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Fatty acid amide hydrolase but not monoacyl glycerol lipase controls cell death induced by the endocannabinoid 2-arachidonoyl glycerol in hepatic cell populations

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ABSTRACT

The endogenous cannabinoids anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG) are upregulated during liver fibrogenesis and selectively induce cell death in hepatic stellate cells (HSCs), the major fibrogenic cells in the liver, but not in hepatocytes. In contrast to HSCs, hepatocytes highly express the AEA-degrading enzyme fatty acid amide hydrolase (FAAH) that protects them from AEA-induced injury. However, the role of the major 2-AG-degrading enzyme monoacylglycerol lipase (MGL) in 2-AG-induced hepatic cell death has not been investigated. In contrast to FAAH, MGL protein expression did not significantly differ in primary mouse hepatocytes and HSCs. Hepatocytes pretreated with selective MGL inhibitors were not sensitized towards 2-AG-mediated death, indicating a minor role for MGL in the cellular resistance against 2-AG. Moreover, while adenoviral MGL overexpression failed to render HSCs resistant towards 2-AG, FAAH overexpression prevented 2-AG-induced death in HSCs. Accordingly, 2-AG caused cell death in hepatocytes pretreated with the FAAH inhibitor URB597, $FAAH^{-/-}$ hepatocytes, or hepatocytes depleted of the antioxidant glutathione (GSH). Moreover, 2-AG increased reactive oxygen species production in hepatocytes after FAAH inhibition, indicating that hepatocytes are more resistant to 2-AG treatment due to high GSH levels and FAAH expression. However, 2-AG was not significantly elevated in $FAAH^{-/-}$ mouse livers in contrast to AEA. Thus, FAAH exerts important protective actions against 2-AG-induced cellular damage, even though it is not the major 2-AG degradation enzyme *in vivo*. In conclusion, FAAH-mediated resistance of hepatocytes against endocannabinoid-induced cell death may provide a new physiological concept allowing the specific targeting of HSCs in liver fibrosis.

1. Introduction

There is increasing evidence that the endocannabinoid system, consisting of arachidonic-acid-derived lipid mediators, termed endocannabinoids, their specific receptors and enzymes that are responsible for endocannabinoid biosynthesis and degradation, is crucially involved in the regulation of hepatic injury and fibrogenesis.

Endocannabinoids evoke a wide spectrum of physiological actions that are mostly mediated through the G-protein coupled cannabinoid receptors CB1 and CB2 [1,2], but can also occur independently of these receptors [3–10]. 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 [11–13]. Moreover, endocannabinoids have also been shown to regulate inflammation, cell death and peripheral lipogenesis [14–17].

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 [9,18,19]. Cannabinoid receptor $2^{-/-}$ mice displayed increased hepatic fibrogenesis in a model of CCl_4-induced liver fibrosis, whereas $CB1^{-/-}$ mice showed reduced fibrogenesis [10,20]. However, the mechanisms by which the endocannabinoid system regulates liver injury and fibrogenesis are not well understood. 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

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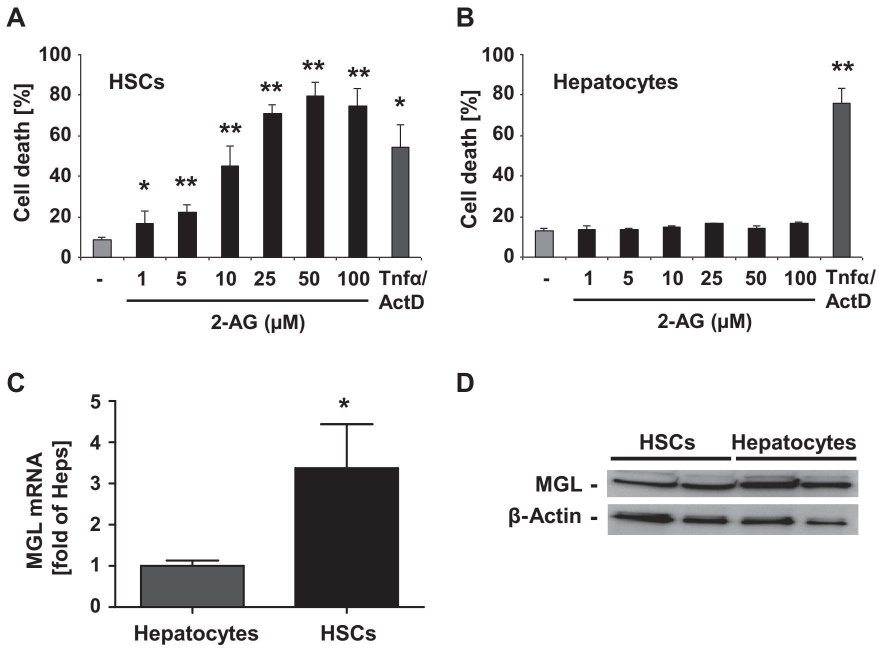

Fig. 1. Different susceptibility to 2-AG-mediated death in HSCs and hepatocytes does not depend on differential MGL expression. (A and B) Serum starved primary mouse HSCs (A), and primary mouse hepatocytes (B) were treated with the indicated concentrations of 2-AG or vehicle (−) for 18 h or the positive control recombinant murine TNFα (30 ng/ml) plus actinomycin D (ActD; 0.2 μg/ml). Cell death was determined by LDH release ($^{*}p < 0.05$ vs. vehicle). (C) mRNA expression in mouse hepatocytes and HSCs was determined by quantitative real time PCR, shown as a fold induction after normalization to 18s ($n = 3$; $^{*}p < 0.05$). (D) MGL protein expression was analyzed in mouse HSCs and hepatocytes by western blotting.

hepatocytes [7,9,18]. Hepatocyte cell death is considered to promote fibrogenesis, whereas elimination of activated HSCs may represent a mechanism to attenuate the fibrogenic response [21]. Interestingly, this selective induction of cell death in HSCs by endocannabinoids occurs independently from the known cannabinoid receptors.

We previously demonstrated that 2-AG, the most abundant endocannabinoid in vertebrate animals, mediated reactive oxygen species (ROS)-triggered, cannabinoid receptor-independent apoptosis in primary HSCs, but not in hepatocytes due to different levels of the antioxidant glutathione in these cell types. However, this factor alone is unlikely to explain the remarkable difference in susceptibility to 2-AG-mediated cell death. To further advance the insights of endocannabinoid signaling during liver injury and fibrosis, we investigated, whether the major 2-AG-degrading enzyme monoacyl glycerol lipase (MGL) or alternative degradation enzymes FAAH, α-β-hydrolase domain (ABHD) 6 or ABHD12 contribute to the differential effects of 2-AG on HSCs or hepatocytes.

2. Material and methods

2.1. Animals and primary cell isolation

Primary HSCs were isolated by a 2-step pronase-collagenase perfusion from livers of male C57BL/6J wild-type (25–30 g, $n = 32$) followed by Nycodenz (Axis-Shield, Oslo, Norway) two-layer discontinuous density gradient centrifugation as described [7–9]. Purity of HSC preparations was 94%, as assessed by autofluorescence at day 2 after isolation. Hepatic stellate cells were cultured on uncoated plastic tissue culture dishes as described, not passaged and considered culture-activated between day 7 and 14 after isolation. Primary mouse hepatocytes were isolated from male $FAAH^{-/-}$ mice or C57BL/6 J $FAAH^{+/+}$ controls [22] ($n = 6$ each) as described previously [8,18]. All animals received humane care and all procedures were approved by the local committees for animal studies (Regierungspräsidium Karlsruhe and LANUV Recklinghausen).

2.2. Cell treatment and detection of cell death

Hepatocytes were kept in serum-free HDM medium for 12 h before experiments. HSCs were serum-starved with serum-free DMEM for 12 h. Cells were treated either with 2-AG (Cayman Chemicals, 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 MGL inhibitors URB602 or JZL184, FAAH inhibitor URB597 (all Cayman) or γ-glutamyl cysteine synthase inhibitor DL-buthionine-(*S*,*R*)-sulfoximine (BSO; Sigma). Cell death was measured by LDH release into the culture medium according to the manufacturer's instructions (Roche, Mannheim, Germany). Apoptosis was visualized by fluorescent microscopy using an annexin V/propidium iodide-staining kit (Roche).

2.3. Adenoviral infection

Adenoviruses expressing MGL, FAAH or GFP have been previously described [8,9]. Hepatic stellate cells were infected with adenoviruses at a multiplicity of infection (MOI) of 250 particles/cell for 12 h, achieving transduction rates of approximately 90%. After further 12 h, cells underwent treatment with 2-AG.

2.4. Measurement of hepatic endocannabinoid levels

The levels of the endocannabinoids anandamide (AEA) and 2-AG were measured by liquid chromatography/mass spectrometry according to Wang et al. [23] in liver tissue from male $FAAH^{-/-}$ mice or C57BL/6J $FAAH^{+/+}$ controls ($n = 4$ each).

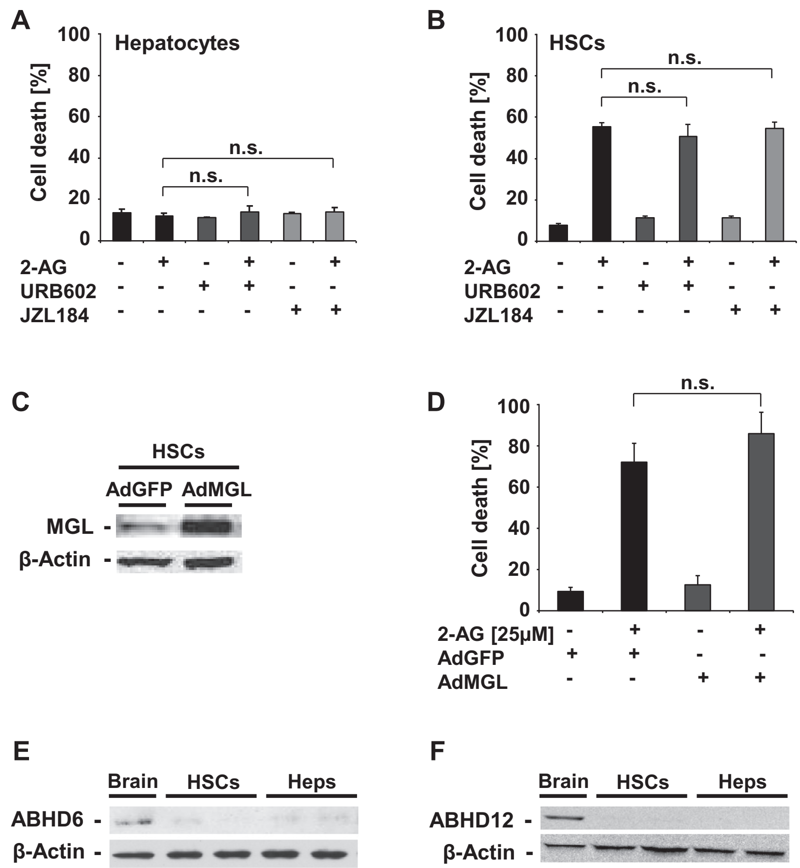

Fig. 2. Monoacyl glycerol lipase, ABHD6 or ABHD12 are not involved in the different cell death susceptibility of HSCs or hepatocytes toward 2-AG. (A and B). Primary mouse hepatocytes (A, 100 μM) or HSCs (B, 25 μM) were treated with 2-AG in the presence or absence of either MGL inhibitor URB602 or JZL184 (10 μM each, 1 h pretreatment). Cell death was determined by LDH assay. (C and D) Primary activated mouse HSCs were infected with adenoviruses expressing MGL or GFP. MGL expression is shown by western blotting (C). 24 h later, cells were treated with 2-AG (25 μM) for 16 h (D). Cell death was determined by LDH release. (E and F) Expression of the alternative 2-AG-degrading enzymes ABHD6 (E) and ABHD12 (F) was analyzed in mouse brain (control), activated primary mouse HSCs or hepatocytes by western blotting.

2.5. Detection of reactive oxygen species

Serum-starved HSCs or hepatocytes were loaded with 4 μM of the redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes-Invitrogen, Darmstadt, Germany) for 30 min at 37 °C, washed, and stimulated with agonists. Reactive oxygen species formation was measured for the indicated time in a multiwell fluorescence plate reader (Fluostar Optima, BMG) using excitation and emission filters of 485 nm and 535 nm, respectively.

2.6. Quantitative real time-PCR analysis

RNA was isolated from serum-starved activated primary HSCs and primary hepatocytes using the TRIzol method (Invitrogen, Carlsbad, CA). After DNAse treatment, RNA was reverse transcribed using random hexamer primers. 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 described [9,18].

2.7. Western blot analysis

Electrophoresis of protein extracts and subsequent blotting were performed as described [8,9,18]. Blots were incubated with anti-MGL, anti-FAAH (both Cayman), anti-ABHD6 (Abcam, Cambridge, UK) or anti-ABHD12 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 overnight at 4 °C. Blots were reprobed with anti-actin mouse antibody (MP Biomedicals, Eschwege, Germany) as an internal control to demonstrate equal loading.

2.8. Statistical analysis

All data represent the mean of at least 3 independent experiments ± SEM, if not otherwise stated. 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. 2-AG induces cell death in primary activated mouse HSCs, but not in primary mouse hepatocytes

We previously showed that 2-AG induced apoptosis in rat and human primary HSCs [9]. To investigate if 2-AG also induces apoptosis in primary mouse HSCs and hepatocytes, we stimulated these cells with different concentrations of 2-AG. As shown in Fig. 1A and B, we found a significant induction of cell death in primary mouse HSCs starting from 1 μM, but not in primary mouse hepatocytes. To examine, whether this remarkable difference in susceptibility was due to a differential expression of the major 2-AG-degrading enzyme MGL, we measured mRNA and protein levels of MGL in these two hepatic cell populations. Surprisingly, MGL mRNA was significantly higher expressed in HSCs than in hepatocytes, but there was no significant difference on the protein level (Fig. 1C and D).

3.2. Monoacyl glycerol lipase, ABHD6 or ABHD12 are not involved in the different susceptibility of HSCs or hepatocytes towards 2-AG-induced cell death

To examine, whether MGL protected hepatocytes from 2-AG-induced cell death, we first treated hepatocytes with the MGL inhibitors URB602 or JZL184 prior to 2-AG exposure (Fig. 2A). MGL inhibition did not sensitize hepatocytes towards 2-AG-induced cell death. Accordingly, pretreatment of HSCs with URB602 or JZL184 did not further aggravate 2-AG-mediated HSC death (Fig. 2B). We next overexpressed MGL in HSCs using an adenoviral vector (Fig. 2C), but again found no effect on 2-AG-induced cell death in HSCs (Fig 2D). Together these results indicate that MGL does not influence the sensitivity of HSCs toward 2-AG-mediated cell death or accounts for the resistance of hepatocytes against 2-AG. The alternative 2-AG-degrading enzymes ABHD6 (Fig. 2E) and ABHD12 (Fig. 2F) were neither detectable in hepatocytes nor in HSCs, making these enzymes unlikely candidates to contribute to the different cell death susceptibility by 2-AG in these cell types.

3.3. Fatty acid amide hydrolase accounts for the different susceptibility towards 2-AG-induced cell death in HSCs and hepatocytes

Mouse hepatocytes expressed high protein levels of FAAH, while almost no FAAH expression was detectable in mouse HSCs (Fig. 3A). Adenoviral overexpression of FAAH (Fig. 3B) rendered HSCs strongly resistant against 2-AG-induced cell death with a decrease of cell death from more than 70% in control cells vs. 24% after AdFAAH expression (Fig. 3C). To confirm this data, HSCs infected with AdGFP or AdFAAH prior to 2-AG treatment were stained with a combination of annexin V, which binds phosphatidylserine in the outer membrane leaflet of apoptotic cells, and propidium iodide, which

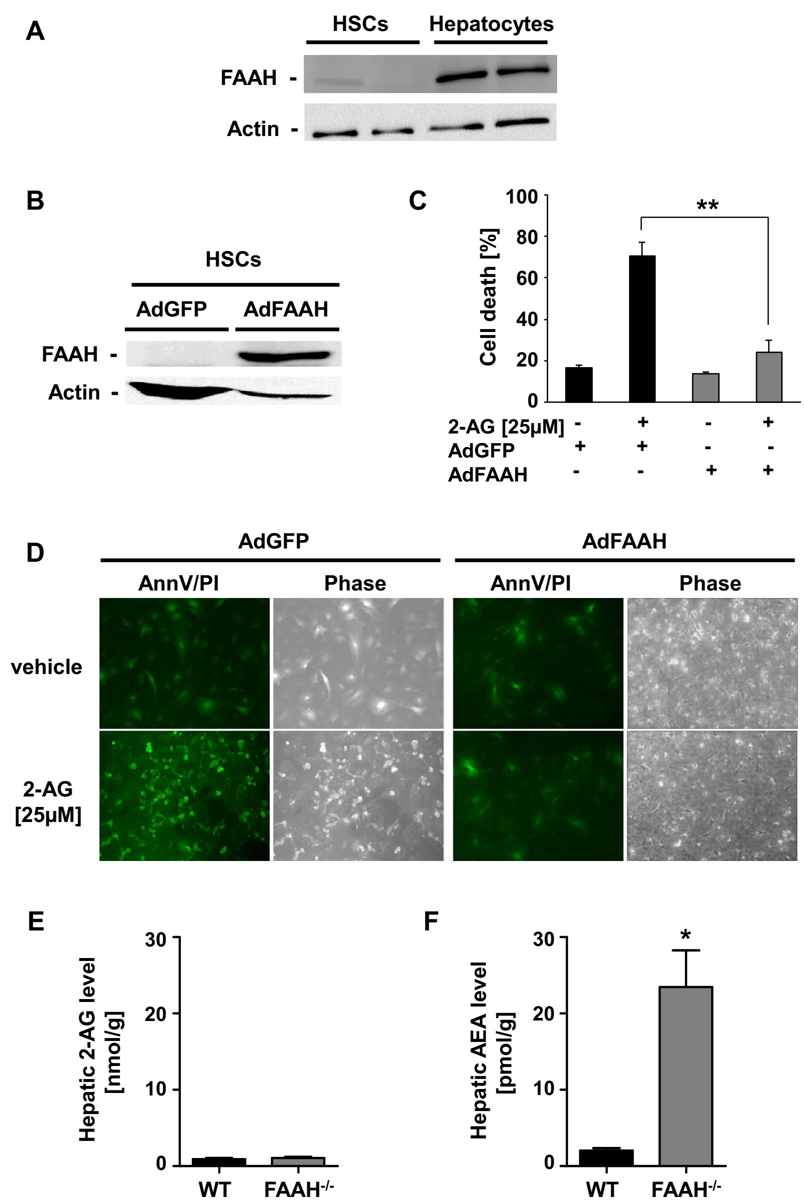

Fig. 3. Differential FAAH expression in hepatic cell populations determines divergent resistance against 2-AG-induced cell death. (A) FAAH protein expression was analyzed in primary activated mouse HSCs and hepatocytes by western blotting. (B and C) HSCs were infected for 12 h with adenoviruses expressing GFP or FAAH (also containing a copy of GFP to mark infected cells). FAAH expression is shown by western blotting (B). 24 h later, cells were treated with 2-AG (25 μM) for 16 h. Cell death was determined by LDH release (C, $^{**}p < 0.001$ vs. AdGFP). (D) HSCs were infected with AdGFP or AdFAAH for 12 h and treated 24 h later with 25 μM 2-AG for 16 h. Apoptotic cell death is indicated by bright green fluorescence of annexin V (AnnV) and typical morphology, necrotic cell death is shown by red staining of the nuclei by PI. (E and F) Endocannabinoids 2-AG (E) and AEA (F) were measured in wildtype or $FAAH^{-/-}$ mouse livers ($n = 4$ each, $^{*}p < 0.05$ vs. wildtype).

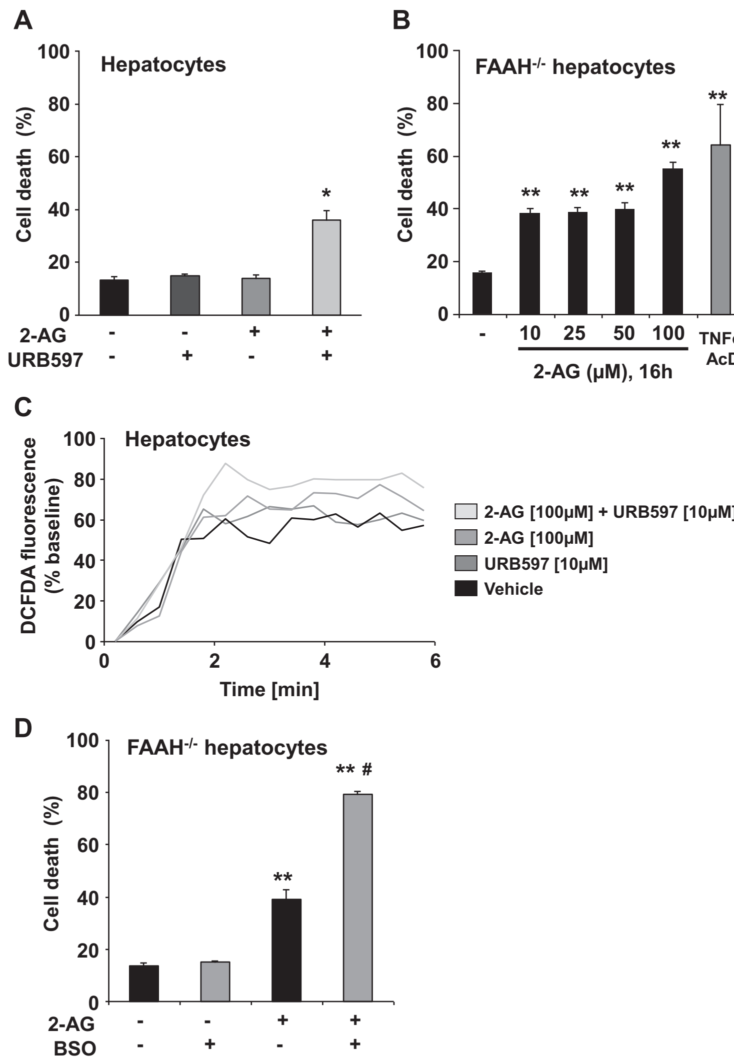

Fig. 4. Pharmacological or genetic inactivation of FAAH renders primary mouse hepatocytes susceptible toward 2-AG-induced cell death. (A) Wildtype hepatocytes were pretreated with vehicle or the selective FAAH inhibitor URB597 (10 µM) for 1 h followed by 2-AG (100 µM) for 24 h. Cell death was determined by LDH release ($^{*}p < 0.05$ vs. vehicle). (B) FAAH$^{-/-}$ hepatocytes were treated with the indicated concentrations of 2-AG for 24 h. Cell death was determined by LDH assay ($^{**}p < 0.001$ vs. vehicle). (C) Wildtype hepatocytes were loaded with CM-H_2DCFDA (5 µM) for 30 min and treated with 2-AG (100 µM) with or without URB597 preincubation. Reactive oxygen species formation was measured in triplicates. The figure is representative of three independent experiments. (D) FAAH$^{-/-}$ hepatocytes were either pretreated with vehicle or BSO (100 µM) to deplete GSH prior to 2-AG (25 µM) for 24 h. Cell death was measured by LDH release ($^{**}p < 0.001$ vs. vehicle, $^{\#}p < 0.05$ vs. 2-AG alone).

indicates cell membrane rupture in necrotic cells. After 2-AG treatment, control HSCs overexpressing GFP showed strong annexin V staining with the typical apoptotic phenotype (phase contrast, Fig. 3D. left panel) in contrast to HSCs overexpressing FAAH (Fig. 3D, right panel). To check, whether FAAH significantly contributes to degradation of 2-AG in the liver, we measured 2-AG levels in livers of wild type and FAAH$^{-/-}$ mice. Lack of FAAH did not influence the intrahepatic level of 2-AG (Fig. 3E; 0.92 ± 0.14 nmol/g in wild type livers vs. 1.07 ± 0.14 nmol/g in FAAH$^{-/-}$ livers) in contrast to intrahepatic AEA levels (Fig 3F; 2.05 ± 0.33 pmol/g vs. 23.47 ± 4.80 pmol/g, resp.).

3.4. Fatty acid amide hydrolase protects hepatocytes from ROS-mediated 2-AG-induced cell death

Next we investigated whether FAAH contributes to the resistance against 2-AG-mediated cell death by pretreating hepatocytes with the specific FAAH inhibitor URB597. URB597 significantly sensitized hepatocytes to the effects of 2-AG with more than 36% cell death vs. no induction of cell death in hepatocytes treated with 2-AG alone (Fig. 4A). We were able to confirm these data with primary hepatocytes isolated from FAAH-deficient mice (Fig. 4B), suggesting that FAAH is indeed critically involved in the resistance of hepatocytes toward 2-AG. Since we previously demonstrated that 2-AG induced cell death via formation of deleterious ROS [9], we sought to investigate whether FAAH expression was involved in hepatocyte resistance against 2-AG-driven ROS generation. Pretreatment with URB597 also increased 2-AG-induced ROS production in hepatocytes, whereas URB597 alone did not significantly increase ROS (Fig. 4C). Pretreatment of FAAH$^{-/-}$ hepatocytes with BSO to deplete the antioxidant GSH significantly increased 2-AG-mediated death with 25 µM from 39% to 79% (Fig. 4D, $p < 0.05$). Thus, FAAH and GSH are main determinants of 2-AG-induced cell death in the liver.

4. Discussion

Endocannabinoids hold the potential to induce cell death in many different cell types, making them interesting tools for treatment of cancer, inflammatory or degenerative diseases [11,15].

Recent studies have established that the endocannabinoid system is involved in the regulation of fibrogenesis in the liver. However, the mechanisms by which endocannabinoids regulate liver injury and fibrogenesis are not well characterized and require further investigation [19].

Endocannabinoids, including AEA, NADA and 2-AG, can selectively induce cell death in HSCs, which are largely responsible for excessive accumulation of extracellular matrix in chronically injured livers [7–10,18]. Selective elimination of HSCs has been linked to the resolution of liver fibrosis, whereas cell death in hepatocytes worsens liver function and enhances fibrogenesis [21]. 2-AG robustly and dose-dependently induced apoptotic cell death in activated HSCs of several species [9], including mouse (see Fig. 1A). During liver injury and fibrogenesis, hepatic levels of 2-AG rise to up to 2.25 μM [9]. This concentration is sufficient to induce cell death in HSCs ([9], see also Fig. 1A). We previously showed that hepatocytes are able to cope with the abundant 2-AG-derived generation of ROS due to significantly higher levels of antioxidants, such as GSH, in comparison to HSCs [9]. This is one reason why hepatocytes are resistant against 2-AG-induced cell death. In this study, we examined, whether the major degradation enzyme for 2-AG, MGL, contributes to the remarkable difference in cellular susceptibility of hepatic cell populations toward 2-AG-induced cell death. It is commonly accepted that 2-AG is primarily hydrolyzed by MGL to arachidonic acid and glycerol [24,25]. However, since 2-AG could also serve as a substrate for FAAH, FAAH-mediated hydrolysis might also play a role in its inactivation [26–29]. We found several lines of evidence indicating that MGL does not significantly contribute to the hepatocellular resistance against 2-AG: (i) both HSCs and hepatocytes express MGL mRNA and protein, (ii) pharmacological blockade of MGL in hepatocytes and in HSCs with two specific inhibitors did not increase their susceptibility toward 2-AG, (iii) adenoviral overexpression of MGL in HSCs did not rescue them from 2-AG-induced death. Instead, we demonstrate that the alternative 2-AG-degrading enzyme FAAH, which degrades AEA with high affinity, accounts for hepatocyte resistance against 2-AG, as (i) FAAH is highly expressed in hepatocytes, but not in HSCs, (ii) adenoviral overexpression in HSCs efficiently rescued these cells from 2-AG-mediated cell death, (iii) pharmacological inhibition or genetic deletion of FAAH led to increased 2-AG-induced vulnerability of hepatocytes. Moreover, lack of FAAH in combination with antioxidant depletion leads to a potentiation of 2-AG-induced cell death, demonstrating the importance of the hepatocyte defense mechanisms of FAAH expression [8] and high GSH levels [9] against endocannabinoid-induced cellular damage.

The recently described alternative 2-AG-degrading enzymes ABDH6 und ABHD12 [30] were not expressed in hepatocytes or HSCs and are thus unlikely to contribute to the remarkable difference between the two cell types in 2-AG-mediated cell death susceptibility.

Moreover, 2-AG can be metabolized effectively by cyclooxygenase-2 (COX-2) [31], which is highly expressed by activated HSCs [32], but not in hepatocytes [33]. Whether this differential expression of COX-2 in these cell types contributes to their discrepant susceptibility toward 2-AG-mediated cell death is currently under investigation.

Interestingly, $FAAH^{-/-}$ mouse livers displayed elevated AEA but not 2-AG levels, confirming that FAAH is the major degrading enzyme for AEA but not for 2-AG. However, during liver injury and fibrogenesis, hepatic AEA and 2-AG levels rise significantly [9,18]. It might be possible, that during diseased states of the organ, FAAH protects against increasing hepatocellular injury caused by rising levels of both deleterious endocannabinoids [29].

In turn, genetic or pharmacological blockade of MGL leads to hepatic accumulation of 2-AG but not of AEA [34,35]. Recently, Cao et al. have shown that pharmacological or genetic inactivation of MGL leads to protection against hepatic damage in diverse models of acute liver injury [34], supporting our finding that MGL does not contribute to protection of hepatocytes against endocannabinoid-induced injury, but even promotes it. On the other hand, we and others were able to show, that blockade of FAAH does not confer hepatic protection but enhances liver damage [8,34]. Thus, we now provide compelling evidence that FAAH not only protects hepatocytes from AEA-, but also from 2-AG-induced cell death.

In conclusion, FAAH-mediated resistance of hepatocytes against endocannabinoid-induced cell death may provide a new physiological concept allowing the specific targeting of HSCs in liver fibrosis.

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