Binding of Cytochrome P450 Monooxygenase and Lipoxygenase Pathway Products by Heart Fatty Acid-Binding Protein†

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ABSTRACT: Arachidonic acid metabolism by lipoxygenases and cytochrome P450 monooxygenases produces regioisomeric hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DHETs), which serve as components of cell signaling cascades. Intracellular fatty acid-binding proteins (FABPs) may differentially bind these nonprostanoid oxygenated fatty acids, thus modulating their metabolism and activities. Vascular cells, which express heart FABP (H-FABP), utilize oxygenated fatty acids for regulation of vascular tone. Therefore, the relative affinities of H-FABP for several isomeric series of these compounds were measured by fluorescent displacement of 1-anilinonaphthalene-8-sulfonic acid (ANS). In general, H-FABP rank order affinities (arachidonic acid > EETs > HETEs > DHETs) paralleled reversed-phase high-performance liquid chromatography retention times, indicating that the differences in H-FABP affinity were determined largely by polarity. H-FABP displayed a similar rank order of affinity for compounds derived from linoleic acid. H-FABP affinity for 20-HETE [apparent dissociation constant (K_d') of 0.44 μM] was much greater than expected from its polarity, indicating unique binding interactions for this HETE. H-FABP affinity for 5,6-EET and 11,12-EET (K_d' of ~0.4 μM) was ~20-fold greater than for DHETs (K_d' of ~8 μM). The homologous proteins, liver FABP and intestinal FABP, also displayed selective affinity for EET versus DHET. Thus, FABP binding of EETs may facilitate their intracellular retention whereas the lack of FABP affinity for DHETs may partially explain their release from cells. The affinity of H-FABP for EETs suggests that this family of intracellular proteins may modulate the metabolism, activities, and targeting of these potent eicosanoid biomediators.

Fatty acid-binding proteins (FABPs)[1] are a family of low molecular mass (15 kDa) cytosolic proteins that bind long-chain fatty acids with nanomolar affinities (*1*). The formation of FABP−fatty acid complexes facilitates the diffusion of the relatively insoluble fatty acids (*2, 3*) and may provide an intracellular fatty acid pool for enzymatic utilization (*4*). Heterologous expression of FABPs in cells and disruption of FABP genes in mice have demonstrated that FABP levels affect fatty acid uptake and flux through the β-oxidation and esterification pathways. For example, heterologous expression of intestinal FABP in mouse L fibroblasts increased triacylglycerol and cholesteryl ester mass, while phospholipid mass remained unchanged (*5*). This selective modulation of fatty acid incorporation suggests that FABPs may target fatty acids to specific metabolic pathways. Cardiac myocytes isolated from heart FABP (H-FABP) disrupted mice showed an ~50% reduction in both the uptake and β-oxidation of palmitate (*6*).

Oxygenated fatty acid metabolites are produced from polyunsaturated fatty acids both nonenzymatically and by the actions of lipoxygenases, monooxygenases, and cyclooxygenases. The lipoxygenase pathway produces a series of hydroxylated isomers known as hydroxyeicosatetraenoic acids (HETEs), while the cytochrome P450 epoxygenase pathway produces four regioisomeric epoxyeicosatrienoic acids (EETs) and their hydration products, the dihydroxyeicosatrienoic acids (DHETs). EETs have numerous biological effects including vasorelaxation produced by modulation of ion channels (*7, 8*), mediation of mitogenesis via tyrosine kinase cascades (*9*), mediation of Ca^{2+} flux (*10*), and antiinflammatory effects in the vasculature (*11*; reviewed in ref *12*). HETEs and EETs are incorporated into membrane phospholipids where they can be released by phospholipases for autocrine and paracrine signaling. Thus, the processes of fatty acid influx/efflux, intracellular transport, and esterification are essential for HETE and EET actions. We postulated, on the basis of Kane et al. (*13*), that as FABPs modulate long-chain fatty acid influx/efflux and distribution among cellular lipid pools, they might also modulate the retention, release, and metabolism of oxygenated fatty acid derivatives. Limited studies of FABP affinity for lipoxygenase pathway products showed that keratinocyte and adipocyte lipid-binding proteins bind several hydroperoxy derivatives with affinities similar to that of arachidonic acid, while

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[1] Abbreviations: ADIFAB, acrylodated intestinal fatty acid-binding protein; ANS, 1-anilinonaphthalene-8-sulfonic acid; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; EODE, epoxyoctadecenoic acid; FABP, fatty acid-binding protein; H-FABP, heart fatty acid-binding protein; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein.

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the affinities for several HETE isomers are from 5- to 12-fold weaker than for arachidonic acid (*13, 14*).

In this report, FABP affinities for the regioisomers of EETs and their hydration products, DHETs, and several isomeric HETEs were determined by competitive displacement of the fluorophore 1-anilinonaphthalene-8-sulfonic acid (ANS). H-FABP was the focus of the binding studies because it is present in vascular cells (*15, 16*), a prominent site of oxygenated fatty acid activity (*12*). We found that H-FABP displayed selective binding for several of the EETs, while DHETs were only weakly bound. This suggests that FABPs may influence the metabolism and signaling functions of these eicosanoids.

EXPERIMENTAL PROCEDURES

Materials. ANS ammonium salt was from Fluka. Type VI hydroxyalkoxypropyldextran and imidazole were from Sigma. K_2HPO_4, KH_2PO_4, and KCl (ultralow fluorescence grade) were from Pan Vera (Madison, WI). *cis*-Parinaric acid was purchased from Calbiochem (La Jolla, CA). Fatty acids were purchased from Caymen (Ann Arbor, MI), except for 20-HETE, which was also obtained from Sigma, and 8(*R*)-HETE, which was purchased from Sigma. Acrylodan-derivatized intestinal fatty acid-binding protein (ADIFAB) was purchased from Molecular Probes (Eugene, OR).

Expression, Purification, and Delipidation of Rat H-FABP. The pET3a-rH-FABP vector containing the entire coding region of rat H-FABP was a generous gift of Jan Glatz (Maastricht University, The Netherlands). H-FABP was expressed in *Escherichia coli* and purified as previously described (*17*) except for the addition of a Mono Q (Pharmacia) anion-exchange column to concentrate the H-FABP-containing fractions. The purity of the pooled Mono Q fractions were analyzed by SDS−PAGE on 15% gels (Bio-Rad, Hercules, CA) and by reversed-phase HPLC on a C_4 silica column (Vydac, Hesperia, CA). Pooled Mono Q fractions (20 μg) were injected onto a C_4 column equilibrated with 10% acetonitrile/0.1% trifluoroacetic acid and eluted with a linear gradient increasing to 80% acetonitrile in 30 min at a flow rate of 1 mL/min (*18*). H-FABP was delipidated with Type VI hydroxyalkoxypropyldextran (Lipidex 1000) as described (*19*), dialyzed into 20 mM HEPES and 20 mM KCl (pH 7.4), and stored at −80 °C at a concentration of 2 mg/mL.

Titration of H-FABP with ANS. ANS was dissolved in water and its concentration determined by $\epsilon_{350} = 4990$ $M^{-1}\cdot cm^{-1}$. H-FABP concentration was determined by two methods, absorbance at 280 nm, ($\epsilon = 14\,000$ $M^{-1}\cdot cm^{-1}$; *20*) and quantitative amino acid analysis. The concentration determined by amino acid analysis agreed within 10% with the value determined by absorbance. ANS binding to H-FABP was studied by measuring fluorescence changes during titration of ANS into a fixed concentration of H-FABP. The ANS emission was monitored with a SLM Aminco 4800 fluorescence spectrophotometer ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 465$ nm, slits 2 nm). H-FABP (1.2 μM) in 2 mL of buffer (20 mM KH_2PO_4 and 80 mM KCl, pH 7.4) was added to a 1 cm path length cuvette at 25 °C with stirring. Aliquots of ANS were added with a syringe, and the fluorescent signal was recorded after a 2 min equilibration period. The fluorescence titration data was analyzed by regression to a one-site binding equation (eq 1, see below).

Displacement Assays with ANS. ANS displacement assays were based on the procedure of Kane and Bernlohr (*21*). A mix of H-FABP (0.4 μM) and a 10-fold excess of ANS (4 μM) in 20 mM KH_2PO_4 and 80 mM KCl (pH 7.4) suitable for ≤10 assays was prepared and kept on ice. For each titration, 2 mL of this mix was added to a cuvette, which was warmed to room temperature and placed into a stirred, thermostated (25 °C) sample holder. Competitor fatty acids were added in increments of 1−4 μL by a syringe equipped with a repeating dispenser (Hamilton, Reno, NV), and the fluorescent signal was averaged for 10 s. The final concentration of ethanol in the cuvette was <0.5%. The cuvette was rinsed with ethanol, dimethylformamide, and 50% nitric acid between titrations. ANS displacement assays with delipidated recombinant rat I-FABP (kindly provided by David Cistola, Washington University) and delipidated recombinant rat L-FABP (kindly provided by Friedhelm Schroeder, Texas A&M University) were performed in the same manner.

Displacement Assays with cis-Parinaric Acid. Parinaric acid displacement assays were based on the method of Stolowich et al. (*22*). A stock of ~300 μM *cis*-parinaric acid was prepared in dimethylformamide and the concentration was determined by $\epsilon_{304} = 78\,000$ $M^{-1}\cdot cm^{-1}$. A mixture of H-FABP (0.45 μM) and *cis*-parinaric acid (0.5 μM) in 50 mM NaH_2PO_4 (pH 7.4) was titrated with fatty acids dissolved in 95% ethanol and the fluorescent signal was recorded 2 min after each addition ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 416$ nm). The fluorescence measurements were corrected by the signal obtained from a titration with ethanol alone.

Analysis of Displacement Data. As detailed above, the displacements were performed under conditions where [ANS] ≫ [FABP]. It is well described that under these conditions the complexity of the resultant titration curves is reduced (*23*), such that they can be fit directly to a generalized, single-site binding equation:

$$F = F_0 - \{[1 + (P_T + L_T)K_a' - [(P_T - L_T)^2 K_a'^2 + 2(P_T + L_T)K_a' + 1]^{1/2}]/2P_T K_a'\}(F_0 - F_\infty) \quad (1)$$

where F is the measured fluorescence, F_0 is the fluorescence in the absence of ligand, P_T is the total protein concentration, L_T is the total titrant ligand concentration, K_a' is the apparent association constant for the titrant ligand, and F_∞ is the fluorescence signal after complete saturation of the protein with ligand. Fitting was performed by nonlinear least-squares regression with a commercial package (SigmaPlot, SPSS Inc., Chicago, IL). The stoichiometric nature of arachidonic acid binding to FABP (see Figure 2) allowed the determination of the parameters P_T and F_∞ through regression analysis to eq 1. These parameters were then constrained for the determination of K_a' for each oxygenated fatty acid titration. The apparent dissociation constant, K_a', was obtained as the inverse of K_a'. This approach allows accurate comparison of relative binding constants for a series of ligands.

Determination of Binding Constants with ADIFAB. Fatty acids in ethanol were concentrated under nitrogen and resuspended in ethanol at a concentration of ~3 mM. Although the ADIFAB assay instructions specified that fatty acids were to be solubilized as sodium salts, this was not

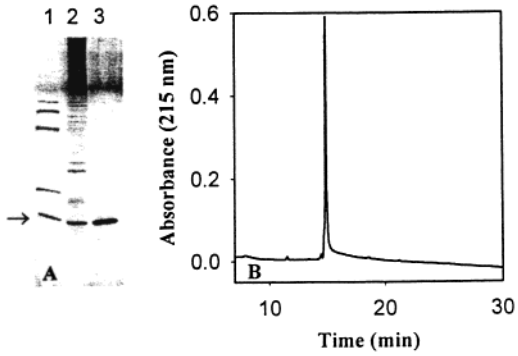

FIGURE 1: Analysis of the purity of recombinant rat H-FABP. H-FABP was expressed in *E. coli* and purified by anion-exchange chromatography. (A) Analysis by SDS−15% PAGE with silver staining. Lane 1, molecular weight markers (arrow indicates 14 kDa standard); lane 2, high-speed supernatant (1 μg) of induced cell lysate; lane 3, pooled fractions (1 μg) from Mono Q anion-exchange chromatography eluted at 40−60 mM NaCl. (B) Reversed-phase HPLC of the pooled Mono Q fractions (20 μg) on a C_4 silica column. A linear gradient of acetonitrile from 10% (0 min) to 80% (30 min) was utilized for elution.

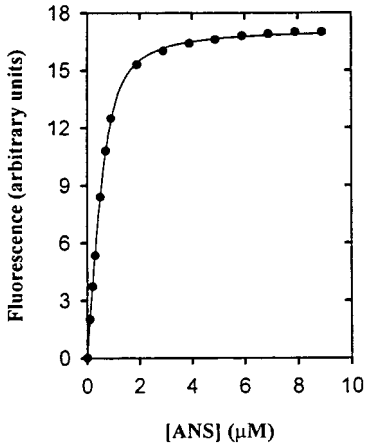

FIGURE 2: Titration of H-FABP with the fluorophore 1-anilinonaphthalene-8-sulfonic acid (ANS). Delipidated rat H-FABP (1.2 μM) was titrated with ANS, and the ANS fluorescence was monitored (λ_{ex} = 380 nm, λ_{em} = 465 nm). No background subtraction was necessary since the fluorescence signal of 10 μM ANS in the absence of protein was 0.1% of the signal obtained when H-FABP was present. The line was obtained by nonlinear fitting to a single-site binding equation.

possible for EET due to the limited quantities and their susceptibility to degradation in NaOH. Also, we found that correction of the emission signals for the slight contribution due to ethanol did not change the K_d values by more than 2%. Titration of ADIFAB with fatty acid was performed to determine ADIFAB binding constants according to the supplier's instructions. Briefly, 0.2 μM ADIFAB in 20 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4 (pH 7.4) at 22 °C was titrated with fatty acid to 4 μM and the emission signals at 432 and 505 nm were recorded (excitation λ = 386 nm). ADIFAB dissociation constants were determined from Hill plots. Dissociation constants for L-FABP binding of arachidonic acid and 11,12-EET were determined according to Richieri et al. (*24*). Briefly, ADIFAB (0.2 μM) and FABP (4 μM) were titrated with fatty acid to 4 μM final concentration and the K_d was determined by Scatchard analysis.

RESULTS

Fluorometric Studies of H-FABP Binding to ANS. Recombinant rat H-FABP was expressed in *E. coli* and purified by anion-exchange chromatography. The H-FABP preparation was found to be homogeneous when analyzed by either SDS−PAGE or reversed-phase HPLC (Figure 1). Figure 2 shows a titration of 1.2 μM H-FABP with ANS. As seen with other FABPs (*21, 25*), ANS binds to H-FABP with saturable enhancement of ANS fluorescence. The data were fit to a single-site binding equation to determine the stoichiometry of binding. We consistently observed a stoichiometry of 0.5−0.6 molecule of ANS per H-FABP. A similar stoichiometry has been previously reported for recombinant rat H-FABP binding of fatty acids (*20*), although a different methodology was employed. As discussed elsewhere (*20, 26*), the deviation of the stoichiometry from unity is probably due to multiple factors including the presence of inactive protein in the recombinant preparations. This uncertainty precludes a reliable determination of H-FABP affinity for ANS.

H-FABP Binding of EETs and DHETs. The relative affinities of H-FABP for the four EET regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET) as compared to arachidonic acid were determined by competitive displacement of ANS (Figure 3A). Arachidonic acid effectively displaced ANS, and a regression analysis yielded a K_d' of 0.05 μM (Table 1). This K_d' agrees well with the value of 0.04 μM obtained by Richieri et al. (*20*) utilizing ADIFAB. The four EET regioisomers displaced bound ANS with variable efficiencies. H-FABP affinities for 5,6-, 11,12-, and 8,9-EET (K_d' values $\sim$ 0.4 μM) were ~8-fold lower than for arachidonic acid, whereas H-FABP affinity for 14,15-EET was 34-fold lower (Table 1). Because many cell types contain epoxide hydro-

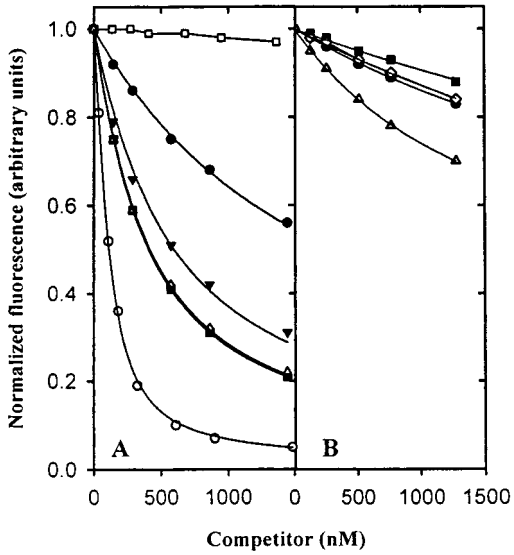

FIGURE 3: Competitive displacement of ANS from H-FABP by EETs and DHETs. Competitors were titrated into a mix of FABP (0.4 μM) and ANS (4 μM). The maximum fluorescence signal for each titration was normalized to 1.0. Lines were obtained by nonlinear regression fitting to a single-site binding equation. Data shown are representative of $n \geq 3$ titrations. (A) Displacement of ANS by arachidonic acid and EETs: Arachidonic acid (○), 5,6-EET (■), 11,12-EET (△), 8,9-EET (▼), 14,15-EET (●), and equivalent volumes of ethanol (□). (B) Displacement of ANS by DHETs: 5,6-DHET (△), 8,9-DHET (●), 14,15-DHET (◇), and 11,12-DHET (■).

Table 1: Binding Analysis of H-FABP[a]

fatty acid	K_d' (μM)	$\Delta\Delta G^o$ (kcal/mol)
arachidonic acid	0.05 ± 0.01[b]	0.00[c]
5,6-EET	0.38 ± 0.02	1.20
8,9-EET	0.56 ± 0.04	1.43
11,12-EET	0.37 ± 0.02	1.18
14,15-EET	1.70 ± 0.07	2.09
5,6-DHET	3.2 ± 0.4	2.46
8,9-DHET	7.5 ± 1.2	2.97
11,12-DHET	14.2 ± 5.2	3.34
14,15-DHET	7.7 ± 1.3	2.98
5-HETE	1.24 ± 0.02	1.90
8-HETE	1.14 ± 0.05	1.85
12-HETE	1.91 ± 0.07	2.16
15-HETE	2.77 ± 0.05	2.38
20-HETE	0.44 ± 0.03	1.29

[a] Displacement assays utilizing ANS and H-FABP were performed and apparent dissociation constants (K_d') were determined by regression fitting as described under Experimental Procedures. [b] K_d' values are presented ± SEM for ≥3 repetitions of the titration. [c] Differences in the free energy of binding relative to arachidonic acid.

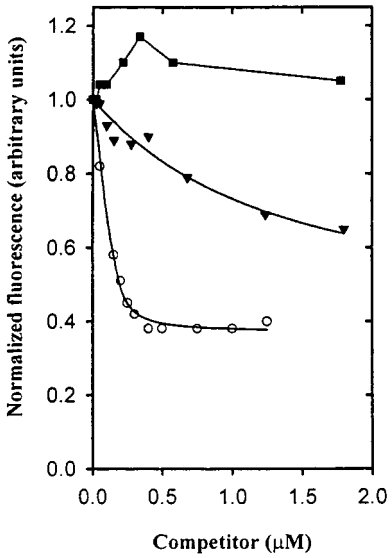

FIGURE 4: Competitive displacement of *cis*-parinaric acid from H-FABP by EET and DHET. Competitors were titrated into a mix of FABP (0.4 μM) and *cis*-parinaric (0.5 μM). The fluorescent signal obtained from *cis*-parinaric acid alone was 0.3 unit. Data shown are representative of duplicate titrations. Displacement of *cis*-parinaric acid by arachidonic acid (○), 14,15-EET (▼), and 14,-15-DHET (■) is shown.

lases that convert EETs to DHETs, the affinities of H-FABP for DHET regioisomers also were measured (Figure 3B). As a group, the DHET regioisomers weakly displaced ANS (K_d' values 3.2−14.2 μM, Table 1), as compared with the EETs.

To validate the relative affinities determined by ANS displacement, displacement assays were repeated with *cis*-parinaric acid (C18:4), a naturally fluorescent fatty acid (Figure 4). An identical rank order of H-FABP affinities was observed (arachidonic acid > 14,15-EET > 14,15-DHET).

Comparative Binding Studies with Liver and Intestinal FABPs. To determine whether the binding profile of other FABPs for cytochrome P450 pathway products would be similar to that of H-FABP, the binding of 11,12-EET and 11,12-DHET by recombinant rat liver FABP (L-FABP) and rat intestinal FABP (I-FABP) was examined. Both L-FABP (Figure 5A) and I-FABP (Figure 5B) displayed the same rank

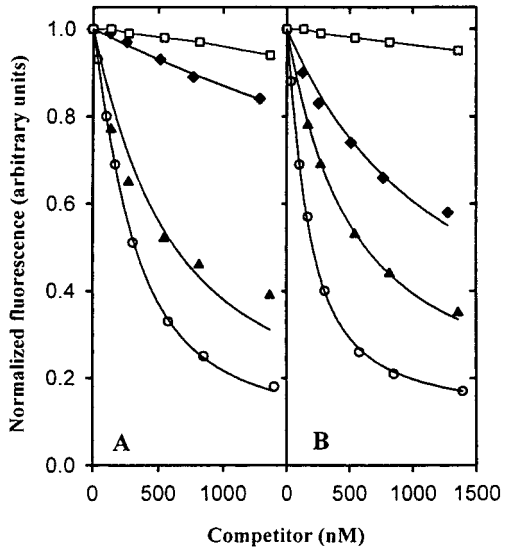

FIGURE 5: EET and DHET displacement of ANS from liver (L-FABP) and intestinal FABP (I-FABP). ANS displacement assays were performed and analyzed as described for Figure 3. Data shown are representative of $n \geq 3$ titrations for L-FABP and $n = 2$ for I-FABP. (A) Displacement curves for L-FABP: Arachidonic acid (○), 11,12-EET (▲), 11,12-DHET (◆), and equivalent volumes of ethanol (□). (B) Displacement curves for I-FABP: Arachidonic acid (○), 11,12-EET (▲), 11,12-DHET (◆), and equivalent volumes of ethanol (□).

Table 2: Comparison of L-FABP Binding Constants Obtained by the ANS Displacement and ADIFAB Methods[a]

fatty acid	ANS	ADIFAB
arachidonic acid	0.16 ± 0.01[b]	0.06[c]
11,12-EET	0.39 ± 0.03	0.26[d]
11,12-DHET	5.7 ± 0.3	nd[e]

[a] Apparent dissociation constants (K_d', micromolar) for L-FABP were determined by ANS displacement as described under Experimental Procedures. For comparison, binding constants were also determined by monitoring ADIFAB fluorescence in the presence of L-FABP and fatty acid. [b] ±SEM for $n = 3$. [c] Average of two determinations. [d] Single determination. [e] ADIFAB binding of 11,12-DHET was too weak (K_d = 150 μM) to determine a K_d for L-FABP.

order of affinity for these compounds as H-FABP (arachidonic acid > EET > DHET). Apparent dissociation constants for I-FABP were arachidonic acid, 0.11 μM; 11,12-EET, 0.47 μM; and 11,12-DHET, 1.34 μM ($n = 2$).

L-FABP binding affinities for arachidonic acid and 11,12-EET were also determined by a direct binding method utilizing ADIFAB. ADIFAB binding constants for arachidonic acid and 11,12-EET were first determined and subsequently L-FABP binding constants were obtained in the presence of ADIFAB. ADIFAB binding constants were as follows: arachidonic acid, $K_d = 0.85$ μM ($n = 2$); 11,12-EET, $K_d = 5.8$ μM ($n = 1$); and 11,12-DHET, K_d = 150 μM ($n = 1$). Table 2 compares the binding constants derived from the ANS displacement and ADIFAB methods. L-FABP affinity for 11,12-EET was ∼4-fold weaker than for arachidonic acid by the ADIFAB method and ∼2-fold weaker by ANS displacement. It should be noted that the stoichiometry of L-FABP is 2 fatty acids/protein; therefore the L-FABP binding constants reported here reflect a mixture of high- and low-affinity sites.

H-FABP Binding of HETEs and HPETEs. H-FABP affinities for the lipoxygenase pathway products, HETEs and

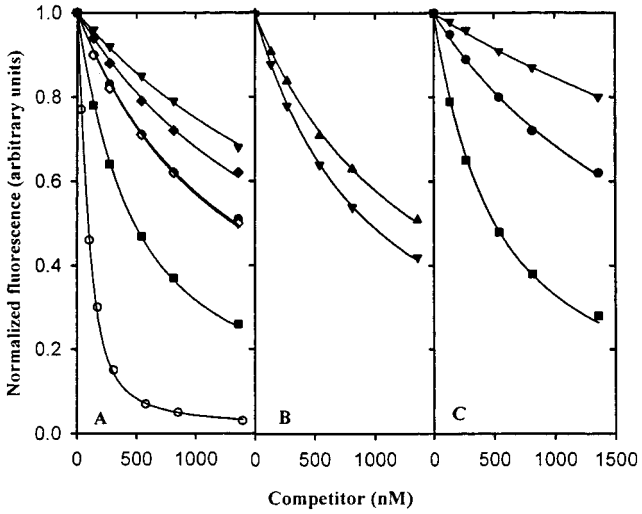

FIGURE 6: Competitive displacement of ANS from H-FABP by HETEs and HPETEs. Conditions were identical to those noted for Figure 3. Data shown are representative of $n = 3$ titrations (A) or $n = 1$ (B, C). Displacement by arachidonic acid (○) is shown for comparison in panel A. (A) Displacement curves for HETEs: 20-HETE (■), 5-HETE (◇), 8-HETE (●), 12-HETE (◆), and 15-HETE (▼). (B) Displacement curves for 8-HETE stereoisomers: 8(R)-HETE (▼) and 8(S)-HETE (▲). (C) Displacement curves for HPETEs: 5(S)-HPETE (■), 15(S)-HPETE (●), and 15(S)-HETE (▼).

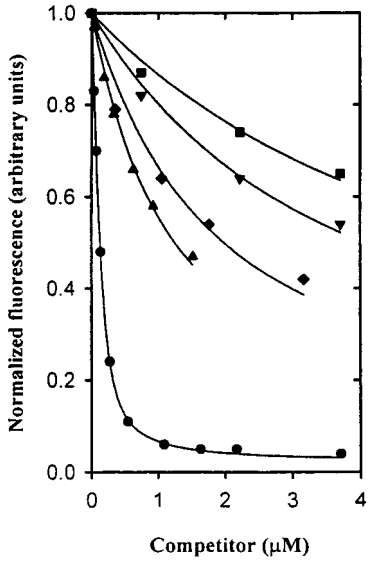

FIGURE 7: Competitive displacement of ANS from H-FABP by epoxide (EODE), hydroxy (HODE), and hydroperoxy derivatives (HPODE) of linoleic acid (C18:2). Conditions were identical to those noted for Figure 3. Each titration was performed once: Linoleic acid (●), 9,10-EODE (▲), 13(S)-HPODE (◆), 13-HODE (▼), 9-HODE (■).

hydroperoxyeicosatetraenoic acids (HPETEs), and the cytochrome 450 monooxygenase-generated 20-HETE were determined. ANS displacement curves for racemic mixtures (*R*/*S*) of 5-, 8-, 12-, and 15-HETE and for 20-HETE are shown in Figure 6A. The affinity of H-FABP for the HETE isomers (Table 1) varied from 8- to 56-fold less than arachidonic acid, and 20-HETE had the highest affinity. H-FABP affinity for the HETEs was roughly intermediate between the tightly bound EETs and the more weakly bound DHETs. Notably, the weakest binding EET isomer (14,15-EET) and HETE isomer (15-HETE) are oxygenated at carbon 15.

The changes in the free energies of binding relative to arachidonic acid are shown in Table 1. The addition of oxygenated substituents to arachidonic acid resulted in average increases of 1.5 kcal/mol for the epoxy-containing EETs, 2.1 kcal/mol for the monohydroxy HETEs (excluding 20-HETE), and 2.9 kcal/mol for the dihydroxy DHETs.

To investigate the possible stereoselectivity of H-FABP binding to HETEs, the binding of 8(*S*)-HETE and 8(*R*)-HETE by H-FABP was measured (Figure 6B). H-FABP was found to have similar affinities for these stereoisomers [8(*S*)-HETE, K_d' of 1.3 μM; 8(*R*)-HETE, K_d' of 0.9 μM]. The binding of several of the lipoxygenase-derived hydroperoxy fatty acids was also investigated (Figure 6C). The affinity of H-FABP for both 5(*S*)-HPETE (K_d' of 0.4 μM) and 15-(*S*)-HPETE (K_d' of 2.0 μM) was higher than for the corresponding reduced products, 5(*R*/*S*)-HETE (K_d' of 1.2 μM) and 15(*S*)-HETE (K_d' of 5.3 μM). Although hydroperoxy groups are fairly chemically reactive, the binding of HPETE to H-FABP was found to be reversible (data not shown).

H-FABP Binding of Oxygenated Products of Linoleic Acid. To assess whether the H-FABP binding profile for oxygenated fatty acids would be the same for 18-carbon compounds, H-FABP affinities for oxygenated derivatives of linoleic acid were determined (Figure 7). H-FABP affinity for linoleic acid (K_d' of 0.03 μM) was the same as that reported by Richieri et al. (*20*). The affinity of H-FABP for the epoxide, 9,10-EODE (K_d' of 1.1 μM), was higher than that of the hydroperoxide, 13(*S*)-HPODE (K_d' of 1.9 μM), and the hydroxy derivatives, 9-HODE and 13-HODE (K_d' values of 6.2 μM and 3.8 μM, respectively). Thus, the rank order of H-FABP affinities for linoleic acid-derived compounds, unmodified fatty acid > epoxide $\cong$ hydroperoxide > hydroxy, was the same as that observed for arachidonic acid-derived compounds. Overall, H-FABP affinity was lower for the linoleic acid-derived compounds as compared to the arachidonic acid-derived compounds.

DISCUSSION

A major finding of this study is that H-FABP has substantial affinity for EETs ($K_d' \sim 0.5$ μM). EETs mediate several responses in the vasculature, including vasodilatation (*7*) and the cytokine-induced inhibition of endothelial cell adhesion molecule expression (*11*). Mediation of these responses involves EET release into the extracellular fluid and uptake by target cells, incorporation into and mobilization from endothelial phospholipids, interactions with membrane lipids and/or membrane-associated proteins (e.g., ion channels, G-proteins), and interactions with cytosolic protein kinases (reviewed in ref *12*). FABPs may play several roles in EET metabolism by acting as intracellular carriers and possibly as chaperones (*4*, *27*, *28*). FABP action as a carrier could partition arachidonic acid and EET away from membranes, possibly increasing their availability to enzymes such as acyl-CoA synthetase, cytochrome P450 epoxygenase, and epoxide hydrolase. FABPs may additionally serve as chaperones to target arachidonic acid or EET to specific membrane domains or enzymes. Recent studies have identified FABP domains that might serve to target FABP to anionic phospholipids (*29*) and to specific enzymes (*30*). Alternatively, FABPs could sequester a portion of the

arachidonic acid or EET, thus prolonging their lifetimes for intracellular signaling. A previous study found that both I-FABP and L-FABP reduced the conversion of arachidonic acid to 15-HETE, presumably by sequestering the substrate (*31*). Likewise, the present results suggest that H-FABP affinity for EET could protect it from conversion to DHET by cellular epoxide hydrolases.

A second finding is that H-FABP affinity for EETs is greater than for DHETs. This result was consistently observed with either ANS or *cis*-parinaric acid displacement assays and with ADIFAB. The conversion of EET to DHET is a prominent pathway of EET metabolism by vascular cells (*32*). DHETs were originally thought to be devoid of activity; however, recent evidence has shown that DHETs also possess vasoactivity (*7*). The selective H-FABP affinity for EET may partially explain the differential retention of EET versus DHET by cultured vascular cells. A study of [^{3}H]11,12-EET uptake in smooth muscle cells demonstrated that most of the radiolabeled material retained by the cells remained as EET (*33*). However, the majority of the radiolabeled material in the medium was DHET, indicating that some of the cell-associated EET was converted by epoxide hydrolases to DHET and rapidly released from the cells. The present results demonstrate that H-FABP affinity for 11,12-EET was 38-fold higher than for 11,12-DHET. Thus, the selective cellular retention of EET as compared with DHET may be related to the higher affinity of the EET for the cytosolic FABP. However, several other factors are also likely to contribute to the selective retention of EETs versus DHETs by cells. One study showed that arachidonic acid and EETs have similar rates of esterification into the phospholipids of liver microsomes, whereas DHETs are poor substrates for the microsomal acylating enzymes (*34*). Thus, the poor retention of DHETs by cells probably results from the combined effects of the weak affinity of FABP for DHETs and their low acylation rate into phospholipids.

Systematic studies of FABP binding of long-chain fatty acids have shown only moderate binding selectivity among the various dietary fatty acids. The range in affinities for a particular FABP type was ~10-fold, such that saturated fatty acids were bound more tightly than unsaturated (*20*, *26*). Analysis of the binding energetics indicated that FABP selectivity is primarily dependent upon the aqueous solubility of the fatty acid, such that FABP affinity decreases with increasing solubility (*26*). In agreement with this analysis, we found that H-FABP binding affinity decreased as the polarity of the oxygen substituent increased: epoxide ≅ hydroperoxy > monohydroxy > dihydroxy. Polarities of the oxygenated fatty acids can be assessed by their retention times on reversed-phase HPLC with octadecylsilyl columns (*35*−*37*). We observed that oxygenated fatty acids with longer retention times (i.e., less polar) were more tightly bound by H-FABP. Additionally, the H-FABP rank order of affinities for the positional isomers of EETs and HETEs was generally correlated with their retention times on reversed-phase HPLC. Thus, our systematic binding study with oxygenated fatty acids supports polarity (i.e., solubility) as a major factor in H-FABP binding selectivity.

20-HETE is a notable exception to the relationship between oxygenated fatty acid polarity and H-FABP affinity. 20-HETE elutes from octadecylsilyl columns before the other HETEs, indicating that 20-HETE is the most polar of the HETEs (*36*). However, H-FABP had a higher affinity for 20-HETE than for the other HETEs. This suggests the existence of unique compensatory interactions between H-FABP binding pocket residues and 20-HETE. 20-HETE is structurally unique among the HETE isomers in two aspects. First, the C20 hydroxyl is 5 carbons away from the nearest double bond, while all other HETE isomers have the hydroxyl adjacent to one of the four double bonds. Second, like arachidonic acid, all 20-HETE double bonds are in the *cis* configuration, while all other HETE isomers have one *trans* and three *cis* double bonds. Most FABPs, including H-FABP (*38*), have a phenylalanine residue(s) that makes multiple contacts with the acyl chain, including interactions with the double bonds of bound polyunsaturated fatty acids (*39*). Interactions with double bonds have been proposed to compensate for losses in binding energy due to the increased solubility of the polyunsaturates (*26*). Thus, the double bond system of 20-HETE may allow compensatory interactions within the H-FABP binding cavity, leading to a higher affinity for 20-HETE as compared to the other HETEs.

Among the lipoxygenase pathway products, H-FABP bound 5(*S*)-HPETE with relatively high affinity (K_d' 0.4 μM). HPETEs are short-lived intermediates that are rapidly reduced by cellular peroxidases to HETEs (*40*). 5-Lipoxygenase production of 5-HPETE is regulated by a feedback loop such that low concentrations of 5-HPETE activate (*41*) and high concentrations inactivate 5-lipoxygenase (*42*). H-FABP could act as a buffer of 5-HPETE feedback regulation, possibly modulating 5-lipoxygenase activity in vascular cells (*43*). The reported binding of HPETEs by keratinocyte lipid-binding protein and adipocyte lipid-binding protein (*13*, *14*) supports a general role for FABPs in the cellular fate of nonesterified hydroperoxy fatty acids.

In conclusion, we have found that FABP has preferential affinity for EETs versus DHETs. These compounds are produced by cells contained in the vascular wall, where they regulate blood flow through autocrine and paracrine mechanisms. The presence of intracellular FABPs in vascular cells and their affinity for cytochrome P450 epoxygenase and lipoxygenase products suggests that this class of lipid-binding proteins may modulate the activities of these eicosanoid mediators.

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