Prostaglandins and Other Lipid Mediators xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

journal homepage: www.elsevier.com/locate/prostaglandins

Original Research Article

Prostaglandins and Other Lipid Mediators (POLM), Special Issue of the 6th European workshop on lipid mediators
Role of Müller cell cytochrome P450 2c44 in murine retinal angiogenesis

Jiong Hu[a,b,1], Alexandra Geyer[a,1], Sarah Dziumbla[a,b], Khader Awwad[a], Darryl C. Zeldin[c], Wolf-Hagen Schunck[d], Rüdiger Popp[a], Timo Frömel[a,b], Ingrid Fleming[a,b,*]

[a] Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Frankfurt am Main, D-60596, Germany
[b] German Center of Cardiovascular Research (DZHK) Partner site Rhein-Main, Frankfurt am Main, Germany
[c] Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA
[d] Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Str 10, 13092 Berlin, Germany

ARTICLE INFO

Keywords:
Docosahexenoic acid
Lipid mediators
Müller glia cells
Retinal angiogenesis
Soluble epoxide hydrolase

ABSTRACT

Polyunsaturated fatty acids (PUFA) and their cytochrome P450 (CYP450) metabolites have been linked to angiogenesis and vessel homeostasis. However, the role of individual CYP isoforms and their endogenous metabolites in those processes are not clear. Here, we focused on the role of Cyp2c44 in postnatal retinal angiogenesis and report that Cyp2c44 is highly expressed in Müller glial cells in the retina. The constitutive as well as inducible postnatal genetic deletion of Cyp2c44 resulted in an increased vessel network density without affecting vessel radial expansion during the first postnatal week. This phenotype was associated with an increased endothelial cell proliferation and attenuated Notch signaling. LC–MS/MS analyses revealed that levels of hydroxydocosahexaenoic acids (HDHA), i.e., 10-, 17- and 20-HDHA were significantly elevated in retinas from 5 day old Cyp2c44$^{-/-}$ mice compared to their wild-type littermates. Enzymatic activity assays revealed that HDHAs were potential substrates for Cyp2c44 which could account for the increased levels of HDHAs in retinas from Cyp2c44$^{-/-}$ mice. These data indicate that Cyp2c44 is expressed in the murine retina and, like the soluble epoxide hydrolase, is expressed in Müller glia cells. The enhanced endothelial cell proliferation and Notch inhibition seen in retinas from Cyp2c44-deficient mice indicate a role for Cyp2c44-derived lipid mediators in physiological angiogenesis.

1. Introduction

The role of cytochrome P450 (CYP) enzymes in the vasculature was initially linked to the acute, endothelium-dependent regulation of tone by mechanisms involving endothelial cell hyperpolarization or an endothelium-derived hyperpolarizing factor [1,2]. Since those first reports it has become clear that metabolites of polyunsaturated fatty acids (PUFAs) generated by CYP enzymes can affect numerous signal transduction cascades as well as angiogenesis (for review see [3]). However, despite the fact that epoxides of arachidonic acid (the epoxyeicosatrienoic acids or EETs) exert potent angiogenic effects *in vitro* [4–7], as well as when applied to control animals e.g. in Matrigel plugs [6–8], there is a lack of evidence for CYP-derived metabolites in the regulation of physiological angiogenesis *in vivo*. Moreover, the angiogenic effects of the ω-6 PUFA epoxides are reportedly reversed in the presence of ω-3 fatty acids [9,10] or cyclo-oxygenase inhibitors [11].

To date, most information about the role of endogenously generated CYP metabolites in angiogenesis has been obtained using animals lacking the soluble epoxide hydrolase (sEH), the enzyme that metabolizes fatty acid epoxides to their corresponding diols. sEH$^{-/-}$ mice demonstrate elevated tissue and circulating levels of EETs [12,13], but

Abbreviations: CYP, cytochrome P450; DHDP, dihydroxydocosapentaenoic acid; DHET, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EDP, epoxydocosapentaenoic acid; EEQ, epoxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; $E_{Na}C$, epithelial sodium channel; EPA, eicosapentaenoic acid; EpOME, epoxyoctadecenoic acid; ERG, ETS-related gene; GCL, ganglion cell layer; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; P, postnatal day; PCL, retinal pigment cell layer; pH3, phosphorylated histone 3; PUFA, polyunsaturated fatty acids; sEH, soluble epoxide hydrolase; VEGF, vascular endothelial cell growth factor

* Corresponding author at: Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Theodor-Stern-Kai 7, D-60590, Frankfurt am Main, Germany.
E-mail address: fleming@em.uni-frankfurt.de (I. Fleming).
[1] *Authors contributed equally.

http://dx.doi.org/10.1016/j.prostaglandins.2017.04.002
Received 21 January 2017; Received in revised form 17 April 2017; Accepted 19 April 2017

rather than being associated with increased angiogenesis, the development of the retinal vascular plexus was attenuated in sEH$^{-/-}$ mice [9]. Similarly, the vascularization of Matrigel plugs and the vascular repair and recovery of blood flow after ischemic injury were attenuated in sEH$^{-/-}$ mice, especially following mobilization of bone marrow-derived progenitor cells [14]. A role for CYP-derived lipid mediators has been demonstrated *in vivo* e.g. in a humanized mouse models e.g. in mice overexpressing CYP enzymes [15,16] but less has been done to assess the role of endogenously expressed CYP enzymes in regulating endothelial cell proliferation and angiogenesis in CYP knockout mice, simply because knocking down one specific CYP enzyme frequently results in the upregulation of another that can functionally compensate for it [17,18].

The aim of this study was to determine the role of endogenous CYP-derived mediators in physiological angiogenesis by targeting the expression of a CYP enzyme using a combination of constitutive and inducible knockout mouse models. Cyp2c44 was chosen as a target given that: it is closely related to the human CYP2C8 and CYP2C9 isoforms [19], the enzyme generates EETs [20], and previous reports linking this enzyme with tumor growth [21] and signaling induced by vascular endothelial cell growth factor (VEGF) [22].

2. Materials and methods

2.1. Chemicals and reagents

All of the fatty acids, i.e., EETs, EpOMEs and HDHAs were obtained from Cayman Chemicals Europe (Tallinn, Estonia). NADPH was from Applichem (Darmstadt, Germany) and tamoxifen was from Sigma Aldrich (Deisenhofen, Germany). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Aldrich. The anti-Cyp2c44 antibody was generated as described [23], and the antibody directed against aquaporin 4 (AQP4) was from Santa Cruz (TX, USA), the antibody against CD31 was from BD biosciences (Heidelberg, Germany), the phospho histone 3 antibody was from Merck Millipore (Darmstadt, Germany), and the anti-ETS-related gene (ERG) antibody from Abcam (Cambridge, UK).

2.2. Animals

C57BL/6 mice (6–8 weeks old) were purchased from Charles River (Sulzfeld, Germany). Floxed Cyp2c44 mice were generated by TaconicArtemis (Cologne, Germany) using C57BL/6 embryonic stem cells for gene targeting. The positive selection cassettes flanked by FRT/F3 sites were deleted by crossing with a ubiquitously expressing FLP1 recombinase strain (TaconicArtemis). The conditional knock out allele was confirmed by PCR with primers flanking the loxP site modified genomic locus using the following primers: 5′-TTATATCATTGCTAGTG GCAATCG-3′, and 5′-GGCAGTTCTCTTCTATGTATGTGC-3′ resulting in a 233 bp product lengths for the wild-type allele and a 384 bp product for the conditional allele (loxP and F3). The Cre dependent recombination (excision) was assessed with PCR using the primer pair 5′-TTATATCATTGCTAGTGGCAATCG-3′ and 5′- TGGAGCTCTGTTCAGT AGTGTTCC-3′ that results in a (305 bp) product for the recombined locus. Genotyping was performed on genomic DNA derived from tail biopsies with the following PCR parameters: 95 °C for 5 min, followed by 35 cycles, at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min. Genotyping PCRs were performed with the following conditions 95 °C for 5 min, followed by 30 cycles, at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min (Supplementary Fig. S1). Floxed Cyp2c44 mice were crossed with Gt(ROSA)26Sortm16(Cre)Arte mice expressing Cre under the control of the endogenous Gt(ROSA)26Sor promoter (TaconicArtemis) to generate mice globally lacking Cyp2c44 (Cyp2c44$^{-/-}$) or with Gt(ROSA)26Sortm(CreERT2)Arte mice (TaconicArtemis) to generate inducible Cyp2c44 knockout (Cyp2c44^{iKO}) mice.

Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the U. S. National Institutes of Health (NIH publication no. 85-23). All experiments were approved by the governmental authorities (Regierungspräsidium Darmstadt: F28_38). Age- and strain-matched animals of both sexes (usually littermates) were used throughout. For the isolation of organs, mice were sacrificed using 4% isoflurane in air and subsequent exsanguination.

2.3. Immunohistochemistry

2.3.1. Retina

Retinas were isolated, fixed in PBS containing 4% PFA for 2 h at room temperature, washed in PBS containing 0.5% Triton X-100, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 1 mmol/L $MnCl_2$ (pH 6. 8), permeabilized using 0.5% Triton X-100 overnight at 4 °C, and blocked with the “mouse on mouse” blocking reagent (Vector Laboratories) before addition of the anti-Cyp2c44 antibody [23] overnight at 4 °C. Afterwards samples were incubated with FITC-labelled Isolectin B4 and a fluorophore-conjugated secondary antibody (Alexa Fluor, Invitrogen, Karlsruhe, Germany).

2.4. Müller cells

Retinas were isolated and digested immediately with collagenase I (100 U/ml, Biochrom, Berlin, Germany) in DMEM/F12 medium for 30 min at 37 °C. The reaction was stopped by the addition of DMEM with 10% FCS and the tissue was further dissociated by gentle trituration using a sterile Pasteur pipette [24]. Cells were fixed and permeabilized by using FIX & PERM cell fixation and cell permeabilization kit (Invitrogen) according to the standard protocol. Antibodies directed against Cyp2c44 and AQP-4 were used at 1:200 and 1:500 dilution, respectively.

2.5. Retinal angiogenesis

Freshly isolated eyes from 2, 5 and 7 day old wild-type and Cyp2c44$^{-/-}$ littermates were fixed in PBS containing 4% formalin for 2 h at room temperature. Thereafter, the retinas were isolated, washed in PBS supplemented with 0.5% Triton X-100, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 1 mmol/L $MnCl_2$ (pH 6. 8) and blocked as well as permeabilized in 1% BSA and 0.5% Triton X-100 overnight at 4 °C. Endothelial cells were visualized using FITC-labelled Isolectin B4 (1:100, Sigma-Aldrich). Samples were visualized using a confocal microscope (LSM 780 scanning confocal microscope; Zeiss, Jena, Germany) and all quantification was performed using high resolution images and Axiovision software (Zeiss) as described [9].

2.6. Endothelial cell culture

Human umbilical vein endothelial cells were isolated and cultured as described [25] and used exclusively after only 1 passage. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki [26] and the isolation of endothelial cells was approved in written form by the ethics committee of the Goethe-University.

2.7. Cell proliferation

Cells were transfected with a control plasmid (pcDNA) or a plasmid encoding Cyp2c44 and after 48 h cells were re-seeded onto 48 well plates (18000 cells) coated with fibronectin (25 µg/mL). Proliferation was assessed with Casy TT cell counter and analyzer system (Roche, Basel, Switzerland) in the absence and presence of VEGF (20 ng/mL) and 20-HDHA (1 µmol/L) in medium supplemented with 5% fetal calf serum (FCS).