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The soluble epoxide hydrolase determines cholesterol homeostasis by regulating AMPK and SREBP activity

Nicole Mangels[a,b], Khader Awwad[a], Annika Wettenmann[a,b], Laila Romagueira Bichara Dos Santos[a,b], Timo Frömel[a,b], Ingrid Fleming[a,b,*]

[a] Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Frankfurt, Germany
[b] German Centre for Cardiovascular Research (DZHK) partner site RheinMain, Germany

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ABSTRACT

Inhibition or deletion of the soluble epoxide hydrolase (sEH) has been linked to reduced cholesterol and protection against atherosclerosis. This study set out to identify sEH substrate(s) or product(s), altered in livers from sEH$^{-/-}$ mice that contribute to these beneficial effects.

In livers and isolated hepatocytes, deletion of sEH decreased expression of HMG CoA reductase, fatty acid synthase and low density lipoprotein receptor. Sterol regulatory element binding proteins (SREBPs) regulate the expression of all three enzymes and SREBP activation was attenuated in the absence of sEH. The effect was attributed to the AMPK-activated protein kinase (AMPK) which was activated in the absence of sEH. Livers from wild-type versus sEH$^{-/-}$ littermates contained significantly higher levels of the sEH substrate 12,13-epoxyoctadecenoic acid, which elicited AMPK activation, while the corresponding sEH product was inactive. Thus, AMPK activation and subsequent inhibition of SREBP can account for the altered expression of lipid metabolizing enzymes in sEH$^{-/-}$ mice.

1. Introduction

Hepatic cytochrome P450 (CYP) enzymes are responsible for the metabolism of xenobiotica and many pharmaceuticals. These enzymes also utilize endogenous ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) as substrates to generate epoxides that have biological activity [1]. Hepatic epoxide concentrations are determined by the availability of the PUFA substrate, the level of CYP enzyme expression, and by their rate of inactivation/metabolism by hydrolysis, membrane incorporation, β-oxidation and chain elongation [2]. The soluble epoxide hydrolase (sEH, gene = *EPHX2*) is, however, the most important epoxide-metabolizing enzyme that generates dihydroxy-fatty acids (or vicinal diols) via its *C*-terminal hydrolase domain [3]. The epoxide hydrolase reaction is frequently viewed as an inactivation step, as the biological activity of the PUFA epoxides is generally reported to be greater than that of the diols [1].

sEH expression is highest in the liver and kidney and while the protective role of sEH inhibition in the kidney has been carefully studied [4,5], much less is known about the role of the sEH in the liver. It is however becoming clear that there are close links between sEH activity and whole body cholesterol metabolism as plasma total cholesterol was significantly decreased in male sEH$^{-/-}$ mice [6], a fact that could at least be partially attributed to decreased hepatic expression of the HMG CoA reductase, the rate limiting enzyme in the synthesis of cholesterol [7]. How the substrates or products of the sEH can affect HMG CoA reductase expression is unknown. However, the expression of the HMG CoA reductase is largely determined by the activity of members of the sterol regulatory element binding protein (SREBP) family that are master regulators of lipid metabolism [8,9]. The aim of the present study was to address the link between sEH and cholesterol, focusing on the role of PUFA epoxides and diols altered in the livers from sEH$^{-/-}$ mice and the potential involvement of SREBP.

2. Materials and methods

2.1. Chemicals and reagents

Williams' Medium E was from Biochrom/Millipore (Darmstadt, Germany) and collagenase II from Worthington (Troisdorf,

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; DHET, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecenoic acid; HMG CoA reductase, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acid; sEH, soluble epoxide hydrolase; SREBP, sterol regulatory element-binding protein.

* 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).

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Germany). The antibodies recognizing phospho-Thr172 AMPK, AMPKα2, phospho-Ser79 ACC, ACC and the HMG CoA reductase in murine samples were from Cell Signaling (New England Biolabs, Frankfurt, Germany), the antibody used to detect HMG CoA reductase in HepG2 cells as well as the antibody against the LDL receptor were from abcam (Cambridge, UK). The antibody against AMPKα1 (1:1000, 8056) was from Eurogentec (Seraing, Belgium), anti-SREBP1 and anti-SREBP2 were from Santa Cruz Biotechnology (Heidelberg, Germany). The sEH was detected using an antibody kindly provided by Michael Arand, (University of Zurich, Zurich, Switzerland). The sEH inhibitor *trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]-benzoic acid (*t*-AUCB) [10] was kindly provided by Bruce D. Hammock (UC Davies, CA). The antibodies directed against γ-tubulin, β-actin and fatty acid synthase were from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Animals

C57BL6 mice were purchased from Charles River (Sulzfeld, Germany) and "floxed"-sEH mice generated by TaconicArtemis GmbH (Cologne, Germany) as described [11], were crossed with animals expressing Cre under the control of the endogenous Gt(ROSA)26Sor promoter (TaconicArtemis GmbH) to generate mice lacking the sEH in all tissues ($sEH^{-/-}$). Genetically modified mice lacking either the AMPKα1 or the AMPKα2 subunits and their respective wild-type littermates were kindly provided by Benoit Viollet (INSERM, U1016, Paris, France) and bred at the Goethe University Hospital animal facility. All animals were housed in conditions that conform to the guide for the care and use of laboratory animals in accordance with EU Directive 2010/63/EU for animal experiments. Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (#F28-25 and FU 1012). For the isolation of organs, mice were sacrificed using 4% isoflurane in air followed by cervical dislocation. Male mice were used exclusively in this study.

2.3. Isolation and culture of murine hepatocytes

Primary hepatocytes were isolated as described previously [12], using a modified two-step digestion method. In brief, after cervical dislocation, disinfection and laparotomy, a 25 G cannula was placed in the inferior vena cava. After dissection of the portal vein, retrograde liver perfusion was performed with pre-warmed Hank's balanced salt solution without Ca^{2+} and Mg^{2+} supplemented with HEPES (15 mmol/L), EDTA (2.5 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL) using a roller pump (10 ml/ min). After 3–5 min the perfusion medium was switched to Hank's containing collagenase type II (250 U/mL). Isolated cells were grown at a density of 2.5×10^5 cells per ml on collagen (50 μg/ ml) coated plates in Williams' Medium E containing heat-inactivated fetal calf serum (FCS, 8%), L-glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL). After 4 h, medium was changed to FCS free medium to keep cell morphology. Cells were lysed one day after isolation or stimulated with insulin. Adenoviral mediated expression of the sEH was performed as described [13,14].

2.4. Cell culture

The human hepatoma cell line HepG2 (ATCC HB-8065; Manassas, VA, USA) was grown in minimal essential medium (MEM) supplemented with FCS (8%), penicillin (100 U/mL), streptomycin (100 μg/mL), non-essential amino acids (0.1 mmol/L) and sodium pyruvate (1 mmol/L).

2.5. Small interfering RNA (siRNA)

For AMPK downregulation, cells were grown to 80–90% confluence and then transfected with specific small interfering RNAs (siRNAs) using the Lipofectamin RNAiMax (Invitrogen, Life Technologies, Carlsbad, CA) according to the manual instructions. Transfection was performed 48 h before analysis. siRNAs were synthesized by Eurogentec (Eurogentec S. A., Seraing, Belgium) to the following human target sequences: AMPKα1 (5′-CCAAGUGGAUAGUAGAACU-3′), AMPKα2 (5′-CCAUCUUCGUCGAAGAAGA-3′) a nonrelated, scrambled siRNA without any other match in the human genomic sequence was used as a control.

2.6. Immunoblotting

Cells or liver tissues were lysed in Triton X-100 buffer and detergent-soluble proteins were solubilized in SDS-PAGE sample buffer, separated by SDS-PAGE and subjected to Western blotting as described [15]. For the analysis of SREBP, Triton X-100 insoluble protein fractions including nuclear proteins were resuspended in SDS-PAGE sample buffer. Proteins were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

2.7. LC–MS/MS epoxide and diol profiling

Liver samples (~50 mg) were extracted twice with ethyl acetate, evaporated under a constant flow of nitrogen, and resuspended in methanol/water (1:2 v/v). The eicosanoid profiles generated were determined with a mass spectrometer (API4000; AB Sciex) operating in multiple reaction monitoring (MRM) mode as described [16]. Chromatography was performed on a Gemini C18 column (150 mm length, 2 mm inner diameter; particle size, 5 μm; Phenomenex). All fatty acid epoxides, diols, and deuterated analogues were obtained from Cayman Europe (Biomol GmbH, Hamburg, Germany).

2.8. RNA isolation and RT-qPCR

Total liver RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Braunschweig, Germany). cDNA was generated by reverse transcription of 1 μg RNA isolated using SuperScript III and oligodT(20) (ThermoFisher Scientific), according to the manufacturer's instructions.

Gene expression was assessed by quantitative real-time PCR using SYBR Green (Absolute QPCR SYBR Green Mix; ThermoFisher Scientific). The relative expression levels of the different genes studied were calculated using the $2^{-\Delta\Delta Ct}$ method with the 18S RNA as a reference. The primer sequences used were: *18S* forward: 5′-CTTTGGTCGCTCGCTCCTC-3′, reverse: 5′-CTGACCGGGTTGGTTTTGAT-3′; *HMG Co A reductase* forward: 5′-AGACTGTGGTTTGTGAAGCCGT-3′, reverse: 5′-CAGTGACAATGTTTGCTGCGTG-3′ as published [17]; *SREBF1* forward: 5′-GTCAAAACCAGCCTCCCAAG-3′ reverse: 5′-GTCCCCGT CCACAAAGAAAC-3′; *SREBF2* forward: 5′-AACACTGACCAGCACCCATA-3′ reverse: 5′-TTTGGCGAGGTCTAGGTCTG-3′; *fatty acid synthase* forward: 5′-TCTGTGCCCGTCGTCTATAC-3′, reverse: 5′-GGAGGTATGCTCGCTTCTCT-3′; LDL receptor forward 5′-CAGAACAAGGAACAA GCCTCCCTCTTTAGCCTTTAACCAGTGATGCAAATGCGGAAAACC-3′: reverse: 5′-CAGTAC ACCAGAATCATATTTACAAAACTGATGAAAGCTGTGCAGGGGAACCAGGAAACT-3′.