Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 461 (2007) 104–112
ABB
www.elsevier.com/locate/yabbi

Catalytic characterization and cytokine mediated regulation of cytochrome P450 4Fs in rat hepatocytes

Auinash Kalsotra [a,*], Sayeepriyadarshini Anakk [a], Chad L. Brommer [b], Yasushi Kikuta [c], Edward T. Morgan [b], Henry W. Strobel [a]

[a] *Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, TX 77225, USA*
[b] *Department of Pharmacology, Emory University, Atlanta, GA 30322, USA*
[c] *Department of Applied Biological Science, Fukuyama University, Fukuyama, Hiroshima 7290292, Japan*

Received 9 December 2006, and in revised form 5 February 2007
Available online 15 March 2007

Abstract

Cytochrome P450 (CYP) 4F mediated leukotriene B_4 (LTB_4) metabolism modulates inflammation during injury and infection. Here we show that in addition to LTB_4, the recombinant rat CYP4Fs catalyze ω-hydroxylations of lipoxin A_4, and hydroxyeicosatetraeonic acids. CYP4F gene regulation studies in primary hepatocytes reveal that pro-inflammatory cytokines interleukin (IL) -1β, IL-6 and tumor necrosis factor (TNF) -α produce a general inductive response whereas IL-10, an anti-inflammatory cytokine, suppresses CYP4F expression. The molecular mechanism behind IL-6 related induction of CYP4F4 and 4F5 is partially signal transducer and activator of transcription 3 (STAT3) dependent. When hepatocytes are subjected to high concentrations of LTB_4 or prostaglandin E_2, lipid mediators of inflammation, only an increase in CYP4F5 mRNA expression is observed. Collectively, the results from isozyme activity and substrate driven CYP4F induction do not support the notion that an autoregulatory pathway could control the excessive concentrations of LTB_4 during an inflammatory challenge to hepatocytes.

Keywords: Cytochrome P450 4F; Eicosanoids; Cytokines; STAT3; Inflammation; Interleukin-6; Leukotriene B_4

Although inflammation is an integral part of the host defense mechanism against pathogens or tissue injury, it often produces a myriad of unwanted complications. Immediately after injury or bacterial infection, polymorphonuclear leukocytes (PMNLs) are activated and recruited to the site of inflammation by soluble mediators causing cell adherence, transcapillary migration and chemotaxis [1]. Eicosanoids such as LTB_4 and hydroxyeicosatetraenoic acids (HETEs) are potent chemotactic agents, which act primarily on PMNLs to enhance their adherence to endothelial tissue [2,3]. Metabolism of LTB_4 by cytochrome P450 (CYP)[1] 4F3 to the 20-hydroxy derivative in the PMNL leads to dramatic loss of its chemotactic and aggregation activity [4,5]. Additionally CYP4Fs are responsible for removal of circulating LTB_4 in the liver, a major organ for this inflammatory mediator inactivation [6–11]. However, little is known about the regulation of hepatic eicosanoid uptake and their subsequent breakdown during either systemic or localized inflammatory conditions.

* Corresponding author. Fax: +1 713 500 0652.
E-mail address: auinash.kalsotra@uth.tmc.edu (A. Kalsotra).

[1] *Abbreviations used:* CYP, cytochrome P450; QRTPCR, quantitative real-time PCR; RXR, retinoic X receptor; PPAR, peroxisome proliferator activated receptor; GR, glucocorticoid receptor; ER, estrogen receptor; HNF, hepatocyte nuclear factor; STAT3, signal transducer and activator of transcription 3; BCA, bicinchoninic acid; BSA, bovine serum albumin; IL-1, interleukin-1; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; YNBD, yeast nitrogen base and dextrose; *TSS*, transcription start site.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.02.027

Unlike CYP4As, which are induced by hypolipidemic drugs and peroxisomal proliferators [12], the CYP4Fs are repressed after these treatments [13–15]. Recently, however, two separate reports confirmed that a*ll-trans*-retinoic acid induces CYP4F3 expression and increases its associated protein activity in differentiating human leukemia-60 cells [16,17]. Christmas et al. have elegantly demonstrated that the *CYP4F3* gene encodes two functionally distinct enzymes that differ only by the selection of exon 4 (CYP4F3A) or exon 3 (CYP4F3B) [18,19]. CYP4F3A and 4F3B are generated from distinct transcription start sites in neutrophils and liver [18] and the alternate usage of exons 3 and 4 results in differential substrate preference and subsequent metabolism [19].

Similar to the human isoforms, the heterologously expressed rat CYP4Fs are quite active against LTB_4 [15,20–24]. The goals of this study were to test the catalytic activities of rat CYP4Fs against the eicosanoid substrates considered to play a role in inflammation. Secondly, we posited that the CYP4F subfamily might be able to modulate the extent of inflammation through control of these mediators. One way to provide support for this hypothesis is to test whether the expression of CYP4Fs changes during an inflammatory response, since these changes may be required to adjust the levels of these inflammatory mediators.

We have previously shown that lipopolysaccharide administration produces an isoform specific response in hepatic and renal CYP4F mRNA expression in both mice and rats [6,25]. The present study investigates CYP4F regulation upon pro- and anti-inflammatory cytokine challenges. We also tested whether *in silico*-predicted binding of IL-6 response element binding proteins such as the signal transducer and activator of transcription 3 (STAT3) contribute to the IL-6 mediated effects on *CYP4F* gene expression. Finally we explored CYP4F participation in an autoregulatory pathway that might control LTB_4 metabolism.

Materials and methods

Materials

Male Fischer 344 (F344) rats (150–200 g) from Harlan Sprague–Dawley (Indianapolis, IN) were used for hepatocyte isolation. 5′ Rapid amplification of cDNA ends by PCR (5′ RACE) kits, *Superscript II* and *Taq* DNA polymerase, Cell culture medium (Waymouth's MB 752/1), insulin, antibiotics, murine recombinant IL-1β, IL-6, IL-10, TNF-α, Type IV collagenase and TA cloning vector kits were purchased from Invitrogen Life Technologies (Carlsbad, CA). Purified LTB_4, prostaglandin (PG) E_2 and arachidonic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). HETEs, lipoxins (LXs) and PGs were obtained from Funakoshi Co. (Tokyo, Japan). STAT3 inhibitor peptide was obtained from EMD Biosciences (San Diego, CA). CYP4F standard amplicons, primers and probes were custom synthesized by IDT DNA Technology Inc. (Coralville, IA). *Saccharomyces cerevisiae,* strain AH22 (*a, leu2, his4, can1, cir*1), was purchased from ATCC (Manassas, VA). The yeast expression vector pAAH5 carrying the ADH1 promoter and terminator was a gift from Dr. B.D. Hall, University of Washington. All other chemicals utilized in the experiments reported here, where not specifically defined, were of reagent grade quality or higher.

Recombinant expression of CYP4Fs in yeast cells

The cDNA clones for rat CYP4Fs were obtained as previously described [15,21]. *Hin*dIII sites were engineered by polymerase chain reaction (PCR) on each end of CYP4F1, 4F4 and 4F5 cDNAs. The purified CYP4F constructs with *Hind*III sites were ligated into TA cloning vector PCR 2.1, transformed into *Escherichia coli* (DH5α) cells and grown on Luria–Bertani–Ampicillin selection media. Positive clones identified by *Eco*RI digestion were sent for sequencing (SeqWright Houston, TX) and the clones containing the complete sequence for each rat CYP4F isoform without any mutations were chosen for expression. The modified CYP4F cDNA fragments with *Hin*dIII sites at both ends were digested from PCR 2.1 and inserted into the *Hin*dIII sites of the pAAH5 vector. The CYP4F constructs were transfected into 100 μL competent yeast cells in the presence of 50% polyethylene glycol (PEG) 400 with a 1 h incubation at 30 °C followed by 5 min incubation at 42 °C. This mixture was plated on sterile YNBD agar plates (0.67% yeast nitrogen base, 2% glucose, 20 mg/L histidine and 2% agar) and incubated at 30 °C for 3 days. Several colonies were picked and cultivated in concentrated SD medium (8% glucose, 5.4% nitrogen base without amino acids, and 160 mg/mL histidine) at 30 °C with continuous shaking at 250 RPM [5].

Microsome preparation

Yeast microsomes were prepared as described previously [5]. Briefly, cells were gently washed three times with ice cold sterile water, centrifuged at 3000*g* and resuspended in ice cold $S_{0.65}$ buffer (0.65 M sorbitol, 10 mM Tris, 0.1 mM EDTA and 0.1 mM DTT) containing a cocktail of protease inhibitors (1 mM PMSF, 1 μg/mL leupeptin and 0.7 μg/mL pepstatin). The resuspended cells were broken by passage through a French press three times at 4 °C. The broken cells were centrifuged at 12,000*g* for 20 min and the supernatant fraction was collected. The supernatant was further centrifuged at 100,000*g* for 45 min, the pellet washed in fresh buffer and again centrifuged at 100,000*g* for 45 min. The final pellet was dissolved in buffer $S_{0.65}$ (1/10 of starting volume) lacking the protease inhibitors. Control microsomes were prepared from yeast transformed with the plasmid without an insert. The total protein concentration in microsomes was determined with the bicinchoninic acid procedure using bovine serum albumin (BSA) as the standard.

Eicosanoid activity assays

The activity assays for the eicosanoid substrates were performed using high performance liquid chromatography (HPLC) methods as previously described [26–31]. LTB_4 ω-hydroxylase activity was determined by incubating the reaction mixture containing CYP4Fs (6–10 pmol), 20 mM Hepes buffer (pH 7.5) including 340 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol (DTT), 1 mM NADPH, and 60 μM LTB_4, in a total volume of 0.1 mL, at 37 °C for 20 min. The reaction products were then extracted with ethyl acetate and measured. LXA_4, LXB_4 and HETE ω-hydroxylation activities were determined using 60 μM concentrations of each substrate by the methods described earlier. Prostaglandin ω-hydroxylating activities were determined in 0.1 M sodium phosphate reaction buffer (pH 7.4) with 100 μM substrates. No significant difference in activities was observed upon addition of cytochrome b5 or cytochrome P450 reductase to the assay system due to the presence of endogenous NADPH-dependent reductases in the microsomes. For kinetic analysis of LTB_4 ω-hydroxylation the reaction mixtures were incubated for 30 min. The activities were in the linear range for the incubation time from 0 to 40 min. The range of substrate concentrations used for the kinetic analysis were 0, 10, 20, 40, 60, 100 and 150 μM. Kinetic parameters were calculated using Hanes–Woolf's plot. Each data point is a mean of at least two independent determinations.

CYP4F transcription start site (TSS) identification by 5′ RACE

To determine the transcriptional initiation site of *CYP4F* genes, the 5′ RACE system was used according to the manufacturer's instructions.