Background: Fatty acid-binding proteins (FABPs) chaperone intracellular transport of lipophilic ligands.

Results: FABP1 and FABP2 differentially promote drug activation of peroxisome proliferator-activated receptor α (PPARα) via ligand-dependent protein-protein interactions.

Conclusion: Drug activation of PPARα is regulated by the presence of different FABPs.

Significance: FABPs may act in a tissue-specific manner to enhance the selectivity of PPARα agonists.

Nuclear hormone receptors (NHRs) regulate the expression of proteins that control aspects of reproduction, development and metabolism, and are major therapeutic targets. However, NHRs are ubiquitous and participate in multiple physiological processes. Drugs that act at NHRs are therefore commonly restricted by toxicity, often at nontarget organs. For endogenous NHR ligands, intracellular lipid-binding proteins, including the fatty acid-binding proteins (FABPs), can chaperone ligands to the nucleus and promote NHR activation. Drugs also bind FABPs, raising the possibility that FABPs similarly regulate drug activity at the NHRs. Here, we investigate the ability of FABP1 and FABP2 (intracellular lipid-binding proteins that are highly expressed in tissues involved in lipid metabolism, including the liver and intestine) to influence drug-mediated activation of the lipid regulator peroxisome proliferator-activated receptor (PPAR) α. We show by quantitative fluorescence imaging and gene reporter assays that drug binding to FABP1 and FABP2 promotes nuclear localization and PPARα activation in a drug- and FABP-dependent manner. We further show that nuclear accumulation of FABP1 and FABP2 is dependent on the presence of PPARα. Nuclear accumulation of FABP on drug binding is driven largely by reduced nuclear egress rather than an increased rate of nuclear entry. Importin binding assays indicate that nuclear access occurs via an importin-independent mechanism. Together, the data suggest that specific drug-FABP complexes can interact with PPARα to effect nuclear accumulation of FABP and NHR activation. Because FABPs are expressed in a regionally selective manner, this may provide a means to tailor the patterns of NHR drug activation in a tissue-specific manner.

Approximately 13% of all Food and Drug Administration-approved drugs interact with nuclear hormone receptors (NHRs) (1). Peroxisome proliferator-activated receptors (PPARs) are NHRs that activate the transcription of genes that encode a range of proteins involved in the regulation of lipid metabolism, differentiation, and inflammation (2). In vertebrates, there are three PPAR isotypes, α, β/δ, and γ (3–5), all of which are therapeutic targets. Despite their importance as therapeutic targets, the development of PPAR ligands is hampered by safety concerns. PPAR activation can promote cell survival and may be oncogenic (6), and the PPARγ agonist troglitazone has been withdrawn due to the emergence of idiosyncratic hepatotoxicity. An understanding of the molecular basis for target specificity is therefore critical, but is complicated by the ubiquitous expression of PPARs.

Because PPARs are normally located in the nucleus, endogenous ligands and PPAR-agonist drugs must traverse the plasma membrane, cytoplasm, and nuclear membrane to acti-
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Variate receptors. Intracellular lipid-binding proteins (iLBPs) are small, soluble proteins that include the sterol carrier proteins, retinol-binding proteins, and cytosolic fatty acid-binding proteins (FABPs). iLBPs bind a range of molecules, including lipids, vitamins, and other poorly water-soluble ligands (7, 8), and can facilitate the cellular and nuclear transport of endogenous PPAR activators. For example, FABP1 expression increases fatty acid delivery to the nucleus (9–11) and enhances ligand-mediated transactivation of PPARα in COS-7 and HepG2 cells (12); cellular retinoic acid-binding protein II translocates to the nucleus on binding retinoic acid, associates directly with the retinoic acid receptor, and thereby facilitates delivery of retinoic acid to its receptor (13–15); adipocyte-fatty acid-binding protein (A-FABP or FABP4) binds the PPARγ agonist troglitazone, promoting nuclear localization and PPARγ activation. Although FABP4 can bind agonists for PPARα, PPARβ/δ, and PPARγ, only PPARγ agonists stimulate nuclear localization of FABP4 (16). This selectivity is attributed to a conformational change in FABP4 on ligand binding that exposes a nuclear localization signal (NLS), facilitating nuclear access of the FABP4-ligand complex via members of the importin family of nuclear transport receptors, of which there are multiple α and β subtypes (17, 18). Similar studies defining a ligand-sensitive NLS on the α1 and α2 helices of FABP5 have also recently been described, where exposure of the FABP5 NLS facilitates nuclear localization and PPARβ/δ activation by linoleic acid and arachidonic acid (19). For the other iLBPs, many of which lack the corresponding NLS-forming residues, the determinants of nuclear access remain largely unknown. In the case of FABP1, which lacks the residues to form an NLS, interaction with PPARα in cultured hepatocytes has been described (11, 12). Immunofluorescence microscopy has also been employed to indicate that FABP1 and PPARα are in close proximity in the nucleus, suggesting a direct interaction (11). However, details of the mechanism of nuclear access of FABP1, the ligand dependence of this interaction, and the potential effect of FABP1 on PPAR activation by PPAR agonist drugs are unknown.

Although PPARs are widely expressed, iLBP expression is tissue-specific (20–22). We propose that the tissue-specific expression of iLBPs mediates selective PPAR activation and may be exploited therapeutically to promote the cellular selectivity of PPAR agonists. For example, enterocytes and hepatocytes play key roles in lipid metabolism, and PPARα agonists exert effects on lipemia via both target sites. However, the expression patterns of FABPs in enterocytes and hepatocytes are markedly different as follows: hepatocytes express FABP1, whereas enterocytes express high levels of FABP1 and FABP2. Whether the capacity of PPAR ligands to selectively interact with FABP1 and FABP2 offers a mechanism to attain selective delivery to NHRs is unexplored.

Herein, we examine the hypothesis that FABP1 and FABP2 differentially control the nuclear transport of PPARα agonists and thereby dictate differential and specific PPAR activation. We compare the ability of FABP1 and FABP2 to bind a range of PPARα agonists, including long chain fatty acids (LCFA) and drugs. We examine the nuclear localization of FABP1 and FABP2 in the absence and presence of PPARα agonists and PPARα activation by PPARα agonists in the absence and presence of FABP. Our results show that although FABP1 and FABP2 are able to bind to both oleic acid and model hypolipidemic drugs (fenofibrate, GW7647), ligand binding results in different patterns of nuclear localization and PPAR activation. FABP2 alone promotes PPARα activation by fenofibrate. Conversely, only FABP1 promotes PPARα activation by GW7647. We find no evidence for FABP1 or FABP2 transport via the classical importin α/β- or the β1-driven pathway. Instead, ligands induce close association of PPARα and FABP and reduce the rate of nuclear egress of FABP, supporting the contention that nuclear localization is mediated by ligand-dependent FABP-PPAR interaction in the nucleus. Thus, FABP1 and FABP2 play differential and ligand-specific roles in NHR activation, and the patterns of drug interaction with intracellular binding proteins such as FABPs may govern the tissue-specific actions of drugs that target NHRs.

Experimental Procedures

Reagents— Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), trypsin, phosphate-buffered saline (PBS), Hanks’ balanced salt solution, propidium iodide, and secondary antibodies conjugated to Alexa 488 and 546 were purchased from Life Technologies, Inc. (Mulgrave, Victoria, Australia). Dimethyl sulfoxide, oleic acid, fenofibric acid, Hoechst stain, bovine serum albumin (BSA), and mouse monoclonal anti-PPARα antibody (clone 3B6) were from Sigma (Castle Hill, New South Wales, Australia). Goat anti-PPARα (C-20) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-FABP1 was from Abcam (Cambridge, UK). Mouse monoclonal anti-FABP2 antibody was a kind gift from Dr. Satoshi Kaiura (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan). X-tremeGENE 9 DNA transfection reagent was from Roche Diagnostics (Castle Hill, New South Wales, Australia). PermaFluor anti-fade reagent and sulfo-NHS-Biotin were from Thermo-Fisher Scientific (Scoresby, Victoria, Australia). Fenofibrate and GW7647 were from Cayman Chemicals (Ann Arbor, MI).

Plasmids—Human FABP2, human FABP1, and human PPARα cDNAs were synthesized by GeneArt (Regensburg, Germany). All three genes were subcloned into the pSG5 mammalian expression vector purchased from Agilent Technologies. Human FABP2 and FABP1 genes were also subcloned into expression vectors containing green fluorescent protein (GFP), pAcGFP-N1 and pAcGFP-C1, from Clontech. The luciferase reporter construct PPRE3–TK–LUC was a kind gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA). The β-galactosidase vector was from Promega (Madison, WI).

FABP1 and FABP2 Expression and Purification—A codon-optimized bacterial expression vector for human FABP1 was synthesized by GeneArt (Regensburg, Germany) and cloned into the plasmid pET-45b (+) between the PmII and BamHI sites. This construct produced recombinant FABP1 with a non-cleavable hexahistidine tag at the N terminus to aid purification. Conditions for expression, purification, and delipidation of FABP1 were identical to those reported previously for human FABP2 (23). The purity of the protein preparation was assessed by SDS-PAGE, and protein concentration was measured by UV-visible spectrometry by recording absorbance at
280 nm ($A_{280}$) using an extinction coefficient of 1490 m$^{-1}$ cm$^{-1}$ (calculated using the ProtParam tool at the ExPASy site). FABP2 was expressed and purified as described previously (23).

Isothermal Titration Calorimetry (ITC) Data—ITC experiments were carried out using an iTC$_{200}$ microcalorimeter (MicroCal®, Malvern UK) with coin shaped sample cell (200 µl) at 37 °C with stirring at 1000 rpm. In the case of fenofibric acid and sodium oleate, which were relatively soluble in sample buffer, the compound was diluted from concentrated stocks (DMSO or methanol) in ITC buffer (20 mM HEPES, pH 8, 50 mM NaCl, 0.5 mM EDTA) and placed in the titration syringe at a concentration of 0.5 or 1 mM, respectively. FABP1 or FABP2 were diluted in the identical buffer containing the same organic solvent and placed in the sample cell at a concentration of 50 µM. Typically 16 serial injections at intervals of 220 s were made with continuous stirring of the solution in the sample cell resulting in ligand concentration in the cell of 5–120 µM. The iTC$_{200}$ control software was used to operate and acquire raw data as power (microcalories/s) versus time (minutes) and processed using the Origin® (7.0) software provided by MicroCal®. For compounds with limited solubility (GW7647 and fenofibrate), concentrated stocks of the compounds in DMSO or N,N-dimethylformamide were diluted in ITC buffer to give a final sample at a concentration of 7 µM (GW7647) and 10 µM (fenofibrate) containing 0.5–5% (v/v) of the organic solvent and placed in the sample cell. In this case, FABPs were diluted in the identical buffer containing the same organic solvent and placed in the syringe at 70 µM for FABP1 titration into GW7647 and 200 and 100 µM for FABP1 and FABP2 titrations into fenofibrate solutions, respectively. Serial injection of FABP1 and FABP2 into the sample cell led to protein concentrations of 0.5–25 µM. In most cases, thermodynamic parameters were calculated by fitting the data to a one-site binding model. For sodium oleate, a two-site sequential/independent binding model was employed.

Cell Culture and Transfections—COS-7 cells were kindly provided by Prof. Phillip Nagley (Monash University, Victoria, Australia). The cells were cultured in DMEM with 4 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% FBS in a 95% air, 5% CO$_2$ atmosphere at 37 °C. For transfections, cells were seeded into 6-well plates at a density of 2 × 10$^5$ cells/well in culture medium devoid of antibiotics. Transfections were carried out using X-tremeGENE 9 DNA transfection reagent according to the manufacturer’s protocol. For immunocytochemical labeling of proteins in situ, cells were grown on sterile glass coverslips in 6-well plates.

Immunofluorescence and Confocal Laser Scanning Microscopy—COS-7 cells were co-transfected with 0.05 µg of PPARα plasmid DNA and 0.3 µg of either FABP2 or FABP1 plasmid DNA. Twenty four hours following transfection, cells were placed in serum-free medium and treated with vehicle or oleic acid (10 µM), fenofibric acid (50 µM), fenofibrate (50 µM), or GW7647 (600 nM) for 24 h. The concentrations employed were determined from the concentration dependence studies. Following treatment, cells were fixed with 3.5% paraformaldehyde for 10 min and washed twice with PBS. Cells were then permeabilized with 0.1% Triton X-100 for 10 min and washed three times with PBS. Nonspecific binding was blocked using 1% BSA (in PBS) for 1 h at room temperature. Cells were washed twice with PBS and then incubated overnight at 4 °C with either mouse anti-FABP2 antibody (1:200 dilution of stocks received from Dr. Kairu) or rabbit anti-FABP1 antibody (2 µg/ml). Cells were washed three times in PBS (15 min) and then incubated with goat anti-mouse or goat anti-rabbit secondary antibody conjugated to Alexa 488 (8 µg/ml) for 3 h at room temperature. After three washes with PBS (15 min each), immunostained cells were incubated with 5 µg/ml propidium iodide for 10 min at room temperature to counterstain the nuclei and then washed three times with PBS for 10 min to remove the excess dye. Coverslips were mounted onto slides using PermaFluor anti-fade mounting medium and kept in the dark at 4 °C. Immunofluorescence was visualized using a Leica SP5 upright confocal laser scanning microscope equipped with a ×60 oil immersion lens (Leica Microsystems, North Ryde, New South Wales, Australia). During image acquisition, the photomultiplier sensitivities were fixed to a level at which bleed-through effects from one channel to the other were negligible in the untreated samples. The same photomultiplier settings used for the untreated controls were used for the treated samples to view changes in fluorescence intensity between the two groups. Quantification of nuclear localization was achieved by measuring the immunofluorescence in the nucleus and the cytosol of individual cells using ImageJ (rsb.info.nih.gov) and calculating the nuclear to cytosolic fluorescence ratio. For each experimental group, ~50–100 cells were analyzed.

Automated INCell Analyzer 2000 High Content Imaging—The INCell 2000 high content imager (GE Healthcare) was used to obtain unbiased images of the localization of GFP-tagged FABP for quantification in live cells. COS-7 cells were co-transfected with 0.05 µg of empty vector (pSG5) or 0.05 µg of PPARα plasmid DNA, and 0.3 µg of GFP-tagged FABP (FABP2-GFP or GFP-FABP1) plasmid DNA in six-well plates. Twenty four hours following transfection, cells were subcultured into black-walled, optically clear 96-well plates, and treated with vehicle (0.1% v/v DMSO) or oleic acid (10 µM), fenofibric acid (50 µM), fenofibrate (50 µM), or GW7647 (600 nM) for 24 h. On the day of imaging, cells were incubated with Hoechst stain (2 µg/ml) for 5 min at room temperature (to stain the nuclei), prior to imaging the live cells in Hanks’ balanced salt solution at 37 °C. The center of each well of a 96-well plate was imaged, and the nuclear redistribution of FABPs was determined by measuring the fluorescence intensity in the nucleus and cytosol of every cell within the field of view using ImageJ. For each experimental repeat, ~50 cells were analyzed. Experiments were performed on four separate occasions.

AlphaScreen Importin Binding Assay—An AlphaScreen™ assay to detect the binding of His-tagged proteins to biotinylated importin was performed as described previously (24). Importins (mouse α2 and β1, equivalent to human α1 and β1) were expressed, as described previously (24), and initially concentrated by ultrafiltration, and the protein concentration was measured using a Bradford assay. Importins (4145 pmol) were subsequently biotinylated using sulfo-NHS-biotin, and the reaction was incubated on ice for 2 h. Free biotin was removed by size exclusion chromatography using a PD-10 column (GE Healthcare, Rydalmere, New South Wales, Australia) according
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to the manufacturer’s instructions, and the resultant biotinylated protein was reconstituted. The concentration of the biotinylated product was determined using a Bradford assay.

The importin α/β heterodimer was freshly generated for binding studies by mixing equimolar concentrations (13.6 μM) of importin α and importin β for 15 min at room temperature in intracellular buffer (440 mM KCl, 20 mM NaHCO₃, 20 mM MgCl₂, 4 mM EGTA, 0.4 mM CaCl₂, 80 mM HEPES, pH 7.4) containing 1 mM DTT. The α/β heterodimer or importin β alone were serially diluted in PBS to give final concentrations between 0 and 60 nM per well. All additions and incubations were performed at room temperature under subdued lighting conditions in 384-well white opaque plates in triplicate. The final total sample volume was 25 μl per well. 2 μl of His-tagged FABPs (FABP1 and FABP2) or T antigen was added to each well to provide final concentrations of 30 nM. 20 μl of biotinylated importin α/β heterodimer was then added at concentrations ranging from 0 to 60 nM, and the plate was incubated for 30 min. Acceptor beads (final 1:200, in 2.5% BSA in PBS) were added to each well and incubated for 90 min. Donor beads (final 1:250) were added, and the plate was incubated for 2 h. Fluorescence was detected using a Fusion-α plate reader (PerkinElmer Life Sciences). Triplicate replicates from two independent experiments were averaged and titration curves fit to a three-parameter sigmoidal model using GraphPad Prism.

Fluorescence Recovery after Photobleaching (FRAP) Experiments—COS-7 cells were co-transfected with 0.5 μg of GFP-tagged FABP plasmid DNA (FABP2-GFP or GFP-FABP1) and 0.05 μg of PPARα plasmid DNA then treated with either 50 μM fenofibrate or 600 nM GW7647 for 6 h at 37 °C prior to imaging on an Olympus Fluoview FV1000 confocal laser scanning microscope using a ×100 oil immersion lens. Multipoint template FRAP experiments were performed by selecting regions of interest (ROIs) in multiple neighboring cells corresponding to either the cell nuclei or an area of the cytoplasm as described previously (25). The ROIs were photobleached (12 scans, 12.5 μs/pixel, 100% laser power), and the fluorescence recovery was monitored (4% laser power, 10 μs/pixel) at 20 s intervals for 500 s. The relative level of fluorescence in the bleached and unbleached regions was quantitated by image analysis using ImageJ. Data are presented as fractional recovery (Froc) of nuclear fluorescence (minus background) relative to the pre-bleach values, where

\[ F(t) = \frac{F_{\text{pre-bleach}}}{F_{\text{max}} - F_{\text{pre-bleach}}} \]

Fb is background fluorescence. For nuclear entry data, the maximal levels of fluorescence recovery (Froc(Fn - b)_{max}) and the half-times (t_{1/2}) of fluorescent recovery were determined by fitting individual values for Froc(Fn - b) (y) against time (t) to a nonlinear equation, where \( y = y_0 + (y_{\text{max}} - y_0) e^{-kt} \). For nuclear egress, the rate constant describing the initial rate of loss of nuclear fluorescence ((Froc(Fn - b))/s⁻¹) was determined by linear regression of the early data points where the rate of recovery of fluorescence was approximately linear.

Transactivation Assays—COS-7 cells in six-well plates (2 × 10⁵ cells/well) were co-transfected with 0.3 μg of plasmid DNA for the luciferase reporter (PPRE5-TK-LUC), 0.15 μg of β-galactosidase plasmid DNA (as a transfection efficiency control), 0.05 μg of either empty vector (pSG5) or PPARα plasmid DNA, and 0.3 μg of either empty vector (pSG5), FABP2, or FABP1 plasmid DNA. Twenty four hours following transfection, cells were treated with vehicle (0.1% v/v DMSO) or oleic acid (10 μM), fenofibric acid (50 μM), fenofibrate (50 μM), or GW7647 (600 nM) for 24 h before cells were lysed and assayed for luciferase activity using the Luciferase Assay System (Promega). In parallel, lysates were assayed for β-galactosidase activity using the β-galactosidase enzyme assay system (Promega) to correct for differences in transfection efficiency. The effect of ligand on PPARα activity was expressed relative to vehicle-treated control, from four independent experiments. Initial experiments assessed the concentration dependence of ligand activation.

Fluorescence was approximately linear.

**Table 1**

Equilibrium dissociation constants for ligand binding to FABP1 and FABP2 determined by ITC

| Ligands | FABP1, K_{D} μM | FABP2, K_{D} μM |
|---------|----------------|----------------|
| Oleate  | 0.58 ± 0.22, 30.6 ± 22.4 | 0.26 ± 0.18 |
| GW7647  | 0.12 ± 0.024 | 1.3 ± 0.57 |
| Fenofibrate | 2.9 ± 1.6 | 0.8 ± 0.33 |
| Fenofibric acid | 1.6 ± 0.10 | 4.4 ± 0.99 |

Data for GW7647 and fenofibrate binding to FABP2 are reproduced from Ref. 23.

**Drug Binding to FABP1 and FABP2**—Drug binding to human FABPs was evaluated using ITC (Table 1). All the PPARα agonists investigated bound to FABP1 and FABP2 with equilibrium
dissociation constant ($K_D$) values in the high nanomolar to low micromolar range, although the affinities of the compounds for the two proteins were somewhat different. Consistent with previously reported results (26–28), oleate bound to the FABPs with nanomolar affinity and at two sites in FABP1 and a single site in FABP2. All other drugs bound to both proteins at a single site.

**Drug Binding to FABP2 and FABP1 Results in FABP Translocation to the Nucleus**—Ligand-induced changes in the intracellular distribution of PPARα, FABP2, and FABP1 were evaluated via differential expression of FABP2 and FABP1 in COS-7 cells. Because neither FABP (Fig. 1A) nor PPARα could be detected in COS-7 cells, the experimental system described herein allowed the study of the effects of expression of different FABPs in the absence and presence of PPARα. Expressed FABPs were localized by confocal microscopy (Fig. 1) and in live cells using high content imaging (Fig. 2). The two datasets are highly consistent, suggesting that no significant bias was introduced through manual analysis of the confocal images. In untreated cells expressing FABP2 and PPARα, FABP2 was detected in the cytoplasm and nucleus (Fig. 1C), with a slight excess in the nucleus. Oleic acid or fenofibrate (24 h) induced an ~2-fold increase in nuclear FABP2 as quantified by confocal microscopy (Fig. 1B) or high content imaging (Fig. 2A). In contrast, fenofibric acid or GW7647 did not affect the localization of FABP2. In cells transfected with FABP2 alone (no PPARα), oleic acid or fenofibrate did not affect the FABP distribution (Fig. 2A). In cells co-expressing FABP1 and PPARα, FABP1 was detected in the cytosol and nucleus in approximately equal amounts (Fig. 1D). Oleic acid, fenofibric acid, fenofibrate, or GW7647 (24 h) all induced a 1.5–2-fold increase in nuclear FABP1 (Fig. 1B, 2B). Similarly to FABP2, there was no effect of ligand treatment on FABP1 localization in the absence of PPARα (Fig. 2B). Thus, PPAR ligands differentially promote the nuclear translocation of FABP1 and FABP2, and this requires the presence of PPARα.

**Ligand-activated Nuclear Relocalization of FABP2 and FABP1 Is Not Mediated by Importin Binding**—There was no detectable interaction between FABP1 or FABP2 and either importin β or the importin α/β complex in either the presence or absence of PPAR agonists (Fig. 2C). As a positive control, T-antigen showed strong importin α/β interactions as expected. Thus, there is no evidence that the classical importins
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![Image 1](https://via.placeholder.com/150)

![Image 2](https://via.placeholder.com/150)

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FIGURE 2. A and B, quantification of ligand-induced changes in the intracellular distribution of FABPs using high content imaging. Images taken from the center of each well of a 96-well plate allowed unbiased quantification of the nuclear redistribution of FABP2-GFP (A) or GFP-FABP1 (B) following treatment with vehicle (0.1% DMSO), oleic acid (10 μM), fenofibric acid (50 μM), fenofibrate (50 μM), or GW7647 (600 nM) for 24 h. The nuclear to cytosolic ratio of fluorescence intensity of every cell within the field of view was calculated using ImageJ. Data shown are mean values ± S.E. from four independent experiments where ~50 cells per experiment were analyzed. *, p < 0.05 versus vehicle control. C, FABPs do not interact with importin β or the importin α/β heterodimer. FABP-importin interactions were measured using an AlphaScreenTM importin binding assay. His-tagged FABP2, FABP1, or T antigen (positive control, all at 30 nM) were incubated with increasing concentrations of biotinylated importin α/β heterodimer (0–30 nM). His-tagged FABP2 and FABP1 (30 nM) were also incubated with increasing concentrations of biotinylated importin β (0–30 nM). Triplicate replicates from two independent experiments were averaged and titration curves fit to a three-parameter sigmoidal model using GraphPad Prism. V, vehicle; OA, oleate; FA, fenofibrate acid; FF, fenofibrate; GW, GW7647.

FIGURE 3. Nuclear FRAP experiments demonstrate that ligand-dependent FABP nuclear accumulation is not likely to be mediated by increased nuclear transport. COS-7 cells co-transfected with PPARα and FABP2-GFP (A–D) or GFP-FABP1 (E–H) were treated with vehicle, 50 μM fenofibrate, or 600 nM GW7647 for 6 h and subjected to photobleaching of the cellular nuclei. Return of fluorescence was monitored every 20 s. B and F, image analysis of digitized images such as those in A and E was performed to determine the fractional recovery of nuclear fluorescence (Frec(Fn – b)). Single typical curves are shown. Curves such as those in B and F were analyzed to determine the maximal Frec(Fn – b) (C and G) and the Frec(Fn – b) t1/2 (D and H). Data represent the mean ± S.E. (n > 15), *, p < 0.05 versus no drug treatment. V, vehicle; FF, fenofibrate; GW, GW7647.

tested here are able to bind either FABP1 or FABP2 and thereby mediate access to the nucleus, suggesting an alternative importin-independent mechanism.

Nuclear Accumulation of FABP2 and FABP1 in the Presence of Drug Reflects Reduced Nuclear Egress Rather Than Enhanced Nuclear Entry—The mechanism of ligand-induced changes to nuclear accumulation of FABP2 and FABP1 was assessed by fluorescence recovery after photobleaching (FRAP). Rates of nuclear entry and nuclear egress of the FABPs were monitored in COS-7 cells co-transfected with PPARα and GFP-tagged FABP2 or FABP1 in the absence or presence of GW7647 and fenofibrate. Effects on nuclear entry were limited. In cells expressing FABP2-GFP (Fig. 3, A and B), addition of GW7647 or fenofibrate resulted in no statistical differences in maximal fractional recovery of nuclear fluorescence (Fig. 3C) or the halftime of fluorescence recovery (Fig. 3D). In cells expressing GFP-FABP1 (Fig. 3, E and F), fenofibrate also had no effects on either maximal fractional recovery (Fig. 3G) or the rate of recovery (Fig. 3H). GW7647 resulted in a small but statistically significant increase in maximal fluorescence recovery (Fig. 3G), although the halftime of recovery was unchanged (Fig. 3H). In contrast, differences in nuclear egress were marked (Fig. 4, A and D) and highly consistent with overall nuclear accumulation observed by high content imaging or confocal fluorescence microscopy. Thus, addition of GW7647 resulted in a significant reduction in the rate of nuclear egress of GFP-FABP1 (Fig. 4, E and F), but it had no effect on the nuclear egress of FABP2-GFP (Fig. 4, B and C). In contrast, fenofibrate resulted in a significant reduction in nuclear egress of FABP2-GFP (Fig. 4, B and C), but it had no effect on GFP-FABP1 (Fig. 4, E and F). Thus, the differences in nuclear translocation observed by confocal fluorescence (Fig. 1) and high content imaging (Fig. 2) appear to primarily reflect reduced nuclear egress of FABP in the presence of drug (Fig. 4) rather than differences in nuclear entry (Fig. 3).
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In cells that did not express PPARα, the ligands had no effect on luciferase activity (Fig. 5). In cells expressing PPARα, but not FABP, oleic acid stimulated a moderate 2-fold increase in luciferase activity relative to untreated cells (Fig. 5A). In cells expressing FABP2 or FABP1 and PPARα, oleic acid further enhanced luciferase activity by 1.8- and 1.6-fold, respectively, compared with the no-FABP controls. Thus, oleic acid promotes transcriptional activation by PPARα in the absence of FABPs, but the presence of either FABP2 or FABP1 magnifies this effect.

Fenofibrate induced a 10-fold increase in luciferase activity in cells expressing PPARα and empty vector (Fig. 5D). In this case, introduction of FABP2 and FABP1 produced different responses. No significant difference was observed between FABP2-transfected cells and no-FABP control cells, although an increase of 1.7-fold was observed in cells transfected with FABP1 when compared with the no-FABP control.

Fenofibrate induced a 10-fold increase in luciferase activity in cells expressing PPARα and empty vector (Fig. 5C). However, a significant further increase of 1.6-fold in luciferase activity was observed in cells that co-expressed FABP2. In contrast, no significant change in luciferase activity was observed in cells co-expressing FABP1.

Treatment of cells with GW7647 induced a 5-fold increase in luciferase activity in the no-FABP control group (Fig. 5B). FABP2 had no additional effects on luciferase activity, whereas FABP1 led to a 1.8-fold increase in luciferase activity relative to no-FABP controls. A summary of FABP-associated PPARα activation under different treatment conditions is presented in Fig. 5E. The data indicate that ligand activation of PPARα is differentially promoted by FABP1 and FABP2. Although all of the test ligands bound both FABP1 and FABP2, only specific combinations of drug and FABP led to enhanced PPARα activation. Thus, drug binding to the FABPs is a necessary but insufficient requirement for the observed potentiation in transcriptional activation.

To validate the experimental system and for comparison with previous data, an equivalent cell system was assembled containing PPARγ and either FABP4 or an empty vector (no-FABP) control. The cells were treated with troglitazone in an analogous fashion to that reported previously (16). Consistent with previous data, troglitazone induced PPARγ activation in vector control cells, and this response increased ~2-fold in cells where PPARγ and FABP4 were co-expressed (data not shown).

To validate the concentration of ligands used to probe PPARα activation and to ensure that the observed effects were not due to differences in fractional occupancy that reflect differences in the $K_D$ value of ligands for FABP1 and FABP2, the concentration dependence of ligand activation in the presence and absence of FABP1 and FABP2 was also explored. The data show consistent trends across a wide ligand concentration range for fenofibrate and GW7647 (Fig. 6).

FABP2 and FABP1 Co-localize with PPARα in a Ligand-specific Manner—FRET was used to quantify ligand-induced interactions between PPARα and FABP2 or FABP1. PPARα was almost exclusively nuclear (Fig. 7). FRET between FABP and

**FABP2 and FABP1 Enhance the Transcriptional Activation of PPARα by Oleic Acid and Hypolipidemic Drugs**—To investigate the impact of FABP2 and FABP1 expression on agonist-stimulated PPARα activation, COS-7 cells were transfected with a reporter construct that produced luciferase under the control of a consensus PPAR-response element (PPRE$_3$:TK-LUC).

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**FIGURE 4.** Cytoplasmic FRAP experiments demonstrate that ligand-dependent FABP nuclear accumulation is due to a significantly decreased nuclear egress. COS-7 cells co-transfected with PPARα and FABP2-GFP (A–C) or GFP-FABP1 (D–F) were treated with vehicle, 50 μM fenofibrate, or 600 nM GW7647 for 6 h and subjected to photobleaching of the cytoplasm. Loss of fluorescence from the nuclei was monitored every 20 s. A, time course of nuclear fluorescence loss. B, FRAP analysis of digitized images such as those in A. C, FRAP analysis of digitized images such as those in B. D, time course of nuclear fluorescence loss. E, FRAP analysis of digitized images such as those in D. F, FRAP analysis of digitized images such as those in E.
PPARα was quantified by acceptor photobleaching and observed as an increase in GFP fluorescence due to "unquenching" following photobleaching of Alexa 546 (Fig. 8).

In cells expressing FABP2-GFP and PPARα, selective photobleaching of PPARα-Alexa 546 resulted in a significant increase in the mean FRET efficiency only for cells treated with fenofibric acid (C), 50 μM fenofibrate (D), or 600 nM GW7647 (E). E summarizes ligand-stimulated activity increases in the presence of FABP relative to the absence of FABP (i.e. PPARα alone). The effect of ligand on PPARα activity was expressed relative to vehicle-treated control. Data shown are mean values ± S.E. from four independent experiments. *, p < 0.05 versus expression of PPARα alone.

FIGURE 5. Ligand-dependent transcriptional activity of PPARα is modulated by FABP2 and FABP1 expression. Transactivation of PPARα in the presence and absence of PPARα and FABP co-expression was assessed using reporter genes following 24 h of treatment of cells with vehicle, 10 μM oleic acid (A), 50 μM fenofibric acid (B), 50 μM fenofibrate (C), or 600 nM GW7647 (D). E summarizes ligand-stimulated activity increases in the presence of FABP relative to the absence of FABP (i.e. PPARα alone). The effect of ligand on PPARα activity was expressed relative to vehicle-treated control. Data shown are mean values ± S.E. from four independent experiments. *, p < 0.05 versus expression of PPARα alone.

FIGURE 6. Concentration dependence of transcriptional activation of PPARα in FABP2- and FABP1-expressing cells treated with fenofibrate and GW7647. Cells were co-transfected with PPARα and empty vector, FABP2, or FABP1. A, cells treated with vehicle alone or increasing concentrations of fenofibrate. B, cells treated with vehicle alone or increasing concentrations of GW7647. All treatments were for 24 h. The effect of ligand on PPARα activity was expressed relative to vehicle-treated control. Data shown are mean values ± S.E. from four independent experiments.

PPARα was quantified by acceptor photobleaching and observed as an increase in GFP fluorescence due to "unquenching" following photobleaching of Alexa 546 (Fig. 8).

FIGURE 7. Ligand-dependent nuclear co-localization of PPARα with FABPs. Cells co-expressing PPARα and FABP2-GFP (A) or GFP-FABP1 (B) were treated with fenofibrate (50 μM) or GW7647 (600 nM), respectively, for 24 h. PPARα was visualized via immunofluorescence using a secondary antibody conjugated to Alexa 546.
brate compared with untreated controls (Fig. 8A, 9A). There was no change in the FRET efficiency in cells treated with GW7647. Thus, only fenofibrate treatment resulted in association between FABP2 and PPARα/H9251, whereas treatment with GW7647 did not. FRET was similarly measured in cells overexpressing GFP-FABP1 and PPARα/H9251. In the case of FABP1, only GW7647 treatment induced FRET between FABP1 and PPARα/H9251 compared with untreated controls, whereas fenofibrate did not (Fig. 8B, 9B). Thus, only GW7647 stimulated association between FABP1 and PPARα.

To validate the FRET signals in the current experimental protocol, a negative control group was transfected only with GFP-FABP (i.e. donor-only). In the absence of Alexa 546, no FRET was observed, and the GFP intensity was consistent before and after photobleaching. To determine the maximal FRET efficiency of the system, cells expressing PPARα/H9251-only were double-immunolabeled using two primary antibodies (mouse and goat) that bound simultaneously to PPARα. The sample was then probed with species-specific secondary antibodies conjugated to either Alexa 488 or Alexa 546. Because the antibodies were both bound to PPARα, the two fluorophores were expected to be in close enough proximity to induce a maximal FRET response. Under these conditions, a mean FRET efficiency of 20% was achieved suggesting that this is the maximum FRET signal attainable with the current experimental system (Fig. 9).

Discussion

To gain access to PPARs, endogenous agonists such as LCFA or hypolipidemic drugs must penetrate the plasma membrane, diffuse across the cytoplasm, and enter the nucleus. The hydrophobic nature of most PPAR agonists (and fatty acids) dictates that partitioning out of the membrane and into the aqueous cytosol is thermodynamically unfavored, presenting a formidable barrier to PPAR activation. To overcome this challenge, intracellular trafficking of endogenous lipophilic molecules such as LCFA is facilitated by interaction with iLBPs, including FABPs (9–12, 16, 29). In tissues where large amounts of lipids are used and stored, nuclear translocation of FABPs is a critical step in the transfer of ligands to PPARs.

Although PPARs are ubiquitous, and endogenous ligands such as LCFA are widely distributed, the expression of FABPs is organ-specific. The importance of FABPs in facilitating nuclear transport and PPAR activation, and the wide variation in patterns of FABP expression, led us to hypothesize that PPAR activation might be differentially controlled by varying FABP expression patterns and that this may be a means of mediating tissue-specific PPAR activation. In particular, although the role of FABP1 in PPARα activation has been described previously, nothing is known about the consequence of FABP2 expression on nuclear localization and PPAR activation, or the differential effects of FABP1 and FABP2 on the ability of hypolipidemic drugs to activate PPAR. This omission is despite the increasing realization that hypolipidemic drugs may act in both the liver
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(when FABP1 dominates) and also in the enterocyte (where FABP2 and FABP1 are both present). The first objective of this study was therefore to explore whether FABP2 was able to direct agonists to PPARα and to effect transcriptional activation in a similar manner to that described previously for FABP1 and PPARα in hepatocytes, FABP4 and PPARγ in adipocytes, and FABP5 and PPARβ/δ in MCF-7 cells (11, 12, 16, 19). Second, we sought to evaluate whether the mechanism of nuclear access was the same as that previously reported. Finally, we aimed to evaluate whether FABP2 and FABP1 mediate similar or different effects on PPAR activation by LCFA or hypolipidemic drugs.

Incubation of FABP2-expressing COS-7 cells with oleic acid promoted an ~2-fold increase in nuclear redistribution of FABP2 compared with untreated cells. However, the effect of incubation with hypolipidemic drugs on FABP2 distribution in cells was ligand-specific. Fenofibrate treatment resulted in an increase in nuclear localization of FABP2, whereas treatment with GW7647 or fenofibric acid had no effect. In contrast, oleic acid and all the fibrate compounds tested led to an increase in the nuclear localization of FABP1. This is despite the fact that all the PPARα agonists bound to both FABP2 and FABP1. For both FABP2- and FABP1-expressing cells, nuclear accumulation was dependent on the presence of PPARα, suggesting that interaction of FABP with PPAR may be responsible for nuclear retention.

For FABP2, the patterns of nuclear localization were consistent with PPARα activation measured with each of the ligands. Thus, oleic acid and fenofibrate were able to activate PPARα more potently in the presence of FABP2, whereas the presence of FABP2 had no effect on the ability of fenofibric acid or GW7647 to activate PPARα. In contrast, FABP1 enhanced the PPARα transcriptional activity of oleic acid, fenofibric acid, and GW7647 but not fenofibrate even though all four ligands resulted in enhanced nuclear localization of FABP1. The observation that FABP1 failed to enhance fenofibrate-mediated PPAR activation suggests that FABP1-mediated shuttling of ligand to the nucleus is a critical step for enhanced PPAR activation, but on its own it is not sufficient. Some correlation was evident between relative binding affinity and the ability of the FABPs to promote ligand activation of PPARα. Thus, the binding affinities of GW7647 and fenofibric acid were higher for FABP1 when compared with FABP2, and for fenofibrate, binding affinity was higher for FABP2 when compared with FABP1, consistent with patterns in the enhancement of receptor activation. However, the differences in binding affinity are moderate, especially for fenofibrate and fenofibric acid (2–3-fold), and absolute affinity overlaps across the series. Thus, GW7647 binds FABP2 with $K_D = 1.3$ μM, but it does not stimulate nuclear localization or PPAR activation, whereas fenofibric acid binds FABP1 with similar affinity ($K_D = 1.6$ μM) but does stimulate nuclear relocation and ligand activation. Fig. 6 also provides evidence of differential patterns of PPAR activation over a wide (3 orders of magnitude) ligand concentration range suggesting simple correlations with receptor occupancy are unlikely.

The enhancement in ligand activation of PPARα described here in the presence of FABP is relatively modest (~2-fold). However, the magnitude of change is consistent with previous studies exploring the effects of different iLBPs on patterns of nuclear receptor activation (16, 30). In the latter studies, differences of this magnitude were subsequently shown to be physiologically significant (30). There is therefore precedent for differences of this size leading to significant downstream changes.

FRAP experiments revealed that the nuclear accumulation of FABP1 and FABP2 in the presence of GW7647 and fenofibrate, respectively, was preferentially a function of reduced nuclear egress in the presence of ligand rather than enhanced nuclear entry. This again was ligand- and FABP-specific. In this case, patterns of reduced nuclear egress of FABPs in the presence of ligand were consistent with the ligand activation studies. Thus, the presence of GW7647, but not fenofibrate, resulted in reduced nuclear egress of GFP-FABP1, whereas fenofibrate, but not GW7647, reduced nuclear egress of FABP2-GFP.

To assess the underlying mechanism of nuclear accumulation, a confocal-based FRET assay was used to probe direct protein-protein interactions between FABP2 or FABP1 and PPARα. Consistent with the PPAR activation assays, the nuclear egress studies, and the dependence of nuclear accumulation on the presence of PPARα, a positive FRET response was observed in cells containing FABP2 and treated with fenofibrate but not GW7647. Similarly, an interaction between FABP1 and PPARα was apparent in cells treated with GW7647 but not with fenofibrate. Thus, FABP1 is able to promote nuclear localization of a range of ligands, including oleic acid, fenofibrate, fenofibric acid, and GW7647, but FABP1-mediated enhancement of PPAR activation is dependent on a direct FABP1-PPARα interaction that is not necessarily facilitated simply by promotion of nuclear localization. In contrast, for FABP2, the observed patterns of nuclear localization, PPAR activation, and PPAR-FABP FRET patterns were well aligned.

Our data indicate that the mechanisms that lead to the nuclear accumulation of FABP1 and FABP2 are distinct from those reported for FABP4 and other iLBPs. Furthermore, nuclear localization of FABP1/2 and its effects on PPAR activation are both ligand-dependent and ligand-specific. A mechanism of ligand-mediated nuclear transport has been reported previously for FABP4 where a putative conformational NLS was identified in the helix-loop-helix region of the protein. In this case, when ligand binding resulted in stabilization of the conformational NLS, nuclear accumulation of FABP4 was observed (17, 18). In contrast, where ligand binding did not stabilize the NLS, no nuclear accumulation was observed. Sequence alignment data suggested the presence of a similar conformational NLS in keratinocyte FABP (FABP5), brain FABP (FABP7), heart FABP (FABP3), and cellular retinoic acid-binding protein II (17, 31), and indeed, recent studies have shown a similar conformational NLS in FABP5 that is stabilized by linoleic acid and arachidonic acid (19). In contrast, residues necessary for formation of a conformational NLS are lacking in FABP1 (17) and FABP2. The absence of an NLS in FABP2 and FABP1 is consistent with the lack of interaction observed between FABP1 or FABP2 and the importins tested here.

FABP2 and FABP1 are small enough to diffuse into the nucleus through the nuclear pore, and the FRAP experiments described here suggest limited differences in the rates or extent
of nuclear entry of these FABPs, regardless of the presence or absence of ligand.

For FABP2, the data are consistent with a ligand-dependent interaction with PPARα, and this is reflected in consistent patterns of nuclear localization and retention, PPAR activation, and FRET between FABP2 and PPARα. It is also consistent with the lack of nuclear localization of FABP1 and FABP2 upon ligand binding in cells that did not express PPARα. Similarly, treatment of cells with GW7647 results in nuclear accumulation of FABP1; activation of PPARα is potentiated by the presence of FABP1, and FRET is observed between FABP1 and PPARα. Thus, it appears that a ligand-dependent interaction with PPARα is driving the observed localization of FABP1 in the nucleus. However, it would appear that FABP1 is able to interact with proteins other than PPARα. In support of this suggestion, FABP1 has previously been shown to interact with PPARγ, and furthermore, direct interactions between other FABPs and proteins involved in lipid metabolism have previously been reported in other cellular models. For instance, selective association of FABP4 with PPARγ (but not PPARβ/δ) and FABP5 with PPARβ/δ (but not PPARγ) has been demonstrated in COS-1 cells (16), and FRET-based assays have shown evidence of FABP4 association with hormone-sensitive lipase in lipocytes derived from primary human embryonic kidney cells (32). It may be the case that ILBPs are able to co-localize with a range of different proteins, although the details of the factors driving these protein–protein interactions are not fully understood.

The current data are consistent with ligand-dependent interaction of FABP1 and FABP2 in the nucleus following free diffusion of FABP through the nuclear pore, ultimately resulting in nuclear retention of FABP and PPARα activation. This interpretation is consistent with data from this study where PPARα was only detected in the nucleus and where accumulation of FABP in the nucleus was due to a reduced rate of nuclear egress in the presence of ligand. However, it has been shown that nuclear receptors, including PPARα, are able to partition between the nucleus and cytosol (33). Therefore, it remains possible that ligand-induced FABP-PPARα interactions occurred in the cytosol followed by nuclear accumulation of the complex.

In summary, we provide evidence that FABP1 and FABP2 promote nuclear localization of their ligands via an importin-independent mechanism, and thereby they enhance activation of PPARα. This occurs in a ligand-specific fashion, and FABP2 and FABP1 have different effects on the ability of different agonists to promote PPARα activation. Thus, the activity of drugs against intracellular targets, in particular NRs, may be modulated by binding to intracellular binding proteins such as FABPs. Organ-specific expression patterns of FABPs may therefore provide a mechanism for the targeted delivery of drugs to NRs in different tissues. This process could be accomplished by designing drugs to interact with specific ILBPs as well as their target NRs.

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