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In Situ Binding of Fatty Acids to the Liver Fatty Acid Binding Protein: Analysis Using 3-[^{125}I]Iodo-4-azido-N-hexadecylsalicylamide

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A photoactivatable radioiodinated fatty acid analogue, 3-[^{125}I]iodo-4-azido-N-hexadecylsalicylamide (^{125}I-AHS) has been synthesized and used to investigate the involvement of cellular lipid carriers in hepatic fatty acid utilization. Photoactivation of Hep G2 internalized ^{125}I-AHS revealed that several cellular proteins were crosslinked with the radiolabeled fatty acid analogue. Three predominant proteins in the membrane fraction of the cell with molecular masses 17, 50 and 127 kDa were crosslinked with the lipid analogue, as determined using autoradiography after SDS-PAGE. Three other proteins in the soluble fraction of the cell, with molecular masses 14, 24 and 35 kDa, were also labeled *in situ*. In contrast to the other labeled proteins, the fatty acid analogue accumulated on the cytoplasmic 14 kDa protein in a time and temperature dependent fashion. The *in situ*-labeled 14 kDa protein was identified from primary rat hepatocytes as the liver fatty acid binding protein by partial purification and its ability to be immunoprecipitated with immunospecific L-FABP antiserum. Collectively the results indicate that fatty acids traverse the plasma membrane and are bound cytoplasmically by the liver fatty acid binding protein, as well as other proteins in the cell. This represents the first demonstration in intact hepatocytes that the liver fatty acid binding protein participates in the process of intracellular fatty acid trafficking, and supports a model in which cytoplasmic lipid carriers solublize fatty acids as a step in their metabolic utilization.

The liver is a site of active lipid metabolism in the body. Hepatocytes take up and secrete lipid in response to the nutritional and hormonal state of the body. Postprandially, hepatocytes actively synthesize and secrete lipid in the form of very low density lipoprotein particles (1, 2). Lipid associated with these particles is derived primarily from *de novo* hepatic lipogenesis and lipids recycled from catabolized chylomicron remnants (3). Postabsorptively, circulating albumin-bound fatty acids released from adipose tissue triacylglycerol stores are rapidly internalized by hepatocytes (4, 5), and represent a major source of energy for hepatocyte metabolism (3, 6). During fasting, fatty acids provide carbon for the elevated hepatic ketone body production seen in

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Abbreviations: FAS, fatty acid synthase; CoA, coenzyme A; FABP, fatty acid binding protein; ALBP, adipocyte lipid binding protein; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylene diaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; AHS, 4-azidohexadecyl salicylamide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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glucose-depleted conditions (6, 7). Although the enzymology of discrete steps in lipogenesis and lipolysis, and the roles of lipoprotein particles and serum albumin in interorgan lipid transfer have been well characterized, the mechanism by which hydrophobic substrates are trafficked within the hepatocyte is poorly understood.

The intracellular movement of fatty acids has been thought to involve cytosolic lipid binding proteins. In 1969 an oganic anion-binding protein was first described as a component of the low molecular weight protein fraction from a soluble liver cellular homogenate (8). The protein was initially characterized *in-vitro* by its ability to noncovalently bind radiolabeled bilirubin and was subsequently found to have a relatively greater affinity for long chain fatty acids and acyl CoAs (9). Further investigations have determined that the binding protein, termed liver fatty acid binding protein (L-FABP), is encoded by a member of a family of intracellular hydrophobic ligand binding protein genes. The family is defined by its conserved sequence identity, intron-exon boundries, tertiary structure of the gene products and their ability to bind hydrophobic ligands. These proteins can be functionally classified into two subpopulations based on their *in vitro* binding properties: those that bind retinoids and those that principally bind fatty acids (10). Proteins in the latter group have been principally characterized in liver, intestine, heart, adipose tissue, mammary tissue, and peripheral nerve tissue.

Despite the extensive *in-vitro* characterization of FABPs, a metabolic role for the these proteins *in-vivo* has not been well defined. Evidence from experiments using manipulations of dietary lipid content to identify a role for L-FABP in hepatic lipid flux has been ambiguous (11,12). Other experminents employing hypolipidemic drugs showed altered L-FABP protein content, binding activity, and mRNA levels consistent with a role for the protein in lipid metabolism (13-15). We have recently used a photoactivatible radioiodinated fatty acid analogue to investigate the involvement of lipid carrier proteins in the intracellular flux of fatty acids in adipose tissue (16). Since both adipocytes and hepatocytes are able to efficiently utilize fatty acids and both tissues express proteins belonging to the FABP family, we undertook the present studies using the analogue to examine the role of L-FABP in hepatic lipid metabolism. In the present paper we demonstrate *in situ*, using isolated primary rat hepatocytes and Hep G2 cells, that the liver fatty acid binding protein participates in the intracellular solublization of fatty acids.

MATERIALS AND METHODS

Synthesis of 4-azido-N-hexadecylsalicylamide (AHS)

AHS was prepared by dissolving hexadecylamine (Fluka Chemical Company) with N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA, Pierce Chemical Company) in ethyl acetate and heating 3-5 min. at 95 °C. The AHS product was isolated using thin layer chromatography and purified by extracting the region of the thin-layer plate containing product with ethyl acetate. Mass spectrometry and fast atom bombardment were used to characterize the purified AHS.

Iodination of AHS

To iodinate AHS, a 20 µl aliquot (approximately 100 nmols) of the thin layer chromatography-purified AHS in ethyl acetate was added to 485 µl of 200 mM borate buffer (pH 8.0) in an Iodogen (Pierce Chemical Company)-coated glass reaction vial. To this was added 5 mCi (2.3 nmols in 10 µl) carrier free sodium iodide (Amersham Corporation). After 15 min at room temperature with occasional agitation, the radioiodinated product was extracted into ethyl acetate. Typically, the iodination procedure yielded 40% radioiodine incorporation into the organic-extractible material.

Cell Culture and Fatty Acid Uptake

Cultures of Hep G2 cells, maintained in Dubelcco's Modified Eagle's Medium (DMEM) containing 15 mM HEPES (pH 7.4), 0.011% sodium pyruvate, and 10% calf serum, were used to characterize hepatocyte fatty acid uptake and to evaluate the participation of intracellular protein carrier(s) in fatty acid trafficking. Hep G2 cells are morphologically similar to parenchymal cells and have been shown to secrete 17 major plasma proteins (17). Additionally, the cells express the human hepatocyte fatty acid binding protein that cross reacts with an antibody developed against the rat liver fatty acid binding protein[1]. Confluent monolayers of Hep G2 cells were preincubated 2 h at 37° in serum-free DMEM prior to fatty acid uptake experiments. The uptake medium was prepared by adding [9,10-^{3}H]oleic acid (5 Ci·mmol^{-1}) in a small volume of ethanol to albumin (ultra pure bovine serum albumin - Boehringer Mannheim) in DMEM to a final 1:1 molar ratio at 100 μM with the carrier albumin. After incubating cells with this medium for 0-5 min, fatty acid uptake was terminated by washing the cell monolayers three times with ice cold 100 mM Tris (pH 7.5), 100 mM sodium chloride, 1 mM EDTA, 0.1 mM phenyl-methyl-sulfonyl fluoride (Buffer A) containing 0.1% albumin and then lysing the cells with 0.5% SDS. The radioactivity contained within the cell lysate was quantitated using a Beckman 3801 Liquid Scintillation Counter, and total cellular protein was determined colorimetrically (18).

Analysis of ^{125}I-AHS Uptake and Protein Labeling

In situ uptake of ^{125}I-AHS and subsequent labeling of cellular components was performed as described for [^{3}H]oleic acid uptake with the following modifications. Uptake was initiated by adding an aliquot of albumin-bound ^{125}I-AHS (1:1 molar ratio 100 μM; 1 x 10^3 Ci/mol) to serum free DMEM and incubating with the cells at 37°C. At various times thereafter the incubation medium was removed and the cell monolayers UV irradiated for 2-3 min at a distance of 2 cm. to crosslink ^{125}I-AHS to cellular proteins as previously described (16). All manipulations prior to irradiation were performed in a darkroom under a photographic red light. After UV irradiation, cell monolayers were washed and homogenized with Buffer A using a Teflon homogenizer. The extract was centrifuged for 30 min at 400,000xg to obtain soluble and membrane components. Protein samples were separated using SDS-PAGE (19) and the rate of ^{125}I-AHS accumulation on cellular proteins between 30 and 300 s was determined by densitometric analysis of autoradiograms from the dried gel.

Immunoprecipitation

To maximize protein labeling for immunological identification, a suspension of primary rat hepatocytes was utilized. A suspension of 3.0 x 10^7 hepatocytes (85% viable at the time of experimentation as determined by trypan blue dye exclusion and cell counting) obtained from collagenase treatment of rat liver was washed twice with serum-free William's E culture medium, and then resuspended into a labeling medium containing 100 μM ultrapure albumin and 0.5 mCi ^{125}I-AHS. After incubating the cells with the fatty acid analogue at 37° for 45 min, the cells were UV irradiated for 10 min to crosslink ^{125}I-AHS to cellular proteins, washed twice in ice-cold Buffer A and then homogenized in a dounce homogenizer. A soluble supernatant fraction was obtained by centrifuging the cell homogenate at 400,000 xg at 4°C for 20 min. The extract was applied to a Sephadex G-50 column and the protein-bound lipid identified by scintillation counting and SDS-PAGE. Fractions containing L-FABP were pooled, concentrated and used for immunological analysis. To immunoprecipitate protein an aliquot of the partially purified material (83 μg) was warmed for 5 min at 37° in the presence of 0.05% SDS, diluted with Buffer A containing 0.1% NP40, and then incubated with anti-LFABP antiserum or nonimmune control serum overnight at 4°. The immune complex was adsorbed with Protein A Sepharose(Pharmacia), washed with Buffer A, separated by SDS-PAGE and analyzed by autoradiography.

RESULTS

Purification of FABP frequently results in the co-purification of a bound lipid, suggesting that these proteins exist with a bound ligand *in vivo* (15,20,21). However, it is not clear if the fatty acids associated with the purified protein were a true reflection of endogenously bound fatty acids or are simply a consequence of the isolation procedure. Because of the difficulty in demonstrating noncovalent fatty acid binding to L-FABP *in situ*, a fatty acid analogue was designed that could be radiolabeled to a high specific activity and could be covalently crosslinked to

[1] J.A. Manning, N.M. Bass, unpublished.

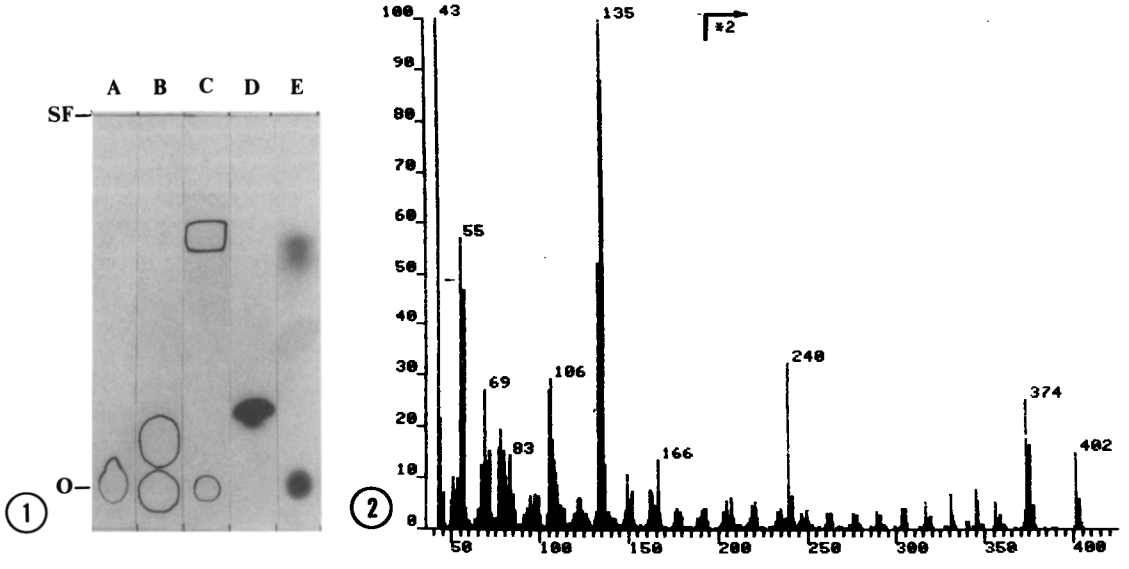

Figure 1. Thin layer chromatographic analysis and purification of 4-azido-N-hexadecyl-salicylamide (AHS). Soluble AHS was purified from the reaction mixture using preparative thin layer chromatography. The entire volume of the reaction mixture was applied to a silica plate [Silica Gel G TLC plate (Fisher Scientific)] and the components were separated using xylene:ethyl acetate (90:10). SF represents the solvent front. Lanes A-C represent material that was visualized by staining with iodine vapor. Lanes D and E are autoradiograms of radioiodinated material. LaneA, hexadecylamine; lane B, NHS-ASA; Lane C, purified AHS; Lane D, ^{125}I-labeled NHS-ASA; Lane E, purified ^{125}I-AHS.

Figure 2. Analysis of 4-azido-N-hexadecylsalicylamide (AHS) using mass spectrometry. AHS was prepared and iodinated as described in materials and methods. The spectrum of AHS shown contains a parent peak corresponding to a molecular mass of 402 (AHS), and four other peaks present in large relative abundance. Other observed masses were consistent with their being the photo decomposition products: amino-hexadecylamine (376), nitreno-hexadecylsalicylamide (374); and ion fragments: hexadecylimine (240), and the substituted phenolic ring (135). Characteristic also of compounds containing an unbranched acyl chain is the ladder of progressively smaller peaks differing in mass by 14.

protein(s) involved in fatty acid utilization. The synthesis resulted from the condensation of N-hydroxysuccinimidyl-4-azidosalicylic acid and hexadecylamine in ethyl acetate. AHS was purified from the reaction mixture using preparative thin layer chromatography. The reaction mixture was applied to silica Gel G plates and developed using a xylene:ethyl acetate (90:10) mixture. Under these conditions AHS demonstrated a relative mobility of 0.70 while N-hydroxysuccinimidyl-4-azidosalicylic acid exhibited a mobility of 0.23, and hexadecylamine remained at the origin (Fig. 1). The area of the silica containing AHS was scraped from the glass plate and the product eluted with ethyl acetate. This compound was rechromatographed identially to ensure its purity and then stored in ethyl acetate at -20°C until further use. AHS was stable for for at least three months.

Using a direct inlet probe, analysis of the purified AHS by mass spectrometry detected a parent peak with a mass of 402 (Fig.2). Other predominant peaks were observed with masses consistent with photo decomposition products or major ion fragments (see legend Fig.2.). Fast atom bombardment mass spectrometry corroborated this finding showing a 403 MH^+ ion (results not shown). The purified, homogeneous AHS was then iodinated for metabolic experiments. Radiochemical purity of the final compound was evidenced by the single spot in the autoradiographic analysis of the thin layer separation (lane E, Fig.1).

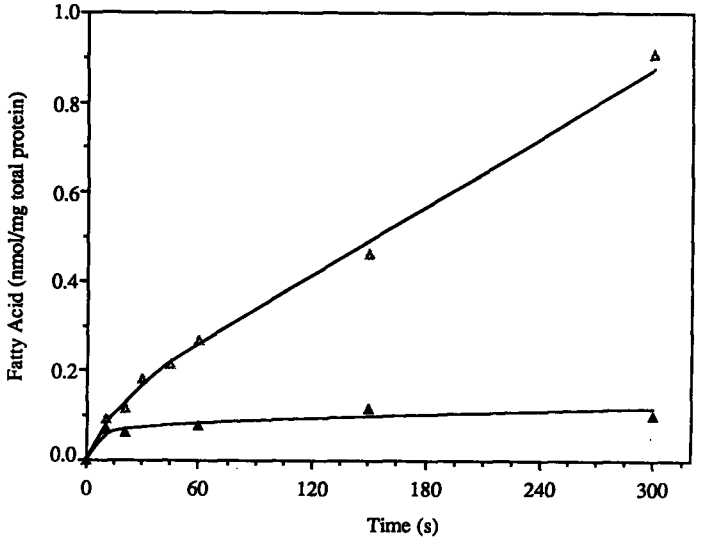

Figure 3. Kinetic Characterization of Hepatocellular Accumulation of [^{3}H] Oleic Acid. Cell-associated radioactivity was determined in Hep G2 cells after incubation of the cell monolayers with [^{3}H]oleic acid:albumin (100 μM, 1:1 molar ratio) at 37° (open symbols) and 4°C (filled symbols). The data are representative of results from four experiments.

In vivo, fatty acids are "delivered" to the liver bound to serum albumin after their release from adipose tissue in the periphery (22). To evaluate fatty acid uptake in Hep G2 cells, tritiated oleic acid and albumin (1:1 molar mixtures at 100 μM) were incubated with cell monolayers for different periods of time and the rate and extent of uptake was assessed. The kinetics of fatty acid uptake were consistently observed to be biphasic (Fig. 3). The biphasic nature of fatty acid uptake suggested that at least two kinetically distinct phenomena occur during this process. The initial rapid accumulation of fatty acid between 0-20 sec was temperature insensitive (Fig. 3) whereas the second phase (20-300 sec) was decreased when uptake was measured at 4°. Under these conditions, fatty acids accumulated in hepatocytes at a rate of 0.38 nmoles·min^{-1}·mg total cell protein^{-1}, as measured between 20-300 s. Similar kinetics have been reported in other cultured cell systems (16).

To investigate the involvement of intracellular carrier proteins in the process of fatty acid uptake, ^{125}I-AHS was employed. When ^{125}I-AHS was incubated with Hep G2 cell monolayers, uptake of the radiolabeled analogue by the cells mirrored that observed with [^{3}H]oleic acid (results not shown). To establish whether the radiolabeled lipid was protein-bound, monolayers were incubated with ^{125}I-AHS, photolyzed with UV light, and cellular protein extracts separated using SDS-PAGE. Proteins crosslinked with ^{125}I-AHS were visualized by autoradiography. As can be seen in Fig. 4, the fatty acid analogue crosslinked a number of proteins in the cell. Three prominent and two less distinct bands were found in the sample containing membrane-associated proteins (17, 50, 127 and 30-35, 70-73 kDa respectively). Three prominent proteins migrating with apparent molecular masses of 14, 24and 35 kDa were found in the soluble fraction of the cell. In addition, a soluble 67 kDa protein was identified as residual carrier albumin that was not completely removed from the cells prior to lysis. It is possible that an undetected soluble hepatic protein of the same apparent molecular mass was labeled.

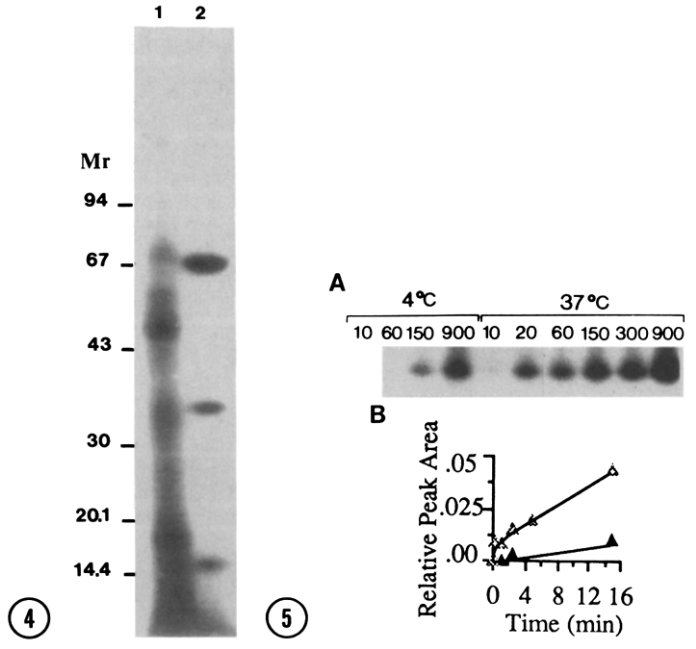

Figure 4. Intracellular distribution of ^{125}I-AHS-labeled proteins. Monolayers of Hep G2 cells were incubated with albumin-carried ^{125}I-AHS in serum-free DMEM at 37° C for 15 min, UV irradiated, washed, homogenized in 200 µl Buffer A and centrifuged at 400,000xg for 30 min. The soluble and insoluble material was recovered and the latter resuspended in a volume of buffer equal that of the soluble material (lane 1, insoluble; lane 2, soluble). The darkened region migrating with the dye front represents uncrosslinked ^{125}I-AHS and ^{125}I-AHS-crosslinked phospholipid.

Figure 5. Time and temperature dependent accumulation of ^{125}I-AHS on a cytoplasmic 14 kDa protein. Hep G2 cell monolayers were incubated with albumin-carried ^{125}I-AHS in serum-free DMEM at 4° or 37° C for up to 300 s. At the indicated times (sec) the incubation medium was aspirated and the cell monolayers UV irradiated, washed, homogenized in 200 µl Buffer A, and centrifuged at 400,000xg for 30 min. A, Equivalent masses of the soluble components were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. Numbers represent time of labeling in seconds. B, Densitometric analysis of ^{125}I-AHS accumulation on the soluble 14 kDa protein after *in-situ* labeling HepG2 cells at 37° C (open symbols) and 4° C (closed symbols).

To determine which (if any) proteins may participate in the intracellular transport of lipid we examined the time and temperature dependence of protein labeling. Kinetic examination demonstrated that only the 14 kDa protein was labeled in a time and temperature dependent fashion. When compared to labeling at 37°C, labeling of the 14 kDa protein in Hep G2 cells was reduced 4-fold at 4°C (Fig. 5). Accumulation of label on the 14 kDa protein was kinetically similar to accumulation of label in the cell and radioactivity associated with this protein constituted 35% of the soluble ^{125}I-AHS-labeled proteins. The identities of the other labeled proteins in the soluble (24, 35 kDa) and membrane fractions of the cell have not been determined. However, by virtue of their apparent selective metabolic labeling, they may associate with fatty acids avidly, or play a role in the transmembrane movement and intracellular solubilization of fatty acids.

The soluble 14 kDa protein labeled with ^{125}I-AHS was partially purified from the supernatant fraction of *in situ*-labeled rat hepatocytes using gel filtration on Sephadex G-50 (Fig. 6). Two peaks of protein-bound radioactivity were directed; a major peak containing 67 kDa

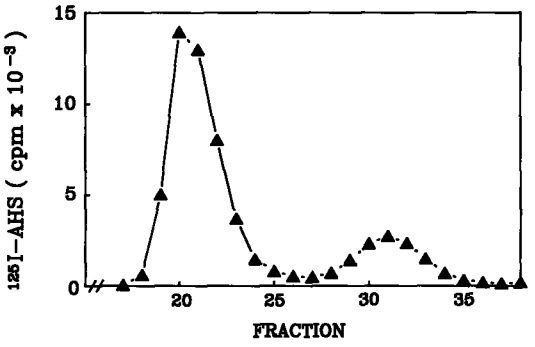

Figure 6. Sephadex G-50 fractionation of ^{125}I-AHS-labeled soluble proteins. The soluble proteins prepared following ^{125}I-AHS metabolic labeling of rat hepatocytes were subjected to fractionation on Sephadex G-50. The amount of radioactivity in each fraction was determined by scintillation counting. The ^{125}I-AHS labeled 14 kDa protein eluted in fractions 28-34.

(albumin) and 33 kDa proteins with a smaller peak containing the ^{125}I-AHS labeled 14 kDa protein. Of the radioactivity contained in the low molecular weight protein fraction, approximately 30% was covalently bound, as determined by trichloroacetic acid precipitation (results not shown). This suggests that even after nonproductive photolysis of ^{125}I-AHS the lipid analogue remains tightly protein bound. To determine if the ^{125}I-labeled 14 kDa protein was L-FABP the partially purified material isolated by gel filtration was immunoprecipitated with antiserum directed against the rat liver fatty acid binding protein (Fig. 7). Immunoprecipitation of the ^{125}I-labeled 14 kDa protein with monospecific antiserum identifed the protein as L-FABP. While the efficiency of immunoprecipitation was quite low, the specificity of antibody reactivity established the radialabeled protein as L-FABP. Specific labeling of L-FABP by ^{125}I-AHS supports the hypothesis that this protein functions *in-situ* as an intracellular fatty acid binding protein, facilitating the intracellular solubilization of fatty acids.

DISCUSSION

The involvement of fatty acid binding proteins in intracellular fatty acid trafficking has been proposed since their first identification in enterocytes and hepatocytes (9,23). Liver FABP

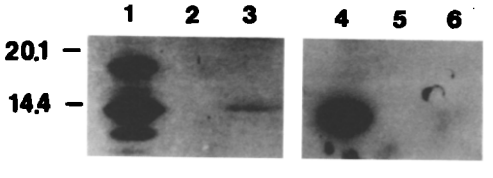

Figure 7. Imunoprecipitation of *in situ*-labeled protein with anti-L-FABP antiserum. An aliquot of Sephadex G-50 fractionated material obtained from *in situ*-labeled isolated rat hepatocytes was warmed for 5 min at 37° C in the presence of 0.05% SDS, diluted with Buffer A plus 0.1% NP40, and then incubated with anti-L-FABP serum or control nonimmune serum overnight at 4° C. The antibody-antigen complex was recovered by adsorption with Protein A-Sepharose, separated by SDS polyacrylamide gel electrophoresis and analyzed by autoradiography. Lanes 1 and 4 represent total low molecular weight proteins. Lanes 2 and 5 are immunoprecipitated proteins using nonimmune serum while lanes 3 and 6 represent immunoprecipitation using anti L-FABP serum. Lanes 1-3 show the protein stained with silver while lanes 4-6 are the corresponding autoradiogram. Numbers to the left indicate molecular mass in kDa.

was initially described as a component of the low molecular weight proteins that coeluted from gel filtration columns with radiolabelled fatty acids after *in-vitro* labeling (9). Therefore, work in the area of fatty acid uptake focused on *in vitro* characterization of this low molecular weight protein fraction.

Since the initial characterization of L-FABP, considerable research in a variety of tissues active in lipid metabolism has defined a gene family coding for homologus proteins, a number of which have been characterized as intracellular fatty acid binding proteins (10,24). However, no definitive evidence from *in situ* or *in vivo* work has defined a specific metabolic role for these proteins. In an attempt to characterize the mechanisms of intracellular fatty acid trafficking we utilized a photoactivatible radioiodinated fatty acid analogue and characterized its cellular uptake in 3T3-L1 adipocytes (16). In doing so we were able to demonstrate the involvement of the adipocyte lipid binding protein in fatty acid trafficking by virtue of *in situ* labeling with this analogue. In the present paper we extend our investigations in hepatocytes to examine the involvement of the liver fatty acid binding protein in intracellular lipid trafficking.

It has been suggested that plasma membranes protein(s) are involved in the process of fatty acid uptake. One 40 kDa protein in the plasma membrane of hepatocytes and adipocytes has been characterized as structurally similar to glutamate-oxalacetate transaminase and suggested to function as a fatty acid transporter (25,26). It has also been reported that an 80 kDa protein in the adipocyte plasma membrane functions similarly (27,28). It is noteworthy that no proteins in the 40 or 80 kDa molecular weight range were labeled by ^{125}I-AHS in the experiments described in the present paper. However the lack of *in situ* labeling of a 40 or 80 kDa species does not exclude the occurance of protein facilitated fatty acid transmembrane flux, but simply that we did not detect a protein of that molecular mass in our experiments. Further analysis is required to determine whether or not the 17, 50, or 127 kDa ^{125}I-AHS-labeled proteins in the membrane fraction of the hepatocytes represent proteins that are involved in fatty acid transmembrane movement.

Hep G2 cell uptake of ^{125}I-AHS was kinetically similar to a chemically unmodified radiolabeled fatty acid. Moreover, by virtue of its ability to metabolically label soluble intracellular proteins, ^{125}I-AHS traversed the plasma membrane. Further, the fatty acid analogue was bound by L-FABP, as determined by immunological methods. These results strongly argue that the L-FABP functions *in-situ* to facilitate the intracellular solubilization and metabolism of fatty acids. Our experiments also point towards a second potential lipid carrier protein of 35 kDa in liver cytosol. The identity of this second protein is unknown, however, the experiments reveal the intriguing possibility of other lipid carriers unrelated to the FABP family. These experiments provide the first substantive evidence for the association of fatty acid derived from cellular uptake with L-FABP *in-situ* (10,15,24,29).

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