Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro

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Insulin resistance can be induced in vivo through elevation of lipid availability. This has been achieved in humans and rats by acute infusion of triglyceride-heparin emulsions to elevate plasma free fatty acids (5, 7–9, 19, 20, 22, 35, 36) and in rodents by chronically feeding a high-fat diet (17, 21, 23, 24, 31, 43). Although increased fatty acid oxidation will decrease glucose oxidation, which can then cause decreased glucose transport/phosphorylation (34), it is becoming increasingly evident that mechanisms other than substrate competition are involved in lipid-induced skeletal muscle insulin resistance (6, 13, 16). Accumulated lipids have been postulated to interact directly with the regulation of glucose transport/phosphorylation and/or glycogen synthesis (36) to alter insulin action. However, it is difficult to study these mechanisms in intact animals because of potential indirect effects driven by alterations in whole body metabolism.

The direct effects of specific fatty acids on skeletal muscle insulin action can be assessed by use of in vitro systems. In the limited number of studies on skeletal muscle in vitro, the direct effects of individual fatty acids have been varied and do not provide convincing evidence that fatty acids can affect insulin action in skeletal muscle. Palmitate may or may not decrease glucose uptake and/or glycogen synthesis (1–3, 15), whereas oleate has been shown to decrease glucose transport or glycogen synthesis (29). In the cases where fatty acids had limited or no effect on insulin action, the incubations may have been too short (1–2 h) for any effects to develop, particularly in light of the time required for triglyceride-heparin infusions to elicit effects in vivo (3–5 h) (7–9, 22, 36).

The primary aims of this study were 1) to determine whether insulin resistance could be directly induced by prolonged incubation of skeletal muscle with individual fatty acids in vitro, 2) to determine the heterogeneity of responses of skeletal muscle to individual fatty acids, and 3) to examine possible relationships between intracellular lipid availability and insulin action to (30, 31), and altered fatty acid composition of membrane phospholipids (40).

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establish potential sites that may be important in the regulation of insulin-mediated glucose metabolism and the development of insulin resistance. The fatty acids studied were palmitate (n-hexadecanoic acid; C16:0), oleate (cis-9-octadecenoic acid; C18:1 n-9), linoleate (cis-9,12-octadecadienoic acid; C18:2 n-6), and α-linolenate (cis-9,12,15-octadecatrienoic acid; C18:3 n-3).

**METHODS**

**Animal Treatment**

Male Wistar rats (ARC, Perth, WA) weighing 220–250 g were housed under controlled conditions (12:12 h light-dark cycle) before experimentation and were fed water and standard laboratory chow (3.5% fat, 76.5% carbohydrate, 20% protein) ad libitum. Some rats were fed a high safflower oil diet (59% fat, 20% carbohydrate, 21% protein) (39) for 3 wk.

**Preparation of Fatty Acids Conjugated to BSA**

Fatty acids were conjugated to fatty acid-free BSA, as described by Svedberg et al. (41). Briefly, 20% (wt/vol) BSA was heated to 45°C, and 200 mM fatty acids in ethanol were added to yield 8 mM fatty acid conjugated to BSA. The 20% BSA solutions were further diluted with incubation buffer to yield 2 mM fatty acid. When fatty acid concentrations of <2 mM were required, an appropriate volume of fatty acid containing BSA was replaced with 20% fatty acid-free BSA (to which ethanol had been added, 1:25 dilution).

**Preparation of Soleus Muscle Strips**

Rats were killed by asphyxiation with CO₂. Each soleus muscle was split longitudinally to obtain multiple strips for incubation in vitro (12, 14). The strips were tied via the tendons to stainless steel clips to maintain resting tension. The muscles were preincubated in media pregassed with 95% O₂-5% CO₂ for 30 min, 4 h, or 6 h in sealed 25-ml conical flasks, as described below. An atmosphere of 95% O₂-5% CO₂ was maintained in all the flasks throughout the preincubation period.

**Muscle Incubations**

**Determination of the time- and concentration-dependent effects of palmitate on glucose uptake and glycogen synthesis.** To determine the time required for palmitate to affect glucose metabolism, muscle strips isolated from chow-fed rats were preincubated at 37°C in Krebs-Ringer bicarbonate buffer (KRB) supplemented with 5.5 mM glucose, 10 mM HEPES, and 5% BSA in the absence or presence of 2 mM palmitate. After this 4-h preincubation, the muscles were blotted and transferred to 3 ml of fatty acid-free incubation media for 1 h for assessment of insulin action as described above.

**Comparison of effects of different fatty acids on glucose uptake, glycogen synthesis, protein kinase B phosphorylation, and LCACoA content.** Muscle strips isolated from chow-fed rats were preincubated for 4 h in KRB supplemented with 5.5 mM glucose, 10 mM HEPES, and 5% BSA in the presence of 0, 0.5, 1, or 2 mM palmitate. After this 4-h preincubation, the muscles were blotted and transferred to 3 ml of fatty acid-free incubation media for 1 h for assessment of insulin action as described above.

**Determination of ATP content of muscle.** Additional incubations were performed so that ATP contents of the muscles could be determined. ATP content was measured spectrophotometrically in neutralized perchloric acid extractions of incubated muscles using a hexokinase and glucose 6-phosphate dehydrogenase coupled assay (4). ATP contents were normal in muscles that had been incubated for the same length of time as those in which insulin action was assessed (5 h control, 4.80 ± 0.28; 5 h with fatty acid, 4.65 ± 0.26 μmol/g wet wt). ATP contents were similar to those in muscle incubated for shorter periods of time (1 h control, 4.25 ± 0.41 μmol/g wet wt), as well as being similar to published values obtained from freeze-clamped rat soleus muscle tissue (26), indicating that the muscle strips remained viable during the experiment.

**Quantification of 2-deoxyglucose-6-phosphate and incorporation of glucose into glycogen.** Muscles were heated at 70°C in 1 M KOH for 15 min to digest the tissue. The digest was split into two 0.2-ml aliquots. The first was neutralized with 25% perchloric acid and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was pipetted onto an anion exchange column (AG 1-X8 resin, acetate form, Bio-Rad). 2-deoxy-[³H]glucose was eluted from the column with 9 ml H₂O and discarded. 2-Deoxyglucose-6-phosphate (2DG-6-P) was eluted with 6 ml HCl, and 2 ml of the eluant were quantified by liquid scintillation counting. Glycogen was precipitated from the remaining 0.2 ml KOH digest (18). The glycogen pellet was digested with amyloglucosidase for 2 h at 37°C in 1 ml 0.25 M acetic acid. The [¹⁴C]glucose incorporated into glycogen was quantified in 0.8 ml of the amylglucosidase digest. The remaining digest was used for quantification of total glucosyl unit content of glycogen by means of a glucose oxidase spectrophotometric assay.

**LCACoA content.** Several soleus muscle strips were pooled to enable determination of LCACoA content from 150–180 mg of muscle tissue. The LCACoAs were extracted using previously published methods (11, 27) and quantified by reverse-phase HPLC.

**PKB activation.** Muscles were homogenized with 20 volumes 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, and 1 mM PMSF.
mM CaCl₂, 2 mM Na₃VO₄, 10 mM Na pyrophosphate, 10 mM NaF, 2 mM EDTA, 1% Nonidet, 10% glycerol, including 2 mg/ml aprotinin, 5 μg/ml leupeptin, and 34 μg/ml phenylmethylsulfonyl fluoride. The samples were solubilized on ice for 1 h, and then insoluble material was removed by centrifugation for 15 min at 13,000 g. The supernatants were subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes for 1 h at 90 V, which were blocked overnight with 2% milk powder in Tris-buffered saline containing 0.025% Tween 20. The membranes were probed with anti-phospho-PKB B-Ser473 and donkey anti-rabbit conjugated with horseradish peroxidase, and then bands were visualized by enhanced chemiluminescence. The membranes were stripped and then reprobed with anti-PKB to quantify total PKB protein.

Data Analysis

All rates are expressed as micromoles per hour per gram wet weight. Statistical differences between groups were assessed by ANOVA and by Fisher’s protected least squares difference (PLSD) using Statview Graphics (Abacus Concepts, Berkeley, CA). P < 0.05 was considered significant.

RESULTS

Time- and Concentration-Dependent Effects of Palmitate on Glucose Metabolism

The time- and concentration-dependent effects of preincubation of soleus muscle with palmitate on the rates of glycogen synthesis and 2DG-6-P accumulation (glucose transport/phosphorylation) are shown in Fig. 1. Preincubation with palmitate decreased the rate of glycogen synthesis in response to 300 μU insulin/ml to ~50% of control muscles (no exposure to palmitate) in a time- (P < 0.005; ANOVA) and concentration-dependent (P < 0.02; ANOVA) manner. The effect was near maximal after 2 h with 2 mM palmitate or 4 h with 1 mM palmitate. There was no effect of palmitate on the rate of glycogen synthesis in the presence of a basal concentration of insulin (10 μU/ml).

Preincubation with 2 mM palmitate decreased both the basal and insulin-stimulated rates of glucose transport/phosphorylation with time, with the decrease becoming significant after 4–6 h (P < 0.05; ANOVA). However, there was no significant effect on the increment in the rate of glucose transport/phosphorylation above basal on stimulation with 300 μU insulin/ml. There was also no concentration-dependent effect of 4-h preincubation with palmitate on either the basal or insulin-stimulated rates of glucose transport/phosphorylation.

Comparison of Effects of Different Fatty Acids on Glucose Metabolism

The effects of 4-h preincubation with 1 mM palmitate (C16:0), oleate (C18:1 n-9), linoleate (C18:2 n-6), or linolenate (C18:3 n-3) on insulin-mediated glucose metabolism are shown in Fig. 2. These preincubation conditions were chosen after the initial experiments with palmitate, because the fatty acid had separate effects on glucose transport/phosphorylation and glycogen synthesis (above and Fig. 1). That is, insulin-
stimulated glycogen synthesis was impaired, but glucose transport/phosphorylation was unaffected. For each fatty acid tested, the rate of glycogen synthesis stimulated with a submaximal insulin concentration (300 μU/ml) was significantly decreased to 50–60% of control (Fig. 2B, P < 0.001 for each fatty acid vs. control). This defect was largely overcome in muscle stimulated with 1,000 μU insulin/ml, with only the rate of glycogen synthesis in oleate-treated muscles being significantly lower than control (P < 0.01). Total glycogen content was similar for each of the fatty acid treatments (control 17.5 ± 1.0; palmitate 16.8 ± 1.5; oleate 17.3 ± 1.4; linoleate 15.6 ± 1.0; linolenate 16.6 ± 1.1 μmol glucosyl units/g wet wt).

In contrast to their effects on glycogen synthesis, 1 mM specific fatty acids had different effects on the rate of glucose transport/phosphorylation (Fig. 2A). Across all groups, there was a significant effect of fatty acid on glucose transport/phosphorylation (P = 0.02; ANOVA). Preincubation of soleus muscles with 1 mM palmitate for 4 h had no effect on the subsequent determination of insulin-stimulated glucose transport/phosphorylation (P > 0.5 for palmitate vs. control at each insulin concentration), as was observed in the initial study. There was no significant effect of linolenate on glucose transport/phosphorylation stimulated with either 10 or 300 μU insulin/ml, although there was an increased response to 1,000 μU insulin/ml (P < 0.05) in muscle preincubated with linolenate compared with control (P = 0.03). Linoleate caused a significant decrease in the rate of glucose transport/phosphorylation (P < 0.03) stimulated with a submaximal physiological insulin concentration (300 μU/ml) without affecting the rate stimulated with either 10 or 1,000 μU insulin/ml. Finally, oleate significantly decreased the rate of glucose transport/phosphorylation in response to 1,000 μU insulin/ml (P < 0.03).

PKB Phosphorylation

The effects of 30 min of insulin stimulation on PKB phosphorylation after preincubation with 1 mM fatty acids are shown in Fig. 3. PKB phosphorylation was increased above a basal level (10 μU/ml) with 300 or 1,000 μU insulin/ml in all muscles. However, the degree of phosphorylation of PKB in muscles stimulated with 300 μU insulin/ml after preincubation with fatty acids was ~50% of control (P < 0.02 for each fatty acid vs. control). There was no difference between groups in PKB phosphorylation in response to 1,000 μU insulin/ml. Thus the impaired phosphorylation of PKB in muscle pretreated with fatty acid and then stimulated with a submaximal physiological concentration of insulin correlates with the impaired glycogen synthesis observed when fatty acid-treated muscle was stimulated with the same insulin concentration (300 μU/ml).

LCACoA Contents

The LCACoA contents of soleus muscle preincubated for 4 h with 1 mM different fatty acids are shown in Table 1. For each of the groups, there was a significant...
increase in the LCACoA species corresponding to the fatty acid in the preincubation medium. There was also a significant increase in total LCACoA content in muscle preincubated with linoleate (P = 0.02) and a tendency toward an increase after preincubation with palmitate or oleate. The total LCACoA was not significantly increased in all cases, because preincubation with some fatty acids not only caused an increase in the corresponding LCACoA but also resulted in a significant decrease in other LCACoA species. For example, oleoyl CoA decreased in muscle incubated with linolenate or palmitate, whereas linolenoyl CoA decreased in muscles preincubated with oleate or linoleate.

There was a significant negative association between the total LCACoA content and the rate of glucose transport/phosphorylation stimulated with a physiological (300 μU/ml) insulin concentration (Fig. 4A, P < 0.03; r² = 0.84). However, because all fatty acids decreased glycogen synthesis compared with control but did not all increase the LCACoA content, there was no significant correlation between the rate of glycogen synthesis and the LCACoA content of muscles (Fig. 4B; P = 0.58, r² = 0.11).

To determine whether increasing the concentration of palmitate and oleate could increase total LCACoA content and decrease the rate of glucose transport/phosphorylation, muscles were preincubated with 2 mM palmitate or oleate for 4 h. The total LCACoA content was increased compared with control (1.06 ± 0.11, 2.18 ± 0.17, and 1.80 ± 0.10 nmol/g wet wt for control, palmitate, and oleate, respectively (P < 0.005), vs. control for each fatty acid). These increased total LCACoA contents at the higher concentrations of palmitate and oleate were associated with decreased rates of glucose transport/phosphorylation stimulated by 300 μU insulin/ml compared with control (3.29 ± 0.19, 1.99 ± 0.38, and 2.23 ± 0.23 μmol·h⁻¹·g wet wt⁻¹ for control, palmitate, and oleate, respectively (P < 0.05), vs. control for each fatty acid).

**Insulin Action in Muscle Isolated from High-Fat-Fed Rats**

The rates of glucose transport/phosphorylation and glycogen synthesis in soleus muscle isolated from chow- or high-fat-fed rats are shown in Fig. 5. There was a significant effect of diet on both the rate of glucose transport/phosphorylation (P < 0.001; ANOVA) and the rate of glycogen synthesis (P < 0.005; ANOVA). The rate of glucose transport/phosphorylation was significantly decreased in response to 300 and 1,000 μU insulin/ml (P < 0.001 and P < 0.01 respectively; Fisher’s PLSD), whereas the rate of glycogen synthesis was decreased in response to 300 μU insulin/ml (P < 0.001; Fisher’s PLSD).

**Table 1. LCACoA content in soleus muscle after 4-h preincubation with 1 mM palmitate, oleate, linoleate, or linolenate**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Palmitoyl-CoA (16:0)</th>
<th>Oleoyl-CoA (18:1)</th>
<th>Linoleoyl-CoA (18:2)</th>
<th>Linolenoyl-CoA (18:3)</th>
<th>Sum of 4 species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.45 ± 0.07</td>
<td>0.31 ± 0.05</td>
<td>0.25 ± 0.04</td>
<td>0.097 ± 0.007</td>
<td>1.02 ± 0.15</td>
</tr>
<tr>
<td>Palmitate</td>
<td>1.09 ± 0.30†</td>
<td>0.15 ± 0.05‡</td>
<td>0.20 ± 0.06</td>
<td>0.089 ± 0.014</td>
<td>1.24 ± 0.29</td>
</tr>
<tr>
<td>Oleate</td>
<td>0.32 ± 0.05</td>
<td>0.50 ± 0.07§</td>
<td>0.33 ± 0.11</td>
<td>0.065 ± 0.008†</td>
<td>1.31 ± 0.18</td>
</tr>
<tr>
<td>Linoleate</td>
<td>0.33 ± 0.05</td>
<td>0.23 ± 0.05</td>
<td>1.01 ± 0.11§</td>
<td>0.068 ± 0.006§</td>
<td>1.63 ± 0.11§</td>
</tr>
<tr>
<td>Linolenate</td>
<td>0.27 ± 0.03</td>
<td>0.09 ± 0.02§</td>
<td>0.30 ± 0.07</td>
<td>0.349 ± 0.034§</td>
<td>1.00 ± 0.13</td>
</tr>
</tbody>
</table>

LCACoA, long-chain acyl-coenzyme A. *P < 0.05; †P < 0.01; ‡P < 0.005; §P < 0.0001 vs. control.
The present study has demonstrated that fatty acids can directly induce insulin resistance in skeletal muscle. Although there was a similar effect of 1 mM of all fatty acids on glycogen synthesis, only some of the fatty acids impaired glucose transport/phosphorylation at this concentration. Thus, the results indicate that glucose transport/phosphorylation and glycogen synthesis may be modulated independently by fatty acids or fatty acid metabolites. The rate of glucose transport/phosphorylation was correlated to the intramuscular LCA-CoA content. However, there was no association between decreased rates of glycogen synthesis and total LCACoA content, indicating that another aspect of lipid metabolism appears to be responsible for the impaired activation of PKB and the subsequent decreased rate of glycogen synthesis.

Preincubation of skeletal muscle with fatty acids produces lipid-induced insulin resistance that has similarities to the insulin resistance that develops in vivo. Direct addition of linoleate to the preincubation medium (Fig. 2) seems to have effects on insulin-mediated glucose transport/phosphorylation and glycogen synthesis that are similar to dietary manipulation of rats by means of a high linoleate diet (Fig. 5). First, the rates of glucose transport/phosphorylation stimulated with a submaximal physiological insulin concentration (300 μU/ml) were decreased by ~25% vs. control in both cases. These results are similar to those of previous studies that have demonstrated decreased insulin-mediated glucose transport/phosphorylation in muscle from high-fat-fed rats studied ex vivo (17, 43). Second, the current study has shown that the rates of glycogen synthesis after stimulation with 300 μU insulin/ml were decreased by 50 and 33%, respectively, compared with insulin-sensitive controls in muscle preincubated with linoleate and in muscle isolated from high-fat-fed rats. Importantly, the degree of impairment in both glucose transport/phosphorylation and glycogen synthesis stimulated with a submaximal physiological insulin concentration (300 μU/ml) was similar to that observed in high-fat-fed rats studied under euglycemic-hyperinsulinemic conditions in vivo (30, 31). Finally, in insulin-resistant muscle induced by either a high-fat diet in vivo or preincubation with linoleate in vitro, there was an increase in LCACoA content (Table 1 and Refs. 30, 31), indicating similar disturbances in lipid metabolism in the two systems.

The time- and concentration-dependent manner by which fatty acids decreased insulin-mediated glucose metabolism in vitro in the present study was similar to that by which elevated plasma fatty acid concentrations affect glucose metabolism in vivo (7, 22). Thus, the fatty acids were probably acting through a similar mechanism in vitro to what they were in vivo. The differential effects of palmitate on the rates of glucose transport/phosphorylation and glycogen synthesis were utilized to design the preincubation conditions of 4 h with 1 mM fatty acid to compare the effects of different fatty acids. An attempt to separate the effects of fatty acids on glucose transport/phosphorylation and glycogen synthesis was made so that alterations in intracellular components could be more closely related to the regulation of glucose transport/phosphorylation or glycogen synthesis.

Alterations in the activation of insulin-signaling intermediates have been implicated in the mechanism by which elevated fatty acid availability leads to the development of insulin resistance (13, 16). In the current study, activation of PKB stimulated with a physiological submaximal insulin concentration was impaired after pretreatment of muscles with each fatty acid. This impaired phosphorylation of PKB was associated with the impaired rate of glycogen synthesis. At a higher, supraphysiological concentration of insulin (1,000 μU/ml), phosphorylation of PKB and the rate of glycogen synthesis were, in general, restored to control levels. Activation of PKB leads to inactivation of glycogen synthase kinase 3 and activation of glycogen synthase (10). Hence, the results of this study are consistent with elevated fatty acids decreasing activation of insulin-signaling intermediates involved in the regulation of glycogen synthase (7–9, 22).

The effects that preincubation with fatty acids had on glycogen synthesis and PKB phosphorylation were...
similar for all fatty acids studied. Thus an increase of fatty acid availability, independent of the fatty acid species, appears to be involved in the mechanism responsible for the impaired PKB phosphorylation stimulated with a physiological insulin concentration. The precise biochemical mechanism involved requires further investigation; however, the absence of a relationship between the decreased rates of glycogen synthesis and an increase in LCACoA content of muscles when all treatment groups are considered appears to dissociate total LCACoA from having a direct role in the regulation of glycogen synthesis activation in skeletal muscle. Other more indirect effects of LCACoA, such as supplying substrate for increases in other lipid pools [e.g., diacylglycerides or ceramide (37)], which may interact with signaling intermediates, could be important.

The significant negative association between the rate of glucose transport/phosphorylation and the LCACoA content suggests that LCACoAs may interact more directly with the regulation of glucose transport/phosphorylation. Triglyceride-heparin infusions that elevate plasma free fatty acids have been shown to inhibit glucose transport/phosphorylation (13, 35, 36). The results of the present study confirm that some fatty acids modulate glucose transport/phosphorylation directly. The differential effect of the fatty acids studied appears to be related to the ability of each fatty acid to raise the total LCACoA content of the muscle. Incubations with 1 mM linoleate increased the LCACoA content and decreased insulin-stimulated glucose transport/phosphorylation. However, increased LCACoA and decreased glucose transport/phosphorylation occurred with palmitate and oleate only at a concentration of 2 mM.

Lipid metabolism may be essential for glucose transport. Experiments in red blood cells demonstrated that glucose transporters can be acylated (28), whereas acyl-CoA synthetase has been shown to colocalize with GLUT-4 vesicles in rat adipocytes (38). It is plausible that an inappropriate increase in specific LCACoA species may interfere with any acylation process. Alternatively, specific LCACoAs have been demonstrated to inhibit hexokinase IV (glucokinase) in liver (42), and it is therefore possible that LCACoAs may also interfere directly with glucose phosphorylation in skeletal muscle. This concept is supported by the study of Nolte et al. (29), in which incubation of muscle with oleate in vitro caused a decrease in insulin-mediated glucose phosphorylation but not in glucose transport.

In summary, the manipulation of lipid metabolism through preincubation of soleus muscle with fatty acids produces insulin resistance that has similar characteristics to the insulin resistance that develops with fat feeding in vivo. Preincubation with 1 mM palmitate, olate, linoleate, or linolenate has provided evidence that all fatty acids decrease the ability of insulin to stimulate glycogen synthesis in skeletal muscle to a similar extent. This appears to be achieved at physiological insulin concentrations through interference with insulin signaling, at least at the level of PKB. On the other hand, similar concentrations of individual fatty acids have different effects on glucose transport/phosphorylation under the conditions used in this study, and these effects are related to the intramuscular LCACoA content. Therefore, it is likely that lipids regulate glycogen synthesis and glucose transport/phosphorylation via different mechanisms, although the exact details of these different mechanisms remain to be elucidated.

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