The Epoxygenases CYP2J2 Activates the Nuclear Receptor PPARα In Vitro and In Vivo

Jessica A. Wray1, Mary C. Sugden2, Darryl C. Zeldin3, Gemma K. Greenwood2, Salma Samsuddin2, Laura Miller-Degraff3, J. Alyce Bradbury3, Mark J. Holness2, Timothy D. Warner1, David Bishop-Bailey1*

1 Translational Medicine and Therapeutics, William Harvey Research Institute, Centre for Diabetes and Metabolic Medicine, Bart’s and the London, Queen Mary University of London, London, United Kingdom, 2 Division of Intramural Research, NEIHS/NIH, Research Triangle Park, North Carolina, United States of America

Abstract

**Background:** Peroxisome proliferator-activated receptors (PPARs) are a family of three (PPARα, -β/δ, and -γ) nuclear receptors. In particular, PPARα is involved in regulation of fatty acid metabolism, cell growth and inflammation. PPARα mediates the cardiac fasting response, increasing fatty acid metabolism, decreasing glucose utilisation, and is the target for the fibrate lipid-lowering class of drugs. However, little is known regarding the endogenous generation of PPAR ligands. CYP2J2 is a lipid metabolising cytochrome P450, which produces anti-inflammatory mediators, and is considered the major epoxygenase in the human heart.

**Methodology/Principal Findings:** Expression of CYP2J2 in vitro results in an activation of PPAR responses with a particular preference for PPARα. The CYP2J2 products 8,9- and 11-12-EET also activate PPARα. In vitro, PPARα activation by its selective ligand induces the PPARα target gene pyruvate dehydrogenase kinase (PDK4) in cardiac tissue. In vivo, in cardiac-specific CYP2J2 transgenic mice, fasting selectively augments the expression of PDK4.

**Conclusions/Significance:** Our results establish that CYP2J2 produces PPARα ligands in vitro and in vivo, and suggests that lipid metabolising CYPs are prime candidates for the integration of global lipid changes to transcriptional signalling events.

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* E-mail: d.bishop-bailey@qmul.ac.uk

Introduction

Exogenous PPAR activators include a number of fatty acids as well as a variety of eicosanoids, HETEs, HODEs, prostaglandins, and leukotrienes. A number of lipid-metabolising pathways have therefore been suggested as sources of PPAR ligands, however none really fully satisfy the criteria required for them to be regarded as ubiquitous endogenous PPAR ligand generators [1,2]. The cyclooxygenase, and 5-, 12/15-lipoxygenase pathways are good examples: prostanoid synthase enzymes and lipoxygenase isoforms have a highly tissue-specific expression pattern that do not fully match those of the PPARs [3]. Phospholipases [3] or lipoprotein lipase [4] can produce PPAR ligands from circulating lipoproteins. However, it is unclear whether these enzymes produce PPAR ligands universally. A very attractive hypothesis is that cytochrome P450 enzymes (CYPs) could provide the link. Similar to related eicosanoids, 8,9-, 11,12- and 14,15-EETs can functionally activate both PPARα ligands from circulating lipoproteins. However, it is unclear whether these enzymes produce PPAR ligands universally. A very attractive hypothesis is that cytochrome P450 enzymes (CYPs) could provide the link. Similar to related eicosanoids, 8,9-, 11,12- and 14,15-EETs can functionally activate both PPARα and PPARγ in vitro. It is not known however, which CYPs act as potential sources of the EETs, or whether CYPs or EETs mediate any functional effects on PPARα in vivo.

There are more than 500 CYP genes primarily associated with the metabolism and detoxification of foreign chemicals. A number of CYPs also catalyze the metabolism of lipids by epoxygenases, lipoxigenases-like, and ω- and ω-1-hydroxylase activities [5]. The CYP2 gene family of epoxygenases has approximately 25 members. CYP2J2 is the only CYP2J family member expressed in man, and it is localised in the heart and vasculature, throughout the gastro-intestinal and respiratory tracts and in the kidney [9,10], where it catalyses the conversion of arachidonic acid via the epoxygenase pathway to anti-inflammatory and vascular-protective EETs [10]. Here we show CYP2J2 activates PPARα in vitro and in vivo.

Results

CYP2J2 activates PPARα in vitro in an autocrine manner

Transient transfection of the CYP2J2 cDNA in HEK293 cells produced significant expression of CYP2J2 protein (Figure 1A). The combination of CYP2J2 with PPARα (Figure 1A), PPARδ or PPARγ (Figure 1B) induced a synergistic activation of PPAR reporter genes, with a marked preference in terms of absolute activity for PPARα activation (Figure 1A). pDR-1 was used as a reporter gene for PPARδ activation due to the reported lack of efficacy for pAGO on PPAR responses [11]. A functional
PPAR was required for this activation, as no significant reporter gene activation was seen in cells co-transfected with vector reporter gene lacking the PPRE (data not shown), or when cells were co-transfected with dominant-negative (DN)-PPARα (Figure 1A). Similarly, the activation of PPAR reporter gene by co-transfection of PPARα and CYP2J2 required active CYP2J2, as the epoxygenase inhibitor SKF525A caused a concentration-dependent inhibition of PPARα-CYP2J2 induced PPAR reporter gene activation (Figure 2). These endogenous products of CYP2J2 act in an intracellular manner, as only when cells are co-transfected so that PPARα and CYP2J2 are co-expressed together in the same cell is a significant synergistic activation of the PPAR reporter gene detected (data not shown).

CYP2J2 activates PPARα and inhibits NFκB activation

PPARα activation inhibits the activation of the pro-inflammatory and survival transcription factor NFκB [1,2]. IL-1β (10 ng/ml) induced NFkB reporter gene activation in HEK293 cells transfected with control plasmid cDNA. In cells transfected with the combination of CYP2J2 and PPARα, IL-1β induced NFkB activation was completely abolished (Figure 3). Inhibiting CYP2J2 with SKF525A (10 μM) restored the ability of IL-1β to activate NFkB in PPARα and CYP2J2 transfected cells (Figure 3).

EETs activate PPARα

8,9- and 11,12-EET at nM concentrations induced activation of PPAR in HEK293 cells in the presence, but not absence of transfected PPARα (Figure 4). The CYP products 14,15-EET, or 5,6-DiHETE, the stable metabolite of 5,6-EET (Figure 4) or the linoleic acid metabolite of CYP2J2 leukotoxin (Figure 5A) had no effect on PPARα reporter gene activation. Although, 14,15-EET had no effect in our hands, consistent with the previous report [5], the CYP4A hydroxylase 14,15-EET metabolite was a potent PPARα activator (Figure 5B). The activation of PPARα responses by 8,9-EET or 11,12-EET was completely reversed when cells were co-transfected with dominant negative DN-PPARα (Figure 4B).

Figure 1. CYP2J2 activates PPAR responses in vitro. CYP2J2 synergises with PPARα (A), PPARβ/δ or PPARγ (B) to induce PPAR reporter gene activation. Dominant-negative (DN-)PPARα co-transfected into cells with CYP2J2 and PPARα abolished the ability of CYP2J2 to activate PPARα (A). HEK293 cells were transfected with PPAR reporter genes (pACO.gLuc for PPARα and –, γ, and pDR-1 for PPARβ/δ), and pcDNA-CYP2J2, pCMX-PPARα, pCMX-PPARβ/δ, or pCMX-PPARγ alone, or co-transfected with CYP2J2 and the individual PPAR (2J2+α, 2J2+δ, and 2J2+γ). All PPAR reporter gene activation studies are represented as fold luciferase from PPAR response element transfection alone (control), normalised to total protein at 20 h post-transfection. Total plasmid DNA for transfections was normalised using pcDNA3.1 throughout. Data represents n = 9−12 replications from 4 separate experiments. * denotes p<0.05 by one-sample t-test between control and transfected cells. Inset (A) is Western blot for CYP2J2 and RT-PCR for PPARα in cells with either mock transfected (−) or pcDNA3.1 or cells transfected with pcDNA-CYP2J2 and pcDNA-mPPARα (+).

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Figure 2. Synergistic activation of PPARα by CYP2J2 requires an active CYP2J2. Cells were co-transfected with PPAR reporter gene, CYP2J2, and PPARα, and treated with the epoxygenase inhibitor SKF525A (0–30 μM). SKF525A caused a concentration-dependant inhibition of PPAR reporter gene activation. Data represents n=9−12 replications from 4 separate experiments.

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PPAR activation induces PDK4 in cardiac tissue in vitro

PDK4 is a tissue specific PPARβ target gene that facilitates fatty acid oxidation by “sparring” pyruvate for oxaloacetate formation [12,13]. The highly selective PPARβ ligand GW6476 induced PDK4 mRNA in mouse cardiac tissue in culture in vitro (Figure 6); an effect which was abolished by co-incubation with the selective PPARβ antagonist GW6471 (Figure 6), or if tissue was used from PPARβ knockout mice (data not shown).

Cardiac-specific CYP2J2 transgenic mice have an elevated PPARβ response during fasting

The fasting response is a model of PPARβ activation in vivo as a decline in insulin levels and/or a rise in lipid fuel availability facilitates PPARβ activation and the up-regulation of PDK4. Moreover, this marked up-regulation of PDK4 expression in response to fasting is absent in PPARβ knockout mice [14]. Therefore, PDK4 is a robust index of PPARβ activation in vivo. Expression of the related proteins PDK1, PDK2 and E1a are not regulated by PPARβ, and were used as controls.

Cardiomyocyte-specific CYP2J2 transgenic (Tr) mice have been generated and have a normal heart anatomy and contractile function [15]. Fed CYP2J2 Tr mice had no altered expression of PDK1, -2, -4 or E1α expression in the heart, kidney, or liver compared to wild type controls (Figure 6; and data not shown). In fasted mice, PDK4 protein expression was selectively up-regulated in the heart (Figure 7A and B), kidney and liver (Figure 7C) of wild type mice. In response to fasting, wild type male mice had an approximate 2–3 fold higher induction of cardiac PDK4 expression than female mice (9.3±2.4 male compared to 3.7±0.9 female; relative fold induction; n = 4–6). The basal PDK4 levels between male and female mice were equivalent, so this gender difference in PPARβ activity/PDK4 expression upon fasting is gender specific.

Upon fasting, male wild type and CYP2J2 Tr mice, had a comparable induction of cardiac PDK4 protein (9.3±2.4 wild type; 7.4±1.3 CYP2J2 Tr; fold expression; n = 4). In contrast, female CYP2J2 Tr exhibited a much greater induction of cardiac PDK4 protein upon fasting compared to wild-type controls (Figure 7). PDK1, PDK2 and E1α protein expression were unchanged by 24 h of fasting in any tissue tested (Figure 6, and data not shown).

Up-regulation of PDK4 expression is linked to a decline in circulating insulin concentrations [13,14,16]. Upon fasting, both
plasma insulin and blood glucose levels fell to equivalent levels in wild type and CYP2J2 Tr mice (Table 1). Fibrate administration is associated with suppression of circulating triglyceride levels [2], however, neither triglyceride nor non-esterified fatty acid concentrations were affected in wild type or CYP2J2 Tr mice (Table 1). Since no systemic metabolic differences were observed, any changes in PPAR response we conclude are due to the local cardiac specific activity of CYP2J2 in the transgenic mouse.

Endogenous CYPs and the cardiac fasting response

The use of pharmacological CYP inhibitors in vivo is complicated due both to lack of specificity of inhibitors and the great heterogeneity in CYP enzymes between species. We did however examine the fasting response in CYP2J5 knockout mice [17], the only murine CYP2J family member where a knockout has been generated. There was however no difference in the circulating blood glucose levels, or the heart, liver or kidney PDK4, or heart E1a expression levels (Table S1) between knockout and wild type male or female mice either under fed or fasted conditions.

Discussion

The nature of endogenous PPAR ligands are still far from clear, as is whether PPARs act as general lipid sensors or whether high affinity ligands exist in the body. Here we show CYP2J2 can act as an endogenous epoxygenase source of high affinity PPAR ligands. When co-transfected together in vitro, CYP2J2 induces PPAR, in particular PPARα, activity. In cardiac-specific-CYP2J2 Tr mice, fasting greatly elevates the PPARα target gene PDK4. These results do not exclude a role for CYP2J2 or other CYPs as regulators of PPARβ/δ or γ. Indeed we found CYP2J2 can activate PPARδ and PPARγ, albeit it to lower absolute levels then...
PPARα in our transfection system) and it is known that lipid CYP products (though not the CYP responsible) are endogenous PPARγ activators, induced by laminar shear of human endothelial cells in vivo [7,8].

Unlike other proposed PPAR ligand-generating enzymes (e.g. 12/15-lipoxygenase; [18]), CYP2J2 did not require additional arachidonic/linoleic acid substrate(s), suggesting a high level of functional coupling between the epoxygenases and PPARs. We also show for the first time a functional in vivo response for a PPAR ligand generating system. Our results do not rule out the role of other enzymes, such as phospholipases [3] or lipoprotein lipases [4] implicated in PPARα ligand generation. These enzymes are likely to produce PPAR ligands in parallel to CYPs, and/or supply free fatty acid substrates for CYPs to utilise.

8,9-EET, and 11,12-EET, but not 14,15-EET activated PPARα. 11,12-EET in contrast to 14-15-EET is highly anti-

Figure 7. CYP2J2 augments PPARα in vivo in the fasting model of PPARα activation. Female wild-type or cardiac-specific CYP2J2 Tr mice were allowed food and water ad libitum, or fasted for 24 h. Figure (A) shows representative western blots for 2 of the 6 animals tested for PDK4, PDK1 (antibody has cross reactivity with PDK4 indicated by changes in the lower band) and E1a in the hearts of wild type (WT) or cardiac-specific CYP2J2 Tr mice (2J2); specific bands are identified by the arrows. Figures show the relative protein expression of PDK4 in the heart (b), kidney, and liver (C as indicated) and PDK1 and E1a in the heart (as indicated) in wild type (WT) and cardiac-specific CYP2J2 Tr (2J2), fed and fasted female mice. Data represents relative densitometry of protein compared to wild type fed controls for n = 4–6 separate animals in each group. Only the PPARα target gene PDK4 was induced on fasting both in the heart and kidney. Upon fasting there was an approximate doubling of PDK4 in the hearts (b), but not the kidney (e), or liver (f) of female cardiac specific CYP2J2 transgenic mice. * denotes p<0.05 by unpaired t-test between the fasting response in wild-type and CYP2J2 transgenic mice.

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Table 1. Blood parameters in fed and fasted wild type and cardiac-specific CYP2J2 transgenic mice (CYP2J2).

|                  | Fed       | Fasted    |
|------------------|-----------|-----------|
|                  | Wild Type | CYP2J2    | Wild Type | CYP2J2    |
| Insulin (μU/ml)  | 13±2      | 11±2      | 3±1*      | 3±1*      |
| Glucose (mM)     | 13±0.3    | 12.5±0.6  | 4.5±0.6*  | 5.5±0.5*  |
| NEFA (mM)        | 1.13±0.04 | 1.12±0.05 | 0.91±0.18 | 0.92±0.14 |
| Triglycerides (mM) | 0.7±0.1  | 0.9±0.1   | 0.5±0.2   | 0.7±0.1   |

Wild type and CYP2J2 mice have similar basal levels of plasma insulin, blood glucose, non-esterified fatty acids (NEFA) and triglycerides. Following 24 h of fasting, plasma insulin and blood glucose dropped in both wild type and CYP2J2 mice to equivalent levels, while non-esterified fatty acids and triglycerides remained relatively unchanged. This data represents the mean±s.e.m. for n = 6 animals per group. * denotes p<0.05 by unpaired t-test between fed and fasted levels.

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Inflammatory and vascular protective [10,19]. Therefore, we propose that PPARγ is a likely anti-inflammatory target for 11,12-
EET and CYP2J2. Indeed we found the combination of PPARγ and CYP2J2 abolished IL-1β induced NFκB activation; a central pro-inflammatory transcription factor and PPARγ target [1,2].

Many EETs, including 14,15-EET, can also act as cellular hyperpolarising agents [9,19], however, since 14,15-EET was inactive in our system, hyperpolarization mechanisms are highly unlikely to be involved. Our results are consistent with previous findings that EETs and some of their metabolites can directly bind and activate PPARγ [5–7]. Although 14,15-EET did not activate PPARγ in our hands, its CYP4A hydroxylase CYP2J2 metabolite 20,14,15-HEET, was the post potent EET product we tested. EETs can be rapidly metabolised by at least 10 different intracellular pathways, and it is estimated that when given exogenously <10% is available free within the cell [9]. Our results therefore do indicate that alternative CYP2J2 products exist or further unknown EET metabolites [5,7,8] are potential endogenous PPARγ activators.

There is considerable species difference between CYPs in man and the mouse. CYP2J2 is the human isoform, in the mouse the situation is far more complex with up to 8 putative homologues (CYP2J5 – CYP2J13; [20]). Since epoxygenases are ubiquitous and potentially have many roles, examining the role of endogenous epoxygenases especially in the mouse is extremely difficult. We therefore chose as our main model the established cardiac specific CYP2J2-Tr mouse. We did however test the recently described CYP2J5 knockout mouse [17], the only CYP2J knockout available. However, we did not detect a change in the fasting response or in PDK4 expression, suggesting a lack of involvement of CYP2J5 in PPARγ ligand generation or the more likely compensation from other mouse CYP2J or CYP2C EET-producing epoxygenases that are present.

The selective augmentation of PDK4 in cardiac-specific CYP2J2-Tr mice occurred only in female mice. The fasting PPARγ response was much stronger in males, and we believe maximally activated. Interestingly, our results are consistent with known gender differences in cardiac PPARγ responses in the mouse. Pharmacological stress of the hearts of PPARγ knockout mice with Etomoxir to prevent mitochondrial fatty acid import, results in cardiac lipid accumulation and a 100% mortality of male mice but only 25% mortality of female mice [21].

In conclusion, in vivo CYP2J2 activates PPARγ without exogenous stimuli. In vitro CYP2J2 does not appear to be rate-limiting as PPARγ target gene (PDK4) expression is only augmented in cardiac-specific CYP2J2 transgenic mouse upon fasting. Therefore, CYP2J2 in vivo is an enzyme apparently quiescent, but capable or responding to changes in lipid availability to generate endogenous PPARγ agonists and thereby integrate transcriptional fasting events. CYP2J2 products activate PPARs, in particular PPARγ in vivo and in vitro. As lipid-metabolising CYP enzymes have a widespread expression, utilise a variety of lipid substrates and produce a large family of oxidised biologically active lipid mediators, we suggest that lipid metabolising CYPs may represent an important source of PPAR ligands throughout the body.

PPARγ is known as a controller of lipid metabolism and inflammation. Linking CYP2J2 and epoxygenases to PPARγ has many potential clinical implications. Variants of CYP2J2 with lower activity are known in some populations to be linked to an increased risk of coronary artery disease [22,23]. Epoxygenases such as CYP2J2 in addition to metabolising arachidonic acid may also regulate xenobiotic drug metabolism. Understanding how epoxygenases are regulated, the mediators they produce, and where they will, give us novel information on biomarkers for dyslipidaemia and inflammation, allow us to understand side-effects of drugs metabolised by epoxygenases, and help us to design novel PPARγ ligands based on the structure of high affinity EETs and their metabolites.

Materials and Methods

Materials

HEK293 cells were from ATCC. pEGFP-N1 and pNFkB-luc were from Clontech. pGL-2 was from Promega (Southampton, UK). pCMXmPPARγ, pACOG-Luc, and h6/29 hPPARγ were gifts from Dr Ruth Roberts (AstraZeneca; Macclesfield, U.K.), pCMX-mPPARδ was from Dr Ronald Evans (Salk Institute, La Jolla, USA), pDR-1 was from Dr Bert Vogelstein (Johns Hopkins University, Baltimore, USA), pCMX-mPPARγ was from Dr Christopher Glass (UCSD, San Diego, USA). Novafector was from VennNova (Pompano Beach, FL, USA). Rabbit polyclonal anti-CYP2J2 [24] and PDK2 [25] were raised as previously described. Anti-PDK4 antibodies were generously provided by Professor Bob Harris (Indiana University, USA). CYP2J2 metabolites were from Cayman Chemical Company (Axzorra, Nottingham, UK). SKF525A was from Biomol (Affiniti Research Products, Exeter, UK). Plasma insulin ELISA was from Mercodia (Uppsala, Sweden). Plasma glucose kits were from Roche Diagnostics (Lewes, East Sussex, UK). WAKO kits for plasma triacylglycerol were from Alpha Labs, (Eastleigh, Hants, UK). ECL reagents, hyperfilm were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Bradford reagents for protein estimation were purchased from BioRad Ltd. (Hemel Hempstead, Hertfordshire, UK). All other reagents were from Sigma (Poole, Dorset, UK).

Cell culture and transfections

HEK293 were maintained in DMEM containing, supplemented with Antibiotic/Antimycotic mix, and 10% FCS; 37°C; 5% CO₂; 95% air. Cells were transfected with Novafector and Luciferase assays performed, essentially as previously described [26] but modified for a 96 well format [27]. Luciferase activity was normalised to cell protein (BCA assay). Global cellular changes, luciferase, and GFP expression were recorded on a Nikon TE2000 inverted florescent microscope, with a SPOT RT digital camera. In some experiments organ culture of mouse cardiac tissue was performed, essentially as previously described [28].
Ethics Statement
All animal studies were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Animal experiments
Cardiace-specific CYP2J2 transgenic mice (α-MHC promoter driven) and littermate wild type C57BL/6J controls [15] along with CYP2J5 knockout mice [27] have been described previously. Animals were allowed food and water ad libitum or fasted for 24 h. In some experiments mice were given SK525A (30 mg/kg; ip) or vehicle (sterile PBS) immediately prior to initiation of the 24 h fasting/non-fasting period.

Immunoblotting and assays
PD1K1, -2, -4 and Ela and CYP2J2 protein levels were determined as previously described [13,24,25]. For animal experiments each representative immunoblot presented are results from a single gel exposed for a uniform duration, and each lane represents a preparation from a different mouse. Plasma immunoreactive insulin concentrations were measured by ELISA, using rat insulin as a standard. Plasma glucose concentrations were determined by a glucose oxidase method. Plasma NEFA and TAG levels were determined spectrophotometrically using commercial kits.

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Author Contributions
Conceived and designed the experiments: MS DZ LMD JAB MH TW DBB. Performed the experiments: JW DZ GG SS MH JAB DBB. Analyzed the data: JW MS GG SS TW DBB. Contributed reagents/materials/analysis tools: DZ DBB. Wrote the paper: JW MS MH DBB.