A direct role for serum albumin in the cellular uptake of long-chain fatty acids

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The interaction of long-chain fatty acids with cells is important for their uptake and metabolism, as well as their involvement in signalling processes. The majority of long-chain fatty acids circulating in plasma exist as complexes with serum albumin. Thus an understanding of the involvement of serum albumin in these processes is vitally important. The effect of serum albumin on the uptake of long-chain fatty acids was studied in 3T3-L1 adipocytes. Serum albumin had a stimulatory effect on oleate uptake at all ratios of oleate:serum albumin tested. Furthermore, the rate of oleate uptake was saturable with increasing concentrations of serum albumin when the oleate:serum albumin ratio, and therefore the concentration of uncomplexed oleate, remained constant. This was not due to uptake being limited by dissociation of oleate from serum albumin, because oleate did not appear to be limiting. Furthermore, at very high ratios of oleate:serum albumin, when the concentration of uncomplexed oleate was predicted to be large relative to the amount of oleate taken up by cells, the rate of oleate uptake was still dependent on the albumin concentration. Serum albumin, covalently labelled with the photoreactive fatty acid 11-*m*-diazirinophenoxy[11-^{3}H]undecanoate, bound to cells in a manner exhibiting both saturable (K_d 66.7 μM) and non-saturable processes. These results indicate that the stimulatory effect of serum albumin on the rate of oleate uptake is due to a direct interaction of serum albumin with the cells and point to an involvement of albumin binding sites in the cell surface in the cellular uptake of long-chain fatty acids.

INTRODUCTION

Long-chain fatty acids are important metabolic substrates for both energy production and lipid synthesis and, as is increasingly apparent, are involved in signalling processes in a variety of mammalian cell types [1–6]. The first steps in all of these processes are the interaction of long-chain fatty acids with the cell surface and their cellular uptake. Non-esterified long-chain fatty acids circulate in the plasma of mammals complexed to serum albumin. Therefore serum albumin plays a potentially important role in affecting the level of cellular uptake of long-chain fatty acids. The involvement of serum albumin in long-chain fatty acid uptake has generally been assumed to be in the solubilization of fatty acids in the aqueous environment to provide a pool of bound fatty acid to replenish uncomplexed fatty acid depleted by cellular uptake [7–17]. In this view, it is assumed that there is no direct interaction between the cells and fatty acid–serum albumin complexes which is relevant for long-chain fatty acid uptake. However, under normal physiological conditions, as well as conditions employed for most cellular long-chain fatty acid uptake assays, the concentration of long-chain fatty acid–serum albumin complexes greatly exceeds the concentrations of uncomplexed long-chain fatty acid [16,17]. Furthermore, serum albumin has been known to associate tightly with cell surfaces [18], suggesting that the amount of albumin-bound fatty acid at the cell surface may be large relative to the amount available as uncomplexed fatty acid.

To test whether cells may directly exploit this large pool of complexed long-chain fatty acids, we have studied the role of serum albumin in long-chain fatty acid uptake by 3T3-L1 adipocytes. These are a murine cell line which differentiates from preadipocytes to adipocytes, acquiring increased levels of oleate uptake [19,20] exhibiting characteristics typical of that in many other mammalian cell types tested [7,19–22]. We report that serum albumin stimulated cellular oleate uptake in a saturable manner at all ratios of oleate:serum albumin tested. This was not due to effects of the serum albumin concentration on the dissociation of oleate from serum albumin. Finally, the binding of fatty acid-labelled serum albumin to intact cells exhibited both a saturable component, with K_d 66.7 μM, and a non-saturable component. These results indicate that serum albumin has a direct role in fatty acid uptake through an interaction with the cell surface, rather than merely a passive role as a carrier of long-chain fatty acids to the general vicinity of the cells. This is important for understanding the mechanisms of long-chain fatty acid uptake and metabolism, as well as signalling events mediated by long-chain fatty acids. Furthermore, it has implications for the understanding of mechanisms by which other ligands carried by serum albumin and related proteins are taken up by cells.

EXPERIMENTAL

Materials

Unless otherwise indicated, all reagents for SDS/PAGE and cell culture were purchased from GIBCO–BRL, except for BSA (prepared from fraction V, essentially fatty acid-free), dexamethasone, insulin and 3-isobutyl-1-methylxanthine, which were obtained from Sigma Chemical Co. [9,10-^{3}H]Oleic acid was purchased from NEN Research Products. 11-*m*-Diazirinophenoxy[11-^{3}H]undecanoic acid (11-DAP-[11-^{3}H]undecanoic acid) was synthesized as described previously [23].

Abbreviation used: DAP, *m*-diazirinophenoxy.
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Measurement of uncomplexed oleate in the presence of BSA

The concentrations of uncomplexed [9,10-^{3}H]oleate in the presence of BSA were measured by the two-phase partitioning method, using heptane as the organic phase [16,24], as described previously [19,25].

Cell culture

3T3-L1 preadipocytes were grown in a humidified atmosphere containing 5.0% CO_2 in Dulbecco's medium containing 10% calf serum, 2.0 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) [19]. Confluent preadipocytes were stimulated to differentiate to adipocytes in the above medium containing fetal-bovine serum instead of calf serum, and supplemented with insulin (0.010 mg/ml), dexamethasone (250 nM) and 3-isobutyl-1-methylxanthine (0.50 mM). After 48 h, the medium was replaced with one lacking dexamethasone and 3-isobutyl-1-methylxanthine, and cells were maintained for a further 3 days before harvesting, as described previously [19].

Long-chain fatty acid uptake

Monolayers from 10 cm plates were washed twice with 5.0 ml of PBS (0.14 M NaCl, 2.7 mM KCl, 15 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4). Cells were released from plates with 5.0 ml of 1.0 mM EDTA in PBS and were considered viable if greater than 90% excluded Trypan Blue [19]. Filtration assays for the uptake of [9,10-^{3}H]oleate (potassium salt) were performed as described previously [19]. Assays were carried out in 1.0 ml of PBS at 1.0 mg of cellular protein/ml. Protein concentrations were determined by the method of Lowry et al. [26]. Rates of oleate uptake plotted versus the concentration of uncomplexed oleate were fitted by the following equation:

$$v = V_{max.}c(K_{0.5}+c)^{-1} + Nc$$

where v is the rate of oleate uptake, $V_{max.}$ is the maximal saturable rate, c is the uncomplexed oleate concentration, $K_{0.5}$ is the oleate concentration at which saturable uptake is half of maximal and N is the coefficient for non-saturable uptake.

Photoaffinity labelling of BSA

BSA and 11-DAP-[11-^{3}H]undecanoate (2.0 mM and 1.0 mM respectively in PBS) were incubated at 37 °C for 60 min. The mixture was photolysed as described previously, resulting in covalent attachment of the photoreactive fatty acid to the BSA [19,25].

BSA binding to cells

The binding of BSA labelled with 11-DAP-[11-^{3}H]undecanoate to intact 3T3-L1 adipocytes was measured as described by Reed and Burrington [27] under conditions which paralleled oleate-uptake assays. Briefly, 3T3-L1 adipocytes (2.0 mg of cellular protein/ml in 50 μl of PBS) were incubated at 37 °C for 5.0 min. An equal volume of 11-DAP-[11-^{3}H]undecanoate-labelled BSA was added, and the incubation was allowed to continue with gentle agitation for 60 s. Cells were pelleted by centrifugation for 10 s in a Beckman Microfuge E, and supernatants were recovered. The pellet surfaces were gently washed with 100 μl of PBS and then centrifuged as described above. Washed pellets were resuspended in 100 μl of 125 mM Tris/HCl, pH 6.8, containing 5.0% SDS, 10% 2-mercaptoethanol, 12% glycerol and 0.0070% Bromophenol Blue. Portions of each pellet and supernatant fraction were analysed by SDS/PAGE, and the amount of radiolabelled BSA was determined from gel slices as described previously [25]. Data plotted as the amount of BSA bound to cells versus the BSA concentration were fitted by the equation given above, where v is the amount of BSA binding, $V_{max.}$ is the maximum saturable binding, c is the BSA concentration, $K_{0.5}$ is replaced by K_d, the dissociation constant for saturable binding, and N is the coefficient for non-saturable binding.

RESULTS

It has generally been accepted that serum albumin does not participate directly in long-chain fatty acid uptake by mammalian cells, but rather serves as a reservoir of complexed fatty acid to replenish the uncomplexed fatty acid taken up by cells [17]. In this view, it is assumed that there is no direct interaction between the cells and fatty acid–serum albumin complexes which is relevant for long-chain fatty acid uptake. Accordingly, uptake rates are usually plotted versus the concentration of uncomplexed oleate in equilibrium with oleate complexed to BSA (determined in separate experiments in the absence of cells) [7,8,10,19–22]. This is shown in Figure 1 for oleate uptake by 3T3-L1 adipocytes. Oleate uptake exhibited both a saturable component (with $K_{0.5}$ 301 nM and $V_{max.}$ 7.00 nmol/min per mg of cellular protein) and a non-saturable component (N of 1.57 min^{-1}; see the Experimental section), as has been generally reported for a variety of mammalian cell types studied [2,8,10,19–22].

However, under normal physiological conditions as well as conditions employed for most cellular long-chain fatty acid uptake assays, the concentration of BSA, and therefore of long-chain fatty acid–BSA complexes, greatly exceeds the concentration of uncomplexed long-chain fatty acid [16,17]. Therefore the data were plotted in Figure 2 as the rate of uptake versus the molar ratio of oleate:BSA. Oleate uptake was linearly proportional to the ratio of oleate:BSA present. Furthermore, when the concentration of BSA was increased from 173 to 500 μM, the slope of the curve increased (from 2.75 to 8.29 nmol/min per mg), indicating that BSA had a stimulatory effect on oleate uptake for each ratio of oleate:BSA, and therefore for each concentration of uncomplexed oleate tested. This effect was

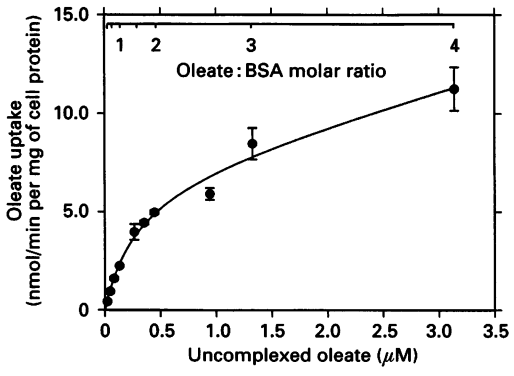

Figure 1 Oleate uptake by 3T3-L1 adipocytes as a function of increasing uncomplexed oleate concentration

3T3-L1 adipocytes were incubated at 37 °C for 20 s with increasing ratios of [9,10-^{3}H]oleate:BSA at a constant concentration of BSA of 173 μM, and oleate uptake was measured as described in the Experimental section. Uptake rates were plotted versus the concentration of uncomplexed oleate measured in equilibrium with BSA at the various ratios (indicated at top of the Figure). The data represent averages ± S.E.M. of three determinations and were fitted by a Michaelis–Menten-type equation containing a term for non-saturable uptake (see the Experimental section).

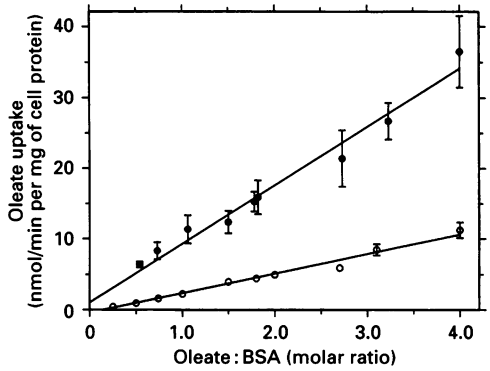

Figure 2 Effect of BSA on the rate of oleate uptake by 3T3-L1 adipocytes at increasing ratios of oleate:BSA

Oleate uptake by 3T3-L1 adipocytes was measured at increasing ratios of oleate:BSA at fixed concentrations of BSA of 173 μM (○) and 500 μM (●) as described in the legend to Figure 1 and in the Experimental section. The data represent averages ± S.E.M. of three determinations.

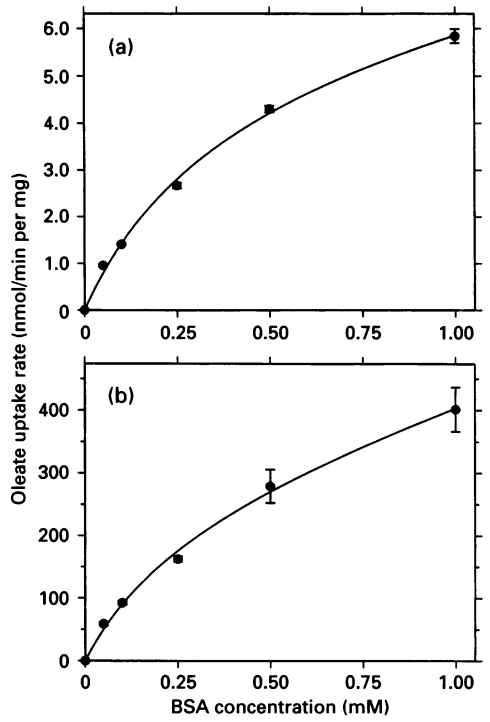

Figure 3 Saturation of oleate uptake by 3T3-L1 adipocytes with increasing concentrations of BSA

The rates of oleate uptake over 1.0 min time-courses were determined as described in the Experimental section. The concentrations of both BSA and oleate were varied so that the oleate:BSA ratio remained constant at (**a**) 0.5:1 or (**b**) 9:1, resulting in a constant concentration of uncomplexed oleate of 47 nM or 188 μM respectively. The results are expressed as the average rates of oleate uptake ± S.E.M. of three determinations.

saturable with the concentration of BSA (Figure 3). Values for $K_{0.5}$ and $V_{max.}$ of 379 μM BSA and 6.21 nmol/min per mg of cellular protein respectively were obtained when oleate uptake was measured at a constant ratio of oleate:BSA of 0.5:1 (Figure 3a). When oleate uptake was measured at a ratio of oleate:BSA of 9:1 (Figure 3b) $K_{0.5}$ and $V_{max.}$ values were 290 μM BSA and 278 nmol/min per mg of cell protein respectively. Results similar

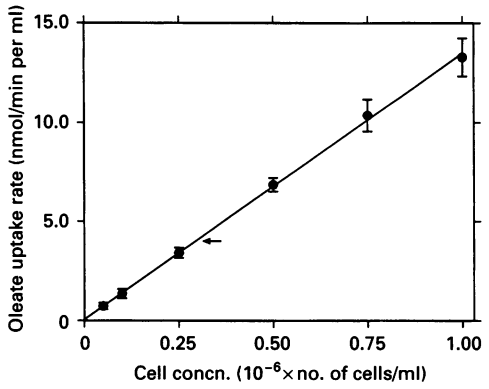

Figure 4 Rate of oleate uptake by 3T3-L1 adipocytes as a function of the cell concentration

The rates of oleate uptake were measured as a function of the concentration of 3T3-L1 adipocytes at a ratio of oleate:BSA of 4:1 in the presence of 46.5 μM BSA as described in the Experimental section. Results are expressed as the average rate of oleate uptake ± S.E.M. of three determinations; 10^6 cells contained 2.35 mg of protein. The arrow represents the point at which the amount of oleate taken up after 1.0 min was equivalent to the total amount of uncomplexed oleate initially present (4.0 μM).

to those of Figure 3(a) have been observed by others with rat hepatocytes, adipocytes and myocytes [10,14,28]. It has been proposed that this saturability was actually the result of long-chain fatty acid uptake at low BSA concentrations being limited by the dissociation of fatty acid from BSA, and that this became non-limiting as the BSA concentration increased [13,14]. In contrast, others have argued that long-chain fatty acid uptake was not limited by dissociation of fatty acids from BSA [15,29–31]. In fact, the dissociation of long-chain fatty acids from serum albumin has been reported to occur on the order of seconds or faster [29–31]. Furthermore, the explanation that the saturability observed in Figure 3(a) is the result of dissociation-limited uptake is not consistent with the results of Figure 3(b). Under the conditions of Figure 3(b) (oleate:BSA 9:1), the concentration of uncomplexed oleate was high (188 μM) and the rate of dissociation from BSA was fast (as oleate was loosely bound to BSA). Therefore, uncomplexed oleate was not likely to be depleted by uptake at any concentration of BSA. This argues that the saturable effect of BSA observed under those conditions was not the result of dissociation-limited oleate uptake at low BSA concentrations.

Further support for this is shown in Figures 4 and 5. It was reasoned that, if oleate uptake was limited by the dissociation of oleate from BSA at low BSA concentrations, then the rate of oleate uptake by 3T3-L1 adipocytes should have decreased as oleate became depleted by uptake and the limiting rate of dissociation from BSA became important. To vary the level of depletion of oleate, uptake was measured at increasing cell concentrations (Figure 4). Oleate uptake was measured at a low BSA concentration (46.5 μM), in the range predicted to result in a limiting rate of dissociation of oleate from BSA (from Figure 3). As the cell concentration was increased, the amount of oleate taken up in 1 min approached and surpassed the amount of uncomplexed oleate initially present (Figure 4). Furthermore, if the dissociation of oleate from BSA was rate-limiting, the time-course of oleate uptake at low BSA concentrations should have appeared biphasic, due to the increasing depletion of oleate with time, whereas the time-course of oleate uptake at high BSA concentrations should have been linear. The time-courses of

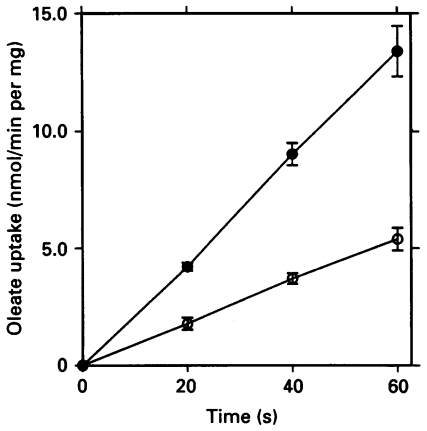

Figure 5 Time-courses of oleate uptake at different concentrations of BSA

Oleate uptake was measured as described in the Experimental section in the presence of either 50 (○) or 500 (●) μM BSA at an oleate:BSA ratio of 2:1, which results in an initial concentration of uncomplexed oleate of 450 nM. Results are expressed as the amount of oleate taken up per mg of cellular protein versus the time (in seconds) of incubation with oleate:BSA, and are means ± S.E.M. of three determinations.

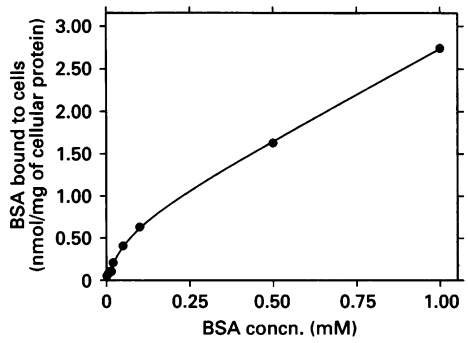

Figure 6 Binding of 11-DAP-[11-^{3}H]undecanoate-labelled BSA by 3T3-L1 adipocytes

The binding of BSA labelled with 11-DAP-[11-^{3}H]undecanoate to 3T3-L1 adipocytes was determined as described in the Experimental section. Typical results are shown and are expressed as the amount of labelled BSA bound to cells versus the BSA concentration. The data were fitted by a Michaelis–Menten-type equation containing a term for non-saturable binding (see the Experimental section).

oleate uptake by 3T3-L1 adipocytes (Figure 5) were linear at both low and high BSA concentrations (50 μM and 500 μM BSA, at a constant oleate:BSA ratio of 2.0:1), even though the level of oleate uptake surpassed the calculated amount of oleate initially available as uncomplexed oleate (0.45 μM at the ratio of oleate:BSA used).

These results demonstrate that the saturable effect of BSA was not due to dissociation-limited uptake, but rather to the concentration of oleate–BSA complexes. To test whether the stimulatory effect of BSA on oleate uptake may be due to a direct interaction between BSA and the cell surface, the cellular binding of BSA labelled with a photoreactive long-chain fatty acid, 11-DAP-[11-^{3}H]undecanoate, was studied. The characteristics of binding of the probe by BSA have previously been shown to resemble the binding of other long-chain fatty acids, such as palmitate and oleate [25]. Figure 6 shows the binding of the covalent fatty acid–BSA complex to 3T3-L1 adipocytes. Binding involved a saturable interaction with a K_d of 66.7 μM BSA and an apparent $V_{max.}$ of 0.675 nmol/bound mg of cellular protein. There was also a clearly non-saturable component to the binding ($N = 2.1$; see the Experimental section). This indicated the existence of a variety of binding sites for BSA in the cell surface, which may be involved in BSA binding during uptake.

DISCUSSION

These results indicate that 3T3-L1 adipocytes can directly utilize BSA-complexed fatty acids as substrates for uptake, and that this involves an interaction between BSA and the cell surface. This is consistent with either a direct transfer of bound oleate from BSA to the lipid bilayer [32] or a cell-surface-mediated stimulation of the dissociation of bound fatty acid from BSA [27,32–34]. Another alternative is that this may represent the binding of fatty acid:BSA complexes to the cell surface at specific sites of transport. In light of this it is interesting that caveolin, a 22 kDa marker of caveolae [35], which are believed to be specialized regions of transport at the plasma membrane [36,37], is exclusively labelled by a photoreactive long-chain fatty acid in the presence, but not in the absence, of BSA (B. L. Trigatti and G. E. Gerber, unpublished work).

The nature of the interaction of BSA with the cell surface is not clear. BSA has been found to bind to a number of surfaces, including those of a variety of cell types (Figure 6; [27,32–34,38,39]), glass, various polymers and air–water interfaces [13]. This suggests that binding is non-specific. In contrast, BSA binding to hepatocytes [27], cardiomyocytes [38] and microvascular endothelium [39] is saturable, with somewhat lower K_d values (0.366, 1.1 and 15.5 μM respectively) than that measured for 3T3-L1 adipocytes (Figure 6). Furthermore, a number of putative BSA receptors have recently been identified in endothelial cell surfaces, where they are believed to be involved in the transcytosis of albumin–fatty acid complexes, a process also thought to involve caveolae [40–45].

These results suggest that models for the mechanism of long-chain fatty acid uptake by mammalian cells should include a component relating to the interaction of fatty acid–serum albumin complexes with the cell surface and the transfer of long-chain fatty acids to the transport apparatus. The nature of the cellular transport apparatus is unclear. It may involve specific proteins thought to bind and transport fatty acids [7,8,11,19–22,25,46]. Alternatively, these proteins may have a regulatory role, and movement of long-chain fatty acids across the plasma membrane may occur by diffusion through the lipid bilayer [15,30,31,47].

The sequestering of albumin at the cell surface should also have major implications for the release of fatty acids from adipocytes during lipolysis. The close association of serum albumin with the cell surface should facilitate the removal of fatty acid from the membrane and enhance the rate of clearance of fatty acids by minimizing the interaction of the uncomplexed fatty acid with the aqueous environment. This should minimize damage to cell membranes due to high aqueous concentrations of uncomplexed fatty acids.

In summary, these results indicate that serum albumin has a saturable stimulatory effect on long-chain fatty acid uptake by 3T3-L1 adipocytes. We propose that this is the result of an interaction between serum albumin and some component of the surface of 3T3-L1 adipocytes. An understanding of the interactions of serum albumin with the cell surface is clearly important for understanding the mechanisms of long-chain fatty acid uptake and release from 3T3-L1 adipocytes. The similarities observed for long-chain fatty acid uptake studied in a variety of mam-

malian cell types suggest that the saturable stimulatory effect of serum albumin on long-chain fatty acid uptake may be a general phenomenon; indeed, similar results have been observed for the effects of BSA on oleate uptake by rat heart myocytes (B. L. Trigatti, N. Fernandes and G. E. Gerber, unpublished work). The wide variety of ligands carried by serum albumin and related carrier proteins such as α-fetoprotein suggests that this effect of serum albumin may be of general importance for the uptake of compounds circulating as complexes with carrier proteins [48,49].

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