Effect of Insulin on Fatty Acid Uptake and Esterification in L-Cell Fibroblasts

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We examined the effects of insulin on fatty acid uptake in L-cell fibroblasts, using cis-parinaric acid to measure uptake rates in the absence of esterification and [3H]oleic acid to measure uptake rates in the presence of esterification. L-cells exhibited both high and low affinity insulin binding sites with Kd of 23 nM and 220 nm and a cellular density of 1.4 and 6.8 × 10⁵ sites/cell, respectively. Insulin in the range 10⁻⁹ to 10⁻⁷ M significantly decreased both the initial rate and maximal extent of cis-parinaric acid uptake by 24 to 30%. Insulin also reduced [3H]oleic acid uptake up to 35%, depending on insulin concentration and decreased the amount of fatty acid esterified into the phospholipids and neutral lipids by 28 and 70%, respectively. In contrast, glucagon or epinephrine stimulated both the initial rate and extent of cis-parinaric acid uptake 18 and 25%, respectively. Because L-cells lack β-adrenergic receptors, the epinephrine effect was not the result of β-receptor stimulation. Hence, insulin altered not only fatty acid uptake, as determined by cis-parinaric and oleic acid uptake, but also altered the intracellular oleic acid esterification. © 1996 Academic Press, Inc.

Key Words: L-cells; cis-parinaric acid; oleic acid; fatty acid uptake; insulin; epinephrine; fatty acid esterification; fluorescence.

Although progress has been made in understanding fatty acid uptake and metabolism in adipocytes (1–3, 27), the mechanism involved in fatty acid uptake and the regulation of this process by insulin is poorly understood. In adipocytes, insulin-induced elevations in cAMP levels inhibit catecholamine-stimulated fatty acid transport (4). However, the mechanisms whereby catecholamines stimulate lipolysis (2–4, 27) and stimulate (14) or inhibit (7) glycerolipid synthesis are still unresolved. In part, the difficulty lies in the fact that fatty acid uptake measurements are complicated by extracellular binding of fatty acids to albumin as well as intracellular fatty acid metabolism.

Attempts to resolve the transport component of fatty acid uptake from the esterification and oxidation components have focused on using fatty acid analogues or probes. Although these approaches have been useful, unnatural fatty acid analogues may not necessarily function as native fatty acids (28, 39, 41). Recently, cis-parinaric acid, a naturally occurring fluorescent fatty acid, has been used to differentiate between these components (17, 18, 29, 33). cis-parinaric acid is poorly esterified (33) and has an initial uptake rate very similar to [3H]oleic acid (29). As demonstrated in previous reports on the biphasic nature of radiolabeled fatty acid uptake (2–4, 27, 42, 43), cis-parinaric acid has a similar biphasic profile (29, 33). The initial rapid linear segment of the biphasic curve reflects predominantly fatty acid uptake, whereas the slower curvilinear component reflects contributions from both fatty acid efflux and intracellular metabolism. Fatty acid uptake into cells has been proposed to occur through the lipid phase (9, 21) and/or through a protein-mediated process (2–4, 16, 26, 27, 32, 38, 36, 42, 43).

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3 Abbreviations used: cis-parinaric acid, 9Z, 11E, 13E, 15Z-octadecanoic acid; BSA, bovine serum albumin; KRHB, Krebs–Ringer–Hepes buffer; PBS, phosphate-buffered saline; PGE-1, prostaglandin E-1.
MATERIALS AND METHODS

Materials. cis-parinaric acid (MW 276 g/mol) was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA, radioimmunoassay grade), epinephrine, oleic acid, and butyloxydihydroxytoluene were obtained from Sigma Chemical Co. (St. Louis, MO). Crystalline porcine insulin (26.8 U/mg) and glucoyclid were kindly provided by Dr. Ronald E. Chance, Eli Lilly (Indianapolis, IN). [3H]Oleic acid was purchased from Du Pont NEN (Boston, MA). Neutral lipid standards were purchased from NuChek Prep (Elysian, MN) or Stearaloids (Wilton, NH). All other chemicals used were of reagent grade or better.

Cells. Mouse L-cells were grown on 75-cm² plastic tissue culture dishes (Corning Corp., Corning, NY) using Higuchi medium (37%) containing 10% fetal bovine serum (GIBCO, Grand Island, NY). For fluorescent fatty acid uptake assays, the cells were harvested with a rubber policeman, washed by centrifugation with phosphate-buffered saline (PBS, 8 mM Na-Phosphate, 130 mM NaCl, 2.7 mM KCl, pH 7.3), and resuspended in the same buffer at a concentration of near 5 x 10⁶ cells/ml and diluted for counting.

cis-Parinaric acid uptake in suspended L-cells. cis-parinaric acid, a naturally occurring fluorescent fatty acid, was used to examine the effects of insulin, glucagon, and epinephrine on fatty acid uptake in L-cell fibroblasts. This recently developed method (17, 18, 29, 33) permits the constant monitoring of cellular fatty acid uptake. Briefly, cis-parinaric acid was added from an ethanolic stock solution (0.1 mg/ml) to 2 ml of constantly stirred cells (150,000 cells/ml) for a final cis-parinaric acid concentration of 1.8 µM. For all assays, sample absorbance at 310 nm was maintained below 0.15 and the ethanol concentration was maintained below 0.01%.

cis-Parinaric acid poorly fluoresces in an aqueous environment, but there is a concomitant increase in fluorescence when cis-parinaric acid enters the cell and associates with a hydrophobic environment. Previous studies have shown that this increase in fluorescence represents cellular fatty acid uptake (17, 29, 33). In these experiments, changes in fluorescence intensity were continuously monitored using an SLM 4800 fluorimeter (SLM Aminco, Urbana, IL) or a PC2 photon counting fluorimeter (ISS Inc., Urbana, IL) using a dual detector T-format. Using a xenon-arc lamp, the sample was excited at 324 nm with emission measured through a GG-375 sharp cutoff filter (an SLM Aminco, Urbana, IL). Using a sharp cutoff filter eliminated highly polarized light. Fluorescence data were collected at a rate of 50 points/min.

For insulin studies, samples were preincubated with 0, 10, 100, or 1000 nM insulin for 5 min prior to the addition of cis-parinaric acid. Epinephrine (5 or 500 µM) or glucagon (10 nM) were added in a similar fashion. None of the compounds caused a significant (>0.1%) quench of cis-parinaric acid fluorescence.

Effects of insulin on [3H]oleic acid uptake. Confluent L-cell monolayers were suspended in 100 mm polystyrene cell culture plates and used to determine the effects of insulin on [3H]oleic acid uptake and esterification. Prior to addition of [3H]oleic acid, the medium was removed and the cells were rinsed twice with PBS buffer, and nonse-
**TABLE I**

<table>
<thead>
<tr>
<th>Endocrine Effects on cis-Parinaric Acid Uptake in L-Cells</th>
<th>% Change in cis-parinaric acid uptake parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (M)</td>
<td>Initial rate</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Insulin 1.0 × 10⁻⁷</td>
<td>-23.4 ± 3.6**</td>
</tr>
<tr>
<td>Epinephrine 5.0 × 10⁻⁴</td>
<td>16.7 ± 4.5*</td>
</tr>
<tr>
<td>Glucagon 1.0 × 10⁻⁸</td>
<td>25.4 ± 3.5**</td>
</tr>
</tbody>
</table>

Note. Control values were 100 ± 3%. The * and ** refer to P < 0.05 and P < 0.01, respectively, compared to control levels as determined by Student’s t test.

**FIG. 1.** Varying concentrations of cis-parinaric acid was added to L-cell fibroblasts (1.5 × 10⁵ cells/ml) and the cells were incubated for 3 min at 37°C. Changes in fluorescence intensity were continually measured to determine cis-parinaric acid uptake. In A, the concentration dependence of the initial rate of fluorescence change is shown. In B, the concentration dependence of the maximum fluorescence change is shown.

Uptake was linear for the first 10–15 s, reaching a plateau 40–60 s after the addition of cis-parinaric acid (data not shown). cis-Parinaric acid uptake was concentration dependent, with the equilibrium fluorescence intensity constant after saturation. The initial rate for cis-parinaric acid uptake was linear up to a concentration of 7.2 μM (Fig. 1A). The changes in maximal fluorescence values at varying cis-parinaric acid concentrations were proportional to the amount of cis-parinaric acid added to the assay up to a concentration of 3.6 μM (Fig. 1B). At higher cis-parinaric acid concentrations, saturation was observed in both maximal uptake (Fig. 1B) and in the initial uptake rate (Fig. 1A).

Insulin effects on cis-parinaric acid uptake. The effect of insulin, glucagon, and epinephrine on fatty acid uptake in the absence of appreciable esterification was determined using cis-parinaric acid. Insulin (100 nM) dramatically reduced both the initial rate and extent of cis-parinaric acid uptake into L-cells (Table I). In contrast, epinephrine (5 μM) or glucagon (10 nM) increased both the initial rate and extent of cis-parinaric acid uptake (Table I). With insulin treatment, both maximal fluorescence and the initial uptake rate decreased in a dose-dependent manner in response to increasing insulin concentrations (Fig. 2). However, at higher insulin concentrations (1000 nM) these curves begin to increase, suggesting that insulin had multiple effects on fatty acid uptake that were concentration dependent.

Insulin effects on oleic acid uptake and esterification. The effects of insulin on fatty acid uptake and esterification were measured using [³H]oleic acid. Similar to the results obtained using cis-parinaric acid, insulin caused a dose-dependent decrease in both the initial rate and extent of [³H]oleic acid uptake in L-cells (Table II). At higher insulin concentrations (1000 nM) the insulin-stimulated decrease in fatty acid uptake was attenuated, suggesting that insulin had multiple effects on fatty acid uptake that were concentration dependent. Insulin (100 nM) decreased the initial rate and extent of [³H]oleic acid uptake by 35% compared to control cells (Table II). A similar reduction was seen using cis-parinaric acid (Table I). Insulin treatment also caused a 28% decrease in [³H]oleic acid esterification into phospholipids and a 59% decrease in [³H]oleic acid esterification into neutral lipids, compared to control cells. Insulin treatment also altered the distribution of [³H]oleic acid between the different lipid fractions. There was a decrease in the proportion of [³H]-

**FIG. 2.** L-cells were preincubated with the indicated concentration of insulin for 5 min at 37°C. The cells were then diluted with PBS to a concentration of 1.5 × 10⁵ cells/ml and cis-parinaric acid (0.5 μg/ml) added. The initial rate (●) and maximum fluorescence change (▲) of cis-parinaric acid was determined. Values represent the mean ± SE, n = 5–6.
TABLE II
Effects of Insulin on [3H]Oleic Acid Uptake in L-Cell Fibroblasts

<table>
<thead>
<tr>
<th>Insulin (nm)</th>
<th>Uptake rate (pmol/min × mg protein)</th>
<th>Maximal uptake (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.4 ± 0.2</td>
<td>102.2 ± 5.2</td>
</tr>
<tr>
<td>10</td>
<td>2.6 ± 0.1*</td>
<td>77.0 ± 2.9*</td>
</tr>
<tr>
<td>100</td>
<td>2.2 ± 0.1*</td>
<td>66.7 ± 4.0*</td>
</tr>
<tr>
<td>1000</td>
<td>2.7 ± 0.03***</td>
<td>80.6 ± 0.8***</td>
</tr>
</tbody>
</table>

Note. Values represent means ± SE, n = 3, except for 1000 nm where n = 2. The * indicates statistical significance from 0 nm, P < 0.01. The ** indicates statistical significance from the 100 nm group, P < 0.05. Cells were preincubated with insulin for 1 min at the indicated concentration and then incubated with [3H]oleic acid (2 μCi) for 30 min.

TABLE III
Effects of Insulin on [3H]Oleic Acid Esterification in L-Cell Fibroblasts

<table>
<thead>
<tr>
<th>Insulin (nm)</th>
<th>Phospholipid</th>
<th>Neutral lipid</th>
<th>Free fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>76.7 ± 3.4</td>
<td>12.7 ± 2.7</td>
<td>12.8 ± 1.7</td>
</tr>
<tr>
<td>10</td>
<td>63.4 ± 4.0*</td>
<td>3.8 ± 0.6*</td>
<td>9.9 ± 2.9*</td>
</tr>
<tr>
<td>100</td>
<td>55.0 ± 3.6*</td>
<td>5.2 ± 0.5*</td>
<td>6.6 ± 1.0*</td>
</tr>
<tr>
<td>1000</td>
<td>65.5 ± 0.9</td>
<td>6.7 ± 0.1</td>
<td>7.9 ± 1.9</td>
</tr>
</tbody>
</table>

Note. Values represent means ± SE and are expressed as pmol/mg protein, n = 3, except for 1000 nm where n = 2. The * indicates statistical significance from 0 nm, P < 0.05. The cells were preincubated with insulin for 1 min at the indicated concentrations and then incubated with [3H]oleic acid (2 μCi) for 30 min.

Adenylate Cyclase Activity in L-Cell Fibroblasts

<table>
<thead>
<tr>
<th>Effect</th>
<th>Specific Activity (pmol/mg protein × 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>82</td>
</tr>
<tr>
<td>NaF</td>
<td>619</td>
</tr>
<tr>
<td>PGE₁ (10 μM)</td>
<td>1748</td>
</tr>
<tr>
<td>Isoproterenol (10 μM)</td>
<td>84</td>
</tr>
<tr>
<td>Isoproterenol (10 μM) + propranolol (1 μM)</td>
<td>64</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>71</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>79</td>
</tr>
</tbody>
</table>

cAMP levels (Table IV). An active adenylate cyclase pathway was confirmed using prostaglandin E₁ (PGE₁) as a positive control. PGE₁ stimulated a 21-fold increase in cAMP levels compared to the basal, epinephrine, and isoproterenol groups. Thus, although epinephrine increased cis-parinaric acid uptake, this increase does not appear to be related to an increase in cAMP levels resulting from β-adrenergic receptor stimulation.

DISCUSSION

The effect of insulin on fatty acid uptake and esterification into cellular lipid pools is unclear (6, 15, 40, 44). To further study this problem, fatty acid uptake and esterification was assessed in L-cell fibroblasts in the presence and absence of insulin. We report that insulin, at concentrations as low as 10 nm, decreased fatty acid uptake by 24–30% in L-cell fibroblasts. Furthermore, insulin decreased the amount of fatty acid esterified into neutral and phospholipids, although insulin appeared to target a greater proportion of esterified fatty acid into the phospholipid fraction. Because normal insulin levels in the circulation range from 0.05–0.3 nm (45), the levels used herein have been defined to be near physiologically relevant levels. Supraphysiological levels of glucagon and epinephrine both stimulated fatty acid uptake. For epinephrine, this increase does not appear to be linked to β-receptor stimulation. Hence, in L-cell fibroblasts insulin decreased fatty acid uptake and esterification.

In the presence of insulin, both cis-parinaric acid and oleic acid uptake into L-cell fibroblasts was significantly decreased. For cis-parinaric acid, the maximal uptake and initial uptake rate were decreased 30 and 23%, respectively, following pretreatment with insulin (100 nm). Because cis-parinaric acid is poorly esterified, this reduction represents reduced cellular fatty acid uptake (17, 29). For oleic acid, a readily esterified fatty acid, both maximal uptake and the initial uptake rate were reduced 35% compared to non-insulin-treated control.
cells. The difference in the magnitude of reduction for both the maximal uptake and initial uptake rate between cis-parinaric acid and oleic acid represents a decrease in oleic acid esterification. Previous studies have shown that the difference in the magnitude of cis-parinaric acid and oleic acid uptake represents the contribution of oleic acid esterification into lipid pools, thereby causing an increase in the magnitude of uptake (29). Conversely, in the present study, a decrease in oleic acid uptake relative to cis-parinaric acid uptake indicates a decrease in not only oleic acid uptake, but also esterification.

Oleic acid esterification was significantly altered by insulin treatment in L-cell fibroblasts. Unlike rat intestinal rings in vitro where insulin did not alter fatty acid esterification (40), in L-cells insulin induced a dose-dependent decrease in oleic acid esterification into both the phospholipids and neutral lipids. Insulin (100 nm) treatment caused a 28% reduction in [3H]oleic acid esterification into total phospholipids and a 60% reduction in [3H]oleic acid esterification into neutral lipids. Insulin also altered the distribution of [3H]oleic acid between the esterified neutral lipids, phospholipids, and free fatty acid fractions. Although the total amount of [3H]oleic acid esterified decreased with insulin treatment, the presence of insulin altered the distribution of esterified [3H]oleic acid. The proportion of esterified [3H]oleic acid was increased in phospholipids and decreased in neutral lipids. The lowest concentration of insulin (10 nm) caused a 60% reduction in the proportion of the total fatty acids taken up that were esterified into the neutral lipid fraction, while this same proportion was increased 10% in phospholipids. This suggests that insulin induced not only a decrease in fatty acid uptake, but also altered the esterification pathways, favoring esterification into phospholipids relative to neutral lipids.

Unlike insulin, both glucagon and epinephrine stimulated an increase in both the initial rate and extent of cis-parinaric acid uptake in L-cells. A similar effect has been reported in adipocytes where exposure to epinephrine (3 μM) increased fatty acid uptake (1, 2). In adipocytes, this epinephrine-stimulated increase in fatty acid uptake appears to be associated with β-adrenergic receptor-linked cAMP formation (2, 3). In L-cells, nearly the same epinephrine concentration stimulated an increase in fatty acid uptake that does not appear to be the result of β-adrenergic receptor stimulation because β-adrenergic receptors that were effectively coupled to adenylate cyclase appear to be absent in L-cells. Although the epinephrine concentration in these studies was supraphysiologic, the glucagon levels were near the maximum levels seen during fasting where glucagon approaches or exceeds 0.2 nm (46). Thus, in L-cells these supraphysiologic levels of glucagon and epinephrine increased fatty acid uptake, with the epinephrine acting through an alternative receptor pathway/mechanism.

The low levels of insulin needed to elicit an effect on fatty acid uptake and esterification suggests a receptor-mediated process. Indeed, L-cells had both low and high affinity insulin receptors. However, the mechanisms whereby insulin caused a decrease in fatty acid uptake are unknown. The effects of insulin at all concentrations were not the same, suggesting a complex set of dose-dependent processes were active. Nonetheless, in L-cell fibroblasts, near physiologically relevant insulin concentrations significantly decreased fatty acid uptake and caused a shift in esterification pathways which favored fatty acid esterification into phospholipids relative to neutral lipids, in spite of an overall decrease in fatty acid uptake.

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