRNA expression. Interestingly, this result corresponds to the activation profile of PPARα by CYP eicosanoids where 14,15-DHET was identified as the strongest activator of PPARα, followed by 11,12-EET-EEZE and 11,12-EET (Chapter 2). Therefore it was

of interest to investigate the role of PPARα in COX-2 potentiation. By using small interfering RNA to decrease PPARα expression, the synergistic effect of PPARα agonists and IL-1β on COX-2 was attenuated. In cells transfected with PPARα siRNA and pre-treated with 14,15-DHET and Wy, COX-2 levels did not increase over IL-1β alone treatment. In 11,12-EET pre-treated cells, COX-2 expression increased over IL-1β treatment alone, but was still attenuated when compared to cells that did not have reduced levels of PPARα. These results emphasize the indispensable role of PPARα in COX-2 potentiation. Since Wy and the CYP eicosanoids increased NF-κB signaling, it was expected that decreasing PPARα expression would only partially attenuate their effects on COX-2 potentiation, as is the case with 11,12-EET. Reports have suggested that activation of NF-κB alone may not be sufficient to up-regulate COX-2 (Davidge, 2001), therefore it may be possible that physiological levels of PPARα are required to cause changes in NF-κB activation, a hypothesis that can be tested by re-examining members of the NF-κB signaling pathway in PPARα siRNA-transfected cells. Moreover, PPARα may be affecting other signaling pathways essential for the regulation of COX-2 expression. In smooth muscle cells, docosahexaenoic acid increased PPARα expression in a p38 MAP kinase-dependent manner; and in cardiac myocytes, PPARα is directly phosphorylated by p38 MAP kinase, further enhancing its activation by cognate ligands (Barger et al., 2001; Diep et al., 2000). The determination of PPARα cross-talk with other signaling pathways may further elucidate its involvement in Wy or CYP eicosanoid-mediated COX-2 potentiation by IL-1β.

To understand the physiological consequences of potentiated COX-2 expression, secreted PGE_2 and $PGF_{1\alpha}$ were determined. In addition, IL-6, an inflammatory cytokine regulated by NF-κB, is used as an indication of NF-κB activation (Libermann and Baltimore, 1990). As expected, Wy, 11,12-EET, and 14,15-DHET further increased the production of IL-6 stimulated by IL-1β. This result is in agreement with the activating effects of Wy and CYP eicosanoids on NF-κB. Surprisingly, prostaglandin levels did not correlate with COX-2 expression. In fact, potentiation of COX-2 by Wy, 11,12-EET, and 14,15-DHET had negative effects on PGE_2 production, and, with the exception of Wy, had no effect on $PGF_{1\alpha}$. Since PGE_2 and $PGF_{1\alpha}$ expression are routinely used as indices of COX-2 activity, the results obtained were puzzling. Several explanations may underlie these observations. PGE_2 and $PGF_{1\alpha}$ are subject to further metabolism into many different prostanoids such as 13,14-dihydro-15-keto PGE_2 and 2,3-dinor 6-keto $PGF_{1\alpha}$ (Hamberg and Samuelsson, 1971; Rosenkranz et al., 1980). The presence of Wy or CYP eicosanoids could affect the efficiency of their metabolism by perhaps altering the function and/or expression of the enzymes responsible for their metabolism. In addition, PGE_2 and PGI_2 synthases may be affected by the activation of PPARα. In cardiac myocytes, PPARγ activation resulted in the inhibition of PGE_2 synthase, although COX-2 expression was also reduced (Mendez and LaPointe, 2003).

It is also important to consider the complex relationship between CYP eicosanoids and COX-2. EETs are subject to metabolism by cyclooxygenases to form epoxy-prostaglandins. Although no reports have shown COX-mediated metabolism of 11,12-EET, both 5,6-EET and 8,9-EET are substrates for cyclooxygenases (Carroll et al., 1993; Homma et al., 1993; Zhang et al., 1992). 5,6-EET is converted to 5-hydroxy-PGI_1

and 5,6-epoxy-PGE_1, while 8,9-EET is converted to 11-hydroxy-8,9-epoxy-EET and 15-hydroxy-8,9-epoxy-EET. Therefore one can imagine that 11,12-EET, or even 14,15-DHET, can act as direct competitors for metabolism by the COX enzymes, thereby reducing the turnover of PGH_2 into PGE_2 or PGI_2. Since an induction of COX-2 levels was evident in the presence of 11,12-EET and 14,15-DHET, it may also be possible that the amount of arachidonic acid is the limiting factor. One study showed that 14,15-EET decreased PGE_2 production in brain microvessel smooth muscle cells, but had no effects on COX-1 or COX-2 mRNA and protein expression (Fang et al., 1998). By increasing the concentration of exogenous arachidonic acid, inhibition of PGE_2 production by 14,15-EET was overcome, leading to the conclusion that 14,15-EET competitively inhibits COX activity.

These explanations however, cannot account for the negative effects of Wy on PGE_2 and $PGF_{1\alpha}$ production, unless Wy is also a substrate for the cyclooxygenases. It has been shown that the upregulation of COX-2 expression by non-steroidal anti-inflammatory drugs is independent of its inhibitory actions on prostanoid production (Meade et al., 1999; Pang et al., 2003). In that sense, the results seen here are not completely surprising. In addition, these results reinforce the notion that caution must be exercised if one attempts to correlate COX-2 expression to its activity.

The characterization of lipoxygenase and cyclooxygenase-mediated arachidonic acid metabolism has been thoroughly investigated in the past decades. The third pathway of arachidonic acid metabolism by cytochrome P450 enzymes is relatively new, but emerging work in the last 20 years illustrates the importance of CYP-derived eicosanoids in myriad biological processes. All three sets of enzymes metabolize the same substrate,

are often localized to the same cell types, and are involved in the regulation of overlapping biological actions such as modifying vascular tone and inflammatory responses. Cross-regulation between these three pathways must exist, yet not many studies have addressed this question. A further understanding of these interactions will more accurately elucidate the fate of arachidonic acid and provide information on the relative abundance of eicosanoids, thereby gleaning insight into the overall biological effects that they can mediate. In this report, we investigated how CYP eicosanoids can affect the expression of COX-2 and showed that in the presence of IL-1β, although COX-2 levels are potentiated by CYP eicosanoids, the production of PGE_2 is inhibited. In the past decade, much interest has been paid to specifically inhibit prostanoid production by COX-2. Since prostanoid production is altered in the presence of CYP eicosanoids, depending on the biological application, it may be of interest to target the formation of EETs and DHETs as well. By examining NF-κB signaling and the role of PPARα, we showed that potentiation of COX-2 is mechanistically mediated by these receptors. These results provide further understanding of COX-2 regulation by CYP eicosanoids, and suggest that these nuclear receptors may also be targeted to obtain therapeutic benefits.

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Chapter 5

Effects of Peroxisome Proliferator-Activated Receptor α Activation on Cytochrome P450 and Soluble Epoxide Hydrolase Expression in the Vascular System

5.1 Introduction

Enzymatic oxygenation of arachidonic acid produces eicosanoids that mediate myriad physiological processes. Increasing attention has been placed on the cytochrome P450 (CYP) pathway of arachidonic metabolism in light of the important role of CYP eicosanoids in the regulation of renal and cardiovascular functions (Roman et al. 2000). Arachidonic acid is metabolized by the CYP-dependent system in two major ways: ω-hydroxylation resulting in the formation of hydroxyeicosatetraenoic acids (HETEs); and epoxidation resulting in the formation of regio- and stereoisomeric epoxyeicosatrienoic acids (EETs) (Kroetz and Zeldin 2002). EETs can be further hydrated into corresponding dihydroxyeicosatrienoic acids (DHETs) by the soluble epoxide hydrolase (sEH) (Zeldin et al. 1993). Regulation of the abundance of these biologically active eicosanoids can be directly achieved by altering the expression and/or activity of the enzymes that are responsible for their formation or degradation.

CYP4A is a major enzyme involved in the ω-hydroxylation of arachidonic acid. In the rat, four CYP4A isoforms have been identified, 4A1, 4A2, 4A3, and 4A8 (Nelson et al. 1996). Extensive evidence points to the CYP4A1, CYP4A2, and CYP4A3 isoforms as the predominant fatty acid ω-hydroxylase in mammalian tissues (Capdevila et al.

2000; Hoch et al. 2000). CYP4A1 displays the highest ω- and ω-1 catalytic activity towards arachidonic acid in recombinant systems (Nguyen et al. 1999) while in renal microvessels, inhibition of CYP4A1 and CYP4A2 have comparable effects on 20-HETE synthesis (Wang et al. 1999). A large body of literature has contributed to understanding of the vasoconstrictive effects and the role of 20-HETE in the myogenic response in multiple vascular beds such as the renal, cerebral, and mesenteric arterioles (Harder et al. 1994; Imig et al. 1996; Ma et al. 1993; Wang et al. 2001). However, in large arteries (>100 μm) and the aorta, 20-HETE has no constrictive effects (Marji et al. 2002)

The regulation of arachidonic acid ω-hydroxylation is mediated in vivo by a variety of factors including the administration of fibrate drugs and diseases such as diabetes and hypertension (Kimura et al. 1989; Kroetz et al. 1998). In rodents, the marked upregulation of CYP4A mRNA and protein in the liver and kidney caused by fibrates and diabetes is mediated by the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα), as the induction of hepatic and renal CYP4A is abolished in the PPARα knockout mice (Kroetz et al. 1998; Lee et al. 1995). ω-Hydroxylase activity can also be altered in disease models of hypertension. In angiotensin II-induced hypertensive rats, 20-HETE formation is increased in the rat renal circulation (Croft et al. 2000). Similarly, increased ω-hydroxylase activity has been detected in the mesenteric artery and kidney in the spontaneously hypertensive rat (Imig et al. 1993; Kroetz et al. 1997; Stec et al. 1996; Zhang et al. 2001). The mechanism whereby the activity and function of CYP4A is altered in these hypertensive models is still not understood. Moreover, investigations into the regulation of CYP4A activity and function in the vascular system are still lacking.

The biosynthesis of EETs has been attributed to multiple members of the P450 family including CYP1A, CYP2B, CYP2C, and CYP2J (Zeldin 2001). EETs are generally regarded as vasodilatory mediators and are putative endothelial derived hyperpolarizaing factors (EDHFs) (Fisslthaler et al. 1999). Increased production of EETs in cardiac myocytes is protective during hypoxic and ischemic conditions (Seubert et al. 2004; Wu et al. 1997; Yang et al. 2001). In a given tissue, several P450s may be involved in the production of EETs depending on the abundance and efficiency of each isoform. In the rat liver and kidney, the majority of the epoxygenases belong to the CYP2C family (Capdevila et al. 2000). In the rat kidney, CYP2C23 is the predominant enzyme responsible for EET formation, although CYP2J3 and CYP2J4 have also been detected (Karara et al. 1993; Yu 2004). In rat cardiac myocytes, the CYP2J3 isoform is highly abundant and has been suggested as the predominant epoxygenase of arachidonic acid (Wu et al. 1997). The expression of CYP2J4 has been reported in rat small intestine (Zhang et al. 1997).

Compared to CYP4A, not much is known about the regulation of CYP2C and CYP2J by PPARα agonists. In male rat liver, CYP2C11 is downregulated after exposure to activators of PPARα (Corton et al. 1998). However studies carried out in the promoter region of CYP2C11 suggested that direct binding of PPARα was not required to result in CYP2C11 suppression (Ripp et al. 2003). Limited data point to both the repression of hepatic CYP2C23 and induction of renal CYP2C23 by PPARα agonists (Muller et al. 2004; Rich and Boobis 1997), implying that the regulation of CYP2C23 by PPARα may be tissue-specific. In addition, hepatic and renal CYP2C expression can be altered under pathophysiological conditions such as fasting, increased dietary salts, and angiotensin II-

induced hypertension, thereby resulting in changes in intracellular EET levels (Holla et al. 1999; Qu et al. 1998; Zhao et al. 2003). Recently, CYP2C11 and CYP2C23 levels were found to be decreased in the renal microvessels and mesenteric arteries of obese Zucker rats (Dey et al. 2004b; Zhao et al. 2005). To date, effects of PPARα agonists on CYP2J expression have not been described. Downregulation of immunoreactive CYP2J2 has been reported during hypoxic conditions and increased renal CYP2J and EET formation were demonstrated in spontaneously hypertensive rats (Yang et al. 2001; Yu et al. 2000a). The mechanism underlying the changes in CYP2C and CYP2J expression has not been explored. It is also of importance to reiterate that although EETs are well-recognized as potent vasodilators and protective mediators in the myocardium, information pertaining to the regulation of their formation by CYPs in the vascular system is limited and requires further investigation.

The biological effects of EETs are likely to be eliminated or attenuated as a consequence of hydration by sEH, therefore sEH plays a critical role in controlling intracellular vasoactive EET levels. sEH is widely distributed in mammalian tissues such as the liver, kidney, intestine, and vascular tissues (Spector et al. 2004). In addition, recent work from our laboratory showed the expression of sEH in renal arteries of varying diameter in rats and humans (Yu 2004; Yu et al. 2004).

The expression of sEH is gender- and age-dependent; it is also highly inducible by clofibrate and testosterone (Pinot et al. 1995). Increasing evidence has illustrated the role of sEH in blood pressure regulation. For example, in spontaneously hypertensive rats, sEH expression and EET hydrolysis were markedly higher compared with normotensive control rats and inhibition of sEH resulted in significantly decreased

systolic blood pressure (Yu et al. 2000b). In both the angiotensin II and DOCA-salt models of hypertension, inhibition of sEH resulted in the reduction of blood pressure (Imig et al. 2002; Loch et al. 2004).

In light of the paucity of information available regarding the regulation of CYP and sEH expression in the vasculature, and the potent biological activities of 20-HETE and EETs, the effects of the prototypic PPARα agonist clofibrate on the protein and mRNA expression of these enzymes were examined in multiple vascular beds in vivo. These studies showed that clofibrate had both inductive and repressive effects depending on the enzyme and tissue examined. Protein data presented in this chapter were obtained from two separate experiments, and discrepancies were evident, emphasizing the preliminary nature of these results. Further experimentation and replications are necessary to confirm the effects of PPARα activation on CYP and sEH expression.

5.2 Materials and Methods

5.2.1 Materials

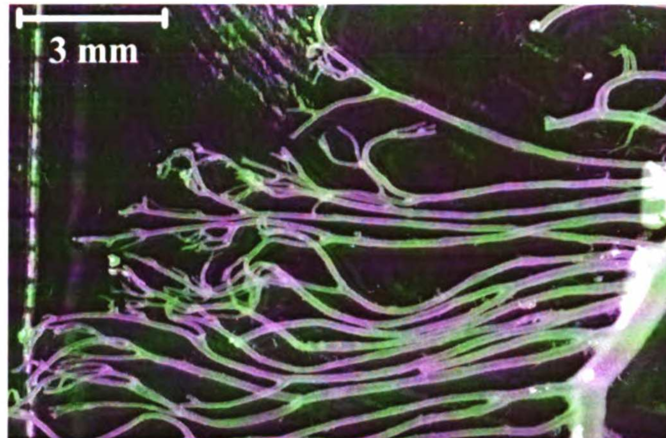
RNA*later* was purchased from Ambion (Austin, TX). The CYP2J4 antibody was a kind gift from Dr. Darryl Zeldin and was used at 1:3000 dilution. Other primary antibodies used in these studies have been described in Chapter 2. All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ) or Sigma Chemical Company (St. Louis, MO).

5.2.2 Animals and Tissue Preparation

All procedures related to the care and treatment of animals were approved by the University of California San Francisco Committee on Animal Research and followed the

National Institutes of Health guidelines. Nine week old Fischer 344 male rats were purchased from Charles River Laboratories (Wilmington, MA). Animals were injected *i.p.* with corn oil or 200 mg/kg clofibrate dissolved in 2.5 ml corn oil for three days. On the fourth day, animals were sacrificed using an *i.p.* injection of Nembutal and an *i.m.* injection of ketamine/xylazine/acepromazine. The abdominal cavities were opened and the vascular system was perfused with ice-cold 0.9% NaCl. For the first study, the liver, heart, and aorta were removed and snap frozen in liquid nitrogen. The kidneys and mesenteric arteries were stored in ice-cold 0.9% NaCl and microdissected on the day of isolation. In the second study, the liver and heart were removed and snap frozen in liquid nitrogen. The aorta and mesenteric arteries were stored in RNA*later* after removal, and kidneys were bisected and stored in RNA*later* for further processing. Connective tissues on the aorta were removed under a microscope and the processed aorta was stored in RNA*later*. For the collection of mesenteric arteries, arteries were removed from connective tissues under a microscope and separated into large or small mesenteric arteries (second order and higher branches, Figure 5.1A). Arteries were placed in RNA*later* after dissection. For the collection of renal interlobar arteries, the renal papilla was removed to expose the microvessels. The interlobar arteries were microdissected and freed from cortical and connective tissue under a dissecting microscope (Figure 5.1B). Dissected microvessels were transferred into ice-cold phosphate buffered solutions and homogenized immediately using a 1 ml duall-style conical tissue grinder (Wheaton Industries, Milville, NJ). To obtain total RNA, the homogenate was processed using the RNAqueous-micro kit (Ambion, Austin, TX). To obtain protein, microvessels were homogenized in lysis buffer containing 50 mM Tris-HCl, 150mM NaCl, and 1%

A.



B.

Interlobular artery
Arcuate artery
Interlobar artery

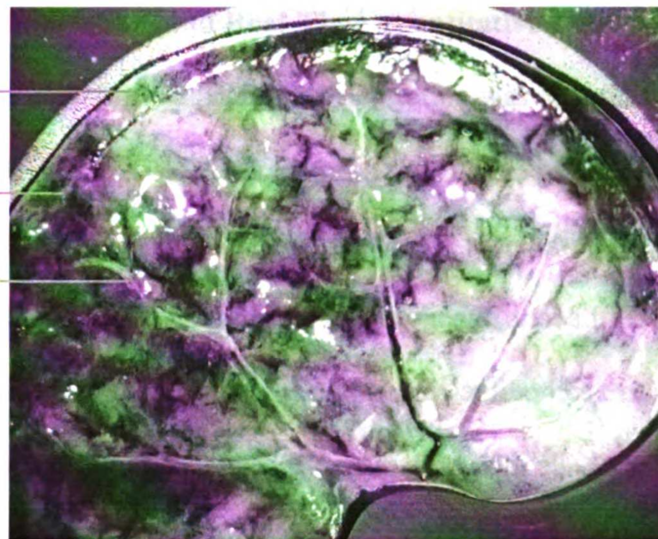


Figure 5.1 Kidney and Mesentric Artery Microdissection. A) Microdissected mesenteric arteries with multiple higher order branches are shown. B) Vessels in a bissected kidney were infused with Trypan Blue to illustrate the location of kidney microvessels (images kindly provided by Dr. Zhigang Yu).

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B.

Figure 5.1 Kidney and Mesenteric Artery Microdissection. A) Microdissected mesenteric arteries with multiple higher order branches are shown. B) Vessels in a bisected kidney were infused with Trypan Blue to illustrate the location of kidney microvessels (Images kindly provided by Dr. Zhigang Yu).

Igepal supplemented with Complete Protease Inhibitor Cocktail Tablets from Roche Applied Science (Indianapolis, IN). All other tissues were cut into small pieces and homogenized using plastic disposable rotor stator generator probes from Omni International (Marietta, Georgia). Protein and total RNA were extracted from microdissected mesenteric arteries that were homogenized in the RNA*later* buffer and processed using the PARIS kit from Ambion (Austin, TX). Protein samples from the liver and heart were obtained by homogenization in lysis buffer, and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). All tissues, extracted protein, and total RNA were stored at -80°C.

5.2.3 Western Immunoblotting and Real-Time quantitative PCR

For Western immunoblotting, protein concentration was quantitated using the BCA method (Pierce, Rockford, IL). Cell lysates (20 μg) were prepared and electrophoresed through NuPage™ 10% Bis-Tris gels and transferred using a wet-transfer method onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Western blots were incubated with a 1:1000 dilution of all primary antibodies except for CYP2J4, which was used at 1:3000 dilution, followed by incubation with alkaline phosphatase- (CYP4A1) or horseradish peroxidase-conjugated (CYP2C11, CYP2C23,CYP2J2, CYP2J4, and sEH) secondary antibodies. Immunoreactive proteins were visualized using an alkaline phosphatase kit (Biorad, Hercules, CA) or an ECL detection kit (Amersham Biosciences, Piscataway, NJ).

For real-time quantitative PCR, reverse transcription is carried out using M-MLV reverse transcriptase (Promega, Madison, WI). Primers and probe sets were designed

pial supplemented with Complete Protease In...

plied Science (Indianapolis, IN). All other ...

homogenized using plastic disposable roto...

International (Marietta, Georgia). Proteins ...

microdissected mesenteric arteries that were ...

processed using the PARIS kit from Ambion ...

liver and heart were obtained by homogeniz...

extracted using TRIzol (Invitrogen, Carlsbad ...

and RNA were stored at -80°C.

5.2.3 Western Immunoblotting and Real-...

For Western immunoblotting, protein ...

BCA method (Pierce, Rockford, IL). Cell ...

electrophoresed through NuPage™ 10% Bis-Tris ...

transfer method onto nitrocellulose membrane ...

blots were incubated with a 1:1000 dilution of ...

which was used at 1:3000 dilution, followed by incubation ...

(CYP4A1) or horseradish peroxidase-conjugated (CYP2C11, CYP2J ...

CYP2J4, and sEH) secondary antibodies. Immunoreactive bands were ...

an alkaline phosphatase kit (Biorad, Hercules, CA) or an ECL detection kit (Amersham

Biosciences, Piscataway, NJ).

For real-time quantitative PCR, reverse transcription was carried out using M-MLV

reverse transcriptase (Promega, Madison, WI). Primers and probe sets were designed

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using Primer Express with the help of Dr. David Ginzinger (UCSF Cancer Center) and are listed in Table 2.1. Probes were labeled with the reporter dye 6-carboxy-fluorescein phosphamidite (FAM) at the 5′-end and the dye quencher Black Hole Quencher at the 3′-end. The CYP2J3 and CYP2J4 primer and probe sets were purchased from Applied Biosystems (Foster City, CA). Reactions were run on an ABI Prism 7700 and cycling conditions were: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of specific transcripts was calculated by the following formula: Relative expression = $2^{-\Delta\Delta Ct}$ where Ct = $(\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{treated} - (\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{control}$.

5.2.4 Statistics

With the exception of the samples used in Figure 5.1, all measurements were performed on samples from individual rats. Results are expressed as mean ±SD from 3 animals. Statistical significance of difference between mean values was evaluated by an unpaired Student's *t*-test. Significance was set at a *p* value of <0.05.

5.3 Results

5.3.1 Preliminary Study: Cytochrome P450 and sEH Protein Expression in the Heart and Various Vascular Beds

An initial study was carried out using pooled samples from four rats to determine the effects of clofibrate treatment on the protein expression of CYPs and sEH in the liver, aorta, heart, mesenteric and interlobar arteries. Since the regulation of CYPs and sEH by clofibrate is well characterized in the rodent liver, the protein expression of these genes in

LightCycler Express with the help of Dr. David ...

listed in Table 2.1. Probes were labeled with ...

fluorescein (FAM) at the 5'-end and the ...

ed. The CYP2B and CYP2J4 primer and ...

Biosystems (Foster City, CA). Reactions ...

conditions were: 95°C for 10 min, followed ...

ed. The relative expression of specific ...

formula: Relative expression = $2^{-\Delta\Delta Ct}$ ...

5.2.4 Statistics

With the exception of the samples ...

performed on samples from individual rats ...

Statistical significance of differences ...

unpaired Student's t-test. Significance was ...

5.3 Results

5.3.1 Preliminary Study: Cytochrome P450 and sEH Protein Expression in the Heart and Various Vascular Beds

An initial study was carried out using pooled samples from ... rats to determine the effects of clofibrate treatment on the protein expression of CYP4As and sEH in the liver, aorta, heart, mesenteric and interlobar arteries. Since the regulation of CYP4As and sEH by clofibrate is well characterized in the rodent liver, the protein expression of these genes in

the liver was first determined as a positive control (Figure 5.2). Although an antibody specific for CYP2J3 was not available, the anti-human CYP2J2 antibody cross reacts with rodent CYP2J and was used to detect total CYP2J3 and CYP2J4 expression (Wu et al. 1996). CYP4A1, CYP2J, and sEH protein expression were makedly induced by clofibrate, whereas the repression of CYP2C11, and CYP2C23 were evident. CYPs and sEH were not detected in the aorta and mesenteric arteries under basal conditions. Upon clofibrate treatment, only the CYP2J immunoreactive protein was detectable in the mesenteric arteries. In the interlobar arteries, CYP4A1, sEH, CYP2C23, and CYP2J4 expression was significantly induced by clofibrate. Clofibrate treatment also resulted in the upregulation of sEH and CYP2C23 in the heart, although the induction was not as

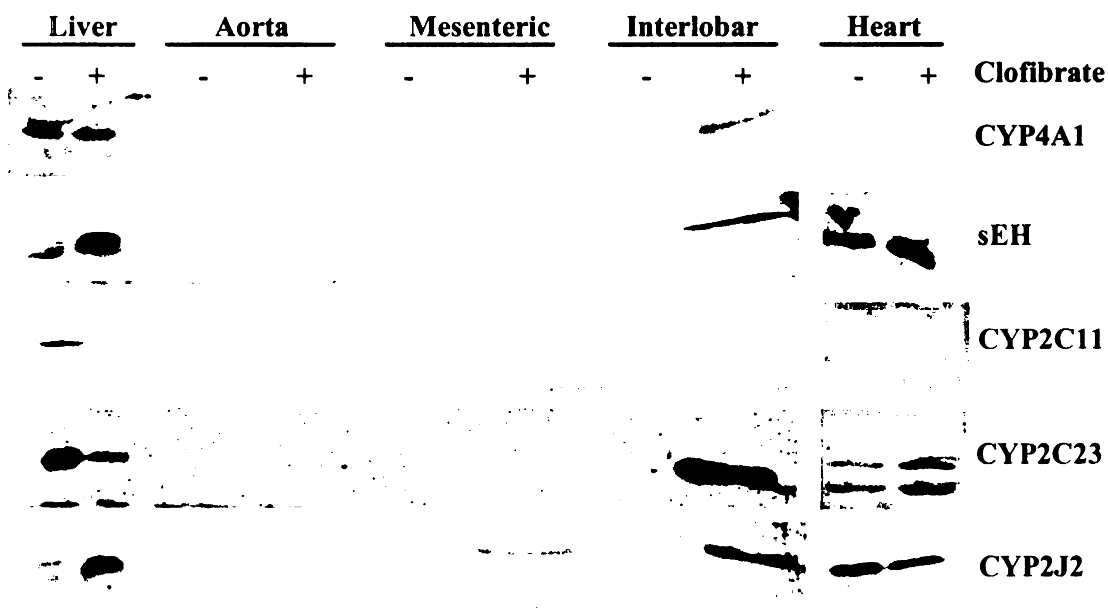


Figure 5.2 CYP and sEH protein expression in Fischer 344 liver, aorta, small mesenteric arteries, interlobar arteries, and heart with or without clofibrate treatment. CYP4A1, sEH, CYP2C11, CYP2C23, and CYP2J2 immunoreactive proteins were detected by Western blotting from pooled protein samples from each treatment group. The CYP2J2 antibody was detects both CYP2J3 and CYP2J4.

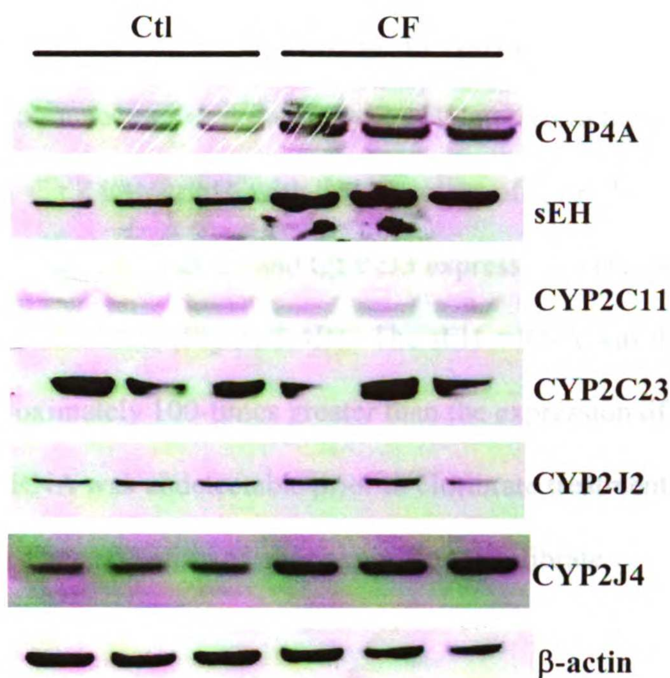
significant as that in the interlobar arteries.

The fact that clofibrate had a significant effect on CYP on sEH expression in the interlobar arteries led to the design of the experiments described in the following figures. It was of interest to be able to demonstrate changes in tissue samples obtained from a single animal as opposed to pooled samples, and have multiple animal replicates. In addition, RNA expression was also examined to determine whether changes at the protein level were mediated in a transcription-dependent manner.

5.3.2 Cytochrome P450 and sEH Expression in the Liver

In the liver, protein expression of CYP4A1 and sEH were markedly induced by clofibrate treatment, however changes in CYP2C11 and CY2C23 levels were minimal (Figure 5.3A). Two immunoreactive proteins were detected with the CYP4A1 antibody with the upper band appearing as a doublet in the induced samples. It has been previously reported that the fastest migrating isoform is CYP4A1, the slowest is CYP4A3, and CYP4A2 migrates at an intermediate rate (Okita et al. 1997). Total CYP2J protein appeared to be slightly induced while CYP2J4 expression was more strikingly increased. Correlating to their protein expression, the mRNA expression of hepatic CYP4A1, sEH, and CYP2J4 were significantly induced after clofibrate exposure while CYP2C11 and CYP2C23 levels were repressed (Figure 5.3B). No change in CYP2J3 transcript expression was observed. It is interesting to note that the basal mRNA expression of CYP2C11 and CYP2C23 are approximately 30-fold higher than the levels of the other CYPs and sEH.

A.



B.

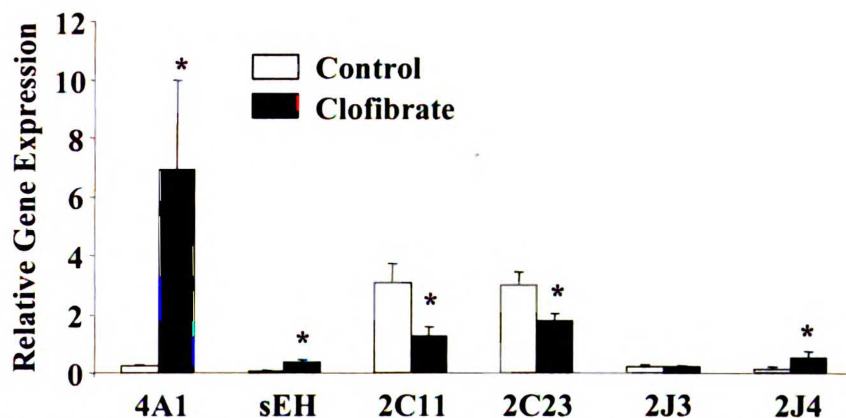
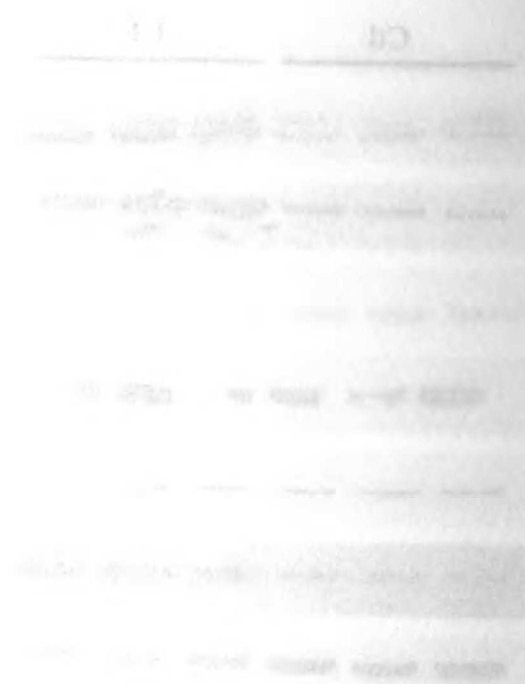


Figure 5.3 CYP and sEH protein and mRNA expression in the Fischer 344 liver after treatment with clofibrate. A) Hepatic CYP4A1, sEH, CYP2C11, CYP2C23, CYP2J2, and CYP2J4 immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated (CF) Fischer 344 rat liver samples. *β*-actin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control ($p<0.05$).

A



B

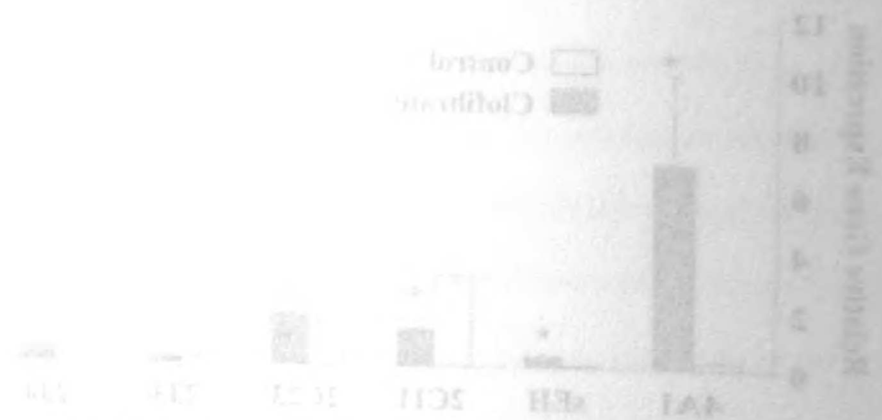


Figure 5.5. CYP and sEH protein and mRNA expression in the liver of rats treated with clofibrate. A) Hepatic CYP4A1, sEH, CYP2C11, CYP2C23, and CYP2J4 immunoreactive proteins were detected by western blotting from three control (Ctl) and three clofibrate-treated (CF) Fischer 344 liver samples. β-actin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control ($p<0.05$).

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5.3.3 Cytochrome P450 and sEH Expression in the Aorta

CYP and sEH immunoreactive proteins were not detected in the aorta by Western blotting (Figure 5.4A). CYP transcripts, with the exception of CYP2J4, were also present only at very low levels, where CYP2C23 and CYP2J3 expression were induced approximately 2-fold by clofibrate (Figure 5.4B). The sEH mRNA was the most abundant and was approximately 100-times greater than the expression of CYP2C3 and CYP2J3. CYP4A1 mRNA was undetectable prior to clofibrate treatment, and no CYP2C11 mRNA was detected before or after exposure to clofibrate.

5.3.4 Cytochrome P450 and sEH Expression in the Interlobar Artery

Renal interlobar arteries were microdissected for the detection of CYP and sEH expression. The position of the interlobar artery is illustrated in Figure 5.1B. Changes in CYP and sEH protein levels were not evident although CYP4A1, sEH, CYP2C23, and CYP2J3 transcripts were induced by clofibrate (Figure 5.5A and B). Clofibrate treatment induced the mRNA expression of CYP4A1 by 13-fold, sEH by 2.5-fold, and CYP2C23 and CYP2J3 by 3-fold. Basal and induced CYP2C23 expression was significantly higher than the expression of other CYPs and sEH.

5.3.5 Cytochrome P450 and sEH Expression in the Small Mesenteric Arteries

Mesenteric arteries were microdissected and further separated into large and small arteries (second order and higher branches) as illustrated in Figure 5.1A. In the small mesenteric arteries, no significant differences in CYP and sEH protein were observed between vehicle- and clofibrate-treated animals (Figure 5.6A). CYP2C11 and CYP2C23

5.5.3 Cytochrome P450 and sEH Expression in [illegible]

CYP and sEH immunoreactive proteins were [illegible]
staining (Figure 5.4A). CYP transcripts, with [illegible]
only at very low levels, where CYP2C23 and [illegible]
approximately 2-fold by clofibrate (Figure [illegible]
abundant and was approximately 100-times [illegible]
CYP2J3. CYP4A1 mRNA was undetectable [illegible]
CYP2C11 mRNA was detected before or [illegible]

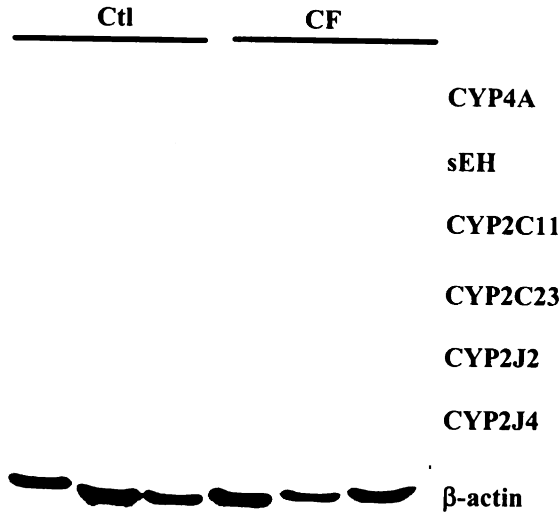
5.5.4 Cytochrome P450 and sEH Expression [illegible]

Renal interlobar arteries were micro[illegible]
expression. The position of the interlobar [illegible]
CYP and sEH protein levels were not evide[illegible]
CYP2J3 transcripts were induced by clofi[illegible]
induced the mRNA expression of CYP4A1 [illegible]
and CYP2J3 by 3-fold. Basal and induced [illegible]
than the expression of other CYPs and sEH

5.5.5 Cytochrome P450 and sEH Expression in the Small Mesenteric Arteries

Mesenteric arteries were microdissected and further separated into large and small arteries (second order and higher branches) as illustrated in (Figure 5.5A). In the small mesenteric arteries, no significant differences in CYP and sEH protein were observed between vehicle- and clofibrate-treated animals (Figure 5.6A). CYP2C11 and CYP2C23

A.



B.

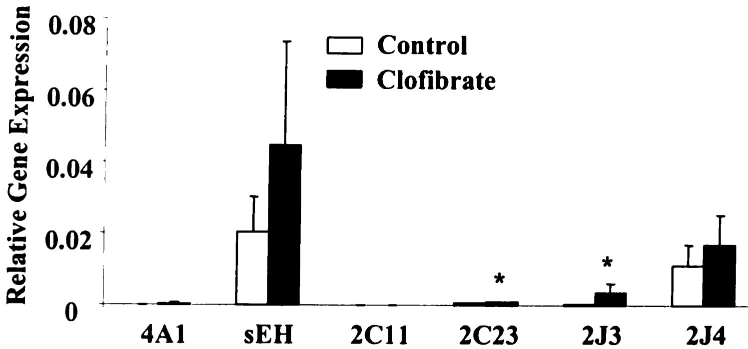
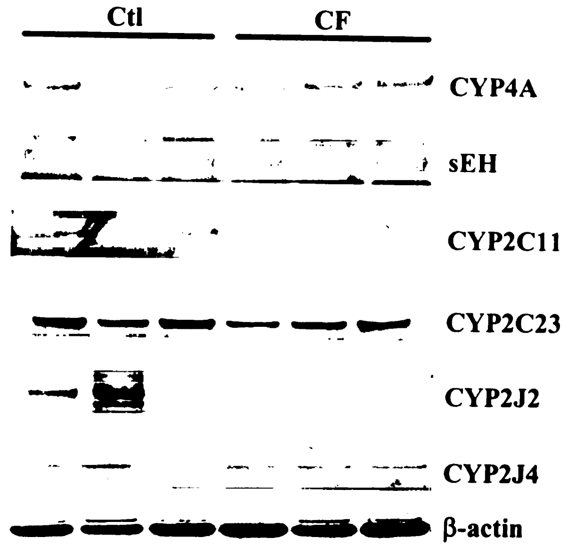


Figure 5.4 CYP and sEH protein and mRNA expression in the Fischer 344 aorta after treatment with clofibrate. A) CYP4A1, sEH, CYP2C11, CYP2C23, CYP2J2, and CYP2J4 immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated (CF) rodent aorta samples. *β*-actin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. CYP2C11 transcript was not detected in either basal or clofibrated-treated samples and uninduced CYP4A1 transcript was not detectable. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control (p<0.05).

A.



B.

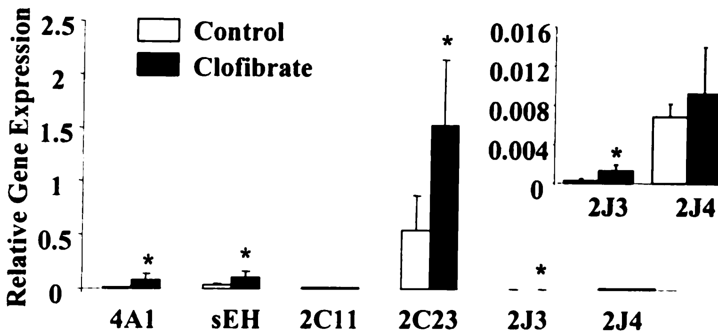


Figure 5.5 CYP and sEH protein and mRNA expression in the Fischer 344 renal interlobar artery after treatment with clofibrate. A) CYP4A1, sEH, CYP2C11, CYP2C23, CYP2J2, and CYP2J4 immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated (CF) renal interlobar artery samples. *β*-actin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. Inset shows in more detail the relative abundance of CYP2J3 and CYP2J4. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control (p<0.05).

A

B

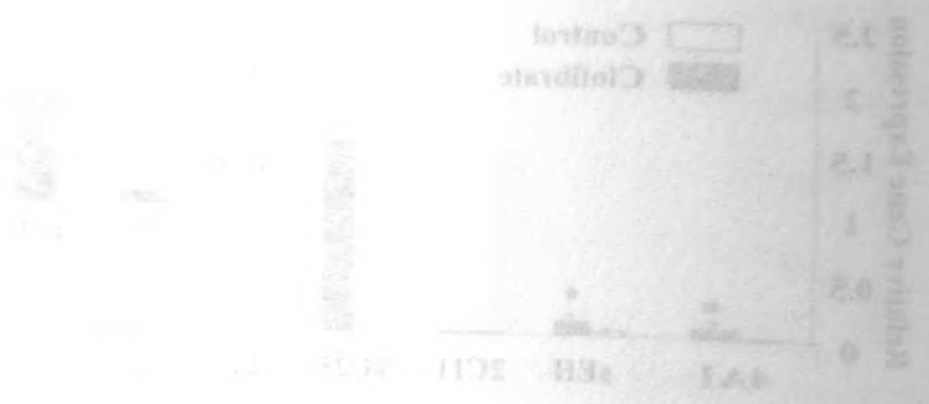


Figure 5.5. CYP and sEH protein and mRNA [illegible] **renal interlobar artery after treatment with clofibrate.** (A) [illegible] **CYP2C23, CYP2J2, and CYP2J4** immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated [illegible] interlobar artery samples. β-actin was used as a loading control. (B) [illegible] RNA was isolated and specific mRNA expression was determined using real time quantitative PCR and normalized to cyclophilin expression. Inset shows in more detail the relative abundance of CYP2J3 and CYP2J4. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control ($p<0.05$).

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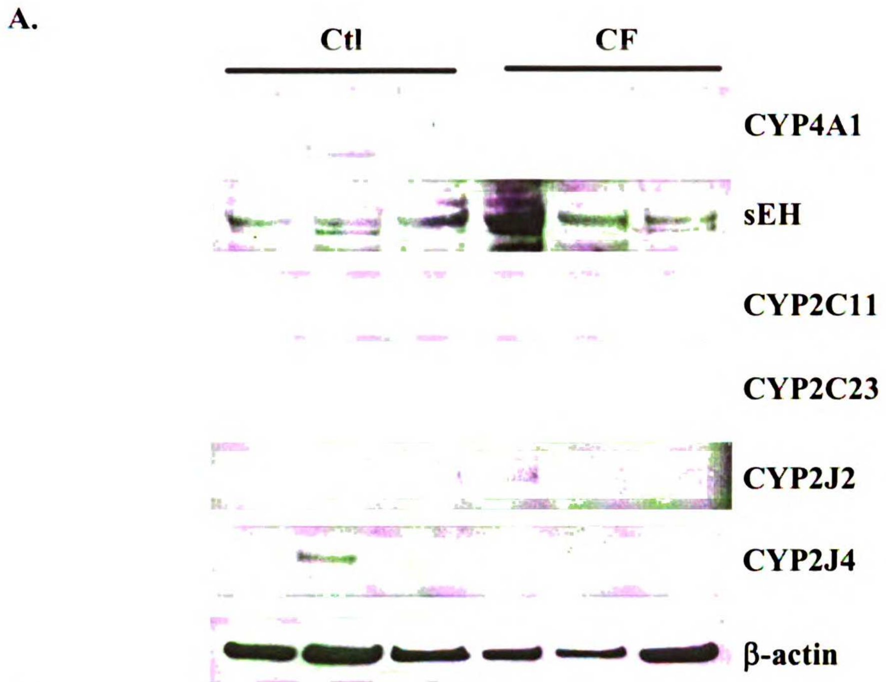
proteins were not detected, whereas low levels of CYP4A and CYP2J immunoreactive proteins were observed in some samples. Examination of their mRNA expression demonstrated that sEH expression was induced by 2-fold after clofibrate treatment (Figure 5.6B). CYP4A1 and CYP2C23 levels were not detectable except in the presence of clofibrate. In addition, sEH and CYP2J4 were both relatively highly expressed in both the basal and treated states. No significant changes in CYP2C11, CYP2C23, CYP2J3, and CYP2J4 transcript levels were observed after clofibrate treatment.

5.3.6 Cytochrome P450 and sEH Expression the Heart

The protein expression of CYP4A1, CYP2C23, and CYP2J4 in the heart was upregulated by clofibrate (Figure 5.7A). sEH levels remained unchanged and CYP2C11 was not detected. Interestingly, the expression of β-actin was markedly downregulated after treatment with clofibrate, therefore the β-tubulin was used as a loading control. The basal mRNA expression of CYP4A1 and CYP2C11 were almost undetectable, but in the presence of clofibrate, they were induced 80-fold and 50-fold, respectively (Figure 5.7B). sEH mRNA was present in 10-1000 times higher abundance than the CYPs, but no induction by clofibrate was evident. Interestingly, clofibrate treatment downregulated CYP2C23 mRNA by 50%. CYP2J3 and CYP2J4 mRNA expression remained unchanged.



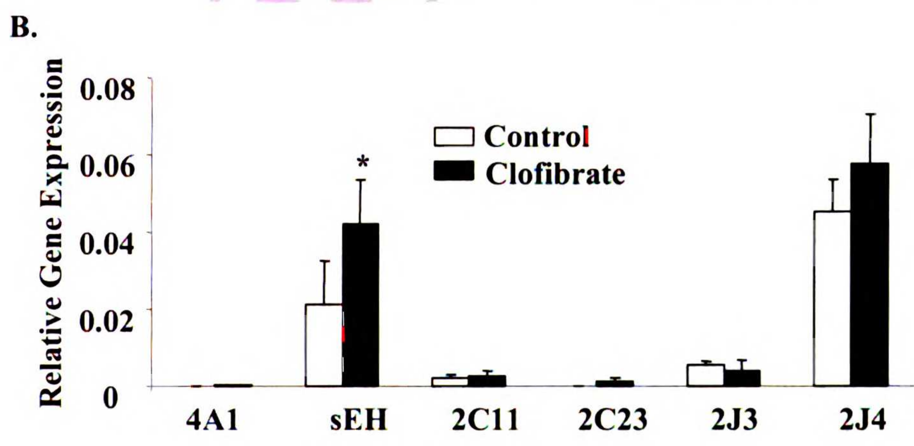
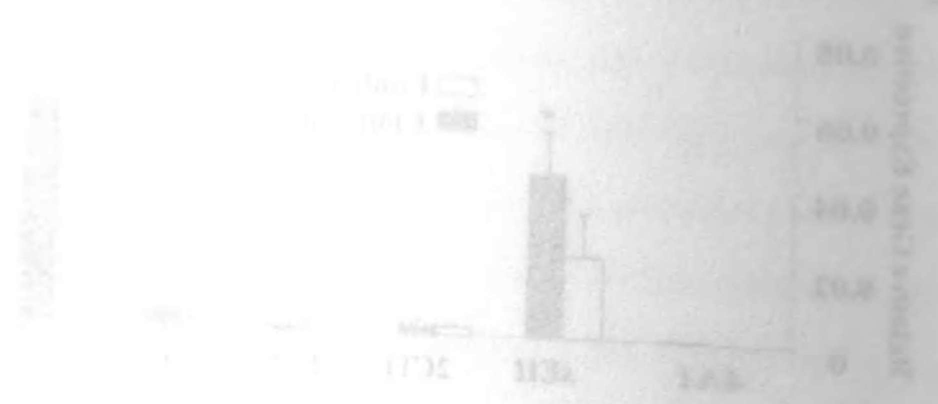


Figure 5.6 CYP and sEH protein and mRNA expression in the Fischer 344 small mesenteric arteries after treatment with clofibrate. A) CYP4A1, sEH, CYP2C11, CYP2C23, CYP2J2, and CYP2J4 immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated (CF) small mesenteric artery samples. *β*-actin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. Basal CYP4A1 and CYP2C23 transcripts were undetectable. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control ($p<0.05$).



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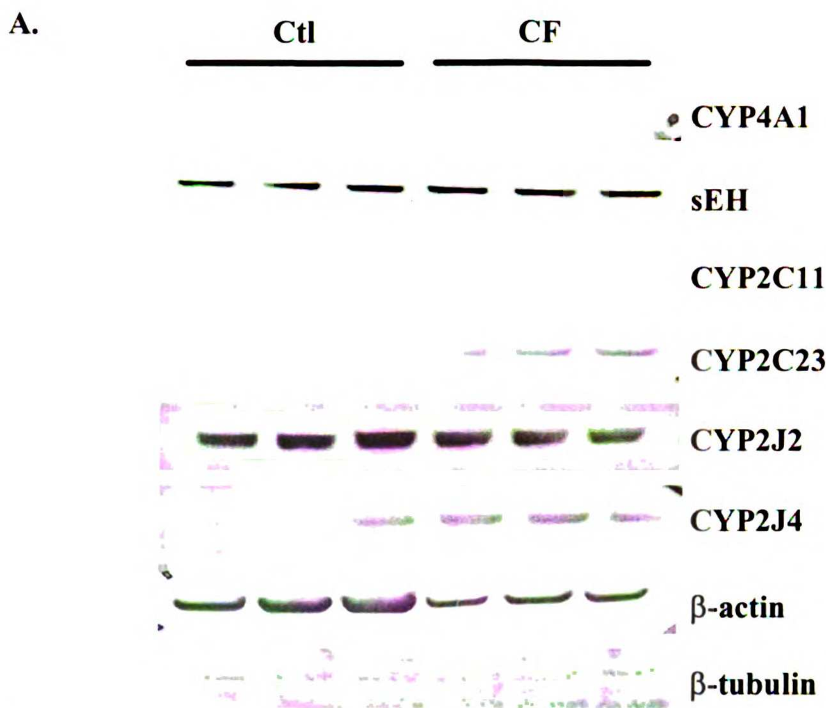


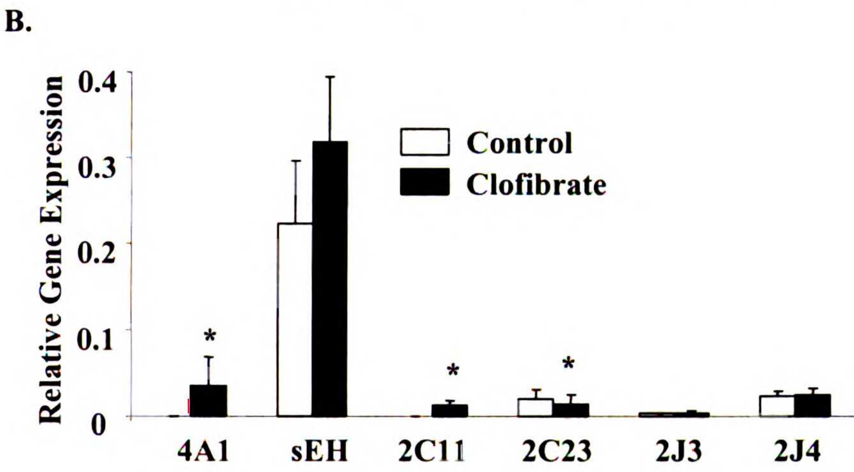
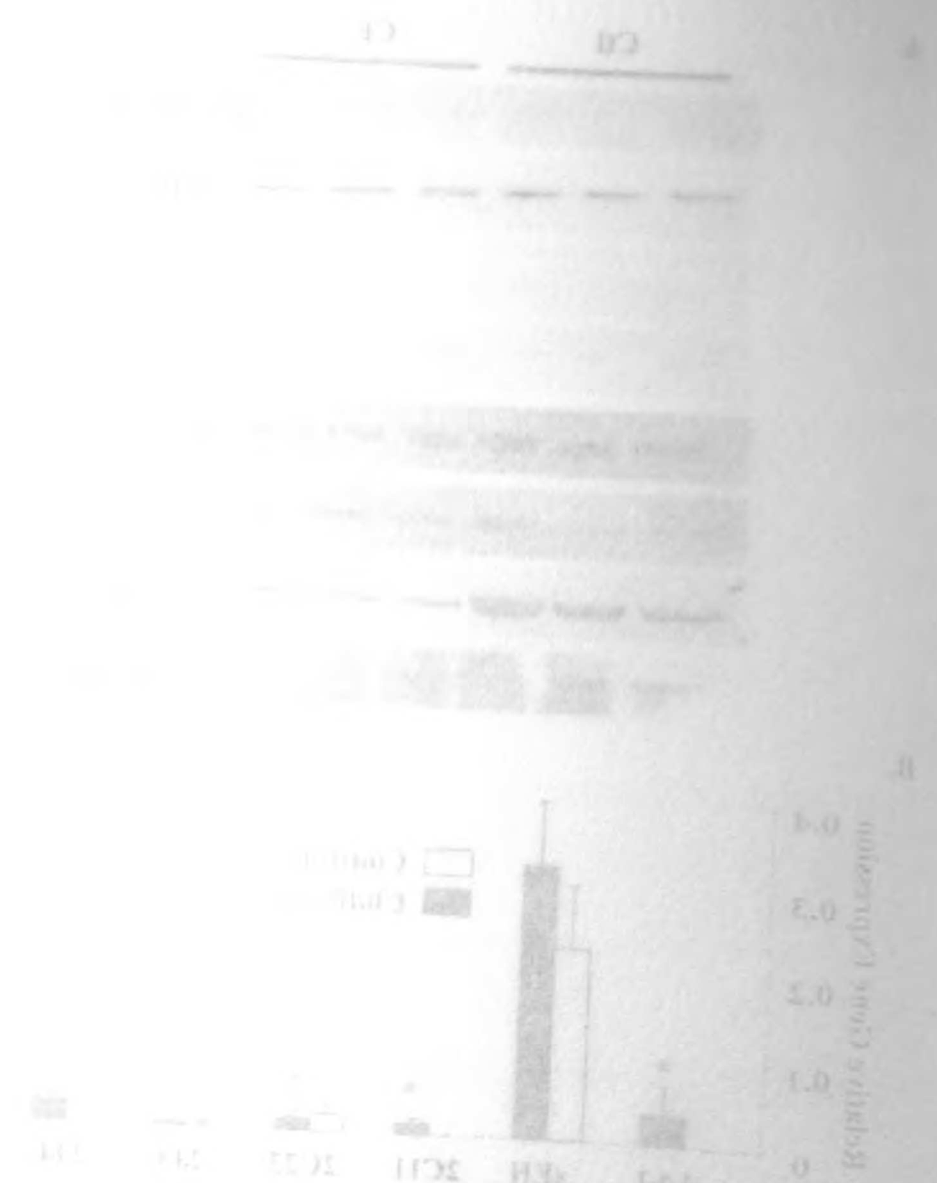


Figure 5.7 CYP and sEH protein and mRNA expression in the Fischer 344 heart after treatment with clofibrate. A) CYP4A1, sEH, CYP2C11, CYP2C23, CYP2J2, and CYP2J4 immunoreactive proteins were detected by Western blotting from three control (Ctl) and three clofibrate-treated (CF) heart samples. *β*-tubulin was used as a loading control. B) Total RNA was isolated and specific mRNA expression was determined using real-time quantitative PCR and normalized to cyclophilin expression. Values were obtained from three individual RNA samples performed in triplicate and are expressed as mean ± SD. * Significant difference over the vehicle control (p<0.05).



5.4 Discussion

The studies described above examined the effects of the PPARα agonist clofibrate on the expression of CYPs and sEH in the liver, heart, aorta, interlobar and mesenteric arteries. In the liver, the expected induction of CYP4A1 and sEH mRNA and protein by clofibrate were clearly evident. CYP4A1 was upregulated to a greater extent compared with the other two CYP4A isoforms, consistent with a previous report showing PPARα-dependent induction of CYP4A (Kroetz et al. 1998). The reported repression of CYP2C11 and CYP2C23 was observed at the mRNA level but was not reflected at the protein level (Corton et al. 1998; Muller et al. 2004). The reason for the lack of repressive effect on hepatic CYP2C11 remains elusive and may be attributed to experimental differences. It has been reported that clofibrate does not have any inductive effects on hepatic CYP2J expression and that the mRNA of CYP2J does not correlate with its protein expression (Wu et al. 1997). However our studies suggested the opposite. Hepatic CYP2J4, and to a limited extent, total CYP2J, were clearly induced by clofibrate treatment, an increase that was also reflected in hepatic CYP2J4 mRNA levels. Curiously, in both the cited report and the present study, the same rat strain and approximately the same clofibrate treatments (250 mg/kg/day for 4 days versus 200 mg/ kg for 3 days in this study) were employed, therefore these factors probably do not account for the discordant results.

In the aorta, only very low levels of CYP mRNA was detected, which was reflected in the absence of corresponding immunoreactive proteins. The absence of CYP4A1 is also consistent with published reports where the biosynthesis of 20-HETE in the rat abdominal aorta was undetectable (Marji et al. 2002). Recently, the metabolism of EETs to DHETs was reported in the human aorta, implying the presence of a functional

Discussion

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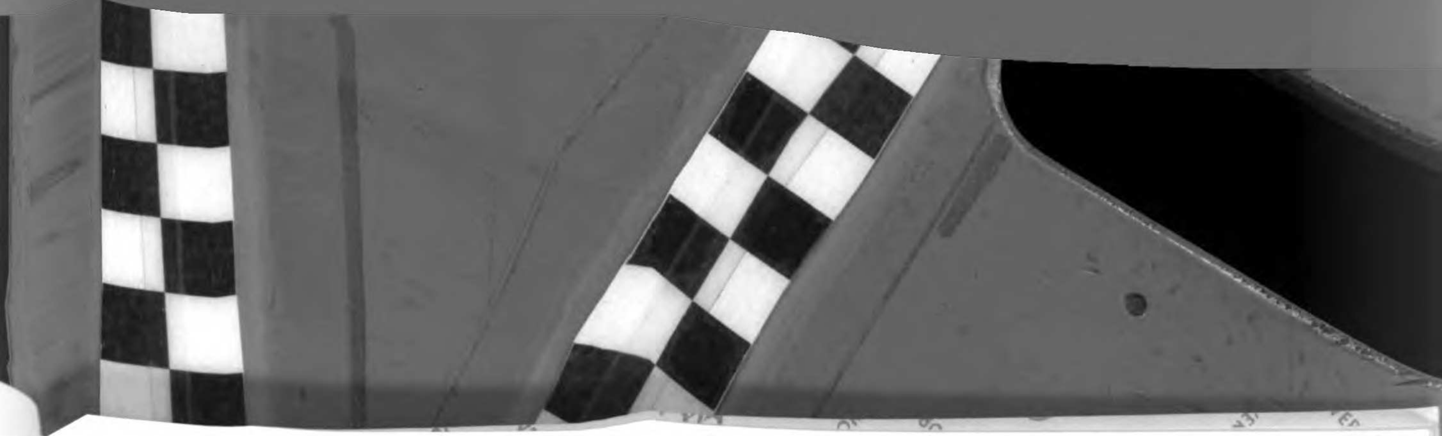

sEH in this tissue (Fang et al. 2004). Although the sEH immunoreactive protein was not apparent in our studies, its mRNA expression was readily observed. The inability to detect the sEH protein may be attributed to the limited sensitivity associated with the detection method, rather that the absence of the protein. Alternatively, there could be species-related differences in sEH expression and the sEH protein may not be present in the rat aorta. An immunoreactive protein detected with the CYP2J2 antibody has been reported in the rabbit aorta, but the current studies did not detect any protein with the CYP2J2 antibody (Pfister et al. 2003). Similarly, in our studies, aortic CYP2C11 protein and mRNA were not observed whereas reports have demonstrated the presence of rabbit CYP2C8 and the female-specific rat CYP2C12 (reported as CYP2C11 in the manuscript) in the aorta (Pfister et al. 2003; Yamaguchi et al. 2001). Since CYP2C11 and CYP2C12 are differentially regulated in a gender-specific manner, differences in their expression within the same tissue can be expected (Sundseth et al. 1992). It should be emphasized that CYP2C and CYP2J expression and regulation in the aorta has only been minimally explored, and additional work will be required to understand their role in EET biosynthesis in the aorta.

20-HETE itself is not produced in quantities enough to exert vasoconstrictive effects in the aorta. However, it can be metabolized by cyclooxygenases to 20-hydroxy-endoperoxides, which exhibit constrictive properties (Escalante et al. 1989). EETs can mediate diverse physiological effects in cells derived from the aorta. Exogenous application of 11,12-EET or the overexpression of CYP2J2 in aortic endothelial cells resulted in the increased expression of tissue plasminogen activator, which plays an important role in regulating vascular thrombosis (Node et al. 2001). In the aortic smooth

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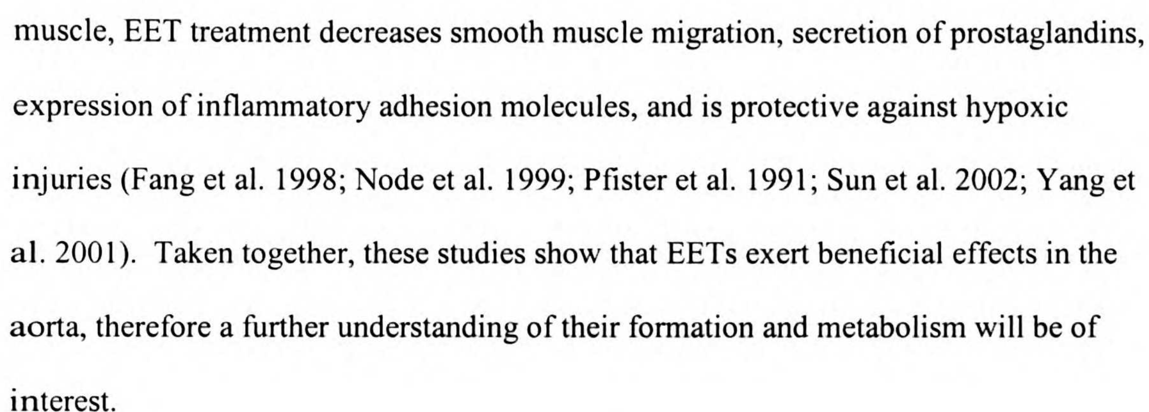

muscle, EET treatment decreases smooth muscle migration, secretion of prostaglandins, expression of inflammatory adhesion molecules, and is protective against hypoxic injuries (Fang et al. 1998; Node et al. 1999; Pfister et al. 1991; Sun et al. 2002; Yang et al. 2001). Taken together, these studies show that EETs exert beneficial effects in the aorta, therefore a further understanding of their formation and metabolism will be of interest.

In the interlobar arteries, changes in CYP and sEH immunoreactive proteins were detected only in one of two experiments conducted. Assuming that the animals obtained were genetically similar, experimental differences may account for the inconsistent results. Interlobar arteries used in the initial study were microdissected on the day the rats were sacrificed, whereas those in the second study were obtained from kidneys stored in the RNA*later* solution for many months (at -80°C). Although degradation of proteins should not occur in tissues immersed in RNA*later*, the possibility exists that CYP and sEH expression were altered after many months or storage. This latter notion is supported, although by no means conclusively, by the fact that only the mRNA, but not protein expression of CYP4A1, sEH, and CYP2J3 were significantly induced by clofibrate. Conversely, since the arteries used in the initial experiment represent the first attempts at vascular microdissection, the observed induction of CYP and sEH expression may result from kidney and tubular contamination. The use of γ-glutamyl transpeptidase in future experiments will be helpful in the determination of microvessel purity (Wang et al. 1999).

The expression of CYP4A1, CYP4A2, and CYP4A3 immunoreactive proteins have been reported in rat interlobar, arcuate, and interlobular arteries and CYP2C11 and

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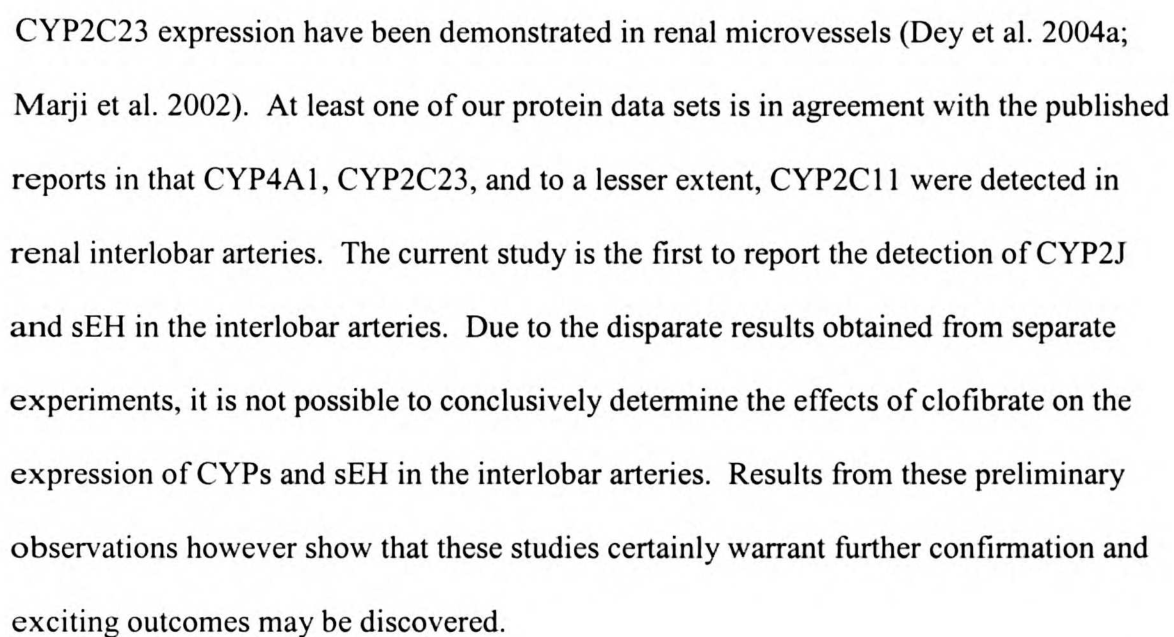

CYP2C23 expression have been demonstrated in renal microvessels (Dey et al. 2004a; Marji et al. 2002). At least one of our protein data sets is in agreement with the published reports in that CYP4A1, CYP2C23, and to a lesser extent, CYP2C11 were detected in renal interlobar arteries. The current study is the first to report the detection of CYP2J and sEH in the interlobar arteries. Due to the disparate results obtained from separate experiments, it is not possible to conclusively determine the effects of clofibrate on the expression of CYPs and sEH in the interlobar arteries. Results from these preliminary observations however show that these studies certainly warrant further confirmation and exciting outcomes may be discovered.

In renal preglomerular vessels, including the interlobar, arcuate, and interlobular arteries, 20-HETE and EETs are important regulators of vascular tone. 20-HETE causes potent vasoconstriction and EETs are second messengers for factors such as angiotensin II and can regulate the diameter of preglomerular arteries and affect glomerular filtration rate (Alonso-Galicia et al. 1999; Cheng et al. 2003; Imig et al. 2001a; Imig et al. 2001b; Kaide et al. 2003). 20-HETE and EETs also contribute to the tubuloglomerular feedback mechanism by acting as paracrine communicators between the macula densa and smooth muscle cells of preglomerular arterioles (Franco et al. 1988; Zou et al. 1994). In the interlobar artery, overexpression of CYP4A and the subsequent increase in 20-HETE production results in augmented endothelial sprouting, implicating 20-HETE in the angiogenic process (Jiang et al. 2004).

In the small mesenteric arteries, the CYP2J4 mRNA and the sEH protein and mRNA were significantly more abundant compared to the other CYPs. The robust expression of CYP4A, CYP2C11, CYP2C23, and CYP2J transcripts and immunoreactive

CYP2C23 expression have been demonstrated in [illegible]

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In the small mesenteric arteries, the CYP4A mRNA and the sEH protein and

mRNA were significantly more abundant compared to the other CYPs. The robust

expression of CYP4A, CYP2C11, CYP2C23, and CYP2J transcripts and immunoreactive

proteins have been reported in rat mesenteric arteries (Zhao et al. 2005). Strain-specific differences may account for the discrepancies observed between the two studies, since Sprague-Dawley rats were used in the previous study and Fischer rats were used in the current study. Moreover, in the present report, the fact that mRNA data correlated with protein expression validates these findings. The high expression of sEH in the mesenteric arteries raises the question of its physiological role in this tissue. In small mesenteric arteries, acetylcholine-induced NO-independent vasodilation has been attributed to the actions of EDHFs, and EETs are putative EDHFs (Campbell et al. 1996; Makino et al. 2000; Wigg et al. 2001). In obese Zucker rats, induction of sEH was evident in the mesenteric arteries compared with lean Zucker rats. By decreasing available EETs, the increase in sEH expression was suggested to be associated with endothelial dysfunction in the mesenteric arteries of the obese rats (Zhao et al. 2005). In the present study, clofibrate increased the mRNA expression of sEH while having only modest effects on the protein. Nevertheless, these results suggest that the possibility exists whereby administration of clofibrate may contribute to mesenteric artery-associated endothelial dysfunction.

In the heart, CYP4A1, CYP2C23 protein and mRNA, and CYP2J4 protein expression were induced by clofibrate treatment. The expression and activity of CYPs have been associated with both positive and negative outcomes resulting from ischemia-reperfusion injury. In the canine heart, 20-HETE production was greatly elevated after myocardial ischemia-reperfusion injury, resulting in increased infarct size (Nithipatikom et al. 2004). In addition, the inhibition of CYP ω-hydroxylase by specific inhibitors markedly reduced myocardial infarct size, implicating 20-HETE as a negative contributor

to this disease. Similarly, inhibition of CYP2C with sulfaphenazole decreased infarct size in the rat heart by diminishing the amount of EETs (Gottlieb et al. 2004). In contrast, pre-incubation of the rat heart with 11,12-EET significantly improves myocardial contractility after ischemia-reperfusion, and in CYP2J2 transgenic hearts, recovery of myocardial function is markedly improved following ischemia and reperfusion compared with wild type hearts (Murohara et al. 1995; Seubert et al. 2004). Therefore, the net effect of increased ω-hydroxylase and epoxygenase expression in the heart by clofibrate is difficult to predict and further studies are needed to provide insight into this question.

The data presented in this chapter represent a preliminary survey of CYP and sEH expression in the vascular system in vivo under both basal and clofibrate-treated conditions. Information on CYP ω-hydroxylase and epoxygenase activities in these tissues is critical and will complement the expression data. In addition, the availability of sensitive detection methods such as high-performance liquid chromatography tandem mass spectrometry will help determine the in vivo concentration and relative abundance of CYP eicosanoids within a given tissue. Additional physiological relevance can also be obtained by employing animal models such as angiotensin II-induced hypertension and spontaneously hypertensive rats. Mechanistic studies can also be performed to determine the role of PPARα in the regulation of vascular CYP and sEH by the use of other PPARα-specific activators and inhibitors, as well as the PPARα knockout mice.

The importance of CYP eicosanoids in humans is starting to emerge and increasing evidence suggests a relationship between cardiovascular diseases and CYP eicosanoid production. Urinary 20-HETE and EET excretion was higher in hypertensive

vasodilators. Similarly, inhibition of CYP2C was sufficient ...

... in rat heart by diminishing the amount of CYP ...

... pre-incubation of the rat heart with ...

myocardial contractility after ischemia-reperfusion ...

recovery of myocardial function is markedly ...

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Therefore, the net effect of increased ω-hydroxylation ...

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The importance of CYP eicosanoids in humans is starting to emerge and

mounting evidence suggests a relationship between cardiovascular diseases and CYP

eicosanoid production. Urinary 20-HETE and EET excretion was higher in hypertensive

than normotensive females (Catella et al. 1990; Ward et al. 2004). In addition, genetic polymorphisms in CYP4A11, CYP2J2, and sEH have been linked to hypercholesterolemia and coronary artery disease (Fornage et al. 2004; Gainer et al. 2005; Sato et al. 2004; Spiecker et al. 2004). Since knowledge regarding the regulation of these enzymes is limited in the vascular system, this work represents an initial step towards understanding the involvement of PPARα in their regulation.

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Chapter 6

Summary and Perspectives

6.1 Summary

Eicosanoids generated from the metabolism of arachidonic acid by cytochrome P450 (CYP) metabolism are mediators of diverse biological functions such as the regulation of vascular tone, renal function, cellular proliferation, and inflammation (Kroetz and Zeldin 2002; Roman 2002). The major products of CYP-catalyzed arachidonic acid metabolism are 20-hydroxyeicosatetraenoic acid (20-HETE) and the regio- and stereospecific epoxyeicosatrienoic acids (5,6-,8,9-,11,12-, 14,15-EETs). 20-HETE formation is catalyzed by the CYP4A and CYP4F family of enzymes, and EETs are formed mainly by the CYP2C and CYP2J enzymes (Karara et al. 1993; Nguyen et al. 1999; Powell et al. 1998; Wang et al. 1999; Wu et al. 1997; Wu et al. 1996; Xu et al. 2004; Zeldin et al. 1996). CYP eicosanoids can be subsequently metabolized into a variety of metabolites via different pathways; one of the major routes is the metabolism of EETs to their corresponding dihydroxyeicosatrienoic acids (DHETS) by soluble epoxide hydrolase (sEH) (Yu et al. 2000; Zeldin et al. 1993).

It has been widely demonstrated that the expression of CYP4A can be induced by a group of chemicals known as peroxisome proliferators (Johnson et al. 2002). Limited evidence also demonstrates the induction of renal CYPC23 and sEH, and repression of hepatic CYP2C by peroxisome proliferators (Corton et al. 1998; Muller et al. 2004; Pinot et al. 1995).

The effects of peroxisome proliferators are unequivocally mediated by the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) since targeted

Chapter 6

Summary and Fu

Summary

Eicosanoids generated from the metabolism

CYP metabolites are mediators of diverse

vascular tone, renal function, cellular pro

al., Roman 2002). The major products

20-hydroxyeicosatetraenoic acid (20-H

epoxyeicosatrienoic acids (5,6-, 8,9-, 11,1

alized by the CYP4A and CYP4F fa

CYP2C and CYP2J enzymes (Karara

Wang et al., 1999; Wu et al. 1997; Wa

the CYP epoxygenases can be subsequent

additional pathways; one of the major

responding dihydroxyeicosatrienoic acids (DH

et al., 2000; Zeldin et al. 1993).

It has been widely demonstrated that the

of chemicals known as peroxisome prolifer

also demonstrates the induction of some CYP

and CYP2C by peroxisome proliferators (Cornu et al. 2004;

et al. 1998)

The effects of peroxisome proliferators are unequivocally mediated by the nuclear

peroxisome proliferator-activated receptor alpha (PPARα) (also targeted

disruption of this receptor attenuated the pleiotropic responses of classic peroxisome proliferators such as clofibrate and Wy 14,643 (Lee et al. 1995). Other PPARα ligands include fatty acids and eicosanoids such as prostacyclin, leukotriene B_4, and hydroxylated EETs (Cowart et al. 2002; Devchand et al. 1996; Forman et al. 1997). PPARα is a member of the PPAR nuclear receptor family that also consists of the PPARγ and PPARδ isoforms. The role of PPARα as a lipid sensor has been well established (Chinetti-Gbaguidi et al. 2005). Recent interest has focused on its role in the cardiovascular system as its activation inhibits inflammatory responses in the vessel wall and the heart (Bishop-Bailey 2000; Huss and Kelly 2004).

The hypothesis of this dissertation is that CYP eicosanoids can mediate their biological effects via the activation of PPARα. This hypothesis is substantiated by several lines of reasoning: 1) the CYP-catalyzed pathway of arachidonic acid metabolism is regulated by peroxisome proliferators (Johnson et al. 2002); 2) related eicosanoids are activators of PPARα (Devchand et al. 1996; Forman et al. 1997); and 3) in the vasculature, treatment with CYP eicosanoids or activation of PPARα both result in the reduction of inflammatory markers via similar cellular mechanisms (Delerive et al. 1999; Delerive et al. 2000; Node et al. 1999).

It was first investigated whether CYP eicosanoids can activate and bind to PPARα, and if so whether they mediate the induction of PPARα-responsive genes (Chapter 2). Activation of PPARγ and PPARδ were also determined. 11,12-EET, 14,15-DHET, and 20-HETE strongly activated PPARα and PPARγ in transactivation assays, whereas PPARδ was most potently activated by 11,12-EET- and 14,15-EET. Electrophoretic mobility shift assays were established to determine whether these CYP eicosanoid can

derivatives of this receptor ascertained the pleiotropic

proliferators such as clofibrate and Wy 14,643

include fatty acids and eicosanoids such as prostaglandins

(FitzGerald et al. 2002; Devchand et al. 1996

member of the PPAR nuclear receptor family

... The role of PPARα as a lipid sensor

(Sugden et al. 2005). Recent interest has

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mobility shift assays were established to determine whether these CYP eicosanoid can

induce the heterodimerization of PPARα with its dimerization partner retinoid X receptor (RXR) and subsequent binding to its response element (PPRE). Results showed that all the CYP eicosanoids can induce PPARα/RXR-specific binding to the PPRE, implying that these eicosanoids are PPARα ligands. Treatment of primary rat hepatocytes with 11,12-EET, 14,15-DHET, and 20-HETE resulted in changes in the expression of PPARα-responsive genes involved in lipid metabolism and transport, indicating that CYP eicosanoids have the ability to affect gene transcription like peroxisome proliferators. In addition, alterations in the mRNA expression of CYPs and sEH responsible for the production and degradation of these eicosanoids were evident, although changes on the protein level were minimal. Results from this chapter demonstrate that CYP eicosanoids are endogenous activators of PPARα, however further work is required to explain their minimal effects on CYP and sEH expression and to determine the relevance in vivo (Figure 6.1).

After the identification of CYP eicosanoids as endogenous PPARα agonists, urea-based inhibitors of the sEH enzyme were shown to be exogenous activators of this receptor (Chapter 3). An adamantyl and a series of cyclohexyl alkanoic acids with varying carbon chain length were tested for their ability to transactivate PPARα and PPARγ. Maximal activation of both receptors was observed with cyclohexyl undecanoic acid urea (CUUA) and cyclohexyl dodecanoic acid urea (CUDA). Shorter chain alkanoic acids displayed decreasing activating effects on PPARα and PPARγ. Adamantyl dodecanoic acid urea (AUDA) showed comparable effects as CUUA and CUDA. CUUA and AUDA induced the formation of a PPARα/PPRE complex in electrophoretic mobility shift assays, implying that they act as PPARα ligands. CUUA and AUDA significantly

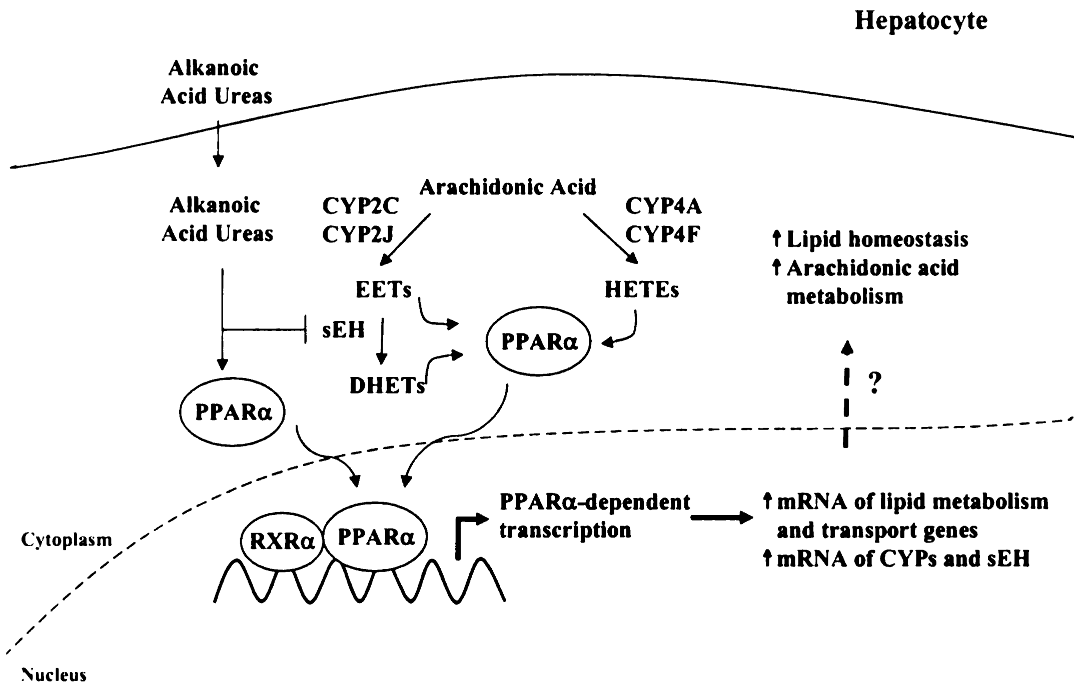

Figure 6.1 Summary of the findings from Chapters 2 and 3. CYP eicosanoids and urea-based alkanoic acids are identified as novel PPARα activators that can induce PPARα-dependent transcription of lipid metabolism and transport genes, as well as CYPs and sEH expression in hepatocytes. The question mark indicates questions raised from these studies of whether the induction of these PPARα-responsive genes leads to increased lipid homeostasis and upregulation of CYP-mediated arachidonic acid metabolism.

induced the expression of PPARα target genes showing that they can function as PPARα agonists. In addition, CUUA and AUDA inhibited platelet-derived growth factor-induced proliferation of human smooth muscle cells by the repression of cyclin D1 expression, thereby resulting in G1 cell cycle arrest. By knocking down the endogenous expression of PPARα in human aortic smooth muscle cells using small interfering RNA, it was determined that PPARα was required, at least in part, in the repression of cyclin D1. Results from this chapter demonstrate that urea-based alkanoic acids are exogenous

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activators of PPARα, and their attenuation of smooth muscle cell proliferation is partially mediated by the actions of this receptor (Figures 6.1 and 6.2).

An increasing number of reports have demonstrated cross-talk between the NF-κB and PPAR signaling pathways (Bishop-Bailey 2000). It was next investigated whether CYP eicosanoids, by activating PPARα, altered the expression of the NF-κB responsive gene, cyclooxygenases-2 (COX-2), in human aortic smooth muscle cells (Chapter 4). Although a previous report demonstrated that PPARα activation in smooth muscle cells attenuated IL-1β-induced expression of COX-2 (Staels et al. 1998), our studies consistently showed that the PPARα activators Wy 14,643, 11,12-EET, and 14,15-DHET potentiated IL-1β effects on COX-2 expression on both the protein and transcript levels. By examining components of the NF-κB signaling cascade including IκBα and phosphorylated IκBα expression, nuclear translocation of p65, and binding of nuclear proteins to an NF-κB response element, it was demonstrated that treatment of smooth muscle cells with PPARα activators in the presence of IL-1β resulted in increased NF-κB activation compared with IL-1β treatment alone. Thus, increased NF-κB signaling provided a mechanistic explanation for the observed potentiation of COX-2 expression by Wy 14,643 and CYP eicosanoids. Using smooth muscle cells that have decreased levels of PPARα by transfection with small interfering PPARα RNA, the synergistic effect of PPARα activators and IL-1β on COX-2 was significantly attenuated, emphasizing the indispensable role of PPARα in COX-2 potentiation. Surprisingly, despite increased COX-2 expression, secreted PGE_2 levels decreased in response to IL-1β and PPARα activator treatments. In contrast, potentiation of IL-6 production was evident, demonstrating increased NF-κB signaling. These results, although initially surprising,

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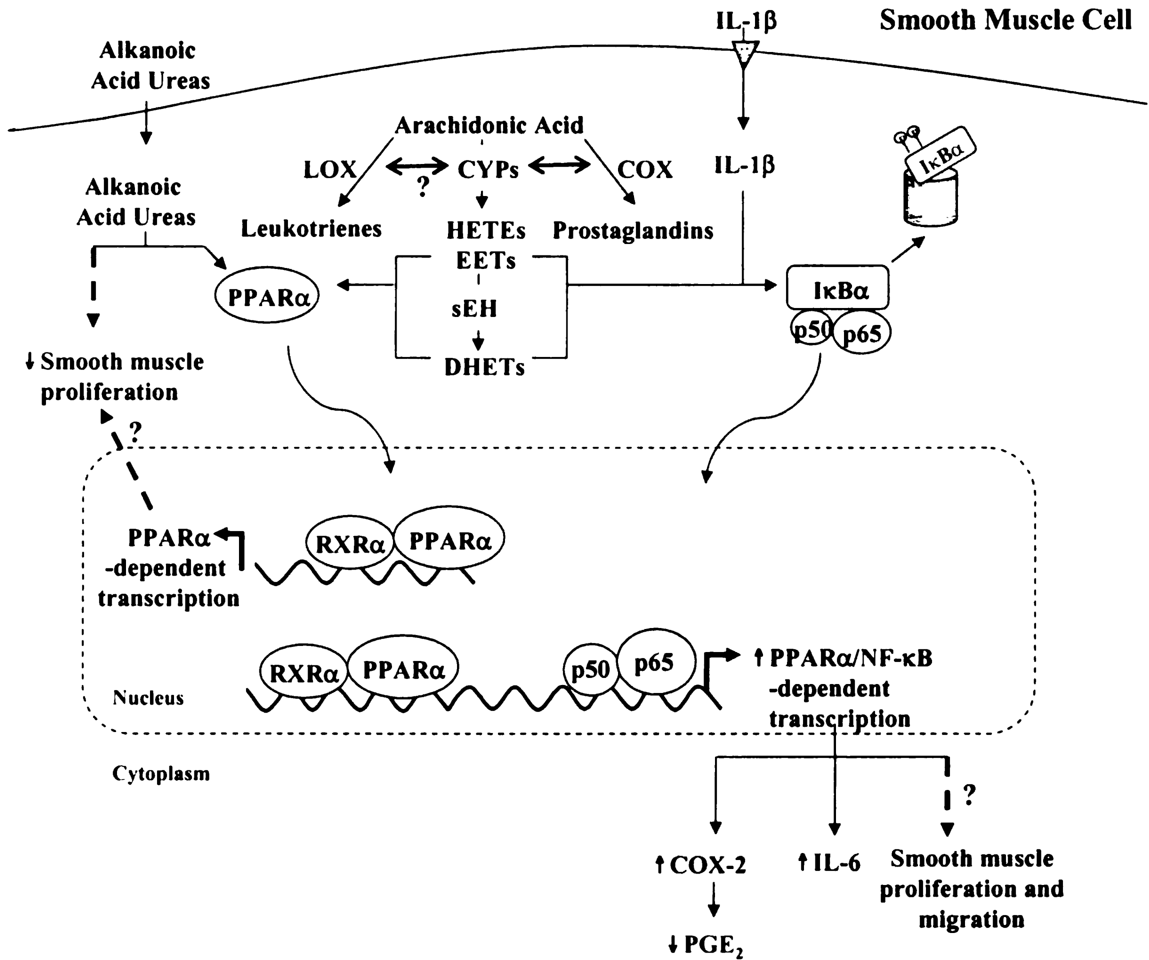

Figure 6.2 Summary of the findings from Chapters 3 and 4. In human aortic smooth muscle cells, alkanoic acid ureas decrease smooth muscle cell proliferation in a PPARα-dependent manner. CYP eicosanoids potentiate the effects of IL-1β on COX-2 expression by increasing NF-κB signaling, an effect that is also PPARα-dependent. PGE_2 production is decreased and IL-6 expression is increased in response to CYP eicosanoids and IL-1β. The question marks indicate the questions raised from these studies of how smooth muscle cell proliferation is inhibited by PPARα activation, whether smooth muscle cell proliferation and migration is altered in the presence of CYP eicosanoids and IL-1β, and whether cross talk exists between the lipoxygenase and the cyclooxygenase or CYP pathways of arachidonic acid metabolism. Cellular processes, rather than specific gene products, are indicated with bold arrows; cross-talk between pathways is indicated with double-headed arrows; factors involved in the transcriptional machinery are circled.

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were reminiscent of COX-2 upregulation and concomitant inhibition of prostanoid production by non-steroidal anti-inflammatory drugs (Meade et al. 1999; Pang et al. 2003). The present studies show that cross-talk between the cyclooxygenase and cytochrome P450 pathways of arachidonic acid metabolism may have important physiological consequences (Figure 6.2).

Results from Chapters 2 and 4 raise the important question of whether CYP and sEH expression are responsive to activators of PPARα in vivo. The regulation of hepatic and renal CY4A and sEH expression are well-documented, however relatively little is known about their regulation in the vasculature (Johnson et al. 2002; Pinot et al. 1995). Therefore, the prototypic PPARα agonist clofibrate was used to examine the effects of PPARα activation on CYP4A1, CYP2C11, CYP2C23, CYP2J3, CYP2J4, and sEH protein and mRNA expression on multiple vascular beds and the heart in vivo (Chapter 5). In general, results show that CYP and sEH levels are responsive to clofibrate treatment in the heart and smaller vessels such as the renal interlobar and small mesenteric arteries. Data presented in this chapter are preliminary and are not always consistent between the two replicate experiments, thus these results require further confirmation.

6.2 Perspectives

PPARs, CYP eicosanoids, and sEH have been receiving increasing attention as potential targets for the treatment of cardiovascular diseases such as dyslipidemia, hypertension, and vascular inflammation (Berger et al. 2005; Chinetti-Gbaguidi et al. 2005; Kroetz and Zeldin 2002; Newman et al. 2005; Yu 2004). Studies presented in this

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dissertation demonstrated the cross-regulation of PPARα and CYP-catalyzed metabolism of arachidonic acid. Although CYPs are highly expressed in the liver, the biological functions of CYP eicosanoids are not clear in this tissue. In this dissertation, it was suggested that CYP eicosanoids have the potential to alter the expression of CYPs, sEH, and fatty acid metabolizing genes in the liver. However, despite potent activation of PPARα by CYP eicosanoids, the reasons underlying the minimal effects of CYP eicosanoids on PPARα-responsive genes are not clear. Availability of the hydrophobic eicosanoids to the cells of interest is always a concern and the recent establishment of liquid chromatography tandem mass spectrometry in our laboratory should be a routine tool to help ascertain their intracellular concentration. The design of CYP eicosanoid analogs that are more stable and easier to handle will also be invaluable in determining their effects.

Smooth muscle cell proliferation is a critical process that is established early during atherosclerosis and can eventually lead to occlusive lesions that result in myocardial ischemia (Sriram and Patterson 2001). Therefore, many therapeutic approaches have been invested in limiting this proliferation. Urea-based alkanoic acids are originally designed as potent inhibitors of sEH and in this thesis, they are shown to be strong activators of PPARα as well. In addition, these alkanoic ureas inhibit cell cycle progression in smooth muscle cells in a PPARα-dependent and non-cytotoxic manner, making them attractive as structural leads. It is not entirely clear if the inhibition of smooth muscle cell proliferation by these compounds is mediated directly or indirectly by PPARα. Further studies, such as the detailed dissection of cell cycle regulators in the presence or absence of PPARα and alkanoic ureas are necessary to determine the

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mechanism of inhibition. It will also be important to examine their potency in atherosclerotic animal models in order to study their utility as therapeutic modulators.

It is curious why not much information is available that pertains to the cross-talk between the three major pathways of arachidonic acid metabolism. We showed that CYP eicosanoids can modify the expression of cyclooxygenases-2 and the production of prostaglandins in smooth muscle cells through a mechanism involving the nuclear receptors NF-κB and PPARα. The actions of CYP eicosanoids on cyclooxygenases-2 expression and activity is reminiscent of the effects of non-steroidal anti-inflammatory drugs, suggesting that CYP eicosanoids act as the body's natural anti-inflammatory compounds, a notion that is supported by studies from other groups (Falck et al. 2003; Node et al. 1999). Other physiological ramifications of this cross-talk such as the effects on smooth muscle proliferation and migration should be explored. In addition, studies detailing the interaction between the CYP and lipooxygenase pathways are also of interest.

The in vivo implications of PPARα activation in the cardiovascular system needs to be addressed by using chemical and genetic tools. In this dissertation, it was shown that PPARα activation caused by a pharmacological agent can result in alterated CYP and sEH expression. It will be important to determine whether the activity of these proteins and the levels of CYP eicosanoids are also modulated, and if so, examine the physiological consequences that are associated with these changes. Recent pharmacogenetic studies have demonstrated the association between CYP/sEH genetic polymorphisms with diseases such as hypertension and hypercholesterolemia (Gainer et al. 2005; Sato et al. 2004; Spiecker et al. 2004). A better understanding of the regulation

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of vascular CYP/sEH expression and activity may be useful for the treatment of these disorders.

In conclusion, we have identified novel endogenous and exogenous PPARα activators which can regulate fatty acid metabolism and vascular function via this receptor. Further studies in vivo delineating their roles in modulating metabolic and vascular diseases is warranted.

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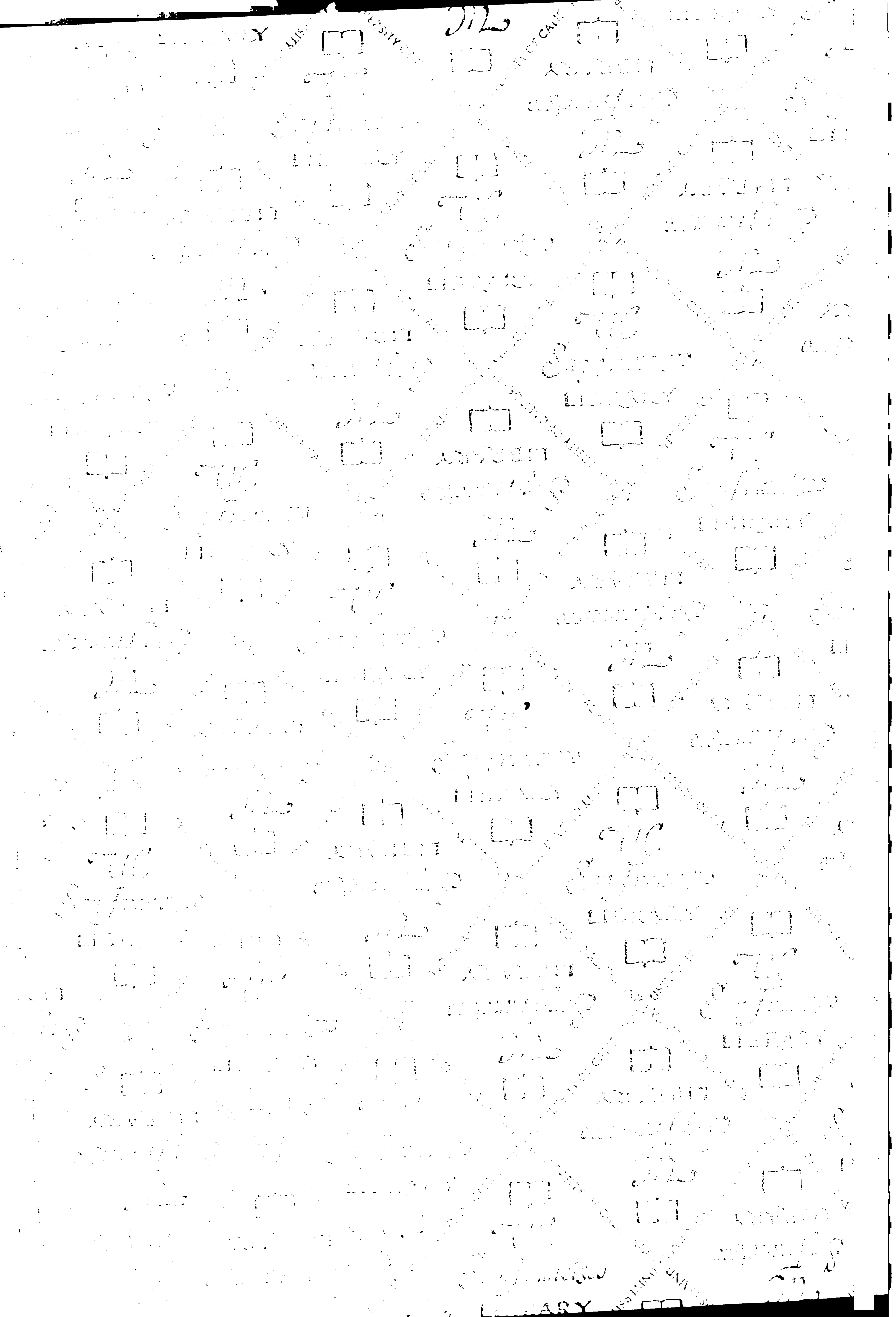

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