Cyclooxygenase-2 contributes to the selective induction of cell death by the endocannabinoid 2-arachidonoyl glycerol in hepatic stellate cells

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Abstract
The endogenous cannabinoid 2-arachidonoyl glycerol (2-AG) is an anti-fibrotic lipid mediator that induces apoptosis in hepatic stellate cells (HSCs), but not in hepatocytes. However, the exact molecular mechanisms of this selective induction of HSC death are still unresolved. Interestingly, the inducible isozyme of cyclooxygenase, COX-2, can metabolize 2-AG to pro-apoptotic prostaglandin glycerol esters (PG-GEs). We analyzed the roles of COX-2 and endocannabinoid-derived PG-GEs in the differential susceptibility of primary activated HSCs and hepatocytes toward 2-AG-induced cell death. HSCs displayed significant COX-2 expression in contrast to hepatocytes. Similar to 2-AG, treatment of HSCs with PGD2-GE dose-dependently induced cell death independently from cannabinoid receptors that was accompanied by PARP- and caspase 3-cleavages. In contrast to 2-AG, PGD2-GE failed to induce significant ROS formation in HSCs, and depletion of membrane cholesterol did not rescue HSCs from PGD2-GE-induced apoptosis. These findings indicate differential engagement of initial intracellular signaling pathways by 2-AG and its COX-2-derived metabolite PGD2-GE, but similar final cell death pathways. Other PG-GEs, such as PGE2-GE or PGF2α-GE did not induce apoptosis in HSCs. Primary rat hepatocytes were mainly resistant against 2-AG- and PGD2-GE-induced apoptosis. HSCs, but not hepatocytes were able to metabolize 2-AG to PGD2-GE. As a proof of principle, HSCs from COX-2−/− mice lacked PDG2-GE production after 2-AG treatment. Accordingly, COX-2−/− HSCs were resistant against 2-AG-induced apoptosis. In conclusion, the divergent expression of COX-2 in HSCs and hepatocytes contributes to the different susceptibility of these cell types toward 2-AG-induced cell death due to the generation of pro-apoptotic PGD2-GE by COX-2 in HSCs. Modulation of COX-2-driven metabolism of 2-AG may provide a novel physiological concept allowing the specific targeting of HSCs in liver fibrosis.

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1. Introduction

The endocannabinoid system, consisting of arachidonic-acid-derived lipid mediators, known as endocannabinoids, their specific receptors and enzymes for endocannabinoid biosynthesis and degradation, is critically implicated in the regulation of hepatic injury and fibrogenesis.

Although the endocannabinoid system is scarcely expressed in healthy liver, endocannabinoid receptors are upregulated and endocannabinoid levels increase significantly during diseased states of the organ [1–3]. Cannabinoid receptor 2−/− mice displayed increased hepatic fibrogenesis in a model of CCl4-induced liver fibrosis, whereas C81−/− mice showed reduced fibrogenesis [4,5]. However, the mechanisms by which the endocannabinoid system regulates liver injury and fibrogenesis are not entirely understood [3]. Endocannabinoids, such as AEA, 2-AG or N-arachidonoyl...
dopamine (NADA) display anti-fibrotic properties in the liver by selectively inducing cell death of activated hepatic stellate cells (HSCs), the main fibrogenic cell type in the liver, but not in hepatocytes [1–3,6–8]. Hepatocyte cell death is considered to promote fibrogenesis, whereas elimination of activated HSCs may represent a mechanism to attenuate hepatic fibrosis [9].

Endocannabinoids evoke a wide spectrum of physiological actions that are mostly effectuated through the G-protein coupled cannabinoid receptors CB1 and CB2 [10,11], but can also occur independently from these receptors [1,2,6,12,13]. Endocannabinoids were initially described in the central nervous system, where they are involved in the control of e.g. food intake, emotions, pain perception, or sleep [14]. Moreover, endocannabinoids have also been shown to regulate inflammation, cell proliferation, cell death and lipogenesis in peripheral organs such as the liver [15–19].

We previously demonstrated that 2-AG, the most abundant endocannabinoid in vertebrate animals, induced reactive oxygen species (ROS)-triggered, cannabinoid receptor-independent apoptosis in primary HSCs, but not in hepatocytes due to different intracellular levels of the antioxidant glutathione and unequal expression of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) in these cell types [1,8]. However, these factors alone do not fully explain the remarkable difference in susceptibility to 2-AG-mediated cell death. Since activated HSCs express significant amounts of the alternative 2-AG-metabolizing enzyme COX-2 [20] in contrast to hepatocytes [21], we sought to investigate, whether this discrepancy of COX-2 expression contributes to the differential susceptibility of 2-AG-induced apoptosis in these two hepatic cell populations.

2. Materials and methods

2.1. Animals and primary cell isolation

Primary HSCs were isolated from livers of balb/c wild-type (25–35 g, n = 30), C57BL/6J wild-type (25–35 g, n = 4), COX-2−/− (25–35 g, n = 4) mice as previously described [1,8], COX-2−/− mice [22] in a C57BL/6J background were kindly provided by Dr. Rolf M. Nüsng (Johann Wolfgang Goethe-University, Frankfurt, Germany). Purity of HSC preparations was 94%, as assessed by autofluorescence at day 2 after isolation. HSCs were cultured on uncoated plastic tissue culture dishes as described. HSCs were not passaged and considered culture-activated between day 7 and 14 after isolation. Primary hepatocytes were isolated from wild-type balb/c mice (25–35 g, n = 6) and cultured as described previously [2,7]. All animals received humane care and all procedures were approved by the local committees for animal studies (Regierungspräsidium Karlsruhe and LANUV Recklinghausen).

2.2. Experimental model of liver fibrosis

Liver fibrosis was induced in male balb/c wild-type mice (30–35 g, n = 4) in a model of cholestatic liver disease by ligating the common bile duct as described [1,7]. Sham-operated animals served as controls (n = 4). Seven or 14 days after surgery the mice were sacrificed and the livers were excised for protein and RNA isolation.

2.3. Cell treatment and detection of cell death

Hepatocytes were kept in serum-free DMEM medium for 12 h before experiments. HSCs were serum-starved with serum-free DMEM for 12 h. Cells were treated either with 2-AG, prostaglandin D2, prostaglandin D2 glycerol ester, prostaglandin E2 glycerol ester, prostaglandin F2α glycerol ester (all Cayman Chemical, Ann Arbor, MI) or vehicle (ethanol; 0.1% final concentration), or actinomycin D (Sigma–Aldrich, Deisenhofen, Germany) plus murine TNFα (R&D Systems, Minneapolis, MN). Where indicated, cells were pretreated with the CB1 antagonist AM251 (Cayman Chemical), CB2 antagonist SR144528 (Sanofi-Aventis, Montpellier, France), pan-caspase inhibitor Z-VAD-FMK (R&D Systems), membrane cholesterol depletor methyl-β-cyclodextrin, or the antioxidant Trolox (both Sigma–Aldrich). Cell death was measured by LDH release into the culture medium according to the manufacturer (Roche, Mannheim, Germany). Apoptosis was visualized by fluorescent microscopy using an annexin V/propidium iodide-staining kit (Roche).

2.4. Measurement of prostaglandin glycerol ester levels

The PGD2-GE concentration in lysates of primary mouse hepatocytes and HSCs was measured by a PGD2-MOX EIA kit according to the manufacturer’s instructions (Cayman Chemical).

2.5. Detection of ROS

Reactive oxygen species formation was measured for the indicated time in a multilowell fluorescence plate reader (Fluostar Optima, BMG) with excitation and emission filters of 485 nm and 535 nm, respectively, using the redox-sensitive dye 5-(and-6)-chloromethyl-2,7’-dichlorodihydrofluorescin diacetate (CMH2DCFDA; Molecular Probes-Invitrogen, Darmstadt, Germany) as described [1,8].

2.6. Quantitative RT-PCR analysis

RNA was isolated from serum-starved activated HSCs and hepatocytes and reverse transcribed as described [1,2,8]. Real time PCR was performed for 40 cycles of 15 s at 95 °C and 60 s at 60 °C using an ABI 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) as shown [1,2,8], and subsequently analyzed with the gene expression assay for PTGS2 (Applied Biosystems; assay no. Mm00478374_m1).

2.7. Flow cytometric analysis

For intracellular staining of COX-2, cells were fixed with 2% paraformaldehyde in PBS with 1% PBS/2 mM EDTA at RT. Afterward, cells were permeabilized with 0.5% saponin, followed by incubation with primary COX-2 antibody (Cayman Chemical) and Alexa647-coupled secondary antibody on ice. The Fc gamma receptor-blocking antibody (clone 2.4G2) was added to prevent non-specific binding. Acquisition and analysis was conducted using a Canto II (BD Bioscience, Heidelberg, Germany) and FlowJo software (Tree Star Inc, Ashland, OR).

2.8. Western blotting

Electrophoresis of protein extracts and subsequent blotting were performed as described previously [1,8]. Blots were incubated with anti-COX-2, (Cayman Chemical); anti-caspase-3 or anti-poly(ADP-ribose) polymerase (anti-PARP, both Cell Signaling Technologies, Beverly, MA). Cytochrome c release was determined in S-100 fractions using anti-cytochrome c (BD PharMingen, San Jose, CA) as described [23]. Blots were reprobed with anti-β-actin antibody (Cell Signaling Technologies) as loading control.

2.9. Statistical analysis

All data represent the mean of three independent
experiments ± SEM. For the determination of statistical significance, unpaired Student’s t-tests were performed using SigmaStat (SPSS, Chicago, IL). P values of ≤0.05 were considered to be statistically significant.

3. Results

3.1. Involvement of COX-2 in 2-AG-induced cell death in HSCs

In parallel to the expression of the HSC activation marker α-SMA, COX-2 mRNA expression increased 54-fold during 14 days in BDL-treated livers (Fig. 1A, B). Interestingly, protein and mRNA levels of COX-2 in activated HSCs were 6.8 times higher compared to hepatocytes (Fig. 1C, D). It was recently shown that 2-AG is first metabolized by COX-2 to unstable PGH₂-GE. PGH₂-GE acts as a substrate for PGD₂, PGE₂-GE or PGE₂, respectively [24]. To check whether these potential metabolites of 2-AG were involved in 2-AG-induced cell death, and to exclude that prostaglandin D₂ (PGD₂), the COX-2 metabolite of arachidonic acid, may also contribute to 2-AG-mediated cell death, primary HSCs were exposed to 100 μM of each substance. Interestingly, only PGD₂-GE induced cell death in primary activated HSCs (Fig. 1E). We were further interested in a possible differential metabolization of 2-AG to PGD₂-GE by

![Image](image_url)
hepatocytes or HSCs. After stimulation of these cell types with 2-AG or vehicle for 16 h (Fig. 1F), no difference in PGD2-GE levels was observed between vehicle-treated hepatocytes and HSCs as well as vehicle-treated and 2-AG-stimulated hepatocytes. However, there was a significant induction of PGD2-GE production in 2-AG-stimulated HSCs reaching a 22-fold higher PGD2-GE level compared to vehicle-treated HSCs and a 15-fold higher PGD2-GE level compared to 2-AG-treated hepatocytes. When we exposed hepatocytes and HSCs to increasing concentrations of PGD2-GE, HSCs showed a significantly higher susceptibility toward PGD2-GE-induced cell death than hepatocytes. Hepatocytes underwent cell death only after exposure to a excessive PGD2-GE concentration of 100 μM, but not after 2-AG in the same concentration. However, since hepatocytes do not significantly express COX-2, it is unlikely that intracellular concentrations of PGD2-GE reach substantial cytotoxic levels in this cell type.
3.2. Comparison between mechanisms of 2-AG- and PGD2-GE-induced cell death in HSCs

Next we investigated whether PGD2-GE accounts for key features of 2-AG-induced HSC cell death. PGD2-GE did not cause significant ROS production in HSCs in contrast to 2-AG (Fig. 2A). Accordingly, antioxidant pretreatment of HSCs by Trolox ameliorated 2-AG-, but not PGD2-GE-induced cell death (Fig. 2B). Moreover, in a previous study we demonstrated that 2-AG required membrane cholesterol interaction in cholesterol-rich lipid rafts to cause cell death in HSCs [1]. PGD2-GE-induced HSC death on the contrary could not be blocked by prior membrane cholesterol depletion by MCD (Fig. 2B). Pretreatment of HSCs with the specific CB1 or CB2 receptor antagonists AM251 or SR144528, respectively, did neither prevent 2-AG- nor PGD2-GE-mediated cell death (Fig. 2B). These data suggest, that PGD2-GE does not require interaction with membrane cholesterol or cannabinoid receptors for cellular uptake and subsequent induction of cell death in HSCs since it is produced intracellularly by COX-2 from 2-AG [2]. PGD2-GE-induced HSC death on the contrary could not be blocked by prior membrane cholesterol depletion by MCD (Fig. 2B). Pretreatment of HSCs with the specific CB1 or CB2 receptor antagonists AM251 or SR144528, respectively, did neither prevent 2-AG- nor PGD2-GE-mediated cell death (Fig. 2B). These data suggest, that PGD2-GE does not require interaction with membrane cholesterol or cannabinoid receptors for cellular uptake and subsequent induction of cell death in HSCs since it is produced intracellularly by COX-2 from 2-AG [2].

Fig. 3. HSCs from COX-2\textsuperscript{-/} mice are resistant against 2-AG-induced cell death. A. Determination of COX-2 expression levels of wild-type (WT; solid lines) and COX-2\textsuperscript{-/} HSCs (dashed lines) by flow cytometric analysis. Isotype controls are shaded in gray. B, C. Activated primary HSCs from wild-type (COX-2\textsuperscript{+/+}, B) and COX-2\textsuperscript{-/-} mice (C) were treated with 50 µM 2-AG for 12 h. PGD2-GE concentration was measured by PGD2 MOX EIA and displayed by fold increase compared to vehicle alone. D. Activated primary HSCs from COX-2\textsuperscript{+/+} and COX-2\textsuperscript{-/-} mice were treated with 50 µM 2-AG for 16 h. Cell death was determined by LDH release. (**p < 0.001 vs. vehicle). E. HSCs from COX-2\textsuperscript{+/+} and COX-2\textsuperscript{-/-} mice were vehicle treated or treated with 50 µM 2-AG for 16 h. Apoptotic cell death was visualized by green fluorescence of annexin V (AnnV). Necrotic cell death was shown by red staining of the nuclei by propidium iodide (PI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Genetic inactivation of COX-2 renders HSCs resistant against 2-AG-induced cell death

To prove the importance of COX-2 in the mechanisms involved in 2-AG-mediated cell death in HSCs, we sought to apply HSCs isolated from COX-2\textsuperscript{-/-} mice and to compare these to wild-type HSCs. First, we confirmed, that activated HSCs isolated from COX-2 wild-type mice indeed expressed significant amounts of COX-2 protein, whereas COX-2\textsuperscript{-/-} HSCs lacked the enzyme completely (Fig. 3A). We then exposed activated wild-type and COX-2\textsuperscript{-/-} HSCs to 2-AG and measured the levels of PGD2-GE production (Fig. 3B, C). In contrast to wild-type HSCs, COX-2\textsuperscript{-/-} HSCs did not produce noticeable amounts of PGD2-GE (Fig. 3C). Thus, COX-2\textsuperscript{-/-} HSCs were resistant against 2-AG-induced cell death in contrast to their wild-type counterparts (Fig. 3D). We further visualized the remarkable difference in cell death susceptibility of wild-type and mitochondrial permeability transition and triggers activation of executioner caspases, such as caspase 3 [23]. Accordingly, HSC treatment with 25 µM of 2-AG or PGD2-GE, respectively, led to caspase 3- and PARP-cleavage, two hallmarks of apoptotic cell death (Fig. 2F). Interestingly, caspase 3 and PARP-cleavage could be blocked both in 2-AG- and PGD2-GE-mediated apoptosis by the pan-caspase inhibitor Z-VAD-FMK (Fig. 2F). Moreover, inhibition of caspase activation by Z-VAD-FMK significantly reduced 2-AG- as well as PGD2-GE-induced HSC apoptosis (Fig. 2G).
4. Discussion

Although liver fibrosis has been regarded as irreversible for a long time, recent studies in animal models and patients have suggested that hepatic fibrosis is, at least to some degree, a reversible process [9]. Cell death of activated HSCs as the key fibrogenic cell population has been linked to the reversal of liver fibrosis. Previously, we and others demonstrated, that endocannabinoids are able to selectively induce cell death in activated HSCs, but not in hepatocytes [1,2,4,6–8] and therefore exhibit antiﬁbrogenic properties. However, the mechanisms by which endocannabinoids regulate liver injury and ﬁbrogenesis are not completely understood [3]. Cell death in activated HSCs by endocannabinoids occurs independently from cannabinoid receptors through interaction with cholesterol-rich lipid rafts in the cell membrane [1,2,6]. In this study we show that 2-AG, the most abundant endocannabinoid in vertebrates, induces a burst of mitochondrial-derived ROS in HSCs, leading to cytochrome C release and subsequent activation of effector caspases such as caspase 3, resulting in apoptotic cell death. Divergence in cell death susceptibility in HSCs and hepatocytes are based on differences in the capability to terminate 2-AG-mediated intracellular events: (i) hepatocytes possess higher levels of antioxidative glutathione to mitigate ROS production and (ii) signiﬁcantly express the endocannabinoid-hydrolyzing enzyme FAAH [1,8]. Thus, the metabolism of endocannabinoids plays a pivotal role in the control of endocannabinoid signaling. Interestingly, besides the hydrolytic degradation pathways of 2-AG by FAAH or monoacyl glycerol lipase (MGL), the molecule may also be metabolized by oxygenases due to their high afﬁnity to the arachidonoyl moiety of 2-AG. In contrast to FAAH, the oxygenase COX-2 speciﬁcally metabolizes 2-AG as efﬁciently as arachidonic acid [24]. Contrarily, the constitutionally active, non-inducible isoform COX-1 is not able to metabolize 2-AG [24]. Here we demonstrate that the expression of COX-2 increased 54-fold during hepatic ﬁbrogenesis and activated HSCs largely contributed to this increase of COX-2 expression. Uprogulation of COX-2 expression during ﬁbrogenesis correlates with increased expression of the HSC acti-vation marker α-SMA (Fig. 1A) and rising hepatic levels of 2-AG [1]. In contrast to hepatocytes, activated HSCs were able to considerably metabolize 2-AG to PGD2-GE, which represents a biologically highly active prostaglandin glycerol ester in endocannabinoid signaling [25]. PGD2-GE appears to be produced intracellularly, since it did not signal through CB1- or CB2-receptors and, in contrast to 2-AG, did not interact with membrane cholesterol. Although PGD2-GE did not generate mitochondrial ROS, it induced the same ﬁnal death signaling pathway including cytochrome C release and activation of executioner caspases like its parental substance 2-AG (see the graphical abstract). The importance of COX-2 as an endocannabinoid-degrading enzyme has been demonstrated, if other degradation enzymes, i.e. FAAH or MGL, are lacking [26]. We have previously shown, that HSCs do not express FAAH, but MGL [7,8]. However, expression of MGL does not rescue HSCs from 2-AG-induced cell death [8]. MGL may rather play a role in terminating PGD2-GE signaling through hydrolyzation [27]. MGL becomes downregulated in the liver during ﬁbrogenesis in contrast to COX-2 [8,28], which may in turn contribute to an increase in PGD2-GE production and thus an increase in 2-AG-induced death of HSCs. Other inﬂammatory cells in the liver, e.g. Kupffer cells also express high amounts of COX-2, but also FAAH and MGL (data not shown) as well as signiﬁcant levels of GSH [29]. Therefore, similar to hepatocytes, Kupffer cells seem to be protected against 2-AG-induced cell death.

Finally, the high resistance of COX-2−/− HSCs against 2-AG-induced cell death conﬁrms the importance of COX-2 in the differential susceptibility of HSCs and hepatocytes towards endocannabinoid-mediated toxicity.

In conclusion, 2-AG selectively induces cell death in activated HSCs due to (i) low levels of glutathione, (ii) low expression of degradation enzymes such as FAAH and (iii) high levels of COX-2, leading to production of pro-apoptotic PGD2-GE. Consequently, modulation of COX-2-driven metabolism of 2-AG may provide a novel physiological concept allowing the speciﬁc targeting of HSCs in liver ﬁbrosis.

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