Δ⁹-Tetrahydrocannabinol Induces Endocannabinoid Accumulation in Mouse Hepatocytes: Antagonism by Fabp1 Gene Ablation

by

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Running Title: FABP1 antagonizes THC induction of hepatic AEA and 2-AG

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Keywords: mouse, hepatocyte, FABP1, THC, endocannabinoid, binding, structure, metabolism
Abbreviations: AEA, n-6 arachidonoylethanolamide (anandamide); 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor-1; DAGLα, diacylglycerol lipase A; DHEA, n-3 docosahexaenoylethanolamide; EC, endocannabinoid; EPEA, n-3 eicosapentaenoylethanolamide; FAAH, fatty acid amide hydrolase; FABP1, liver fatty acid binding protein-1; FABP3, fatty acid binding protein-3; FABP5, fatty acid binding protein5; FABP7, fatty acid binding protein-7; FATP2, 4, and 5, fatty acid translocase protein-2 ,4, and 5; HSP70, heat shock protein 70; LCFA, long chain fatty acid; LCFA-CoA, long chain fatty acyl CoA; LC-MS, liquid chromatography/mass spectrometry; LKO, FABP1 gene ablated mouse on C57BL/6NCr background; 2-MG, 2-monoacylglycerol; MGL, 2-monoacylglycerol lipase; NAE, N-acylethanolamide; NAFLD, non-alcohol fatty liver disease; NAPE, N-acylphosphatidylethanolamide; NAPEPLD, N-acylphosphatidylethanolamide phospholipase-D; OEA, oleylethanolamide; 2-OG, 2-oleoylglycerol; PEA, palmitoylethanolamide; 2-PG, 2-palmitoylglycerol; PPARα, peroxisome proliferator-activated receptor alpha; SCP-2, sterol carrier protein-2; SCP-x, sterol carrier protein-x; Δ9-THC, Δ9-Tetrahydrocannabinol; WT, wild-type C57BL/6NCr mouse.
ABSTRACT

Phytocannabinoids such as Δ⁹-tetrahydrocannabinol (Δ⁹-THC), bind and activate cannabinoid receptors (CB)—thereby ‘piggy-backing’ on the same pathway endogenous endocannabinoids (EC). The recent discovery that liver fatty acid binding protein-1 (FABP1) is the major cytosolic ‘chaperone’ protein with high affinity for both Δ⁹-THC and EC suggests that Δ⁹-THC may alter hepatic EC levels. Therefore, the impact of Δ⁹-THC or EC treatment on endogenous ECs such as N-arachidonylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) levels was examined in cultured primary mouse hepatocytes from wild-type (WT) and liver fatty acid binding protein-1 (Fabp1) gene ablated (LKO) mice. Δ⁹-THC alone or 2-AG alone significantly increased AEA and especially 2-AG levels in WT hepatocytes. LKO alone markedly increased AEA and 2-AG levels. However, LKO blocked/diminished the ability of Δ⁹-THC to further increase both AEA and 2-AG. In contrast, LKO potentiated the ability of exogenous 2-AG to increase hepatocyte level of AEA and 2-AG. These and other data suggest that Δ⁹-THC increases hepatocyte EC levels at least in part by upregulating endogenous AEA and 2-AG levels. This may arise from Δ⁹-THC competing with AEA and 2-AG binding to FABP1, thereby decreasing targeting of bound AEA and 2-AG to degradative enzymes, FAAH and MAGL, to decrease hydrolysis within hepatocytes.
INTRODUCTION

Since the discovery of key elements of the endocannabinoid system in liver and its roles in non-alcoholic fatty liver disease (NAFLD), much research has focused on development of agonists/antagonists of this system. Cannabinoid (CB) receptors, together with their endogenous endocannabinoid (EC) ligands, i.e. N-arachidonylethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG), constitute a novel system for modulating not only behavior, satiety, pain and inflammation (1-7), but also hepatic fat accumulation (8-10). Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the main psychotropic component of cannabis, binds and activates CB receptors—thereby ‘piggy-backing’ on the endogenous EC pathway (11-13).

Several studies indicate that cannabis use or Δ⁹-THC treatment promote steatosis and are associated with development of NAFLD (14, 15). Similarly, activation of the endogenous EC system to increase AEA, 2-AG, and CB1 is linked to NAFLD (8-10). CB1 is upregulated in patients with NAFLD (10) (9). Finally, studies with gene targeted mice reveal that CB1 activation is essential for NAFLD development (8, 9, 16). The mechanism whereby CB1 induces hepatic lipid accumulation involves downstream upregulation of sterol regulatory element binding protein 1c (SREBP1c) which in turn induces transcription of de novo lipogenic enzymes (8, 14). However, it is not known if Δ⁹-THC elicits its steatotic effects in liver only by directly activating hepatic CB1 and/or also by indirectly upregulating the liver endogenous EC activators of CB1 (AEA, 2-AG). In support of the latter possibility, Δ⁹-THC treatment increases circulating levels and brain levels of ECs (AEA, 2-AG) in humans and rodents (17, 18). However, nothing is known regarding the impact of Δ⁹-THC on hepatic EC levels or the mechanism(s) whereby this may occur.

Recent novel discoveries suggest mechanistic involvement of fatty acid binding protein-1 (FABP1) in the hepatic endocannabinoid system (19-21). FABP1 binds both phytocannabinoids (Δ⁹-THC) as well as endocannabinoids (AEA, 2-AG) with high affinity and is the major cytosolic ‘chaperone’ protein for transport/targeting of these lipid ligands to intracellular metabolic and regulatory sites (19-21). Moreover, the level of hepatic FABP1 protein is markedly upregulated in animal models of NAFLD (22, 23) and...
human NAFLD (24), especially in human subjects expressing the highly prevalent FABP1 T94A variant (26-38% minor allele frequency; 8.3±1.9% homozygous) (rev. in (21). While the human FABP1 T94A variant protein differs only modestly from its wild-type FABP counterpart in affinities for endocannabinoids (AEA, 2-AG) (25) as well as a variety of other ligands (26-29), the conformation of FABP1 T94A variant is much less responsive to ligand-induced change (25, 26) which in turn diminishes its ability to enter nuclei, interaction with, and activation of peroxisome proliferator activated receptor-α (PPARα) therein (29).

To begin to address these issues, the effect of Δ⁹-THC treatment on ECs and proteins in the EC system was examined in cultured primary hepatocytes from male WT C57BL/6N mice and from liver fatty acid binding protein (Fabp1) gene ablated mice (LKO) on the same background. Hepatic levels of ECs (AEA, 2-AG) as well as non-arachidonic acid N-acylethanolamides (NAE) and 2-monoacylglycerols (2-MGs) were determined by liquid chromatography/mass spectrometry (LC-MS) while hepatic proteins in the EC system were quantitated by western blotting. The results showed that Δ⁹-THC treatment induced hepatic accumulation of AEA and 2-AG—effects antagonized by loss of FABP1.
MATERIALS AND METHODS

Materials. n-6 arachidonoylethanolamide (AEA), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), n-3 docosahexaenoylethanolamide (DHEA), n-3 eicosapentaenoylethanolamide (EPEA), 2-arachidonoylglycerol (2-AG), 2-oleoylglycerol (2-OG), and 2-palmitoylglycerol (2-PG) were purchased from Cayman Chemical (Ann Arbor, MI). Deuterated AEA-d4, OEA-d2, PEA-d4, DHEA-d4, EPEA-d4, and 2-AG-d8 were also from Cayman Chemical (Ann Arbor, MI). The phytocannabinoid Δ9-tetrahydrocannabinol (Δ9-THC, also called dronabinol) was also acquired from Cayman Chemical (Ann Arbor, MI).

The following antibodies to liver proteins involved in the hepatic endocannabinoid system were obtained commercially as follows: anti-fatty acid amide hydrolase (FAAH, sc-26427), anti-N-acylphosphatidylethanolamide phospholipase-D (NAPE-PLD; sc-163117), anti-fatty acid transport protein 4 (FATP-4; sc-5834), rabbit polyclonal anti-monoacylglyceride lipase (MAGL, sc-134789), anti-diacylglycerol lipase α (DAGLα; sc-133307), and liver-type fatty acid binding protein (FABP1, L-FABP; sc-16064) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-fatty acid transport protein 4 (FATP-4; sc-5834), polyclonal anti fatty acid transport protein 2 (FATP2, ab83763) and specific monoclonal anti-mouse heat shock protein-70 (HSP70; ab2787) were from Abcam (Cambridge, MA). Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, MAB374) was from Millipore, Inc (Billerica, MA). Rabbit polyclonal anti-sterol carrier protein-2 recognizing both Sfp-2/Sfp-x gene products (58 kDa SCP-x, and 13.2 kDa SCP-2) was prepared as in (30). All solvents and reagents used were of the highest commercial grade available.

WT Fabp1 and Fabp1 gene ablated mice. Male WT and Fabp1 gene ablated (LKO) mice were obtained similarly as described earlier (31, 32). During maintenance of the mouse colony, all mice were fed a standard rodent chow mix [5% calories from fat; D8604 Teklad Rodent Diet, Teklad Diets (Madison, WI)] and were maintained in barrier cages on ventilated racks at 12-hr light/dark cycle in a temperature controlled facility (25°C) with ad libitum food and water until study initiation. Mice were sentinel
monitored quarterly and confirmed free of all known rodent pathogens. Experimental protocols for animal use were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

**Isolation and culture of primary mouse hepatocytes.** Male WT and LKO mice 5-7 mo. old were maintained, housed, and fed as described above and primary hepatocytes isolated as described earlier (32-34). Briefly, mice were euthanized by CO₂ asphyxiation, livers excised, and perfused with Buffer A [10 mM HEPES, pH 7.4 in calcium/magnesium-free Hank’s buffered saline solution (HBSS), gentamycin sulfate (1 mg/ml medium), and 0.5 mM EGTA]. To release the hepatocytes, the livers were then perfused with Buffer B [Buffer A without EGTA, supplemented with 5 mM CaCl₂, Collagenase type IV (Sigma, at concentration of 100 unit/ml], and 5% FBS. To facilitate hepatocyte release, the liver capsule was gently palpated during perfusion. Primary hepatocytes were then washed twice in cold DMEM with 5% fetal bovine serum, purified with Percoll gradient (33), plated on collagen-coated dishes at 3×10³ cells/100 mm culture dish, and cultured overnight at 37°C in a CO₂ incubator as described in the cited papers.

**Treatment of cultured primary mouse hepatocytes with phytocannabinoid (Δ⁹-THC) and endogenous (2-AG) agonists of cannabinoid receptor (CB1).** A time course and dose response treatment was performed as follows. Primary mouse hepatocytes were isolated from WT mice, and cultured overnight as described above. The hepatocytes were washed with PBS and further incubated with 2µM THC/0.015% BSA, 20µM THC/0.15% BSA, 40µM THC/0.3% BSA in Puck’s buffer for 0-2 h time as indicated in the Figure legends in a 37°C incubator as described (32-34). These concentrations were chosen to be in the range of those used previously with a variety of hepatoma and other cell lines (35-37). The concentrations of BSA were chosen based upon an approximate 1:1 binding stoichiometry at the µM concentrations of Δ⁹-THC and 2-AG (34-36). The control was incubated in Puck’s buffer with the same amount of MeOH vehicle and 0.15% BSA (without Δ⁹-THC). The 20µM Δ⁹-THC concentration was chosen as used previously (37). Multiple culture dishes of primary mouse hepatocytes were incubated at 20µM THC/0.15% BSA in Puck’s buffer for 1 hour in a 37°C incubator as described (32-34). The control was incubated in Puck’s buffer with the same amount of MeOH vehicle and 0.15% BSA (without Δ⁹-THC).
For treatment with endogenous CB1 agonist, hepatocytes were incubated similarly as above except for without or with 2-AG (1μM)—a low concentration that does not produce adverse effects in cultured primary hepatocytes (38, 39).

**Lipid extraction from cultured primary mouse hepatocytes.** Hepatocytes were removed from the culture plate and stored in sealed microtubes at -80°C as described earlier (32, 40, 41). For lipid analysis, the hepatocytes in each microtube were thawed on ice, vortexed, and contents subsequently transferred to an ice-cold Dounce homogenizer. The microtube were then rinsed with 1 ml of ice cold KPD (20 mM potassium phosphate, pH7.4, 1 mM DTT) to remove any residual hepatocytes. This solution was also vortexed and combined with the previous hepatocyte/buffer mixture into the Dounce homogenizer. This hepatocyte/buffer mixture was homogenized using ten up/down strokes of the Dounce homogenizer. For protein quantification, an aliquot (200 µL) of the homogenate was removed and placed in a separate microtube as described previously (19, 40). The following internal standard mixture was added: 4000 pg each of AEA-d4, OEA-d2, PEA-d4, EPEA-d4, and 40000 pg of 2-AG-d5 to the remaining homogenate in the Dounce homogenizer. Extraction of the lipids from the hepatocyte homogenate was performed as described previously (40). The final lipid extract was dried under N₂ and then dissolved in 60 µL of acetonitrile with the addition of 60 µL of H₂O. Each of the samples were then purged with N₂ gas and stored at -80 °C.

**Liquid chromatography/mass spectrometry (LC-MS) analysis of N-Acylethanolamides (NAE) and 2-monoacylglycerols (2-MG) in cultured primary mouse hepatocytes.** The lipid extracts (above) were resolved to determine NAE and 2-MG levels as quantitated by LC-MS analysis in the Protein Chemistry Laboratory (directed by Dr. Larry Dangott at Texas A&M University) as described (19, 42). An external standard bracket to determine linear range was performed of 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, or 50.0 ng of AEA, OEA, PEA, DHEA, EPEA, 2-AG, 2-OG, and 2-PG, respectively. Individual NAE or 2-MG values were expressed based on hepatocyte homogenate mg protein.
Impact of Δ⁹-THC treatment on protein levels in the endocannabinoid (EC) system of cultured primary hepatocytes from livers of WT and LKO mice. To determine if the altered endocannabinoid levels in wild-type (WT) and/or LKO hepatocytes treated with Δ⁹-THC resulted from altered expression of proteins in the endocannabinoid system, hepatocytes were homogenized, protein determined, 2-10 μg protein loaded/resolved by SDS-PAGE, bands of interest identified by western blotting, and quantitated as described (32, 40, 41). Hepatocyte levels of the following groups of proteins involved in the liver EC system were determined by Western blotting: i) Synthetic enzymes: N-acylphosphatidylethanolamide phospholipase-D (NAPE-PLD), diacylglycerol lipase A (DAGL-A), ii) Degradative enzymes: fatty acid amide hydrolase (FAAH), N-acylethanolamide-hydrolyzing acid amidase (NAAA), and 2-monoacylglycerol lipase (MAGL), iii) CB receptors: cannabinoid receptor-1 (CB1), iv) Cytosolic transport/chaperone proteins: fatty acid binding protein-1 (FABP1), heat shock protein-70 (HSP70), and sterol carrier protein 2 (SCP-2) (recognizes 58 kDa SCP-x as well as 13.2 kDa SCP-2), and v) Membrane fatty acid translocases involved in uptake of arachidonic acid (ARA)—the precursor from which EC are derived: fatty acid transport protein 2 (FATP-2), fatty acid transport protein 4 (FATP-4). GAPDH was used as internal loading control. Individual protein bands on western blots were quantitated by densitometric analysis by ImageJ software (NIH, Bethesda, MD) and normalized to GAPDH as in (42). Representative western blots were then cropped and inserted (separated by a white line/space) into figure panels as in earlier publications.

Statistical analysis. Values represent the mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed with the Newman-Keuls post-hoc analysis using Sigma Plot software (Systat, San Jose, CA). P-values of \( p < 0.05 \) were considered statistically significant and were denoted by a # (FABP1 KO vs wild-type) or * (Δ⁹-THC treated vs untreated of the same genotype).

RESULTS
Impact of Δ⁹-Tetrahydrocannabinol treatment on hepatocyte level of endogenous CB1 agonists N-arachidonylethanolamide (AEA) and 2-arachidonoylgllycerol (2-AG): time and Δ⁹-THC concentration dependence. Hepatocytes express primarily cannabinoid receptor CB1 (43) which is activated by both phytocannabinoids (e.g. Δ⁹-THC) and endocannabinoids (e.g. AEA, 2-AG) (12, 44). Likewise, the major liver cytosolic lipid binding/‘chaperone’ protein (i.e. liver fatty acid binding protein-1, FABP1) has high affinity for both Δ⁹-THC and endocannabinoids (AEA, 2-AG) (19-21). Therefore, the possibility that Δ⁹-THC treatment may indirectly impact CB1 activation by altering the level of the AEA and 2-AG was examined.

Wild type (WT) cultured primary mouse hepatocytes were incubated with increasing Δ⁹-THC (2-40 μM) and time (up to 2 h) and hepatocyte AEA and 2-AG level determined as in Methods. Δ⁹-THC treatment elicited a concentration-dependent increase AEA level at all time points examined (Fig. 1A). Likewise, increasing Δ⁹-THC generally increased hepatocyte 2-AG level (Fig. 1B). Based on these findings, a 20 μM Δ⁹-THC concentration and 1 h incubation time was chosen as set of non-saturating conditions to determine statistical significance of the impact of Δ⁹-THC on AEA and 2-AG levels as described in the following sections.

Δ⁹-Tetrahydrocannabinol treatment increases cultured primary hepatocyte level of AEA: impact of Fabp1 gene ablation.

Since liver fatty acid binding protein (FABP1) has high affinity for endocannabinoids as well as Δ⁹-THC (19-21), the possibility that loss of FABP1 protein (i.e. Fabp1 gene ablation, LKO) may alter the ability of Δ⁹-THC to increase levels of AEA, an endogenous CB1 agonist, was examined in cultured primary hepatocytes from wild-type WT and LKO mice.

Treatment of WT hepatocytes with Δ⁹-THC alone significantly increased AEA nearly 30% (Fig. 2A). LKO alone (in the absence of Δ⁹-THC) increased WT hepatocyte AEA by nearly 2-fold (Fig. 2A). However, Δ⁹-THC treatment of LKO hepatocytes did not significantly increase AEA level (Fig. 2A).

Taken together, the above data indicated that both Δ⁹-THC alone and, even more so, LKO alone
significantly increased WT hepatocyte level of AEA. However, LKO blocked the ability of Δ⁹-THC to further increase AEA content in LKO hepatocytes.

**Effect of Δ⁹-Tetrahydrocannabinol treatment on hepatocyte levels of non-arachidonic acid containing NAEs (OEA, PEA, DHEA, EPA).** Non-ARA containing NAEs [N-palmitoylethanolamide (PEA), N-oleoylethanolamide (OEA), N-docosahexaenoylethanolamide (DHEA), N-eicosapentaenoylethanolamide (EPEA)] and 2-MGs [2-oleoylglycerol (2-OG), 2-palmitoylglycerol (2-PG)] do not directly bind/activate CB receptors. Nevertheless, such NAEs and 2-MGs can act as ‘entourage’ molecules that enhance the effects of AEA and/or 2-AG by competing either with transporters or the enzymes mediating the inactivation of endocannabinoids or by enhancing binding/action of ECs such as AEA and 2-AG on CB receptors (45-53).

Δ⁹-THC treatment alone did not significantly affect NAE levels in cultured primary hepatocytes from WT mice (Fig. 2B-E). LKO alone increased hepatocyte level of EPEA by 1.8-fold (Fig. 2E), but did not significantly alter that of OEA (Fig. 2B), PEA (Fig. 2C), or DHEA (Fig. 2D). LKO completely blocked the effect of Δ⁹-THC on increasing hepatocyte level of EPEA (Fig. 2E).

Thus, Δ⁹-THC alone did not significantly alter WT hepatocyte levels of NAEs. In contrast, LKO alone selectively increased hepatocyte level of EPEA regardless of the presence or absence of Δ⁹-THC.

**2-arachidonoylglycerol treatment increases hepatocyte levels of AEA: Impact of Fabp1 gene ablation.**

To determine if the Δ⁹-THC-induced increase in hepatocyte AEA was specific to this exogenous phytocannabinoid CB1 activator, the effect of the endogenous CB1 agonist 2-arachidonoylglycerol (2-AG) was examined. Liver fatty acid binding protein (FABP1) has high affinity for the endogenous CB1 receptor agonist 2-AG (19-21). Therefore, the impact of 2-AG and/or FABP1 on hepatocyte level of AEA was examined in cultured primary hepatocytes from wild-type WT and Fabp1 gene ablated (LKO) mice.

Treatment of WT hepatocytes with 2-AG significantly increased the level of AEA (Fig. 3A). In contrast, LKO alone (in the absence of 2-AG) increased AEA by 2.2-fold (Fig. 3A). Furthermore, LKO
conferred on 2-AG the ability to increase WT hepatocyte AEA level (Fig. 3A).

With regards to the non-ARA containing NAEs, 2-AG treatment of WT hepatocytes increased the level of EPEA by 2-fold (Fig. 3E) while not changing that of OEA, PEA, or DHEA (Fig. 3B-D). LKO alone likewise increased WT hepatocyte level of EPEA by 2-fold (Fig. 3E), but not that of OEA, PEA, or DHEA (Fig. 3B-D). Finally, LKO blocked the ability of 2-AG to increase hepatocyte level of EPEA (Fig. 3E) and did not affect levels of OEA, PEA, or DHEA (Fig. 3B-D).

These data indicated that 2-AG treatment alone increased WT hepatocyte AEA level analogous to \( \Delta^9 \)-THC-induced AEA increase (Fig. 2A). LKO alone significantly increased hepatocyte level of AEA and moreover conferred on 2-AG the ability to increase hepatocyte AEA content. This was in marked contrast to LKO blocking of the ability of \( \Delta^9 \)-THC to further increase AEA content therein (Fig. 2A).

Impact of \( \Delta^9 \)-Tetrahydrocannabinol and \( Fabp1 \) gene ablation on hepatocyte levels of the endocannabinoid 2-arachidonoylglycerol and non-arachidonic acid containing 2-monaclylglycerols (2-OG, 2-PG).

Since FABP1 exhibits high affinity for 2-AG as well as (\( \Delta^9 \)-THC) (19, 20), the effect of \( \Delta^9 \)-THC on cultured primary hepatocyte levels of the 2-monoacylgllycerols was examined as described in Methods.

\( \Delta^9 \)-THC treatment significantly increased WT hepatocyte level of 2-AG 1.8-fold (Fig. 4A). LKO alone increased 2-AG level even more, i.e. by 3.3-fold (Fig. 4A). However, LKO impaired the ability of \( \Delta^9 \)-THC to increase hepatocyte 2-AG by nearly half (Fig. 4A).

With regards to the non-ARA containing 2-MGs, \( \Delta^9 \)-THC treatment significantly increased WT hepatocyte level of 2-OG by 1.7-fold (Fig. 4B) while also increasing that of 2-PG by 1.5-fold (Fig. 4C). LKO alone increased levels of 2-OG even more by 3.5-fold (Fig. 4B), but not that of 2-PG (Fig. 4C). However, LKO did not further potentiate the ability of \( \Delta^9 \)-THC to increase hepatocyte 2-OG (Fig. 4A) or 2-PG (Fig. 4C) as compared to their \( \Delta^9 \)-THC-treated WT counterparts.

Thus, \( \Delta^9 \)-THC treatment alone in WT hepatocytes and even more so LKO alone markedly increased hepatocyte levels of 2-AG much more than those of AEA (Fig. 3A). Furthermore, both \( \Delta^9 \)-THC treatment
alone in WT hepatocytes and LKO alone markedly increased hepatocyte levels of 2-OG and 2-PG—in marked contrast to effect of Δ⁹-THC treatment on the non-ARA containing 2-MGs which were not further increased by LKO (Fig. 2).

Impact of 2-arachidonoylglycerol and Fabp1 gene ablation hepatocyte level of 2-arachidonoylglycerol and non-arachidonic acid containing 2-monoacylglycerols.

It is not known if the ability of Δ⁹-THC- to markedly increase hepatocyte 2-AG and other 2-MGs noted above was unique to this exogenous phytocannabinoid CB1 agonist or also shared by the endogenous CB1 agonist 2-AG. Therefore, the impact of exogenous 2-AG on hepatocyte levels of 2-AG and non-ARA containing 2-MGs was examined in cultured primary hepatocytes from wild-type WT and LKO mice as described in Methods.

2-AG treatment alone significantly increased 2-AG levels by 16-fold in WT hepatocytes (Fig. 5A). LKO alone also increased 2-AG level to the same extent, i.e. 15-fold (Fig. 5A). Further, LKO markedly potentiated the ability of exogenous 2-AG increased hepatocyte 2-AG content to the level--over 5-fold more than in 2-AG treated WT hepatocytes (Fig. 5A).

With regards to the non-ARA containing 2-MGs, 2-AG treatment and/or LKO selectively impacted only 2-oleoylglycerol (2-OG, Fig.5B), but not 2-palmitoylglycerol (2-PG, Fig. 5C) level. While 2-AG alone had no effect on WT hepatocyte 2-OG level, LKO alone increased hepatocyte 2-OG level by 4-fold (Fig. 5B). In contrast, LKO diminished by 50% the ability of 2-AG to increase 2-AG level in the hepatocytes (Fig. 5B). Neither 2-AG alone, LKO alone, nor both together significantly affected the 2-PG level in the hepatocytes (Fig. 5C).

Taken together, these data showed that 2-AG treatment markedly increased hepatocyte levels of 2-AG several-fold in WT hepatocytes—in marked contrast to Δ⁹-THC which induced 2-AG much less in WT hepatocytes (Fig. 4A). LKO exacerbated this 2-AG-induced increase by several fold in hepatocyte 2-AG level—again in marked contrast to LKO diminishing the ability of Δ⁹-THC to increase 2-AG (Fig. 4A). The simple explanation for this may be that the exogenous agent became sequestered in membranes, accounting
for the significant 2-AG increase that would be unrelated to the presence of intracellular trafficking proteins. This possibility was addressed in a control experiment wherein cultured primary mouse hepatocytes were incubated without and with a solution of 900 nM 2-AG and 100 nM 2-AG-d8 similarly as described herein for unlabeled 2-AG (54). While hepatocyte level of both unlabeled 2-AG and 2-AG-d8 was increased similarly in WT hepatocytes, this effect was exacerbated by LKO.

**Impact of Δ⁹-Tetrahydrocannabinol and Fabp1 gene ablation on hepatocyte protein levels of enzymes in endocannabinoid synthesis and degradation:** Liver fatty acid binding protein (FABP1) is the major hepatic cytosol binding/’chaperone’ protein for both Δ⁹-THC and ECs such as AEA and 2-AG (19, 21, 25, 55). Competition between these ligands may potentially impact hepatocyte protein levels of the enzymes in EC synthesis and degradation, or CB1 receptor. Therefore, cultured primary hepatocytes from wild-type (WT) and FABP gene (Fabp1) ablated (LKO) mice were treated with or without Δ⁹-THC and western blotting of the respective proteins was performed as described in Methods.

Δ⁹-THC differentially impacted hepatocyte protein levels of the AEA and 2-AG synthetic enzymes NAPEPLD and DAGL. Neither Δ⁹-THC nor LKO significantly altered hepatocyte protein level of NAPEPLD—the key enzyme in AEA and NAE synthesis (Fig. 6A). Δ⁹-THC alone did not alter protein level of DAGL, the key enzyme in 2-AG and 2-MG synthesis, in WT hepatocytes (Fig. 6B). In contrast, the DAGL protein level in LKO hepatocytes was decreased regardless of the presence or absence of Δ⁹-THC (Fig. 6B).

Δ⁹-THC alone also differentially impacted hepatocyte protein levels of AEA and 2-AG degradative enzymes, FAAH and MAGL. Δ⁹-THC alone significantly increased the protein level of FAAH, the major AEA and NAE hydrolysis enzyme, in cultured primary hepatocytes from WT mice—an effect abolished by LKO (Fig. 6C). In contrast, neither Δ⁹-THC nor LKO significantly altered the protein level of MAGL (degrades 2-AG and 2-MGs) in hepatocytes.

Finally, neither Δ⁹-THC nor LKO altered protein level of CB1 (major cannabinoid receptor in hepatocytes (Fig. 6E).
Overall, these data indicated that the Δ⁹-THC-induced higher levels of AEA (Fig. 3) and 2-AG (Fig. 5) in WT hepatocytes were not associated with any upregulation of synthetic enzymes (NAPEPLD, DAGL) or downregulation of degradative enzymes (FAAH, MAGL). On the contrary, Δ⁹-THC-induced upregulation of FAAH would have been expected to decrease rather than increase AEA level. It appears Δ⁹-THC enhanced hepatocyte levels (to varying degrees of significance) of all acylethanolamides in WT hepatocytes (Fig 3). Perhaps this is due to modulation of other lipid metabolic systems by Δ⁹-THC, e.g. COX and LOX isoforms. Likewise, the LKO-induced higher levels of AEA (Fig. 3) and 2-AG (Fig. 5) were also not associated with any upregulation of synthetic enzymes (NAPEPLD, DAGL) or downregulation of degradative enzymes (FAAH, MAGL). In fact, some downregulation of DAGL occurred in the LKO (both without and with Δ⁹-THC) possibly because of significant accumulation of 2-AG in the LKO as compared to the WT. Some evidence indicates that DAGL synthesis of 2-AG occurs ‘on demand’ (56). While these possibilities contextualize the findings, it is beyond the scope of the present work to experimentally resolve all potential mechanisms.

Effect of Δ⁹-Tetrahydrocannabinol and Fabp1 gene ablation on hepatocyte protein levels of membrane transport/translocase proteins involved in uptake of arachidonic acid from which AEA and 2-AG are derived: While the mechanism(s) whereby Δ⁹-THC, AEA, and 2-AG undergo uptake/reuptake across membranes is not yet clear, uptake of fatty acids such as ARA (precursor of AEA and 2-AG) occurs at least in part by membrane associated fatty acid transport/translocase proteins (e.g. FATP2, FATP4) (57). Since Δ⁹-THC and LKO both increased hepatocyte levels of AEA and 2-AG, western blotting was performed to determine the impact of Δ⁹-THC on protein levels of FATP2 and FATP4 therein. Neither Δ⁹-THC nor LKO significantly altered hepatocyte protein levels of FATP2 or FATP4. Thus, the Δ⁹-THC-induced increase in hepatocyte AEA (Fig. 3) and 2-AG (Fig. 5) was not associated with altered expression of membrane translocases involved in ARA uptake.

Effect of Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) and Fabp1 gene ablation on hepatocyte protein levels cytosolic proteins that bind/’chaperone’ Δ⁹-THC, AEA, 2-AG and/or ARA: Several liver
cytosolic proteins bind/’chaperone’ lipidic ligands such as ARA [FABP1 (58, 59)], AEA [FABP1 (19-21) (58), SCP-2 (60), HSP70 (61)], 2-AG [FABP1 (19-21), SCP-2 (60)], and Δ⁹-THC [FABP1 (19-21)]. Therefore, western blotting was performed to determine the impact of Δ⁹-THC on protein levels of these cytosolic binding/’chaperone’ proteins in cultured primary hepatocyte isolated from livers of wild-type (WT) and fatty acid binding protein gene (Fabp1) ablated (LKO) mice.

In cultured primary hepatocytes isolated from livers of WT mice, Δ⁹-THC treatment alone did not significantly alter protein level of binding/’chaperone’ proteins FABP1 (Fig. 7C), HSP70 (Fig. 7D), or SCP-2 (Fig. 7E). In contrast, LKO alone modestly increased hepatocyte protein level of HSP70 (Fig. 7D), but not SCP2 (Fig. 7E). However, LKO differentially impacted the ability of Δ⁹-THC to alter protein levels of these proteins in cultured primary hepatocytes—decreasing that of HSP70 (Fig. 7D) which was offset by an increase in SCP-2 (Fig. 7E). Δ⁹-THC has now been shown to bind not only FABP1 (19, 21) but also other members of the FABP family (e.g. FABP3, FABP5, and FABP7) (37) with moderate affinities. Since liver also expresses the intestinal form of FABP, i.e. FABP2, it seems plausible that concomitant upregulation of FABP2 also may play some transport role for endocannabinoid and/or cannabinoid in hepatocytes. However, despite a significant 35% increase in upregulation of the intestinal fatty acid binding protein (FABP2) in the LKO hepatocytes (data not shown), FABP2 represents a very small constituent in the liver—much lower than that of FABP1 (62). Furthermore, FABP2 has not been found to bind monoacylglycerols (63), although its binding affinities for endocannabinoids and Δ⁹-THC have not been directly ascertained.

Thus, the Δ⁹-THC-induced higher levels of AEA (Fig. 3) and 2-AG (Fig. 5) in cultured primary hepatocytes from livers of WT mice did not appear to be associated with overall altered expression of cytosolic binding/’chaperone’ proteins in hepatocytes from livers from either WT or LKO mice.

DISCUSSION
Since the discovery that key elements of the endocannabinoid system are present in liver and are involved in non-alcoholic fatty liver disease (NAFLD) (9, 64), much research has focused on development of agonists/antagonists of this system. $\Delta^9$-THC, the main psychotropic component of cannabis, binds and activates cannabinoid receptors (CB)—thereby ‘piggy-backing’ on the endogenous EC pathway (11-13). Although liver expresses both CB1 and CB2 receptor subtypes, their cellular distribution differs significantly such that hepatocytes contain primarily CB1 (43). CB1 binds and is activated by both phytocannabinoids (e.g. $\Delta^9$-THC) as well as endogenous endocannabinoids (e.g. AEA, 2-AG (12, 44). However, it is not known if phytocannabinoids such as $\Delta^9$-THC or endocannabinoids such as 2-AG themselves may indirectly impact CB1 by concomitantly altering levels of other endogenous EC in liver hepatocytes. This possibility is suggested by recent findings that the most prevalent cytosolic lipidic ligand binding protein in hepatocytes, i.e. liver fatty acid binding protein-1 (FABP1), has high affinity for both phytocannabinoid (e.g. $\Delta^9$-THC) and endocannabinoid (e.g. AEA, 2-AG) agonists of CB1 (19-21, 65).

Therefore, the impact of exogenous $\Delta^9$-THC and 2-AG treatment on hepatocyte AEA and 2-AG levels was examined in cultured primary mouse hepatocytes from wild-type (WT) and liver fatty acid binding protein-1 gene ($Fabp1$) ablated (LKO) mice. The data provide the following new insights:

First, the phytocannabinoid CB1 agonist $\Delta^9$-THC significantly increased AEA and 2-AG level in WT hepatocytes. Consistent with this finding, phytocannabinoids [i.e. $\Delta^9$-THC, cannabidiol (CBD)] increase levels of AEA and 2-AG in blood and brain of humans and rodents (17, 18) (37). Since CB1 has similar affinity for AEA as for $\Delta^9$-THC (44), this suggests that $\Delta^9$-THC may at least in part exert its activating effect on CB1 by increasing the hepatocyte’s endogenous level of AEA. $\Delta^9$-THC even more dramatically increased WT hepatocyte level of 2-AG by 2-fold more than AEA. Despite CB1’s weaker affinity for 2-AG than for either AEA or $\Delta^9$-THC (44), 2-AG is about 3-fold more potent than AEA at CB1 (8, 66, 67). While the 2-AG-induced increase in WT hepatocyte level of 2-AG may be attributable at least in part to increased 2-AG available for uptake, 2-AG had no effect on the non-ARA containing 2-MGs, i.e. 2-OG, 2-PG, in WT hepatocytes. Taken together, these novel observations showed that exogenously added $\Delta^9$-THC
as well as 2-AG, increased WT hepatocyte level of AEA and even more so 2-AG. Although the hepatocytes were incubated with about 20-fold higher concentration levels than typically observed in mouse serum after either intravenous injection of 3mg/kg or inhalation of 20mg of Δ⁹-THC (68), uptake did not appear saturated with respect to concentration.

Second, loss of FABP1 (i.e. Fabp1 gene ablation) alone increased AEA and 2-AG levels in cultured primary mouse hepatocytes by more than 2-fold. This finding is physiologically significant since LKO also significantly increased AEA and 2-AG in mouse liver, albeit to a smaller extent near 30% (19). In addition, LKO concomitantly increased WT hepatocyte levels of EPEA and 2-OG by >2- and 4-fold, respectively. A similar effect, albeit also of smaller magnitude was also observed in livers of LKO mice (19). The significance of LKO’s impact on the non-ARA containing NAE (i.e. EPEA) and 2-MG (i.e. 2-OG) lies in their ability to indirectly alter the effectiveness of CB1 agonists. While non-ARA containing NAEs (OEA, PEA) and 2-MGs (2-OG, 2-PG) do not directly bind/activate CB receptors, they represent ‘entourage’ molecules that may enhance the effects of AEA by competing either with transporters or the enzymes mediating the inactivation of endocannabinoids or by enhancing binding/action of ECs such as AEA (45-52). In contrast, the EPA-derived EPEA displaces AEA and 2-AG from cell membranes to reduce AEA and 2-AG release by synthetic enzymes (53). In fact, EPA supplementation in humans and animals decreases 2-AG and AEA in brain and plasma (53). Since LKO elicits a several-fold larger increase in hepatocyte 2-OG than EPEA, this would suggest potential net potentiation of CB1 agonists.

Third, LKO blocked/diminished the ability of Δ⁹-THC to increase both AEA and 2-AG, but in contrast, potentiated the ability of 2-AG to increase hepatocyte level of AEA and 2-AG. The reasons for the opposite effects of LKO on Δ⁹-THC and 2-AG ability to impact hepatocyte AEA and 2-AG are not completely clear. One possibility is based on differences in CB1’s and FABP1’s affinities for these ligands. For example, CB1 binds Δ⁹-THC with nearly 10-fold higher affinity than for 2-AG (44). On the other hand FABP1 binds 2-AG with 10-fold higher affinity than for Δ⁹-THC (19). An alternate possibility may relate to mechanistic difference in uptake of Δ⁹-THC and 2-AG. Nearly 90% of oral cannabinoid undergoes first-pass removal
by the liver (69-73) by a yet poorly understood mechanism (72-76). Although the mechanism of endocannabinoid (AEA, 2-AG) uptake across the plasma membrane is also not completely clear (74, 75), AEA uptake appears to be driven by intracellular degradative enzymes (37, 76). Much less is known about 2-AG uptake, except that it is saturable and blocking 2-AG hydrolysis does not alter the rate of 2-AG uptake (76, 77).

A potential mechanism whereby FABP1 may be involved in mediating the above effects of Δ⁹-THC and 2-AG on hepatocyte levels of AEA and 2-AG is shown in a proposed schematic model (Fig. 8). This model is based on the fact that Δ⁹-THC, AEA, and 2-AG are all highly lipophilic molecules that are highly associated with membranes as shown in a proposed schematic model. Within the hepatocyte, the lipophilicity of Δ⁹-THC, AEA, and 2-AG requires soluble binding/‘chaperone’ proteins that facilitate cytosolic transport to intracellular target sites (e.g. release, reuptake, degradation). This is analogous as has been demonstrated for FABP5 and FABP7 in cultured transfected cells (75, 78-82). Since FABP1 is the most prevalent high-affinity liver cytosolic binding protein for both Δ⁹-THC and endocannabinoids such as AEA and 2-AG (19-21, 65), FABP1 may similarly function in this role in hepatocytes (see middle portion of Figure 8). In support of this possibility, FABP1 is known to enhance uptake, cytosolic transport, and targeting of other lipophilic ligands (e.g. fatty acids) to oxidative organelles in cultured primary mouse hepatocytes (31, 40) and transfected cells overexpressing FABP1 (83-86). The fact that Δ⁹-THC inhibits uptake of AEA (37) suggests that inhibition of AEA internalization (for subsequent degradation) by Δ⁹-THC treatment may account at least in part for the increase hepatocyte AEA level. Furthermore, once Δ⁹-THC translocates across the WT hepatocyte plasma membrane the FABP1 would bind/facilitate Δ⁹-THC desorption into cytosol as well as trafficking to intracellular sites for metabolism (ER) or excretion (bile). It is important to note that Δ⁹-THC is itself not an inhibitor of AEA hydrolysis by FAAH (37). Although FABP1 binds 2-AG and AEA more strongly than Δ⁹-THC (19-21, 65), AEA and 2-AG levels in WT hepatocytes (shown herein) and WT liver (shown earlier (19), are normally very low. Thus, once sufficient Δ⁹-THC is taken up it would displace FABP1-bound AEA and 2-AG (see left side of the schematic in Figure
This in turn would decrease the quantity of AEA and 2-AG trafficking/targeting to intracellular degradative sites—thereby increasing hepatocyte level of AEA and 2-AG as was indeed observed herein. WT hepatocyte treatment with 2-AG significantly increased 2-AG but did not statistically increase AEA. However, LKO markedly enhanced the impact of 2-AG by increasing both AEA and 2-AG.

In summary, the studies presented herein show for the first time that phytocannabinoids such as Δ⁹-THC not only directly bind/activate CB1 receptors in WT hepatocytes, but also indirectly activate CB1 receptors by increasing WT hepatocyte levels of both major endocannabinoids (AEA, 2-AG). The latter effect was likely due to internalized Δ⁹-THC displacing these endogenous CB1 activators from FABP1 binding sites and thereby decreasing their hydrolysis by FAAH and MGL. Fabp1 gene ablation LKO alone more markedly increased AEA and 2-AG levels since loss of this major AEA and 2-AG binding protein would be expected to decrease even further the targeting of these endocannabinoids for hydrolysis. In contrast, treatment of WT hepatocytes with the endocannabinoid 2-AG did not increase AEA level but only increased that of 2-AG—likely due to increased availability of 2-AG for uptake rather than any reduction in 2-AG transport to degradative sites. However, relatively little is known about uptake of 2-AG (76, 77).

Finally, LKO potentiated the ability of both Δ⁹-THC and 2-AG to increase hepatocyte level of AEA and 2-AG. Taken together, these and other data suggested that Δ⁹-THC increases hepatocyte EC levels at least in part by competing for binding to FABP1—a cytosolic ‘chaperone’ that facilitates targeting of bound EC to intracellular degradative enzymes.

Acknowledgements: This work was supported in part by TxAgriLife Research Stimulus Funds (FS).

Notes: The authors have no conflicts of interest with this article’s contents, which is solely the responsibility of the authors and does not necessarily represent the official views of TxAgriLife.
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Figure 1. Time and concentration dependence of Δ⁹-THC impact on hepatocyte levels of AEA and 2-AG. Cultured primary hepatocytes were isolated from wild-type (WT) mice, plated on culture dishes, incubated with increasing Δ⁹-THC (2-40 μM) for increasing time (0-2 hour), as described in Methods. Lipids were then extracted followed by analysis and quantitation by LC-MS as described in Methods. A. N-arachidonoylethanolamide (AEA); B. 2-arachidonoylglycerol (2-AG). n=1.
Figure 2. Effect of Δ⁹-THC and Fabp1 gene ablation on hepatocyte levels of AEA and non-ARA containing N-acylethanolamides. Cultured primary hepatocytes were isolated from WT or LKO mice, plated on culture dishes, incubated with Δ⁹-THC (20 μM) for 1 hour, as described in Methods. Lipids were then extracted followed by AEA and NAE analysis and quantitation by LC-MS also as described in Methods. A. N-arachidonylethanolamide (AEA); B. N-oleylethanolamide (OEA); C. N-palmitoylethanolamide (PEA); D. N-docosahexaenoylethanolamide (DHEA); E. N-eicosapentaenoylethanolamide (EPEA). Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 3. Effect of 2-arachidonoylglycerol and Fabp1 gene ablation on hepatocyte levels of AEA and non-ARA containing N-acylethanolamides. Hepatocytes were isolated from WT or LKO mice, plated on culture dishes, and incubated with 2-AG (1 μM) for 1 hour, lipids extracted, and analyzed by LC-MS to determine AEA and NAE levels as described in Methods. A. N-arachidonylethanolamide (AEA); B. N-oleoylethanolamide (OEA); C. N-palmitoylethanolamide (PEA); D. N-docosahexaenoylethanolamide (DHEA); E. N-eicosapentaenoylethanolamide (EPEA). Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 4. Effect of Δ⁹-THC and Fabp1 gene ablation on hepatocyte levels of 2-AG and non-ARA containing 2-monoacylglycerols. Hepatocytes were isolated from WT or LKO mice, plated on culture dishes, incubated with Δ⁹-THC (20 μM) for 1 hour, as described in Methods. Lipids were then extracted followed by 2-AG and 2-MG analysis and quantitation by LC-MS also as described in Methods. A. 2-arachidonoylglycerol (2-AG); B. 2-oleoylglycerol (2-OG); C. 2-palmitoylglycerol (2-PG). Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 5. Effect of 2-arachidonoylglycerol and Fabp1 gene ablation on hepatocyte levels of 2-AG and non-ARA containing 2-monoacylglycerols. Hepatocytes were isolated from WT or LKO mice, plated on culture dishes, incubated with 2-AG (1 μM) for 1 hour, as described in Methods. Lipids were then extracted followed by 2-AG and 2-MG analysis and quantitation by LC-MS also as described in Methods. A. 2-arachidonoylglycerol (2-AG); B. 2-oleoylglycerol (2-OG); C. 2-palmitoylglycerol (2-PG). Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 6. Effect of Δ⁹-THC and Fabp1 gene ablation on hepatocyte levels of proteins involved in endocannabinoid synthesis, degradation, and action. Cultured primary hepatocytes were isolated from WT or LKO mice, plated on culture dishes, incubated with Δ⁹-THC (20 μM) for 1 hour, as described in Methods. Hepatocytes were then washed, homogenized, protein determined, and aliquots used for SDS-PAGE and Western blotting as we described (79, 87) to determine levels of the following proteins: A. 46 kDa NAPE-PLD, B. 120 kDa DAGL-α, C. 63 kDa FAAH, D. 33 kDa MAGL, and E. 53 kDa CB1. Insets show representative western blots of the respective protein (upper blot) and the gel-loading control protein (37 kDa GAPDH, lower blot). Relative protein was normalized to internal control and WT was set to 1. Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 7. Δ⁹-THC and Fabp1 gene ablation impact hepatocyte protein levels of membrane proteins and cytosolic proteins involved in uptake and cytosolic binding/’chaperoning’ of bound Δ⁹-THC, AEA, 2-AG, and/or fatty acids such as ARA. All conditions were as in legend to Fig. 7 except that western blot analysis was performed to determine levels of: A. FATP2; B. FATP4; C. FABP1; D. HSP70; E. SCP2. Insets show representative western blots of the respective protein (upper blot) and the gel-loading control protein (37 kDa GAPDH, lower blot). Relative protein was normalized to internal control and WT was set to 1. Values represent the mean ± SEM, n=4-6. #p<0.05 vs WT in the same treatment groups; *p<0.05 vs untreated of the same genotype.
Figure 8. Proposed pathway regulating cultured primary mouse hepatocyte levels of endocannabinoids: impact of phytocannabinoids (Δ⁹-THC), 2-AG, and Fabp1 gene ablation. In serum, the highly lipophilic Δ⁹-THC, AEA, and 2-AG, and are bound by albumin (and lipoproteins) (34, 35, 61, 88, 89). After binding to hepatocyte cannabinoid receptor (CB1) and/or entering the plasma membrane (90), these ligands are translocated across the plasma membrane by as yet unknown mechanism(s) with most evidence suggesting rapid spontaneous transbilayer migration (74, 75, 91). Due to their lipophilicity, these ligands require cytosolic binding/‘chaperone’ proteins for trafficking to intracellular sites of degradation/hydrolysis (21, 37, 79). In liver hepatocytes the liver fatty acid binding protein-1 (FABP1) is the most highly prevalent cytosolic lipophilic ligand binding/‘chaperone’ protein—recently shown to serve this function for phytocannabinoids such as Δ⁹-THC and endocannabinoids such
as AEA and 2-AG (19-21, 55). FABP1 then transports the bound AEA primarily to endoplasmic reticulum for hydrolysis by fatty acid amine hydrolase (FAAH) to yield arachidonic acid (ARA) and ethanolamine. Similarly, FABP1 also transports bound 2-AG for targeting to monoacylglycerol hydrolase (MGL), localized in cytosol and associated with lipid droplets (LD), to yield ARA and glycerol. The released ARA is then available for subsequent metabolism, i.e. oxidation (mitochondria, peroxisomes) or utilized for synthesis of ARA-containing phospholipids (PL) and triacylglycerols (TG). As shown in the left side of the figure, Δ⁹-THC competes with FABP1-bound AEA and 2-AG to displace them from FABP1 binding site—thereby increasing hepatocyte AEA and 2-AG levels. As shown in the right side of the figure, 2-AG similarly competes with FABP1-bound AEA and 2-AG to displace them from FABP1 binding site—thereby increasing hepatocyte AEA and 2-AG levels. Since 2-AG uptake is not driven by intracellular hydrolysis (77), continued uptake of 2-AG further exacerbates hepatocyte level of 2-AG.