METHOD TO ASSESS FATTY ACID ETHYL ESTER BINDING TO ALBUMIN

CATHERINE A. BEST1,2*, MICHAEL LAPOSATA2, VERONIA G. PROIOS1,2 and ZBIIGNIEW M. SZCZEPIORKOWSKI1,3

1Department of Biomedical Sciences, Northeastern University, Boston, MA 02115, USA, 2Division of Laboratory Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA, 3Department of Pathology, Dartmouth-Hitchcock Medical Center and Dartmouth Medical School, Lebanon, NH 03756, USA

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Abstract — Aims: To develop a method to assess the relative binding of fatty acid ethyl esters (FAEE) and free fatty acids (FFA) to albumin, and to determine if binding affinity is related to fatty acid chain length and or degree of saturation. Methods: Radiolabelled ethyl-[14C]oleate -bound to albumin was challenged with various ratios of FFA to FAEE. The displacement of ethyl-[14C] oleate by FFA was visualized and quantitated through a combination of native-PAGE, autoradiography, and liquid scintillation counting (LSC). Results: As the ratio of FFA to FAEE increased from 0:1 to 12:1, for all fatty acids tested (myristate, palmitate, stearate, oleate, linoleate, and arachidonate), ethyl-[14C] oleate displacement increased, when expressed as radioactivity (in DPM) as a percentage of control. In contrast, ethyl oleate did not displace stearate or oleate from albumin at molar ratios up to 5:1 (FAEE:FFA). Conclusions: The method developed gave reproducible visualization of noncovalent binding of radiolabelled FAEE to albumin. The combination of native-PAGE and autoradiography LSC works well in assessing the binding properties of albumin and radiolabelled FAEE. The data indicate preferential binding of FFA over FAEE to albumin with six different FFA displacing FAEE to an approximately equal extent.

INTRODUCTION

Fatty acid ethyl esters (FAEE) are neutral hydrophobic non-oxidative metabolites of alcohol (ethanol), and are formed by the esterification of ethanol and endogenous fatty acids. They have been implicated as mediators of ethanol-induced organ damage and have potential clinical utility as a marker for ethanol intake (Laposata and Lange, 1986; Szczepiorkowski et al., 1995; Werner et al., 1997; Laposata, 1998; Best and Laposata 2003a ). Serum FAEE levels correlate with ethanol levels and persist for at least 24 h following ethanol ingestion (long after ethanol is no longer detectable) (Doyle et al., 1996). Albumin is one of the main carriers of FAEE in vivo (Doyle et al., 1994; Bird et al., 1996). FAEE have been shown to transfer between albumin and lipoproteins in in vitro experiments (Bird et al., 1996). Ethyl palmitate (E16:0) and ethyl oleate (E18:1) are the predominant serum FAEE species (Doyle et al., 1994; Doyle et al., 1996). The percentage of E16:0 and E18:1 of total FAEE is higher than the corresponding percentage of palmitate (16:0) and oleate (18:1) of total fatty acids (free) in serum (Doyle et al., 1994). The observed greater concentrations of E16:0 and E18:0 compared with their respective unesterified fatty acids, and relative to other FAEE species, may be the result of differential enzyme specificities or different affinities for transport binding (albumin binding) (Newsome and Rattray, 1966).

Albumin is the most abundant plasma protein (32–53 mg/mL, 0.53–0.75 mM). Under physiological conditions albumin carries between 0.1 and 2.0 mol of fatty acid per mol protein (Curry et al., 1999). The entire mass of serum free fatty acids (FFA) turns over within approximately 2 min (Demant et al., 2002). Albumin also functions to maintain colloidal osmotic pressure and plays a role in the distribution and metabolism/detoxification of many biologically active compounds. In addition to fatty acids, albumin binds a wide array of endogenous and exogenous substances including hormones such as thyroxine, drugs, and drug metabolites such as warfarin, and the focus of this paper, FAEE, respectively (Newsome and Rattray, 1966; Spector, 1975; Bird et al., 1996; Curry et al., 1999; Demant et al., 2002).

Given that albumin binding is thought to be dominated by the lipophilicity of the bound compound, it is expected that similar molecules will have similar interactions with binding proteins (Kratchovil et al., 2002). Therefore, we hypothesized that non-esterified FFA and FAEE compete for binding sites on albumin. The aim of the current study was to develop a method to assess FAEE-protein binding, and to evaluate the relative binding of FFA and FAEE to albumin.

MATERIALS AND METHODS

Materials
Radiolabelled [1-14C] triolein (112.0 mCi/mmol) and [1-14C] oleate (56.0 mCi/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). Unlabelled ethyl oleate, ethyl stearate, myristate, palmitate, stearate, oleate, linoleate, and arachidonate were purchased from Nu-Chek Prep (Elysian, MN). Essentially fatty acid-free bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St Louis, MO). BSA was used in these binding studies because of its use in many such studies, and because BSA and human serum albumin (HSA) share similar physicochemical properties and are similar in structure (Spector, 1975; Curry et al., 1998). All reagents used for the PAGE were purchased from Bio-Rad Laboratories (Richmond, CA).

Synthesis and isolation of radiolabelled FAEE
Ethyl-[1-14C] oleate was synthesized from [1,14C] triolein (112.0 mCi/mmol) and ethanol. Radiolabelled triglycerides...
were dried under nitrogen and resuspended in 0.6 mL dichloromethane. Fatty acids were transesterified as described by Turk et al. (Turk et al., 1986) using absolute ethanol in place of methanol. Briefly, the radioactive triglyceride was incubated in 0.5 M KOH in ethanol for 45 min at room temperature. The reaction was terminated by addition of 6 M HCl, and the FAEE were extracted into dichloromethane. The dichloromethane extracts were concentrated under nitrogen and then resuspended in 300 μL of hexane. The FAEE were then isolated by Bond-Elut solid phase extraction (SPE) (as described below).

**Bond-Elut solid phase extraction (SPE)**

A modified SPE method described by Kaluzny et al. was used to isolate FAEE (Kaluzny et al., 1985). The aminopropyl-silica columns (Bond-Elut LCR, Varian Diagnostics, CA) were placed on a Vac-Elut vacuum apparatus (Analytchem International, Varian Diagnostics, CA) set at 10 kPa. The column was pre-conditioned with 4 mL of dichloromethane and then isolated by Bond-Elut solid phase extraction (SPE) (as described below).

**Thin-layer chromatography**

The synthesized FAEE were checked for purity by a method developed, with minor adjustments, by Kinnunen and Lange (Kinnunen and Lange, 1984). A 75:5:1 ratio of petroleum ether-diethyl ether-deionized water was used. The TLC plate (silica gel 60 thin layer plate, Merck Darmstadt, Germany) was heated for 1 h in the oven at 75°C and then allowed to cool to room temperature. After application of samples and standards, the plate was secured in the TLC tank, and the solvent was allowed to migrate for 30 min. The area corresponding to FAEE was identified by comparison to known standards of FAEE on the silica gel plate visualized by iodine vapour in the standard lane. The FAEE were then scraped and the radioactivity was quantitated by scintillation counting in a Beckman LS 5000 TD (Beckman-Coulter Inc., Fullerton, CA). The purity of synthesized FAEE was assessed by thin-layer chromatography (TLC) as described below (Kinnunen and Lange, 1984).

**Binding of FAEE to albumin**

The ethyl-[1-14C] olate in hexane (200 μL) was dried under nitrogen, then resuspended in an equal amount of ethanol (200 μL) and aliquoted for binding studies. Ethyl-[1-14C] olate (25 μL), plus BSA (135 μL for a 1:1 molar ratio with FAEE) and 10 mM Tris–HCl, pH 7.4, 150 mM NaCl buffer (65 μL) were placed into siliconized test tubes. The contents were then vortexed for 30 s and incubated at 37°C for 15 min. This step was repeated once. FFA (20 μL), with increasing FFA to FAEE molar ratios of 0:1 to 12:1 were then added to the mixture. The contents were vortexed for 30 s and incubated at 37°C for 15 min, after which the tubes were again vortexed for 30 s and incubated at 37°C for an additional 15 min. After incubation, the samples were analysed by native-PAGE as described below.

**Gel electrophoresis**

After incubation, the samples were subjected to electrophoresis on 8–16% Tris–HCl gradient pH 7.4 minigels (Bio-Rad Laboratories, Richmond, CA) with a 4% stacking area. According to the method by Laemmli, native-PAGE was performed under constant voltage (80 volts) for 2.5 h (Laemmli, 1970). The gels were then dried for 1 h at 70°C in a FBGD45 Gel Dryer (Fisher Scientific, Pittsburgh, PA).

**Autoradiography**

After native-PAGE was completed, the gels were dried for 1 h at 70°C. To visualize and quantitate the radioactivity, the dried gels were exposed to Kodak X-OMat AR film at 80°C for 14 days. The films were developed on a Kodak M35A X-OMAT Processor (Kodak, Wilmington, MA).

**Recovery of the radioactivity from dried gels**

The films were developed and used to outline the lanes and row segments on the dried gels. Each lane and row segment (~0.2 mm in height) of the gel was cut with careful attention to the labelled bands shown on the film and radioactivity measurement in each cut segment.

**Competitive binding of FFA and FAEE to albumin**

To assess the relative affinity of FAEE and FFA to albumin, a number of competitive binding studies were performed. We investigated a range of ratios of FAEE to albumin and the 1:1 molar ratio of radiolabelled ligand to BSA was used because it optimized the recovery of radioligand. At lower molar ratios less FAEE was recovered. At ratios greater than 1:1 of FAEE to albumin, the 66 kDa band was distorted.

**FFA added to radiolabelled FAEE saturated albumin**

Ethyl-[1-14C] olate bound to BSA (20 mg/mL; 1:1 molar ratio) was incubated with various fatty acids (myristate, palmitate, stearate, oleate, linoleate and arachidonate) in increasing molar ratios from 1:0 to 1:12 (FAEE to FFA). The mixtures (radiolabeled FAEE, BSA, buffer and FFA) were vortexed and incubated at 37°C for an additional 30 min. Following this additional incubation period, the samples were subjected to native-PAGE, the gels were dried and exposed to x-ray film (as described above). The remaining radioactivity from FAEE still complexed to the protein was then quantitated through autoradiography and scintillation counting. This technique provided a means to visualize albumin-ethyl-[1-14C] olate binding.

**FAEE added to radiolabelled FFA saturated albumin**

Radiolabelled [1-14C] olate bound to BSA (20 mg/mL; 1:1 molar ratio) was challenged with various ratios of ethyl oleate (E18:1) and ethyl stearate (E18:0) in 1:0.2, 1:1 and 1:5 ratios (FFA to FAEE). The FAEE were added to the radiolabelled FFA, BSA, and buffer mixtures, vortexed and incubated at 37°C for an additional 30 min. Following this incubation
period the samples were subjected to native-PAGE, the gels were dried and exposed to x-ray film. The relative amounts of complexed protein-radioligand were then quantitated through autoradiography and LSC. This technique provided a means to visualize albumin-[1-14C] oleate binding.

Coomassie blue staining

Staining was performed according to Sambrook et al. (Sambrook et al., 1989) with minor alterations. The gel was stained for ½ h with 0.1 % Coomassie blue R-250 in fixative [40% methanol, 10% glacial acetic acid (v/v)] solution. The gel was then destained with a 40% methanol, 10% glacial acetic acid solution for 1.5 h to remove any residual background. The gel was then dried for 1 h at 70°C in a FBGD45 Gel Dryer (Fisher Scientific, Pittsburgh, PA).

Statistical analysis

Results were expressed as mean ± standard error (SEM) and were calculated from at least two separate experiments for each test condition. Regression analysis was used to assess potential correlations.

RESULTS

We developed a method utilizing native-PAGE, autoradiography, and LSC to measure noncovalent binding of radiolabelled FAEE to albumin. Consistent with previous BSA native-PAGE studies, albumin is detected at 66 kDa as well as in an associated doublet bands, observed higher in the gels, which is polymerized BSA (Tanaka et al., 2001). Coomassie staining demonstrated that albumin mobility was invariant with FFA binding (stained gel not shown). The gels in Fig. 1 show that FFA readily displaced albumin bound radiolabelled FAEE. As the molar ratio between FFA and ethyl-[1-14C] oleate increased, from 0:1 to 12:1, a corresponding increase in ethyl-[1-14C] oleate displacement was observed, as evidenced by a progressive decrease in band intensity at 66 kDa, as well as a decreased intensity in the polymerized BSA bands, in lanes 1–7. Lane 1, the control lane (with a molar ratio of FFA to FAEE of 0:1), has the most intense banding patterns (highest FAEE DPM), and lanes 7 and 8 have little observable banding patterns (lowest FAEE DPM). The molar ratios of FFA to FAEE in lanes 7 and 8 are 7:1 and 12:1, respectively. Ethyl-[1-14C] oleate was displaced approximately equally, when expressed as a percentage of the total, by all FFA tested including myristate (14:0), palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), and arachidonate (20:4).

The first and last panel in Fig. 1, corresponding to the gels testing the displacement of E18:1 by 14:0 and 20:4, show the darkest and most intense banding patterns, at all molar ratios tested (including 1:0 FAEE:FFA). We believe that these variations in band intensity are due to technical differences involved in the autoradiography process, such as differences in the quality of the intensifying screen utilized, or in the age of the photographic paper used, rather than being an indication of enhanced E18:1:albumin binding by these fatty acids. Some residual radioactivity was retained in the stacking gel area. This is probably unbound (free) FAEE, as the FAEE (neutral and hydrophobic molecules) can form droplets in the hydrophilic milieu.

Figure 2 shows the quantitative data derived from the gels (representative gels are shown in Fig. 1). Data points represent the mean ± SEM calculated from at least two separate experiments. Panels A–F correspond to the various FFA used to challenge ethyl-[1-14C] oleate–BSA binding (A, myristate; B, palmitate; C, stearate; D, oleate; E, linoleate; and F, arachidonate, respectively, n = 2 for A, B, C, and F, n = 3 for E and F). The data show that the dissociation of ethyl-[1-14C] oleate from albumin following the addition of FFA is most significant when the challenging concentrations of FFA are 7- to 12-fold greater than the concentration of ethyl-[1-14C] oleate. The relative capability of FFA to displace ethyl-[1-14C] oleate did not reflect differences in their solubility, chain length, or degree of saturation. The displacement of ethyl-[1-14C] oleate from albumin by saturated fatty acids (myristate, palmitate and stearate), as evidenced by a decrease in the recovered radioactivity at 66 kDa and within the polymerized BSA, is shown in Fig. 3A. The r-values for the correlation between ethyl-[1-14C] oleate recovered bound to albumin and the ratio of saturated FFA added ranged from r = 0.837 to r = 0.949. The addition of 1 molar myristate, palmitate, and stearate, (saturated FA) resulted in the displacement of 16, 14, and 30%, respectively, of ethyl-[1-14C] oleate from albumin.

The displacement of ethyl-[1-14C] oleate from albumin by unsaturated fatty acids (oleate, linoleate and arachidonate) is...
shown in Fig. 3B. The $r$-values for the correlation between ethyl-[1-$^{14}$C] oleate recovered bound to albumin and the ratio of unsaturated FFA added ranged from $r = 0.889$ to $r = 0.943$. The addition of 1 molar oleate, linoleate, and arachidonate (unsaturated FA) resulted in the displacement of 25, 10, and 21%, respectively, of ethyl-[1-$^{14}$C] oleate from albumin.

In contrast, Fig. 4 shows that at a 5:1 molar ratio of FAEE to FFA, [1-$^{14}$C] oleate was not displaced from albumin by either ethyl oleate (E18:1) or ethyl stearate (E18:0). An increase in the ratio of FAEE to $^{14}$C-FFA, from 0.2:1 to 5:1 had no effect on the [1-$^{14}$C] oleate binding to albumin. In the corresponding gels (not shown), the band intensity of the $^{14}$C-FFA-albumin plus FAEE lanes were no different from control lanes (radioactive FFA-albumin only as [1-$^{14}$C] oleate–BSA), indicating no FAEE induced changes in [1-$^{14}$C] oleate albumin binding. At the same ratio of FFA to FAEE (5:1),
<70 and <40% of the ethyl-[1-14C] oleate was recovered with albumin when challenged by 18:0 and 18:1, respectively, indicating that at this molar ratio a significant amount of FAEE is displaced by FFA (see Fig. 3).

**DISCUSSION**

The combination of native-PAGE, autoradiography, and LSC works well in characterizing the binding properties of BSA and radiolabelled FAEE. Furthermore, this study is physiologically relevant because, like BSA, we also observed HSA-14C FAEE binding (results not shown). The data in this report show that FAEE are easily displaced from albumin by FFA, and conversely that FAEE are unable to displace FFA from albumin in vitro and at the concentrations tested (Figs 2 and 4, respectively). We observed that with the addition of increasing FFA to ethyl-[1-14C] oleate-bound albumin, decreasing amounts of radioactive FAEE were recovered with albumin. This indicates that FAEE are bound to albumin much less avidly than FFA, and that FFA easily displace FAEE from albumin.

These findings are consistent with previous reports that suggest that albumin has a stronger affinity for FFA compared to FAEE (Bird et al., 1996; Bird et al., 1997; Chang and Borensztajn, 1999). This is probably because while FAEE and FFA are structurally similar FAEE do not exist in an ionized form. Thus, FAEE do not have the same electrostatic interactions as the charged form of FFA. It is likely that mixed electrostatic and hydrophobic interactions more rigidly secure FFA to albumin, compared to FAEE. The FFA, unlike FAEE, are anchored to albumin binding sites via electrostatic interactions between the FFA carboxylate anion head group and tyrosine, arginine, and serine residues on albumin (Curry et al., 1999). These fatty acid-protein interactions appear to be common among a number of fatty acid binding proteins and FFA (Hamilton, 2004).

Given that albumin has multiple binding sites, FFA and FAEE may simultaneously bind albumin by occupying different hydrophobic pockets. It is likely that FAEE occupy the ‘lower affinity binding sites’, while the FFA preferentially occupy the ‘higher affinity binding sites’. This would explain the data shown in Fig. 2. It may take up to 5–12 moles of FFA to displace 1 mole of radiolabelled FAEE from albumin, given that multiple binding sites with varying affinities are most likely being filled. Each binding site has a distinct chemical environment and conformation for the FFA (Demant et al., 2002), or FAEE ligand molecule. This is consistent with other in vitro experimental observations that report finding 6–13 moles FFA per mole of BSA (Spector et al., 1969).

The binding of certain FFA may cause subtle conformational changes within the albumin molecule that expose additional hydrophobic binding cavities to FAEE (Spector et al., 1969). This may account for the increased binding of E18:1 to albumin, up to approximately 140% of control, following...
the addition of low concentrations of myristate and palmitate as seen in Fig. 2A and B (compared to 2C–F).

The lack of a linear correlation between FAEE displaced and FFA added, as illustrated in Fig. 2A–F, is consistent with the Karush model of FFA/ligand binding to albumin (Karush, 1954; Spector et al., 1969). The Karush model assumes that binding sites are modified and created as binding occurs. Thus the non-linearity reflects the high degree of complexity involved in albumin-ligand binding and may be accounted for by allosteric and independent interactions (site-ligand, ligand-ligand and site-site) that mediate differential binding to the heterogeneous sites by the various FFA and FAEE species.

Typically serum FAEE concentrations are low relative to serum albumin concentrations and FFA levels. The physiological concentration of FAEE detected in the serum following ethanol intake, in healthy volunteers who drank to legal intoxication, ranged from ~1.8 to 3.8 μM (Doyle et al., 1996; Best et al., 2003b). While the average total FFA in serum, in adults following a 12 h fast, ranges from 170–950 μM (Sambrook et al., 1989). In cases of liver and pancreas damage, the level of FAEE may increase disproportionately, relative to FFA and albumin concentrations, because FAEE synthase activity is elevated in patients with organ damage (as determined by elevated liver and pancreatic enzymes) (Laposata and Lange, 1986; Aleryani et al., 1996) and because certain disease states (those resulting in hepatocyte damage) may also have reduced albumin levels (Demant et al., 2002).

The observations made in this study have potential significance in that displacement of FAEE by FFA from albumin may promote FAEE delivery into tissues where it may induce toxic effects. Also, this study highlights the potential dependence of FAEE bioavailability to tissues on plasma FFA metabolic and nutritional status; such that in circumstances of high FFA levels and low albumin levels, the delivery of FAEE to cells will be favoured.

FAEE may also interact (cooperatively or competitively) with other toxins or drugs that are bound by albumin. FAEE–albumin binding may mediate ethanol-induced organ damage by affecting unbound circulating concentrations, disposition, bioavailability, and toxicity of FAEE as well as other drugs. Future studies to determine if FAEE–albumin binding changes the molecular interaction between other drugs/toxicants and albumin binding sites are warranted.

We also investigated retinal binding protein (RBP)–FAEE binding. Under our experimental conditions, RBP—radiolabelled FFA binding was observed, while RBP–radiolabelled FAEE binding was not (data not shown). Previous reports have only shown interphoto-receptor retinoid binding protein (IRBP) FFA binding. IRBP is thought to be an intracellular fatty acid carrier and the binding can be either covalent (35%) or noncovalent (65%) (Bazan et al., 1985; Lin et al., 1997). Additional investigations may more precisely characterize the binding of FFA to RBP and other intracellular binding proteins.

The potential clinical utility of this assay should also be investigated. It has been suggested that FAEE species distribution may be useful in distinguishing heavy drinking from social drinking patterns (Soderberg et al., 2003). If a specific FAEE distribution profile proves to be a reliable biomarker of heavy drinking then this albumin–FAEE binding assay may assist in the identification of individuals at increased risk for alcoholism. Ultimately the assay may aid in the identification of individuals requiring intervention, as well as in evaluating the effectiveness of therapeutic interventions.

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REFERENCES


