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Fatty acid binding protein-1 (FABP1) and the human FABP1 T94A Variant: Roles in the Endocannabinoid System and Dyslipidemias

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Abstract

The first discovered member of the mammalian FABP family, liver fatty acid binding protein (FABP1, L-FABP), occurs at high cytosolic concentration in liver, intestine and in the case of humans also in kidney. While the rat FABP1 is well studied, the extent these findings translate to human FABP1 is not clear—especially in view of recent studies showing that endocannabinoids and cannabinoids represent novel rat FABP1 ligands and FABP1 gene ablation impacts the hepatic endocannabinoid system, known to be involved in non-alcoholic fatty liver (NAFLD) development. Although not detectable in brain, FABP1 ablation nevertheless also impacts brain endocannabinoids. Despite overall tertiary structure similarity, human FABP1 differs significantly from rat FABP1 in secondary structure, much larger ligand binding cavity, and affinities/specificities for some ligands. Moreover, while both mouse and human FABP1 mediate ligand induction of peroxisome proliferator activated receptor-α, (PPARα), they differ markedly in pattern of genes induced. This is critically important because a highly prevalent human SNP (26–38% minor allele frequency and 8.3±1.9% homozygous) results in a FABP1 T94A substitution that further accentuates these species differences. The human FABP1 T94A variant is associated with altered body mass index (BMI), clinical dyslipidemias (elevated plasma triglycerides and LDL cholesterol), atherothrombotic cerebral infarction, and non-alcoholic fatty liver disease

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(NAFLD). Resolving human FABP1 and the T94A variant's impact on the endocannabinoid and cannabinoid system is an exciting challenge due to the importance of this system on hepatic lipid accumulation as well as behavior, pain, inflammation, and satiety.

Keywords

Liver; fatty acid binding protein (FABP1); triglyceride

INTRODUCTION

Liver fatty acid binding protein (FABP1, L-FABP), the first discovered member of the FABP family (1–4), is highly a prevalent soluble protein in rodent (2–6% of cytosol protein; 200–400 μM) and even more so in humans (7–10% of cytosolic protein (700–1000 μM) liver cytosol (5, 6). Nevertheless, most studies of FABP1 structure, ligand specificity and function have focused on the rat and murine FABP1. Although the human (7, 8) and rat (9, 10) FABP1 share in common an overall tertiary structure composed of a ten-β-sheet β-barrel along with two α-helices and turns between them, nearly 20% of the amino acid sequence of human FABP1 is non-identical to that of the rat FABP1 (11). In fact, nearly half of these amino acid substitutions are non-identical nonconservative replacements (11). As a result, the secondary structure of the human FABP1 is less α-helical (12), has higher thermal stability (12), and differs in conformational flexibility and mode of LCFA binding (7, 13–16). Further, recent X-ray and NMR studies show that the binding cavity of human FABP1 is larger and is the largest of any mammalian FABP which suggests potential differences in ligand affinity, specificity, and/or function (7, 8, 13, 14, 17). Rat and human FABP1 are unique among the FABP family in terms of both the size of its binding cavity as well as much broader ligand specificity. Unlike other FABPs, the binding cavity of FABP1 is much larger—accommodating up to two lipophilic ligands rather than only one (7, 8, 13, 14, 17, 18).

More important, FABP1 has much broader ligand specificity. For example, rat FABP1 binds both straight- and branched-chain LCFA (19–21), long chain fatty acyl CoA (LCFA-CoA), acyl-carnitines, LCFA oxidation products, prostaglandins, lysophospholipids, and many other LCFA-like lipophilic ligands (rev. in (4, 5, 9, 22, 22–27)). Rat FABP1 also accommodates a single larger molecule (e.g. cholesterol, bile acid), thereby functioning as the primary cytosolic chaperone for secretion of bile acids and HDL-derived cholesterol into bile (6, 28–34). While early studies of the human FABP1 confirmed significant qualitative overlap in specificity for many lipophilic ligands with that of the rat FABP1, specificity of the human FABP1 was even broader as indicated by the binding of steroid hormones (testosterone, estradiol), fatty alcohols (eicosanol, retinol), retinoic acid, and vitamins (D_3, E, K_1) (12, 27, 35–37). Importantly, direct comparison of the ligand binding affinities of the human and rat FABP1 within the same study showed that the human FABP1 has slightly higher affinities for saturated LCFA (palmitic and stearic acids) and monounsaturated LCFA (oleic acid), 2.2-fold higher affinity for oleoyl-CoA, and 3 to 200-fold higher affinities for lysophosphatidic acid, 1-palmitoyl-2-oleoyl phosphatidic acid, and fenofibric acid (12, 37). In contrast, while both human and rat FABP1 bind cholesterol, the human FABP1 has 3.5-

fold weaker affinity for cholesterol as compared to rat FABP1 (38, 39). Taken together these findings indicate the limitations of assuming similar ligand specificities and/or specificities for the human FABP1 based on those established for the rat FABP1. This caveat is consistent with the structural differences between the human and rat FABP1 binding cavities noted above. Due to its ability to bind fibrates and a broad variety of other xenobiotics, FABP1 is a target of active therapeutic interest (7, 8, 13–16, 40–42). Yet, the above studies underscore the need to examine not just rodent liver and hepatocyte functional models but also extend them to the respective human FABP1, liver, and hepatocytes.

HUMAN AND MURINE FABP1 ENHANCE LCFA UPTAKE

While there have been no reports of complete loss of FABP1 in humans, the impact of human and murine FABP1 expression level has been examined in a variety of tumor cell lines including cloned human HepG2 hepatoma cells, transfected ‘Chang liver’ cells overexpressing human FABP1, and transfected L-cell fibroblasts overexpressing FABP1. Rat FABP1 overexpression in cultured mouse L-cell fibroblasts stimulates fatty acid uptake and trafficking (43–47). Likewise, the expression level of human FABP1 in human liver-derived HepG2 cells correlated directly with uptake of radiolabeled monounsaturated LCFA (48). Overexpression of human FABP1 in ‘Chang liver’ cells also stimulates uptake of LCFA (49). Conversely, the impact of complete loss of FABP1 has been studied extensively in mouse FABP1 gene ablated models. FABP1 gene ablation inhibits uptake of a variety of fluorescent saturated fatty acids (NBD-stearic acid, C18:0; BODIPY-C16) and/or radiolabeled saturated fatty acids (C18:0), branched-chain saturated (phytanic acid), and monounsaturated fatty acids (C18:1) *in vivo* (50, 51) and in cultured primary mouse hepatocytes (52–54). Concomitantly, FABP1 ablation decreased liver cytosol LCFA binding capacity >80% in vivo (50) and decreased cytosolic transport/diffusion 2-fold (52). LCFA are membrane-bound, and cytoplasm is 10-fold more viscous than aqueous due to cytoskeleton, organelles, and proteins (55). FABP1 overcomes these barriers by desorbing membrane-bound LCFA into the cytosol and decreasing ‘tortuosity’ of diffusional paths (55). It should be noted that FABP1 gene ablation was not compensated for by upregulation of other liver cytosol LCFA binding proteins (SCP-2, FABP7, FABP3, FABP2, FABP5, CRABP1, CRABP2, FABP4) or membrane LCFA transport proteins (50, 53, 56, 57).

HUMAN AND MURINE FABP1 INDUCE LCFA OXIDATION

FABP1 directly targets LCFA-CoA to oxidative organelles for oxidation. FABP1 ablation inhibits LCFA β-oxidation *in vitro* (58), in mouse hepatocytes (52, 54) and decreased serum β-hydroxybutyrate (*in vivo* LCFA β-oxidation) in mice (2, 59). Rat FABP1 binds and alters CPT1 conformation to transfer bound LCFA–CoA into mitochondria for β-oxidation (58, 60). Conversely, rat FABP1 overexpression increased LCFA targeting to mitochondria and peroxisomes for oxidation (52).

Recent studies *in vitro*, transfected cells, and cultured primary mouse and human hepatocytes have established that both human and murine FABP1 also elicit longer term impact on LCFA oxidation by facilitating ligand activation of nuclear receptors such as PPARα and HNF4α. Ligand (LCFA, n-3 polyunsaturated LCFA, fibrates) binding to human

and murine FABP1 redistributes the FABP1 into the nucleus, thereby also co-transporting the bound ligands into the nucleus, a process impaired by FABP1 gene ablation (61–63). Within the nucleus these FABP1s directly bind to and alter PPARα conformation (17, 61, 64–66)--thereby facilitating transfer of FABP1-bound ligand to PPARα for transcriptional activation FABP1 gene ablation or chemical inhibition, like PPARα gene ablation, abolishes ligand (fibrates, n-3 polyunsaturated fatty acids) activation of PPARα transcription of multiple genes involved in LCFA uptake (FATP), intracellular transport (FABP1), and oxidation (CPT1A, CPT2, ACOX1) in cultured primary mouse hepatocytes (61, 62, 67). Concomitantly, FABP1 ablation decreased/abolished the ability of synthetic (fibrate) and natural (branched-chain LCFA) peroxisome proliferators to lower serum and hepatic TAG (57, 68), but also exacerbated toxicity of dietary PPARα agonists (52, 57, 69, 70).

Finally, it is important to note that in view of the differences in human and rodent FABP1 structures and ligand specificities noted above, fibrate and other activators of PPARα do not induce the same target genes in human compared to mouse cultured primary hepatocytes. While there is significant overlap in inducing transcription of target genes in LCFA oxidation, nearly half of the ligand-induced PPARα target genes differ between human and mouse cultured primary hepatocytes (71, 72). Recently, it was also shown that rodent FABP1 binds and potentiates ligand (LCFA-CoA) activation of hepatocyte nuclear factor 4α (HNF4α), another nuclear receptor involved in hepatic LCFA and glucose metabolism (73).

FABP1's ROLE IN HEPATIC LIPID ACCUMULATION

Rat FABP1 *in vitro* and overexpression in cultured L-cell fibroblasts markedly enhanced long chain fatty acid (LCFA) intracellular targeting to endoplasmic reticulum for esterification (43–46, 74–77). Conversely, all FABP gene ablated mouse models generated to date have exhibited increased TAG accumulation in liver *in vivo* (50, 78–81) and in hepatocytes (52–54, 80). Hepatic TAG accumulation in FABP1 gene ablated mice was not associated with altered intestinal fat absorption and food intake was only slightly or not increased (2, 81, 82). It is important to note that these findings with primary hepatocytes in culture and liver *in vivo* differed significantly from cultured transformed cell models. For example, overexpression of human FABP1 enhanced LCFA targeting to TAG to elicit TAG accumulation in transfected human 'Chang liver cells' (49). Contrary to their name, however, human 'Chang liver' cells are not of hepatic origin but instead are derived from human cervical cancer cells (83).

FABP1 impacts hepatic lipid accumulation not only by decreasing hepatic LCFA β-oxidation (see above), but also in part by its ability to influence biliary secretion of HDL-derived cholesterol and alter bile acid profile (34, 84, 85). FABP1 gene ablation decreases hepatic uptake and biliary secretion of high density lipoprotein (HDL)-derived NBD-cholesterol (34). Furthermore, FABP1 ablation significantly decreases hepatic bile acid concentration while increasing biliary bile acid and altering biliary bile acid composition towards increased hydrophobicity and lower indices of cholesterol solubility in biliary bile. Concomitantly, FABP1 ablation increases serum TAG (2, 39, 59, 80, 86, 87)—associated not only with reduced hepatic LCFA oxidation but also reduced VLDL clearance by lipoprotein lipase (LPL) but not increased hepatic VLDL secretion (80).

HUMAN AND MURINE FABP1 INTERACT WITH THE ENDOCANNABINOID PRECURSOR ARACHIDONIC ACID

The endogenous endocannabinoids (EC) such as arachidonoylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG) are both derived from arachidonic acid (ARA)-containing phospholipids. Although FABP1 is not detectable in brain, recent studies suggest that hepatic FABP1 may impact EC formation not only in liver but also in brain by regulating plasma availability (88, 89).

FABP1 has high affinity for arachidonic acid (ARA, C20:4n-6), the precursor of phospholipids from which endocannabinoids AEA and 2-AG are derived. Human as well as rat FABP1 bind ARA with higher affinity than saturated and monounsaturated fatty acids (18, 47, 90). Direct comparison in a single study using an ANS fluorescence displacement assay showed that human and rat FABP1 both bind ARA with high affinity, $K_i$ds of 0.113±0.006 and 0.110±0.006 μM, respectively (37). Rat FABP1 affinity for ARA was confirmed by direct binding A5C, a novel metabolizable fluorescent ARA developed in collaboration with Dr. Bill Smith (U. Michigan), that was bound with high affinity, (K_d=77±6nM) (47). NBD-ARA, a novel NBD-ARA probe (Fig 2A) developed in collaboration with Drs. W. Shaw, S. Burgess, and S. Li (Avanti). FABP1 exhibited two NBD-ARA binding sites with average affinity of K_d=0.66±0.06 μM (Fig 2B) (89). Taken together with the high level of human and rat FABP1 in liver cytosol, these findings suggest FABP1 is a major contributor to hepatic cytosolic ARA binding capacity—analogous to its comprising >80% of cytosolic binding of other LCFA (50, 90). Nearly 3/4 of LCFA binding sites are occupied in native FABP1 isolated from rat liver (90). Consistent with FABP1's higher affinity for ARA than saturated or unsaturated fatty acids, ARA comprises 25% of the total FABP1 bound LCFA despite the fact that other LCFAs are much more prevalent in liver (90). As shown below, FABP1 gene ablation has important consequences not just for liver but also brain levels of ARA-containing endocannabinoids.

FABP1 enhances uptake of the endocannabinoid precursor arachidonic acid (ARA) (47). Although nothing is known about the impact of human FABP1 on ARA uptake, the impact of rat FABP1 overexpression has been examined in murine L-cell fibroblasts. Overexpressing rat FABP1 in L-cells increased the uptake of *cis*-parinaric acid (43, 44, 91, 92). While *cis*-parinaric acid, the first naturally-occurring fluorescent LCFA discovered (93), has four double bonds as does ARA, neither the 18-carbon chain length nor the methyl-terminal location of the double bonds tetraene reflect that of ARA (47). In contrast, both the chain-length and the double bond localization of A5C much more accurately reflect those of ARA (47). Rat FABP1 overexpression increased ARA uptake as shown by real-time multiphoton imaging of A5C (47) and by uptake of radiolabeled [^{3}H]-ARA (47). FABP1 enhanced initial rate, decreased half-time, and increased maximal binding capacity. Although human FABP1 exhibits the same affinity for ARA as does rat FABP (37), how the structural differences will impact human FABP1's ability to enhance ARA uptake is not known.

HUMAN AND MURINE FABP1 ROLES AS ENDOCANNABINOID ‘CHAPERONES’

The endogenous ECs [arachidonoylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG)] derived from ARA-containing phospholipids together with their cannabinoid (CB) receptors constitute a novel system for modulating behavior, pain, inflammation, and satiety (94–100) as well as hepatic lipid accumulation (101–104) by central and/or peripheral mechanisms. FABPs present within brain neuronal and other cells (i.e. FABP3,5,7) have been shown to bind and act as brain cytosolic binding proteins of endocannabinoids (AEA, 2-AG) and cannabinoids (THC, CBD) (105, 106). These brain FABP3,5,7s act as ‘chaperones’ that facilitate re-uptake and targeting of the respective bound ligands to degradative enzymes present in brain organelles (endoplasmic reticulum, mitochondria, lysosomes) or cytosol for metabolism (107–109). Alternately, the brain FABP3,5,7s may also ‘chaperone’ the ECs to the nucleus for regulating nuclear receptors (110). In contrast, until recently the identity of major EC ‘chaperone(s)’ in liver was not clear (89).

Little is known about how the very hydrophobic, highly-membrane associated endocannabinoids (AEA, 2-AG) traffic within hepatocytes from sites of synthesis for extracellular release, uptake/reuptake, or to intracellular sites for hydrolysis/degradation (102, 111). A *cis*-parinaroyl-CoA displacement assay developed by our laboratory (112, 113) suggested for the first time that endocannabinoids and phytocannabinoids bind to rat FABP1 (89). The endocannabinoids AEA and 2-AG both displace rat FABP1-bound *cis*-parinaroyl-CoA (Fig. 1A). Analysis of multiple binding curves yielded K_is of 0.40±0.02 and 0.205±0.003 μM, respectively (89). However, the lack of a suitable fluorescent-labeled AEA and 2-AG assays has been a major limitation in more directly demonstrating AEA and 2-AG binding to FABP1 or other FABPs. This limitation was recently overcome by the development of novel synthetic fluorescent NBD-AEA and NBD-2-AG analogues in collaboration with scientists at Avanti Polar Lipids, Inc. (Fig. 2A). These probes for the first time allowed direct determination of rat FABP1’s binding affinity for these endocannabinoids in a direct binding assay (89). Rat FABP1 affinities for NBD-AEA and NBD-2-AG, K_ds of 0.80±0.20 and 0.25±0.05 μM, respectively (Fig. 2B), were in the same range as that for NBD-ARA with K_d of 0.66±0.06 (Fig. 2B). However, the fractional saturation binding curves indicated that each molecule of rat FABP1 protein bound only a single NBD-AEA or NBD-2-AG as compared to two molecules of NBD-ARA (Fig 2B).

In contrast, human FABP1 bound AEA in a different manner from that observed with the rat FABP1. While AEA did not displace human FABP1 bound *cis*-parinaroyl CoA (Fig. 1B), nevertheless AEA did displace another fluorescent ligand, i.e. 11-(dansylamino)-undecanoic acid (DAUDA), which was bound by human FABP1 albeit more weakly than *cis*-parinaroyl CoA (Fig 1B). Rat FABP1 also binds DAUDA with affinities in a similar range as does human FABP1 (26, 114). Taken together, these findings indicate that although both human and rat FABP1 bind AEA, they likely differ significantly with regards to affinity and localization of the bound AEA within the respective binding sites.

Similarly, little is known about how the equally hydrophobic, highly-membrane associated phytocannabinoids and synthetic cannabinoids are taken up and traffic within hepatocytes from sites of uptake to intracellular sites for metabolism or secretion at the bile canaliculus. The *cis*-parinaroyl-CoA displacement assay suggested that FABP1 may also serve this function (89). Cannabidiol displaced rat FABP1-bound *cis*-parinaroyl-CoA with K_d of 0.58±0.06 μM (Fig. 1C). Rat FABP1 also exhibited high affinity for the psychoactive tetrahydrocannabinol and a variety of synthetic cannabinoid agonists and antagonists (89). Taken together with FABP1's very high cytosolic concentration (5, 6), these findings suggest FABP1 as a major 'chaperone' protein in the liver. Further, the high affinity of FABP1 for cannabidiol suggests that FABP1 may contribute significantly to the very high (90%) first-pass removal of oral cannabinoid (115–119).

FABP1 also binds non-ARA containing potentiating 'entourage' (EC*) N-acylethanolamides and 2-monoacylglycerides. Although N-oleoylethanolamide (OEA) and N-palmitoylethanolamide (PEA) do not directly bind/activate CB receptors, they nevertheless act as 'entourage' lipids that potentiate AEA (and/or 2-AG) activity by increasing their affinities for CB receptors or decreasing their enzymatic degradation (120–125). Rat FABP1 bound these 'entourage' NAE (OEA, PEA) and 2-MG (2-OG, 2-PG) with similar or weaker affinities than for AEA and 2-AG (89). The observation that rat FABP1 binds 2-OG confirms earlier NMR, Lipidex 1000 radioligand competition, and Tyr quenching assays (126, 127). Further, studies with FABP1 gene ablated mice showed that murine FABP1 is the major ^{3}H-2-OG binding protein in mouse liver cytosol (126). In contrast, nothing is known regarding the human FABP1 interaction with such potentiating 'entourage' EC* ligands.

Real-time imaging established that the NBD-labeled AEA and 2-AG as the first potentially useful endocannabinoid analogues for visualizing the uptake, intracellular trafficking, and targeting of these molecules by living cells. L-cells take up NBD-ARA, NBD-AEA and NBD-2AG as shown by representative images (Fig 3E, F, G). Analysis of multiple cells over time revealed showed biphasic uptake curves approaching a maximum for each probe (Fig. 3A). While the initial rates of uptake of these probes was in the order NBD-2-AG > NBD-ARA > NBD-AEA (Fig 3D), the overall half-time of uptake of for both NBD-AEA and NBD-2-AG was longer than that of NBD-ARA (Fig 3C). Furthermore, maximal uptake of NBD-AEA and NBD-2-AG was at least 1.6-fold higher than that of NBD-ARA (Fig. 3B). It should be noted that the half-time of NBD-ARA uptake (Fig. 3C) was in the same range as that of radiolabeled and our earlier A5C fluorescent ARA analogue (47, 128–130)—suggesting that the relative differences in kinetics between NBD-AEA and NBD-2-AG versus NBD-ARA uptake accurately reflect those of unlabeled ARA. These novel analogues now allow real-time determination of the impact of: i) FABP1 overexpression on AEA and 2-AG uptake in murine L-cells; ii) FABP1 gene ablation on hepatic uptake of AEA and 2-AG *in vivo* or by cultured primary hepatocytes; iii) human T94A variant on uptake of AEA and 2-AG in cultured primary human hepatocytes; iv) FABP1 on both hepatic and brain uptake of ARA, AEA and 2-AG.

HUMAN AND MURINE FABP1 IMPACT LIVER ENDOCANNABINOIDS

The functional significance of endocannabinoids and the CB1 receptor in liver was first established by Kunos et al (101, 131–133). Hepatic CB1 (and CB2) are markedly upregulated in non-alcoholic liver disease (NAFLD) (101–103), while CB1 is upregulated in alcoholic liver disease (AFLD) (102, 104) and in response to high-fat diet-induced obesity (102, 104). Concomitantly, hepatic AEA and 2-AG levels are also elevated in NAFLD, while 2-AG (but not AEA) is elevated in AFLD, and AEA (but not 2-AG) is elevated in response to high-fat diet (101–104). Despite these advances, little is known about hepatic factors contributing to these alterations in the hepatic endocannabinoid system. One possible candidate protein is the hepatic FABP1 which not only binds AEA and 2-AG (Figs 1,2) but loss of FABP1 also elicits hepatic lipid accumulation *in vivo* (50, 78–81) and in hepatocytes (52–54, 80).

Indeed, FABP1 gene ablation markedly increases hepatic levels of arachidonic acid containing endocannabinoids (EC) such as AEA and 2-AG (Table 1) (89). This increase in hepatic AEA and 2-AG may contribute to hepatic TAG accumulation by a SREBP1 mediated mechanism (Fig 4) (2, 39, 59, 80, 86, 87). CB1 receptor agonists induce SREBP1 that in turn induces transcription of lipogenic enzymes *de novo* such as acetyl-CoA carboxylase and fatty acid synthase (Fig 4) (134). Concomitantly CB1 agonists reduce LCFA oxidation by inhibiting adenylate cyclase and AMPK activity (134). Alternately loss of FABP1 may reduce transfer of AEA into the nucleus wherein AEA would normally bind to enhances PPARα activation (135) (Fig. 4). It is therefore important to extend these findings toward the human FABP1 and its role regulating the endocannabinoid system and thereby fatty liver disease.

FABP1 gene ablation also increases hepatic levels of non-arachidonic acid containing N-acylethanolamides or 2-monoacylglycerols (EC*). Hepatic levels of OEA and PEA (34±7 and 60±10 pmol/g liver, respectively) in male wild-type C57BL/6N mice are about 3–5 fold higher than that of AEA (Table 1) (89). FABP1 gene ablation nearly doubled the hepatic level of the even more highly prevalent (nmol/g vs pmol/g) 2-oleoyl-glycerol (2-OG) which is a finding not compensated for by decreased expression of the much less prevalent OEA and PEA (Table 1) (89). The loss of FABP1 could also contribute to hepatic TAG accumulation by its impact on hepatic EC* levels. By reducing OEA, this would increase the SREBP1 pathway to increase lipogenesis while decreasing lipolysis and fatty acid oxidation through the PPARα pathway, likely through GPR119 (136–140). FABP1 gene ablation would reduce cotransport of EC* ligands (OEA, PEA, 2-OG, 2-PG) into the nucleus for transfer to and activation of PPARα or alternately through G protein-coupled receptors other than CB1/CB2 (GPCR*s) (Fig. 5).

Although the non-arachidonic acid containing EC* have no agonist activity at CB1 or CB2 receptors, they are nevertheless bioactive (120). This has led to the suggestion that EC* may directly activate as yet unknown receptor(s) (120). Alternately, it has been proposed that the EC* may act indirectly by enhancing the action of endogenous AEA or 2-AG by increasing their affinity for CB receptors and/or decreasing AEA or 2-AG degradation—possibilities termed ‘entourage’ effects (120, 120–125). However, there is not universal agreement with

the ‘entourage’ idea since it is clear that OEA and PEA have alternative targets. For the sake of simplicity in this review, however, the term potentiating ‘entourage’ EC* will be used.

MECHANISM(S) WHEREBY FABP1 IMPACTS LIVER ENDOCANNABINOIDS

While the mechanism(s) whereby FABP1 gene ablation raises hepatic levels of EC (AEA, 2-AG) and some EC* (2-OG) remains to be determined, these increases were not associated with compensatory changes in protein levels of enzymes, receptors, or ‘chaperones’ in the hepatic endocannabinoid system (89). One possibility is based on the fact that FABP1’s high affinity for AEA and 2-AG which suggests potential role(s) for FABP1 in AEA and 2-AG reuptake for ‘chaperoning’ and targeting to degradative enzymes (Fig. 4)—analogous to roles exhibited by brain FABPs 3,5, and 7 (106, 107, 110). Consistent with this possibility increased level of FABP1 enhances (5, 46, 55, 141), while FABP1 gene ablation markedly decreases (52), the cytosolic transport/diffusion of other bound ligands (e.g. NBD-stearic acid). Similar considerations may be proposed for the FABP1 gene ablation-induced increase in hepatic 2-OG (Fig 5) since rat FABP1 has been shown to increase by 7-fold the transfer of a fluorescent 2-OG to model membrane phospholipid vesicles *in vitro* (126). Thus, loss of FABP1 would reduce AEA, 2-AG, and 2-OG ‘chaperoning’ towards hydrolytic/degradative enzymes such as fatty acid amide hydrolase (FAAH) localized in endoplasmic reticulum, mitochondria, and lipid droplets (142–144), N-acylethanolamide acid amide hydrolase (NAAA) in lysosomes (human but not rodent liver) (143, 145), and monoacylglycerol lipase (MAGL) localized primarily in cytosol at much lower levels in liver than other tissues (142). Less clear is the role of putative as yet to be identified plasma membrane AEA and 2-AG binding/translocase proteins and/or contributions by endocytic reuptake of AEA or 2-AG bound to CB receptors. Finally, it must be noted that the above pathway does not appear account for why FABP1 decreased levels of the more prevalent OEA and PEA or did not change levels of the less prevalent 2-PG. However, a possible explanation for the discrepancy my lie in the finding that other ligands which also bind to FABP1 selectively alter or do not alter its conformation in response to ligand binding (12, 37). In turn ligand-dependent alterations in FABP1 conformation may, depending on the specific ligand, either facilitate or prevent or have no impact on FABP1 intracellular redistribution and/or interaction with other proteins (e.g. PPARα, CPT1) (12, 37, 58, 63).

MURINE FABP IMPACTS BRAIN ENDOCANNABINOID SYSTEM

The presence of cytosolic fatty acid binding proteins (FABP 3, 5, 7), established over 20 years ago (146–153), led to the recent pioneering studies of Deutsch and coworkers identifying these FABPs as endocannabinoid (AEA, 2-AG) ‘chaperones’ for reuptake/intracellular targeting to endoplasmic reticulum for hydrolysis/degradation (105, 106, 108, 110). Ablating or inhibiting FABPs present in brain cytosol (especially FABP3) reduces brain ARA uptake (required for AEA formation)/AEA degradation (154–158). However, it is not completely clear if the impact of ablating or inhibiting these ‘brain’ FABPs is attributable only to their loss/inhibition in brain. For example, FABP3 is also highly prevalent in heart and skeletal muscle, while FABP5 is also found in epidermal cells, mammary gland, liver, kidney, lung, and adipose tissue (3, 159, 160). Likewise, the chemical BMS309403 inhibits the FABP3 and 5 localized in brain and these other tissues as well as

FABP4 found in adipose tissue (110). Interestingly, FABP3 gene ablation also diminishes heart uptake of ARA, the precursor of ARA-containing phospholipids from which AEA and 2-AG are synthesized (161). The fact that the liver FABP1 binds the arachidonic acid (ARA) (see above), but is not expressed or detected in brain (156, 162, 163), offers the opportunity to resolve the impact of this extra-CNS FABP on the brain endocannabinoid system (Fig. 5).

LC/MS analysis of brain endocannabinoids of male C57BL/6N mice either expressing or ablated in the liver FABP1 revealed that indeed FABP1 has a role in regulating brain endocannabinoid levels (88). FABP1 gene ablation markedly increased brain levels of both AEA and 2-AG (Table 2). Concomitantly, FABP1 ablation even more markedly increased brain levels of all the potentiating 'entourage' N-acylethanolamides (OEA, PEA) and 2-monoacylglycerols (2-OG, 2-PG) (Table 2). Again, these increased levels of endocannabinoids (AEA, 2-AG) and their highly prevalent potentiating 'entourage' lipids (OEA, PEA, 2-OG, 2-PG) were not due to altered brain protein levels of brain CB1 receptors or enzymes in endocannabinoid synthesis/degradation.

While the mechanism(s) whereby liver FABP1 gene ablation increases brain AEA and 2-AG levels remains to be resolved, one possibility may lie in the role of FABP1 in hepatic clearance of ARA from plasma to reduce bioavailability for ARA uptake by the brain (Fig. 5) (88). This mechanism is based on the fact that the brain ARA-containing phospholipids (from which AEA and 2-AG are synthesized) are largely derived from ARA taken up from plasma (164, 165). Yet, ARA availability for brain uptake is significantly diminished by high hepatic clearance rate (166–168). Human and rat FABP1 have high affinity for ARA as well as 18:2, n-6 which can be metabolized to ARA in liver, but much less so in brain (18, 37, 90). Overexpressing FABP1 in mouse L-cell fibroblasts increased ARA and ARA analogue uptake (43, 47, 91, 92) more than that of other LCFA (43, 44, 46, 47, 50, 53, 91). Both rodent and human liver cytosolic levels of FABP1 are very high (2–10% of cytosolic protein; 0.1–1.0mM) (2, 5, 6, 37). In fact the hepatic cytosol FABP1 protein concentration is nearly 20 to 100-fold higher than that of all three FABPs (i.e. FABP3, 5, 7) in brain cytosol (146–153). This suggests the liver may very effectively compete with brain for ARA uptake from plasma (Fig. 5). Indeed, nearly half of plasma ARA undergoes hepatic clearance which significantly diminishesavailability for brain uptake (166–168).

Whether a similar explanation may hold for the non-arachidonic acid derived 'entourage' EC* (PEA, OEA, 2-OG, 2-PG) is less clear since brain does not need to derive the palmitic acid and oleic acid from plasma for synthesis of palmitic acid and oleic acid containing phospholipids from which the above 'entourage' EC* are derived. It is important to note, however, that FABP1 does also bind palmitic and oleic acids, albeit with lower affinity than ARA (18, 47, 90). Furthermore, the uptake of these and other non-ARA fatty acids is increased in FABP1 overexpressing L-cell fibroblasts and correlates directly with FABP1 level in human HepG2 liver cells (5, 43, 44, 46, 48, 92). Conversely, FABP1 gene ablation decreases non-ARA uptake by cultured primary mouse hepatocytes (52–54) and *in vivo* (50, 79, 80). Full testing of this hypothetical scheme (Fig. 5) and differentiating these possibilities will require future studies with iv injected labeled ARA, palmitic acid, and oleic acid.

Much remains to be done with regards to potential functional consequences of FABP1 on the brain endocannabinoid system. The brain endocannabinoids (AEA, 2-arachidonoylglycerol), the cannabinoid receptors, and the potentiating 'entourage' N-acylethanolamides and 2-monoacylglycerols constitute a novel system for modulating behavior, pain and inflammation, food intake, and weight gain (94–100). Since high endocannabinoid levels produce analgesia (108), the FABP1 gene ablation-induced increase in brain AEA level may decrease pain sensitivity (Fig 4). In contrast, the non-ARA containing EC* ligands have opposing influences on food intake and weight gain by differentially impacting LCFA synthesis *de novo* versus oxidation (Fig 5). For example, increased level of the cannabinoid receptor-1 (CB1) agonist AEA increases food intake and LCFA synthesis *de novo* by a SREBP1 mediated mechanism (102) (Fig 4), while increased OEA decreases food intake and weight gain by a mechanism involving induction of PPARα transcription of LCFA oxidative genes (134, 137, 169) (Fig. 5). FABP1 gene ablated mice showed unaltered or slightly increased food intake (39, 57, 81, 86, 170, 171)—suggesting the opposing effects of AEA and OEA on food intake were offset since both were increased in parallel by FABP1 gene ablation. The net effect of FABP1 gene ablation mediated changes in brain endocannabinoid levels on other brain functions remains to be elucidated. Since ablation or inhibition of FABPs 3,5, and 7 in brain is known to markedly impact such parameters (108, 172), whether FABP1 gene ablation alters behavior, pain and inflammation remains an intriguing possibility.

FABP1 In Human Health: Impact of the human FABP1 T94A Variant

Increasing evidence points to a role for FABP1 in human health. Hepatic FABP1 level is environmentally responsive, e.g. high fat diet, chronic alcoholism, sex, PPARα agonists. While to date there have been no reports of human genetic variants resulting in complete loss of FABP1, a SNP in the FABP1 gene promoter region is associated with decreased FABP1 and decreased serum TAG (173). Conversely, a SNP in the FABP1 coding region is associated with increased FABP1 level, altered FABP1 conformation/function, human dyslipidemias and non-alcoholic fatty liver disease (NAFLD) (63, 174–176, 176, 177). .

FABP1 is upregulated in both human and rodent models of non-alcoholic fatty liver diseases (NAFLD) (178–182). Upregulation of FABP1 may mitigate the deleterious effects of high LCFA load by: i) preventing LCFA lipotoxicity through binding oxidized and reactive LCFA species (182–189); and ii) partitioning of potentially lipotoxic LCFAs into stable TAG *in vivo* (190). However, as FABP1 becomes depleted, NAFLD progresses to non-alcoholic steatohepatitis (NASH) (180, 183–187). The human FABP1 directly interacts with human PPARα to facilitate ligand transfer/activation of PPARα transcription of genes in LCFA metabolism, especially oxidation (12, 17, 37, 63, 191). Dysregulation of PPARα is associated with diabetes, cardiovascular disease (CVD), obesity, and NAFLD (182, 191, 192). A human PPARα-V227A variant exacerbates alcohol-induced plasma and liver lipid abnormalities (193, 194).

Interest in the role of the human FABP1 in health has markedly increased since the discovery of several SNPs in the human FABP1 gene. For example, a common polymorphism in the human FABP1 gene promoter region (rs2919872) leads to decreased

FABP1 promoter transcriptional activity, decreased FABP1, and decreased plasma TAG in human subjects (173). However, the impact of this single nucleotide polymorphism (SNP) in the human FABP1 promoter region on hepatic TAG accumulation and NAFLD has not been reported. In contrast, a SNP in the coding region of human FABP1 results in a T94A substitution—one of the most prevalent polymorphisms in the FABP family, occurring with 26–38% minor allele frequency and 8.3±1.9% homozygous in the human population (MAF for 1000 genomes in NCBI dbSNP database; ALFRED database) (175–177, 195–198). Impact on the FABP1 T94A variant on whole phenotype, however, is somewhat variable. Several studies correlated T94A variant expression with decreased body mass index (BMI) and waist circumference (174, 175), no change in BMI (195, 196), or increased BMI (176). This variation in whole body phenotype may be associated with the genetic diversity among the different human populations studied. Nevertheless, the expression of the FABP1 T94A variant is associated with clinical dyslipidemias including elevated plasma TAG (174, 175), increased LDL cholesterol (175, 176), and atherothrombotic cerebral infarction (177). With regards to liver phenotype, expression of the human FABP1 T94A variant also elicits non-alcoholic fatty liver disease (NAFLD) (176) and hepatic TAG accumulation concomitant with increased total FABP1 level in cultured primary human hepatocytes (63). Interestingly, the lipid lowering drug fenofibrate binds to both murine and human FABP1 to alter FABP1 conformation and thereby interaction with and activation of PPARα transcriptional activity (12, 17, 41, 42, 63). Fenofibrate, the most commonly prescribed fibrate in the US and Canada (199), lowers serum lipids in both wild-type FABP1 and T94A variant expressing human subjects, but in the FABP1 T94A variant expressers levels are not lowered to baseline (174). Until recently, however, the mechanism(s) whereby this single amino acid substitution in human FABP1 alters its function and responsiveness to fibrates or other drugs remained unclear. While it was initially thought that the T94A substitution results in complete-loss-of-function (i.e. ligand binding ability) analogous to L-FABP gene ablation (49), the following sections demonstrate that the human FABP1 T94A substitution results in an altered structure, structural response to ligand binding, and function rather than loss-of-function.

Molecular Characterization of the Human FABP1 T94A Variant

All previous structures of the recombinant human FABP1 protein were fortuitously derived from cDNAs that each encoded the human WT T94T L-FABP (200–202). In contrast, a commercially available human FABP1 cDNA (OriGene Technologies, Rockville, MD) actually encodes for the human FABP1 T94A variant mutant rather than the wild-type (12). While the number of clones is limited, nevertheless one out of four (i.e. 25%) encoding the FABP1 T94A variant is consistent with the high frequency of the FABP1 T94A variant in the human population (26–38% minor allele freq.; 8.3±1.9% homozygous; MAF for 1000 genomes in NCBI dbSNP database; ALFRED database) (175–177, 195–198). Circular dichroism reveals that the secondary structures of the recombinant human WT FABP1 T94T (obtained by site-directed-mutagenesis of the FABP1 T94A variant cDNA) and the FABP1 T94A variant proteins show key significant differences (12). The non-conservative substitution of a medium sized, uncharged, polar T residue by a smaller, nonpolar, aliphatic A residue at position 94 significantly increases α-helical structure, decreases β-sheet structure, decreases thermal stability, but conversely increases resistance to unfolding by

urea. Temperature and chemical denaturation access different aspects of protein stability (12, 203, 204). Thus, the human FABP1 T94A variant represents an altered structure mutation.

While the T94A substitution did not impact the specificity of the human FABP1 protein for a broad variety of ligands, it nevertheless alters affinities for several important ligands (12, 37, 38, 63). T94A substitution does not or only slightly alters FABP1 affinities for long chain fatty acids (saturated, monounsaturated, or polyunsaturated), oleoyl-CoA, lysophosphatidic acid, palmitoyl-oleoyl-phosphatidic acid, n-3 polyunsaturated LCFA (EPA, DHA) PPARα agonists or fibrate PPARα agonists (fenofibrate, fenofibric acid). On the other hand, T94A substitution increases affinity of human FABP1 for cholesterol by 3-fold as demonstrated with a NBD-cholesterol fluorescence binding assay and by cholesterol isothermal titration microcalorimetry (ITC) (38). LCFA binding alters the secondary structure of the human FABP1 WT protein, generally increasing the proportion of α-helical and unordered structures while decreasing that of β-sheet (12, 37, 70). T94A substitution markedly attenuated the ability of the LCFA ligands to alter human FABP1 secondary structure. Likewise, while fenofibric acid (the active metabolite of fenofibrate) also increases the α-helical and unordered structure of human FABP1 WT protein. T94A substitution significantly diminished this response. Fibrate-induced conformational change in human FABP1 is an essential component for human FABP1/PPARα interaction and potentially function (191). Thus, the altered structure of the human FABP1 T94A variant results in an altered ligand-affinity functional mutation rather than loss-of-function.

Functional Impact of the Human FABP1 T94A Variant Expression on Lipidic Ligand Uptake and Metabolism in Cultured Primary Human Hepatocytes

Expression of the human FABP1 T94A variant differentially impacts fatty acid and cholesterol uptake in cultured primary human hepatocytes. FABP1 T94A variant expressing cultured primary human hepatocytes exhibited decreased uptake of poorly metabolizable (fluorescent NBD-stearic acid) and metabolizable ([9,10–^{3}H]-stearic acid) long chain fatty acid (63). Similarly, uptake of radiolabeled palmitic acid by transfected ‘Chang’ liver cells was increased by overexpression of human WT FABP1, but not vector with T94A variant or empty vector (49). Thus, although the affinity of human FABP1 T94A variant for LCFA did not differ from that of the human WT FABP1, nevertheless the T94A substitution decreased LCFA uptake. While the molecular basis for the reduced LCFA uptake exhibited by T94A expressing hepatocytes and transfected cells is not known, it was not attributed to decreased levels of plasma membrane and other intracellular membrane LCFA transport protein. Instead, the finding that mouse FABP1 directly interacts with the plasma membrane fatty acid translocase protein-5 (FATP5) in cultured primary mouse hepatocytes (53) suggests that the altered structure and/or a attenuated conformational response changes FABP1 T94A response to ligand binding (12, 37) may decrease FABP1 T94A interaction with FATP5 and thereby reduce LCFA uptake.

In contrast, human FABP1 T94A substitution oppositely impacts lipoprotein-mediated cholesterol uptake in cultured primary human hepatocytes. Unlike LCFA taken up via membrane fatty acid transport proteins (FATP), lipoprotein cholesterol is taken up via hepatocyte cell surface receptors for LDL and HDL (38). T94A substitution enhances

lipoprotein mediated cholesterol uptake which is consistent with its 3-fold higher affinity for cholesterol (38). FABP1 T94A substitution increased cultured primary human hepatocyte uptake of NBD-cholesterol from NBD-cholesterol labeled HDL much more than from LDL (38). Likewise, human FABP1 T94A variant expression in cultured primary human hepatocytes or overexpression of human FABP1 T94A variant (but not human WT FABP1) in cultured 'Chang' liver cells increases cholesterol accumulation (49, 63). Consistent with these findings, FABP1 T94A variant expressing human subjects exhibit elevated plasma levels of low-density lipoprotein (LDL) cholesterol (175, 176) concomitant with increased CVD (174, 175), and atherothrombotic cerebral infarction (177).

Expression of the human FABP1 T94A variant elicits lipid accumulation in cultured primary human hepatocytes and livers in vivo. Human subjects expressing the FABP1 T94A variant have increased incidence of non-alcoholic fatty liver disease (NAFLD) as evidenced by ultrasound analysis (176). However, while ultrasound visualizes lipid droplets within liver cytoplasm, it does not actually resolve the types of lipids accumulated therein (205). In contrast, chemical analysis of cultured primary human hepatocyte lipids established that FABP1 T94A variant expression induces accumulation of neutral lipid, especially TAG and cholesteryl esters (CE), a process exacerbated by high LCFA load (63). Accumulation of TAG and CE is consistent with NAFLD in human subjects (206). In contrast, overexpressing human FABP1 T94A variant did not alter TAG mass in human 'Chang liver' cells (49). The discrepancy between the impact of FABP1 T94A variant expression in cultured primary human hepatocytes versus 'Chang liver' cells may lie in the fact that 'Chang liver' cells are derived from human cervical cancer cells rather than human liver (83). Taken together, these data were consistent with cultured primary human hepatocytes providing a useful model for examining the mechanism(s) whereby the human FABP1 T94A variant elicits NAFLD.

FABP1 T94A variant expression increases anabolic mechanism(s) to induce neutral lipid accumulation. Neutral lipid (TAG, CE) accumulation in cultured primary human hepatocytes is associated with upregulation of total liver FABP1. This possibility is supported by earlier studies *in vitro* showing that WT FABP1 stimulates glycerol-3-phosphate acyltransferase (GPAM), the rate limiting enzyme in lipogenesis (74, 77, 207), acyl-CoA cholesterol acyltransferase (ACAT) (208, 209), as well as increases mRNA expression of downstream enzymes in lipogenesis (*GPAM*, *LPIN2*) in heterozygotes, decreases mRNA expression of microsomal triglyceride transfer protein (*MTTP*), increases secretion of ApoB100 but not TAG. TAG accumulation is not due to increased LCFA uptake, lipogenesis *de novo* (ACC1, FASN), or the alternate monoacylglycerol acyltransferase (MOGAT) pathway in lipogenesis. Thus, T94A-induced neutral lipid accumulation is associated, at least in part, with increased total FABP1 protein for stimulating neutral lipid synthesis, but less able to load neutral lipids on apoB for secretion (63).

Conversely, FABP1 T94A variant expression impairs catabolic mechanism(s) that would reduce neutral lipid accumulation. Increased neutral lipid accumulation in FABP1 T94A variant expressing human hepatocytes is also attributed at least in part to decreased LCFA β–oxidation (63). T94A substitution decreases β–oxidation of [9,10–^{3}H]-stearic acid by 70% and 40% in heterozygotes and homozygous T94A hepatocytes (63) which is consistent with the decreased β-oxidation in development of NAFLD (205). Impaired LCFA β-oxidation is

not due to reduced transcription of LCFA β-oxidative enzymes (CPT1A, CPT2, ACOX1) whose mRNA levels actually increased (63). Instead decreased LCFA β–oxidation was associated with decreased translation of the rate limiting enzyme CPT1A mRNA into CPT1 protein (63). Consistent with this finding, miRNA microarray analysis (Phalanx Biotech Group (San Diego, CA) reveals that T94A increases the level of miR-34a (not shown). miR34a decreases the protein level of CPT1A (rate-limiting enzyme in mitochondrial LCFA β-oxidation) (210) and miR-34a is highly increased in human NAFLD (210, 211).

FABP1 T94A variant expression also impairs ligand-induced PPARα transcription of LCFA β-oxidative enzymes in human hepatocytes (63). While fibrate PPARα agonist efficacy in NAFLD is unclear (212), Very long chain polyunsaturated fatty acids, i.e. VLCn-3PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce lipogenesis *de novo* (decrease SREBP1c, activate ChREBP) and increase LCFA β-oxidation (activate PPARα) (213–215). T94A impairs fenofibrate and VLCn-3PUFA mediated PPARα transcription of LCFA β-oxidative enzymes (12, 37, 63), suggesting fenofibrate may be less effective in lowering hepatic TAG in T94A subjects. Similarly, fenofibrate is less effective in lowering elevated plasma TAG to basal levels in these T94A variant expressing individuals (174, 175). The decreased ability of the FABP1 T94A variant to mediate ligand activation of PPARα transcriptional activity is attributed at least in part to reduced ability of these ligands to induce redistribution of the FABP1 T94A variant into the nucleus for interaction with and activation of PPARα therein (54). These impaired functions of the T94A variant correlate with FABP1 T94A altered protein structure and reduced protein structural response to ligand binding as noted in the preceding sections.

Does the Human FABP1 T94A Variant Impact the hepatic Endocannabinoid System?

NAFLD in the human FABP1 T94A variant population may at least in part be associated with an altered endocannabinoid system. While underlying causes of NAFLD are unclear (216), genetic variation and environment contribute to the incidence of NAFLD (190, 205, 212, 215, 217–223). Genome wide array studies (GWAS) of NAFLD estimate a 39% heritability of liver lipid accumulation as a continuous trait after controlling for age, gender, race, and BMI (219). The highly prevalent human L-FABP T94A variant (175–177, 195–198) is associated with TAG accumulation in liver (NAFLD) (176), primary hepatocytes (63), and serum (174, 175, 224). Hepatic levels of endocannabinoids and/or receptors (CB1 and/or CB2) of the endocannabinoid system are elevated in NAFLD (101–103), alcoholic liver disease (AFLD) (102, 104), high-fat diet-induced obesity (102, 104), and in response to cannabis with CB1 agonists (e.g. HU-210) (102–104) or CB2 selective agonists (e.g. JWH-133) (102, 104). Expression of the human FABP1 T94A variant markedly induced transcription of key enzymes in AEA and 2-AG synthesis (NAPEPLD, DAGLα) and degradation (FAAH1) as well as their target cannabinoid receptor-1 (CB1) in cultured primary human hepatocytes (Fig 6). These effects are specific since T94A expression has no effect on other AEA and 2-AG degradative enzymes, FAAH2 or MAGL (Fig 6). While the net effect of these opposing influences on AEA and 2-AG levels in the cultured primary human hepatocytes remains to be shown, the 3- and 10-fold increased mRNA levels of enzymes for AEA and 2-AG synthesis (NAPEPLD, DAGLα) concomitant with much smaller or no increase in degradative enzymes (FAAH1, FAAH2, MAGL), suggests

increased levels of these endocannabinoids as well as their ‘potentiating’ chaperones. This in turn increases hepatocyte TAG accumulation (63, 176) .

These finding may have important implications for current therapies for NAFLD in human subjects. One approach to reducing TAG levels in NAFLD is to induce PPARα target genes in hepatic fatty acid β-oxidation (63, 176, 225, 226) and lipoprotein metabolism (72, 227–229). In NAFLD individuals, not segregated by T94A or other genotype, fibrate PPARα activators do not uniformly lower TAG and CVD risk (230, 231). Fibrates act by multiple mechanisms, of which many are mediated through PPARα (192, 232–234). For example, fibrates bind and activate PPARα transcription of key genes of LCFA β-oxidation (CPT1, CPT2, ACOX1), LCFA uptake (FATP, L-FABP), and plasma VLDL TAG hydrolysis (LPL) (235–238). PPARα interacts (directly or via cross-talk) with other lipid-regulating genes (HNF4α, LXR, FXR, ANGPTL4); and additional pleiotropic effects. It is important, however, to recognize that fibrates also induce transcription of enzymes involved in LCFA synthesis, desaturation, elongation, and TAG formation *de novo* (235–238). Fibrates alter endoplasmic reticulum fatty acid composition to enhance cleavage/release of mature SREBP1c which in turn induces nuclear expression of genes involved in LCFA synthesis *de novo* and TAG formation (235–238). Partitioning of LCFA- or glucose-derived acetyl-CoA toward oxidative versus synthetic pathways will determine the net effect on hepatic TAG and treatment outcome (235, 239, 240). Even if the net effect of fibrate in human FABP1 WT expressers results in more LCFA catabolism than synthesis *de novo*, however, the available evidence suggests that fibrates may be much less effective in lowering hepatic TAG to treat NAFLD than in lowering serum TAG in T94A expressers (12, 37, 63, 174).

CONCLUSIONS

The discovery of FABP1 nearly forty years ago was followed by elucidation of the rodent FABP1s structure, function *in vitro*, and more recently physiologically in gene ablated mice. Yet, FABP’s impact on human health is only beginning to be appreciated. Major strides in this regard include the first structural characterizations of the human FABP1, the novel discovery that FABP1 may be the major hepatic endocannabinoid and cannabinoid binding protein, and growing recognition of the highly prevalent human FABP1 T94A variant’s roles in hyperlipidemia and NAFLD. Since NAFLD is also associated with upregulation of hepatic endocannabinoids, it is important to resolve how the T94A variant impacts the endocannabinoid system and transcriptional mechanisms of lipogenesis *de novo*. This would facilitate development of new nutraceutical approaches to better target elevated TAG in this group, obese subjects and diabetics. One possible candidate is the very long chain n-3 fatty acids (EPA and DHA). While EPA and DHA induce PPARα transcription activity of LCFA oxidative genes, they concomitantly accelerate degradation and/or reduce nuclear distribution of SREBP1c and ChREBP. This decreases SREBP1c (213–215) and ChREBP (241–245) transcription of lipogenic genes which thereby decreases hepatic TAG and NAFLD in human subjects not segregated by FABP1 genotype. Another possibility is suggested by cannabinoid receptor (e.g. CB1) inhibitors that may block the SREBP1c mediated lipogenesis to lower hepatic lipid accumulation (Fig 4). Since FABP1 appears to be involved in the cannabinoid as well as endocannabinoid pathway, it will be important to determine the impact of the FABP1 T94A substitution thereon. In any case, FABP1 (7, 13,

14, 16, 40–42) and the nuclear receptors it impacts, i.e. PPARα (41, 42, 47, 246–248), SREBP1c (213–215), and ChREBP (241–245), continue to be current active therapeutic targets for lipid lowering.

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Abbreviations

ARA	C20:4n-6 arachidonic acid
ACC	acetyl-CoA carboxylase
AEA	N-arachidonoylethanolamide (anandamide)
ALB	albumin
ACOX1	acyl-CoA oxidase 1, palmitoyl
2-AG	2-arachidonoylglycerol
CB1	cannabinoid receptor-1
CB2	cannabinoid receptor-2
FAS	fatty acid synthase
***cis*-PnCoA**	*cis*-parinaroyl-CoA
CPT1A	carnitine palmitoyl transferase IA, liver
CPT2	carnitine palmitoyl-CoA transferase II
DAGLα	diacylglycerol lipase-α
DAGLβ	diacylglycerol lipase-β
DAUDA	11-(dansylamino)-undecanoic acid
DHA	C22:6n-3 docosahexaenoic acid
EC	arachidonic acid containing endocannabinoids (AEA, 2-AG)
EC*s	non-ARA containing N-acylethanolamides and 2-monoacylglycerols
EPA	C20:5n-3 eicosapentaenoic acid
DGAT2	diacylglycerol O-acyltransferase 2
FABP1	liver fatty acid binding protein or FABP1

FABP1 T94T	wild type (WT) human FABP1
FABP1 T94A	human FABP1 T94A variant
FABP3	heart fatty acid binding protein-3
FABP4	adipocyte fatty acid binding protein
FABP5	epidermal fatty acid binding protein
FABP7	brain fatty acid binding protein
FABP KO	FABP1 gene ablated mouse on C57BL/6NCr background
FAAH	fatty acid amide hydrolase
FF	fenofibrate
LCFA	free unesterified long chain fatty acid
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial
GPCR*	G protein-coupled receptors other than CB1/CB2
HDL	high-density lipoprotein
GPR119	G protein-coupled receptor 119
HNF4α	hepatocyte nuclear factor-4α
LCFA	long chain fatty acids, unesterified
LCFA-CoA	long chain fatty acid-CoA thioester
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein-C
LDLR	Low-Density Lipoprotein (LDL) Receptor
LPL	lipoprotein lipase
LSCM	laser scanning confocal microscopy
MAGL	monoacylglycerol lipase
MTTP	microsomal triglyceride transfer protein
NAAA	N-acylethanolamide acid amide hydrolase
NAFLD	non-alcohol fatty liver disease
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NBD-stearic acid	[12-N-methyl-(7-nitrobenz-2-oxa-1,3-diazo)-aminostearic acid]

NBD-ARA	NBD-arachidonic acid or [20-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] arachidonic acid
NBD-AEA	NBD-N-arachidonoylethanolamide or [20-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] arachidonoylethanolamide
NBD-2-AG	NBD-2-arachidonoylglycerol or 2-[20-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] arachidonoyl glycerol
NBD-cholesterol	22-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-amino)-23, 24-bisnor-5-cholen-3β-ol
OEA	oleoylethanolamide
2-OG	2-oleoylglycerol
PEA	palmitoylethanolamide
2-PG	2-palmitoylglycerol
PL	phospholipid
PPARα, -β/δ, or -γ	peroxisome proliferator-activated receptor alpha, beta/delta, or gamma
SCP-2	sterol carrier protein-2
SCP-x	sterol carrier protein-X
SNP	single nucleotide polymorphism
SRB1	Scavenger receptor class B member 1
SREBP1c	sterol regulatory element binding protein-1c
SCD1	stearoyl CoA desaturase
TAG	triacylglycerol
VLDL	very low-density lipoprotein
WT	wild-type C57BL/6NCr mouse.

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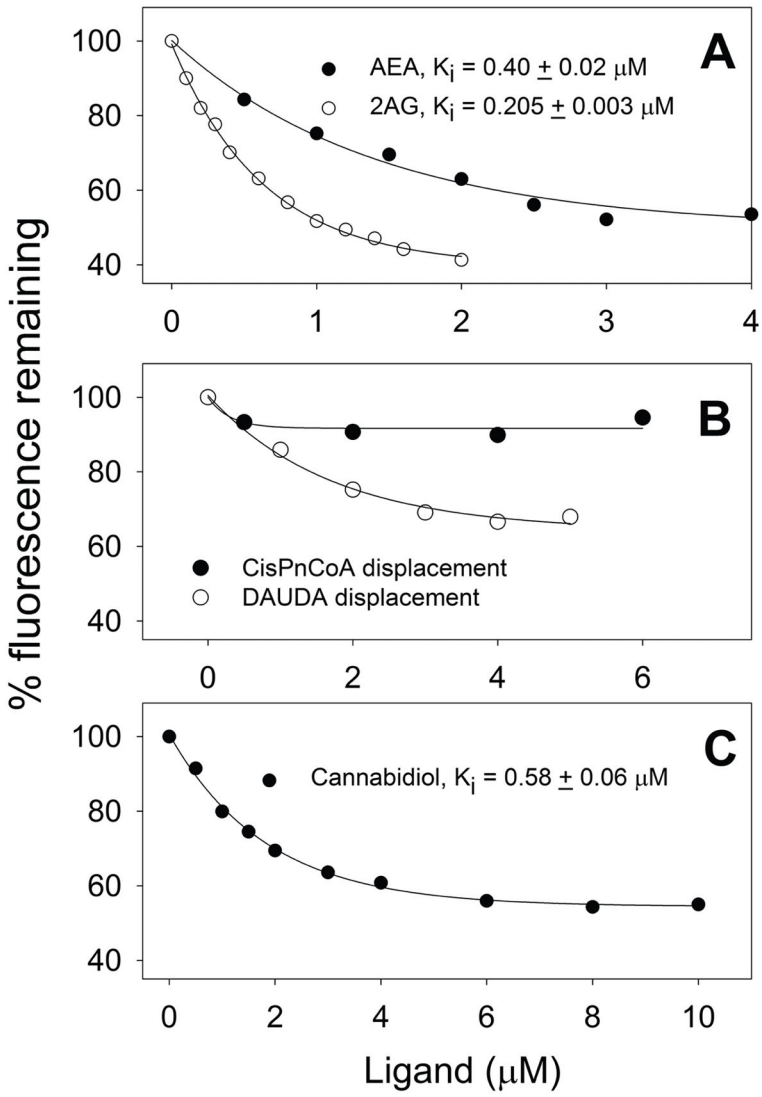

Figure 1. Binding of endocannabinoid (AEA, 2AG) and phytocannabinoid (cannabidiol) to rat and human wild-type FABP1: fluorescent ligand displacement assay

Binding of endocannabinoids (AEA and 2-AG) and a phytocannabinoid (cannabidiol) to rat or human WT FABP1 was measured by displacing bound *cis*-PnCoA and monitoring *cis*-PnCoA fluorescence decrease as in (112, 113) and/or by displacing bound 11-(dansylamino)-undecanoic acid (DAUDA) as in (26, 114). *Cis*-parinaroyl CoA (cisPnCoA) (112, 113) and DAUDA (26, 114) are only weakly fluorescent in buffer, but its fluorescence increased dramatically upon binding to FABP1. The complex of FABP1 (500nM) with the respective fluorescent ligand (500nM) in 10mM phosphate buffer was titrated with displacing ligand: Panel A. Rat FABP1/*cis*-parinaroyl-CoA with AEA (0–6μM) or 2-AG (0–2μM); Panel B: Human FABP1/*cis*-parinaroyl-CoA or FABP1/DAUDA with AEA (0–6μM); Panel C: Rat FABP1/*cis*-parinaroyl-CoA with cannabidiol (0–10μM). CisPnCoA fluorescence (Ex 304nm, Em 425nm) decrease was recorded at 24°C using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Palo Alto, CA). Fluorescence signals from *cis*-PnCoA with increasing amount of displacing ligand were used as background and

subtracted. EC_{50} was obtained from curve fitting of the displacement curves. K_i was calculated according to the equation $EC_{50}/[cisPnCoA]_{total} = K_i/K_d$ where $[cisPnCoA]_{total}$ = 500nM and K_d = 228 ± 18nM is the dissociation constant of *cis*-PnCoA binding to rat L-FABP. DAUDA fluorescence (Ex 330nm, Em 510nm) decrease was recorded at 24°C. Fluorescence signals from DAUDA with increasing amount of AEA were used as background and subtracted. Kd and Ki were determined similarly (data not shown). K_is calculated from multiple displacement curves were presented as mean ± SE (n=3).

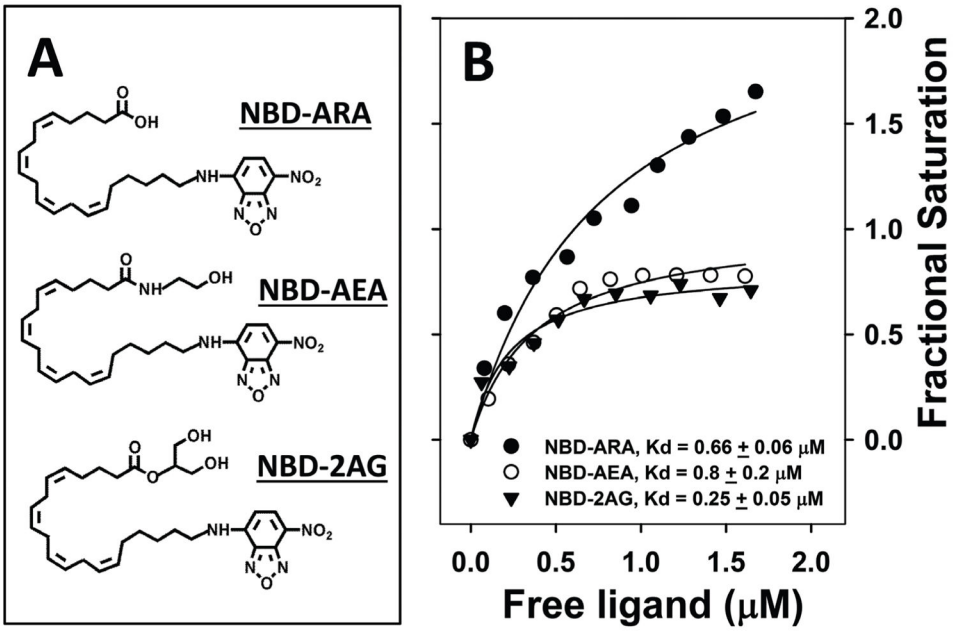

Figure 2. Direct binding of NBD-labeled ARA, AEA, and 2AG to rat FABP1

Panel A: Structures of NBD-arachidonic acid (NBD-ARA), NBD-arachidonoyl-ethanolamide (NBD-AEA) and NBD-arachidonoyl-2-glycerol (NBD-2-AG). Panel B: Binding of the NBD labeled endocannabinoids AEA, 2AG and their precursor ARA to rat L-FABP was measured based on the fluorescence increase of NBD group upon binding to the hydrophobic binding pocket as in (89). Briefly, NBD fluorescence emission spectra were obtained by scanning from 515–600nm with 490 nm excitation. Forward titrations (500nM L-FABP titrated with 0–2.5μM total ligand), and reverse titrations (100nM NBD-labeled ligands titrated with 0–3μM FABP1 were performed. Signals from corresponding NBD labeled ligands without FABP1 were used as background and subtracted from each data point. From the curve fitting of the reverse titration, the fluorescence intensity (at emission wavelength 540nm) of NBD-labeled ligand (per nM) when fully bound to FABP1 was calculated. This parameter was then used to calculate the fractional saturation and free ligand concentration in forward titration. Binding curves were constructed by plotting fractional saturation (Y) *vs* free ligand concentration (X), from which K_d and B_{max} were calculated by curve fitting. K_d was the mean ± SE (n=3).

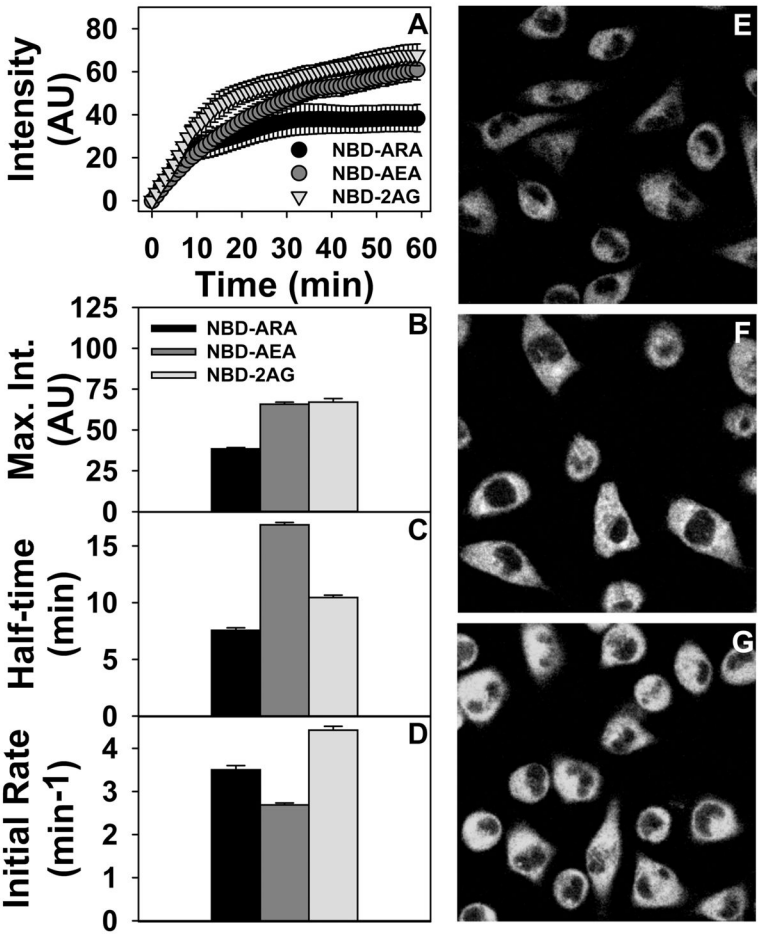

Figure 3. Cellular uptake of NBD-ARA, AEA, and 2-AG

NBD-ARA, NBD-AEA, and NBD-2-AG uptake (A), maximal uptake (B), half-time of uptake (C) and initial rate of uptake (D) by L-cell were measured by confocal imaging in L-cells similarly as for NBD-18:0 (46, 53). Values represent the mean ± SEM, n=20. Panels E, F, and G show representative fluorescent images of NBD-ARA, NBD-AEA, and NBD-2-AG uptake at 60 min, respectively.

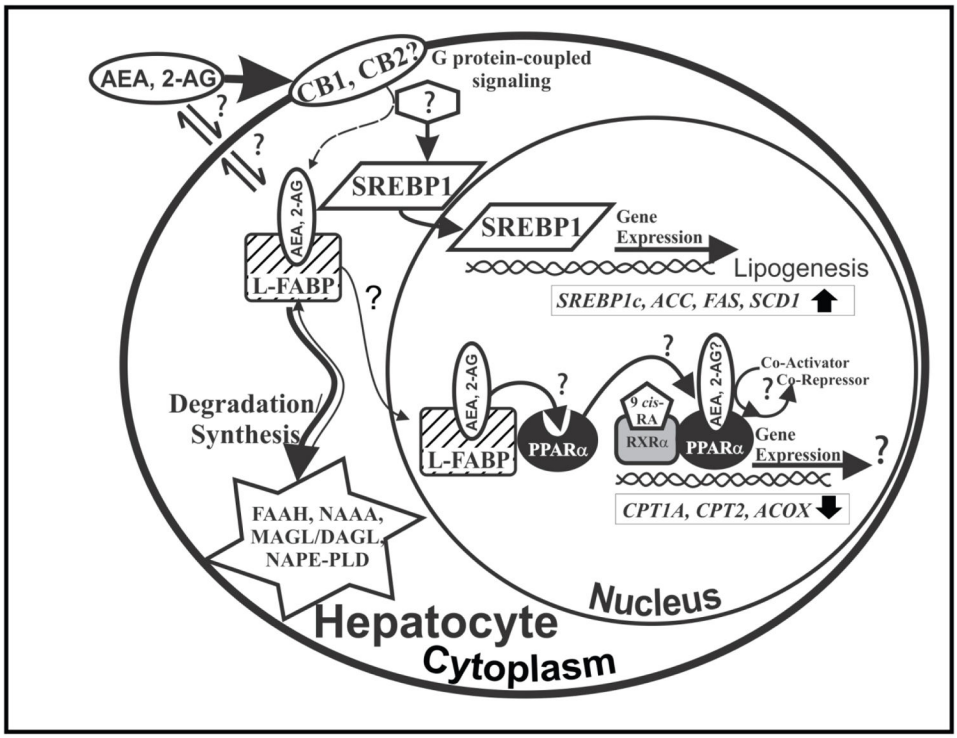

Figure 4. Schematic of FABP1's role in endocannabinoid (AEA, 2-AG) trafficking and function in primary hepatocytes

By binding anandamide (AEA) and 2-arachidonoylglycerol (2-AG), FABP1 may influence key aspects of the hepatic endocannabinoid system: A). FABP1 may facilitate AEA and 2-AG release/solubilization into the cytosol after their enzymatic cleavage/synthesis from plasma membrane phospholipids by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase α and β (DAGLα and DAGLβ). FABP1 may or may not facilitate bound AEA and 2-AG for transport/efflux across the plasma membrane and activation of CB receptors on the exofacial leaflet and/or intracellular sites for degradation/ hydrolysis. FABP1 is known to enhance the cytosolic transport of other bound ligands (5, 46, 52, 141, 251). B). FABP1 may facilitate the re-uptake of AEA and 2-AG from the plasma membrane after these lipophilic ligands cross the plasma membrane by diffusion or via G protein-coupled cannabinoid receptor 1 (CB1). Reuptake of 2-AG may also occur via G protein-coupled endocannabinoid receptor (CB2). CB2 is expressed only in embryonic liver and in diseased conditions such as fatty liver (252, 253). CB1 and CB2 activation has been linked to diet-induced hepatic steatosis, primary biliary cirrhosis, chronic hepatitis, and alcoholic liver (101). CB1 activation in mice enhances lipogenesis through the sterol regulatory element binding protein-1c (SREBP1c) pathway which induces transcription of multiple genes in lipogenesis such as SREBP1c itself, acyl CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl CoA desaturase (SCD1) (132). C). Upon FABP1-mediated release of plasma membrane and/or CB receptor bound AEA or 2-AG into the cytosol, FABP1 may transport the bound AEA (and 2-AG?) into the nucleus for PPARα activation. Although anandamide has been shown to bind PPARα and enhance PPARα activation (135), liver fat accumulation in hepatic steatosis results in decreased LCFA oxidation—likely through saturation and/or inhibition of the PPARα pathway by the increasing fat load or

damage by inflammation (101). D) FABP1 may also transport the bound AEA to the degradative enzyme fatty acid amide hydrolase (FAAH) localized with smooth endoplasmic reticulum, mitochondria, lipid droplets, and more rarely at the cell membrane (142–144) by a process analogous to that established for other FABP family members (FABP3,5,7) in brain (106–110, 144). In human liver FABP1 may also transport AEA to/from lysosomes, where it is degraded by N-acylethanolamide acid amide hydrolase (NAAA) (143, 145). Finally, FABP1 may transport bound 2-AG for degradation by monoacylglycerol lipase (MAGL), an enzyme found at lower levels in liver than brain or other tissues where it is localized diffusely in cytosol and less so in membranes without overall compartmental preference (142).

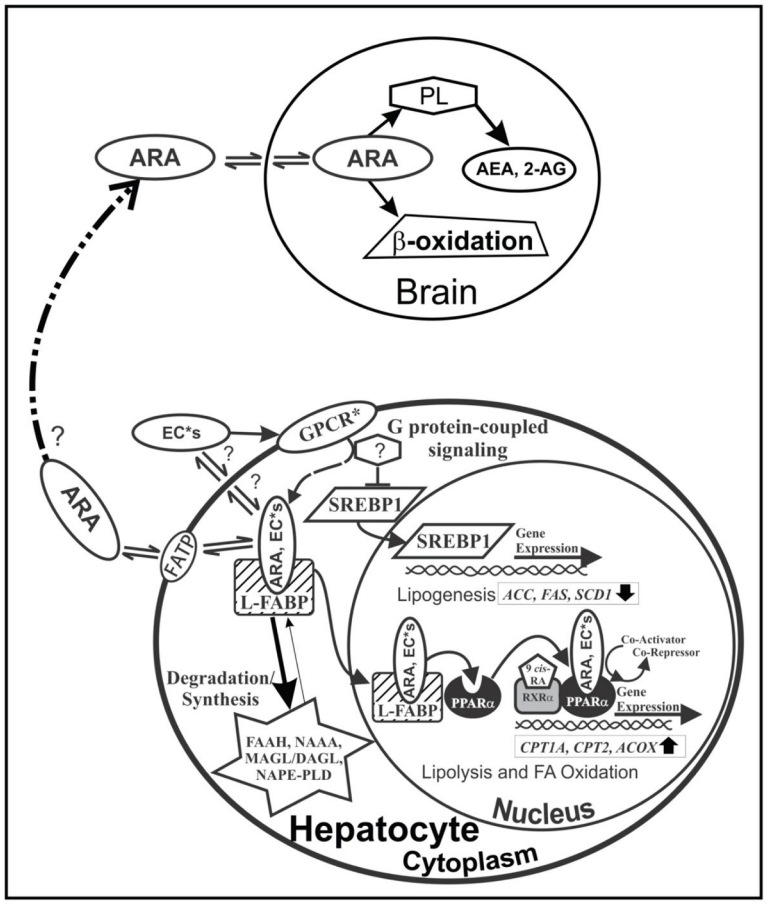

Figure 5. Schematic of FABP1's role in arachidonic acid (ARA) and EC* (OEA, PEA, 2-OG, 2-PG) targeting/trafficking

A). FABP1, not detectable in brain, binds and enhances uptake of arachidonic acid (ARA) translocated by fatty acid translocase protein (FATP) in the plasma membrane in cultured cells and likely also in hepatocytes. Within hepatocytes the FABP1 may facilitate transport/ targeting of bound ARA to endoplasmic reticulum for incorporation into phospholipids from which AEA and 2-AG are subsequently derived. B) Alternately, FABP1 mediated hepatic uptake may diminish plasma availability for ARA for uptake and conversion into phospholipids from which AEA and 2-AG are derived in brain. Most brain ARA is derived from plasma and rapid hepatic ARA clearance accounts for nearly 50% of ARA removal from the blood. C) By binding other non-ARA containing N-acylethanolamides and 2-monoacylglycerols (EC*s) such as oleoylethanolamide (OEA), palmitoylethanolamide(PEA), 2-oleoylglycerol (2-OG), or 2-palmitoylglycerol (2-PG), FABP1 may influence their synthesis release at the endoplasmic reticulum, transport for efflux at the plasma membrane, re-uptake from the plasma membrane, and targeting for degradation in the endoplasmic reticulum analogous to those of AEA and 2-AG (Fig. 4). D) By binding other EC*s, FABP1 may exert effects on the SREBP1 lipogenic and PPARα oxidative pathways opposite to those of AEA and 2-AG (Fig. 4). For example, the EC* ligands may exert their effects through G protein-coupled receptors other than CB1/CB2 (GPCR*s). For instance, OEA is known to suppress the SREBP1 pathway to reduce

lipogenesis while enhancing lipolysis and fatty acid oxidation through the PPARα pathway, likely through activation of GPR119 (136–140, 169). Conversely, FABP1 transports bound ARA and/or EC* into the nucleus for interaction with and induction of PPARα.

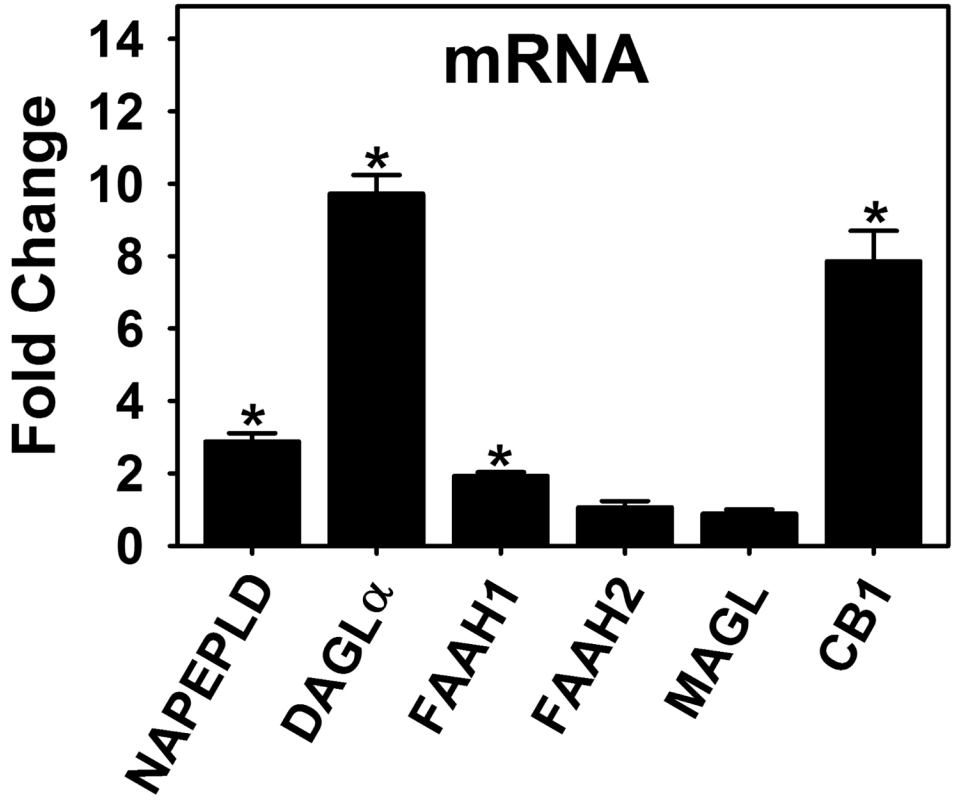

Figure 6. Human FABP1 T94A variant expression induces transcription of enzymes and receptors in the endocannabinoid system

Primary human hepatocytes as in Fig 6 were cultured as in Fig 6 followed by followed by determination of mRNA levels encoding the human N-acylphosphatidylethanolamide phospholipase-D (NAPE-PLD), diacylglycerol lipase-α (DAGLα), fatty acid amide hydrolase-1 (FARAH1), fatty acid amide hydrolase (FARAH2), monoacylglycerol lipase (MAGL), and cannabinoid receptor-1 (CB1) similarly as for other human mRNA transcripts (213–215). Values are expressed as the fold-change in the ratio of respective mRNA in human FABP1 T94A variant (T94A)/mRNA in wild-type human FABP1. Data are the means ± SEM (n = 7); *p<0.05 for T94A vs WT.

Table 1

Effect of FABP1 gene ablation on N-acylethanolamide and 2-monoacylglycerol levels in male mouse liver

Male C57BL/6N wild-type (WT) and FABP1 gene ablated mice (8 wk old) placed on a phytol-free, phytoestrogen-free control chow diet for 4 weeks, fasted overnight, and then livers removed/flash frozen and stored at −80°C as in (89).

Endocannabinoid	Wild-type (WT)	FABP1 Gene Ablated
	N-acylethanolamides (pmol/g Liver)	
AEA	13 ± 1	20 ± 2 *
OEA	34 ± 7	20 ± 3 *
PEA	60 ± 10	11 ± 1 *
	2-monoacylglycerols (nmol/g Liver)	
2-AG	0.16 ± 0.02	0.37 ± 0.04 *
2-OG	1.4 ± 0.2	2.6 ± 0.2 *
2-PG	0.18 ± 0.03	0.16 ± 0.01

N-acylethanolamides were extracted and analyzed by LC/MS using deuterated internal standards (Cayman Chemical) as in (89, 249) to determine levels of: AEA, arachidonoyl ethanolamide; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide. 2-Monoacylglycerides were extracted similarly, but deuterated internal standards (Cayman Chemical) and LC/MS solvent conditions were modified as in (89, 108) to quantitate liver determine: 2-AG, 2-arachidonoyl monoacylglycerol; 2-OG, 2-oleoyl monoacylglycerol; 2-PG, 2-palmitoyl monoacylglycerol. Values represent the mean ± SEM, n=6–7.

* $p<0.05$, one-way ANOVA for FABP1 gene ablated vs wild-type.

Table 2

Effect of FABP1 gene ablation on N-acylethanolamide or 2-monoacylglycerol levels in male mouse brain. All conditions were as in Table 1, except that LC-MS was used to identify and quantify each N-acylethanolamide or 2-monoacylglycerol as in (88, 250).

Endocannabinoid	Wild-type (WT)	FABP1 Gene Ablated
	N-acylethanolamides (pmol/g Brain)	
AEA	13 ± 2	25 ± 2 *
OEA	70 ± 10	200 ± 20 *
PEA	74 ± 9	130 ± 20 *
	2-monoacylglycerols (nmol/g Brain)	
2-AG	14 ± 1	46 ± 3 *
2-OG	4.8 ± 0.4	19 ± 1 *
2-PG	6.2 ± 0.8	9.5 ± 0.4 *

AEA, arachidonoyl ethanolamide; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide, 2-AG, 2-arachidonoyl monoacylglycerol; 2-OG, 2-oleoyl monoacylglycerol; 2-PG, 2-palmitoyl monoacylglycerol. Results are presented as pmol lipid/g brain for N-acylethanolamides and as nmol lipid/g brain for 2-monoacylglycerols (mean ± SEM, n = 6–7);

* $p < 0.05$ for FABP1 gene ablated vs wild-type (WT).