Oleate protects against palmitate-induced insulin resistance in L6 myotubes

Dan Gao, Helen R. Griffiths* and Clifford J. Bailey

School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

(Received 12 February 2009 – Revised 29 April 2009 – Accepted 26 May 2009 – First published online 22 July 2009)

Oleate has been shown to protect against palmitate-induced insulin resistance. The present study investigates mechanisms involved in the interaction between oleate and palmitate on insulin-stimulated glucose uptake by L6 skeletal muscle cells. L6 myotubes were cultured for 6 h with palmitate or oleate alone, and combinations of palmitate with oleate, with and without phosphatidylinositol 3-kinase (PI3-kinase) inhibition. Insulin-stimulated glucose uptake, measured by uptake of 2-deoxy-D-[3H]glucose, was almost completely prevented by 300 μM-palmitate. Cells incubated with oleate up to 750 μM maintained a significant increase in insulin-stimulated glucose uptake. Co-incubation of 50–300 μM-oleate with 300 μM-palmitate partially prevented the decrease in insulin-stimulated glucose uptake associated with palmitate. Adding the PI3-kinase inhibitors wortmannin (10−7 mol/l) or LY294002 (25 μM) to 50 μM-oleate plus 300 μM-palmitate significantly reduced the beneficial effect of oleate against palmitate-induced insulin resistance, indicating that activation of PI3-kinase is involved in the protective effect of oleate. Thus, the prevention of palmitate-induced insulin resistance by oleate in L6 muscle cells is associated with the ability of oleate to maintain insulin signalling through PI3-kinase.

NEFA: Insulin resistance: L6 muscle cells: Phosphatidylinositol 3-kinase

Insulin resistance, which describes an impaired response to physiological concentrations of insulin, is strongly associated with obesity and type 2 diabetes, and contributes to cardiovascular risk[1–3]. Excess accumulation of saturated lipid, especially in skeletal muscle which is the major site of insulin-stimulated glucose uptake, is an important factor in the development of insulin resistance[4–6]. Since insulin signalling to regulate glucose uptake in muscle is mediated largely through a pathway dependent upon phosphatidylinositol 3-kinase (PI3-kinase) and Akt phosphorylation, the effects of lipids on this pathway provide an important focus for the study of lipid-induced insulin resistance.[7–9].

Insulin resistance caused by cellular lipid accumulation is mainly associated with SFA[10]. Palmitate, one of the most abundant SFA, representing about 30% of the total NEFA in human plasma, has been shown to induce insulin resistance in cultured skeletal muscle cells and adipocytes[11,12]. By contrast, oleate, representing about 90% of the monounsaturated NEFA and 30% of the total NEFA in human plasma, has not been reported to cause significant insulin resistance.

Recent studies have shown that combination of oleate with palmitate can reverse palmitate-induced alterations in insulin signal transduction[13–15]. These studies also suggested that the oleate:palmitate ratio may influence the protective effect of oleate. However, these studies have not examined the effect of oleate on palmitate-induced insulin resistance at the level of glucose uptake.

The present study examines whether oleate protects against palmitate-induced insulin resistance at the level of glucose uptake in cultured rat L6 muscle cells. The study also investigates whether the PI3-kinase pathway is involved, and whether the morphology and viability of the cells are affected by palmitate and/or oleate.

Materials and methods

Materials

The L6 rat skeletal muscle cell line was obtained from the European Culture Collection (Porton Down, UK). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), an antibiotic and trypsin-EDTA were purchased from Cambrex (Verviers, Belgium). Sodium palmitate, sodium oleate, fatty acid-free bovine serum albumin (BSA), sodium pyruvate, PI3-kinase inhibitors (wortmannin and LY294002), trypsin blue solution and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Poole, UK). 2-Deoxy-D-[3H]glucose (555 GBq/mmol) was from Amersham International (Little Chalfont, Bucks, UK).

Cell culture

L6 myoblasts were grown in DMEM containing 5% heat-inactivated FBS, 25 mM-d-glucose, 1 mM-sodium pyruvate,
1 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) as described previously\(^{(16)}\). Cells were maintained at 37°C with humidified 95% air and 5% CO\(_2\). Experiments were undertaken in twenty-four-well plates seeded from preconfluent flasks with 5 × 10\(^4\) cells in 1 ml. The cells were grown to 70–80% confluence and the medium was changed to DMEM containing 0.5% FBS for 24 h which induces rapid differentiation and fusion of these myoblasts into myotubes\(^{(16)}\).

**Conjugation of fatty acids to bovine serum albumin**

A stock solution of palmitate (200 mmol/l) or oleate (100 mmol/l) was prepared by dissolving sodium palmitate or sodium oleate into 70% ethanol and 0.1 M NaOH as described previously\(^{(17)}\). Fatty acids were then complexed with 5% fatty acid-free BSA to a concentration of 5 mmol/l at 37°C, stirring for 4 h and adjusted to pH 7.4. After sterilising through a 0.2-μm filter, both solutions were stored at 4°C for no longer than 2 weeks. A control solution was prepared by mixing 70% ethanol and 0.1 M NaOH with 5% BSA in the absence of fatty acids.

**Incubation with fatty acids and phosphatidylinositol 3-kinase inhibitors**

L6 myotubes were incubated with various concentrations of palmitate (50–300 μmol/l) or oleate (50–750 μmol/l) alone, and combinations of 300 μM-palmitate with 50–300 μM-oleate for 6 h. Palmitate (300 μmol/l) induces a time (0–6 h)-dependent reduction in insulin-stimulated glucose uptake which is maximal at 6 h and does not induce significant toxicity\(^{(18)}\). Further experiments to investigate any involvement of the PI3-kinase pathway in the effects of fatty acids were undertaken with and without the PI3-kinase inhibitors wortmannin (10\(^{-7}\) mol/l) for 6 h or LY294002 (25 μmol/l) for 1 h 10 min at 37°C. The presence of wortmannin at 10\(^{-7}\) mol/l elicited a reduction of insulin-stimulated glucose uptake by L6 muscle cells over 6 h, from 140.5 (SEM 4.8) to 105.5 (SEM 3.2) % of non-insulin-stimulated glucose uptake \((P<0.001; n = 8)\). Similarly, pre-incubation of L6 muscle cells with LY294002 for 10 min (25 μmol/l) reduced insulin-stimulated glucose uptake by L6 muscle cells from 150.1 (SEM 11.5) to 93.3 (SEM 4.6) % of non-insulin-stimulated glucose uptake \((P<0.001; n = 9)\). Control cells received BSA and/or other vehicles as appropriate.

**2-Deoxy-D-glucose uptake**

2-Deoxy-D-glucose uptake was undertaken immediately after completion of the 6 h incubation with fatty acids in which insulin (10\(^{-8}\) mol/l) had been added for the last 1h. L6 myotubes were then washed with glucose-free Krebs–Ringer bicarbonate buffer at 22°C and incubated with 0.5 ml of this buffer supplemented with 0.1 mM-2-deoxy-D-glucose and 2-deoxy-D-[\(^{3}H\)]glucose (3700 Bq/ml) for 10 min at 22°C. After washing cells three times with ice-cold Krebs–Ringer bicarbonate buffer, cells were lysed with 0.5 ml of 1 M NaOH and radioactivity was counted in 5 ml Hi-safe 3 scintillant using a Packard 1900 TR liquid scintillation counter (Packard, Chicago, IL, USA). Uptake of 2-deoxy-D-glucose was expressed as the percentage compared with control (100%), which was typically 5–8 pmol/10\(^5\) cells per min for basal uptake of 2-deoxy-D-glucose in the present studies as reported previously\(^{(19)}\).

**Cell viability assays**

**Trypan blue exclusion assay.** For the trypan blue exclusion assay, L6 myotubes in twenty-four-well plates were gently washed with PBS, and trypsinised with 100 μl trypsin-EDTA for 2 min and neutralised with 100 μl DMEM with 10% FBS. After mixing with 0.4% trypan blue solution, the live cells were counted on a Neubauer haemocytometer (AO Scientific, Buffalo, NY, USA). Cell viability was expressed as percentage of live cells.

**Caspase-3 activity assay.** L6 myotubes in six-well plates were gently washed twice with ice-cold PBS. After application of lysis buffer (10 mM-2-amin-2-hydroxymethyl-propane-1,3-diol-HCl, pH 7.5, 130 mM NaCl, 1% TritonX-100, 10 mM-NaH\(_2\)PO\(_4\), 0.4 mM-phenylmethylsulfonylfluoride, 0.2 mM-NaF, 0.2 mM-Na\(_3\)VO\(_4\), leupeptin (0.3 mg/ml)) on ice for 30 min, cells were centrifuged at 10 000 g for 5 min. The supernatant fraction was collected and caspase-3 activity was measured using a fluorescent caspase-3 substrate II (7-amino-4-methylcoumarin, N-acetyl-l-aspartyl-l-glutamyl-l-valyl-l-asparagic acid amide; Ac-DEV-D-AMC\(^{(20)}\)) and protein content was measured by bichinonic acid protein assay\(^{(21)}\).

**Phase-contrast microscopy**

To assess cell morphology, L6 myotubes were photographed under a phase-contrast microscope (Olympus, Southend-on-Sea, Essex, UK) at magnification × 20.

**Statistical analysis**

Data are expressed as mean values with their standard errors, and 2-deoxy-D-glucose uptake is expressed as percentage compared with control (100%). Statistical analyses were performed by one-way ANOVA with Tukey–Kramer post hoc tests. \(P<0.05\) was considered significant.

**Results**

**Effect of palmitate or oleate alone on glucose uptake**

Initial experiments were conducted to characterise the effect of palmitate and oleate alone on basal and insulin-stimulated glucose uptake by L6 myotubes. Since palmitate was...
conjugated to BSA, the control and the oleate incubations were conducted with the same concentration of BSA. L6 myotubes were incubated with palmitate (50–300 μmol/l) or oleate (50–750 μmol/l) alone for 6 h. Both palmitate and oleate at the concentrations tested showed no significant effect on basal glucose uptake compared with controls (Fig. 1(a), (b)). When BSA-treated control cells were stimulated with 10⁻⁶ M-insulin for 1 h, this produced a significant increase in glucose uptake by about 40–50 % which is a maximal insulin-induced effect for these cells over this time period (data not shown). However, when L6 myotubes were incubated with palmitate for 6 h, there was a concentration-dependent decrease in insulin-stimulated glucose uptake. Insulin-stimulated glucose uptake was almost completely prevented by exposure to 300 μM-palmitate (Fig. 1(a)). Therefore, this concentration of palmitate was chosen to induce insulin resistance in subsequent studies.

Oleate alone caused a small concentration-dependent decrease in insulin-stimulated glucose uptake. The highest oleate concentration tested (750 μmol/l) did not completely prevent insulin-stimulated glucose uptake (Fig. 1(b)).

Olate protects L6 cells from palmitate-induced insulin resistance

The effect of oleate on palmitate-induced insulin resistance was examined by co-incubating increasing concentrations of oleate (50, 150 and 300 μmol/l) with 300 μM-palmitate for 6 h with and without 10⁻⁶ M-insulin stimulation for the last 1 h. Whereas 300 μM-palmitate alone abolished insulin-stimulated glucose uptake, co-incubation with oleate (50 μmol/l) partially restored (to 75 %) insulin-stimulated glucose uptake in the presence of 300 μM-palmitate, but co-incubation with 50 μM-BSA (control) did not exert any protective effect against the inhibition of insulin-stimulated glucose uptake by palmitate. The protective effect of oleate against palmitate-induced insulin resistance was not further affected by increasing the oleate concentration to 300 μmol/l (Fig. 2).

Effects of fatty acids on L6 cell viability

To test whether fatty acids have adverse effects on L6 myotubes, membrane integrity was assessed by trypan blue exclusion, and mitochondrial-reducing capacity was measured by an MTT assay.

Cells incubated with 300 μM-oleate alone did not show any significant difference in membrane integrity compared with BSA controls. With 300 μM-palmitate, there was a slight reduction in membrane integrity compared with BSA controls (viability of 74 (SEM 4) % vs. 88 (SEM 3) %; P < 0.001). Co-incubating cells with 50–300 μM-oleate prevented the slight reduction in membrane integrity with 300 μM-palmitate (Fig. 3).

The overall pattern in mitochondrial-reducing activity was similar to that of membrane integrity. Palmitate (300 μmol/l) decreased mitochondrial-reducing activity by more than 50 % (P < 0.001), whereas the same concentration of oleate (300 μmol/l) caused a 25 % decrease in mitochondrial-reducing activity compared with BSA controls. Addition of 150 μM- and 300 μM-oleate with 300 μM-palmitate partially

![Fig. 1. Effect of palmitate (a) and oleate (b) alone on basal (●) and insulin-stimulated (●) 2-deoxy-D-glucose (2-DG) uptake by L6 myotubes. L6 myotubes were incubated with bovine serum albumin (BSA) as control or BSA with palmitate (50–300 μmol/l) and oleate (50–750 μmol/l) alone for 6 h at 37°C. Insulin (10⁻⁶ mol/l) was added for the last 1 h of incubation. Data are the means of three independent experiments performed in triplicate, with standard errors represented by vertical bars. Mean value was significantly different from that of the same treatment without insulin addition: ** P < 0.01, *** P < 0.001. Mean value was significantly different from that of the insulin-stimulated BSA-only-treated control (0 μM-palmitate): † P < 0.05, ††† P < 0.001.](https://www.cambridge.org/core/terms, https://doi.org/10.1017/S0007114509990948)
(b) L6 myotubes were incubated with synthetic peptide 7-amino-4-methylcoumarin, in lysates was measured as the release of coumarin fluorescence from the mitochondrial-reducing activity (P < 0.001). ††† Mean value was significantly different from that of the Pa-only treatment (P < 0.001).

To exclude the possibility that BSA (a fatty acid carrier protein) could contribute to the protection by oleate, it was noted that the loss of spindle shape caused by palmitate was not prevented by co-incubating with increasing concentrations of BSA (Fig. 5(g), (h), (i)).

Phosphatidylinositol 3-kinase activity in palmitate- and oleate-treated L6 cells

To investigate the underlying mechanism of the protective effect of oleate against palmitate-induced insulin resistance in L6 myotubes, the involvement of PI3-kinase activation was examined by incubating L6 myotubes with two different types of PI3-kinase inhibitors: wortmannin which binds to the catalytic subunit (P110) and LY294002 which binds to the regulatory subunit (P85) of PI3-kinase[22]. Both wortmannin (10−7 mol/l) and LY294002 (25 µmol/l) slightly reduced basal glucose uptake by 10 % (P < 0.05) and prevented insulin-stimulated glucose uptake by L6 myotubes (Fig. 6(a), (b)). The reduction in insulin-stimulated glucose uptake by 300 µM-palmitate was partially prevented by co-incubating with 50 µM-oleate as tested earlier (Fig. 2). With the addition of wortmannin (10−7 mol/l) or LY294002 (25 µmol/l), the improvement in insulin-stimulated glucose uptake by 50 µM-oleate in 300 µM-palmitate-treated cells was significantly reduced (P < 0.001) (Fig. 6(a), (b)).

Discussion

In the present study, the effect of oleate on palmitate-induced insulin resistance at the level of glucose transport was investigated in L6 myotubes. Treating L6 cells with 300 µM-palmitate for 6 h almost completely abolished insulin-stimulated glucose uptake. Oleate (up to 750 µM) produced only a slight reduction in insulin-stimulated glucose uptake. Oleate prevents the palmitate-induced alteration in L6 cell morphology

There was a distinct difference between the effects of palmitate and oleate alone on L6 myotube morphology. When L6 myotubes were incubated with 300 µM-palmitate, the muscle cells lost their spindle shape (Fig. 5(b)), whereas cells incubated with the same concentration of oleate maintained a similar shape to BSA controls (Fig. 5(c)). When 50 µM-oleate was co-incubated with 300 µM-palmitate, the spindle shape was retained (Fig. 5(d)). This protective effect of oleate was not further enhanced when the oleate concentration was raised to 150 and 300 µmol/l (Fig. 5(e), (f)).

Oleate prevents the palmitate-induced alteration in L6 cell morphology

Fig. 3. Effect of palmitate (Pa) and oleate (Oa) on viability measured as (a) membrane integrity by trypan blue exclusion and (b) caspase-3 activity in cell lysates. (a) L6 myotubes were incubated with 300 µM-Pa and 300 µM-Oa alone and combinations of 300 µM-Pa with various concentrations of Oa (50, 150, 300 µmol/l) or bovine serum albumin (BSA) for 6 h at 37°C. Viable cells were counted after trypsinisation. (b) L6 myotubes were incubated with BSA, 300 µM-Pa and 300 µM-Oa alone for 6 h at 37°C. Caspase-3 activity in lysates was measured as the release of coumarin fluorescence from the synthetic peptide 7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (Ac-DEVD-AMC). Data are the means of three independent experiments performed in triplicate, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the Pa-only treatment (P < 0.001).

Fig. 4. Effect of palmitate (Pa) and oleate (Oa) on mitochondrial-reducing activity measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. L6 myotubes were incubated with 300 µM-Pa and 300 µM-Oa alone and combinations of 300 µM-Pa with various concentrations of Oa (50, 150, 300 µmol/l) or bovine serum albumin (BSA) for 6 h at 37°C. Data are the means of three independent experiments performed in triplicate, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the Pa-only treatment (P < 0.001). ††† Mean value was significantly different from that of the BSA-only treatment (P < 0.001).
glucose uptake. However, addition of 50 μM-oleate reduced the inhibitory effect of 300 μM-palmitate on insulin-stimulated glucose uptake. This protective effect was not further increased with increasing concentrations of oleate up to 300 μmol/l but required the activity of PI3-kinase.

Fatty acid-induced insulin resistance has been extensively studied in vitro using skeletal muscle cells (13,20). In L6 cells palmitate (300 μmol/l) reduced insulin-stimulated glucose uptake acutely (6 h), confirming previous studies with these cells (23).

Two distinct fatty acid metabolites formed from oversupply of lipids to skeletal muscle have been implicated in the development of skeletal muscle insulin resistance: ceramide and diacylglycerol (24). Palmitate is an important precursor of de novo synthesis of ceramide (25), and ceramide strongly inhibits insulin action (26,27), mainly by disrupting activation of Akt (protein kinase B) and reducing translocation of GLUT into the cell membrane (28-30). Studies of lipid-induced insulin resistance in muscles of animals and human subjects support a role of diacylglycerol in the activation of the protein kinase C0–NF-κB pathway (31). This involves serine phosphorylation of insulin receptor substrate-1 and an associated reduction of PI3-kinase activity (32).

Although oleate alone (up to 750 μmol/l) slightly reduced insulin-stimulated glucose uptake by L6 myotubes, only 50 μmol/l oleate was required to prevent 300 μM-palmitate-induced insulin resistance. This observation is consistent with a recently published study in which 100 μM-oleate reversed 500 μM-palmitate-induced insulin resistance in C2C12 myotubes; this effect involved altered phosphorylation of Akt (15). Using the PI3-kinase inhibitors, wortmannin and LY294002, we have demonstrated that the effect of oleate to partially reverse palmitate-induced insulin resistance requires PI3-kinase activity, which is an insulin-signalling intermediate between insulin receptor substrate-1 and the 3-phosphoinositide-dependent kinase that regulates Akt. It is anticipated that reactivation of this pathway will restore GLUT-4 translocation and glucose transport (33). It has been reported previously that long-term exposure to oleate can stimulate the activation of PI3-kinase in MDA-MB-231 cancer cells (34). Since the present study involves only 6 h exposure to oleate, the effect of oleate on PI3-kinase is unlikely to be a direct effect on gene expression. Also, oleate alone did not increase insulin-stimulated glucose uptake. It has been proposed that palmitate-induced insulin resistance could be mediated via protein kinase C0-induced
serine-phosphorylation to suppress insulin receptor substrate-1, which then reduces PI3-kinase activity\(^{(32,35)}\). Activation of protein kinase C\(\theta\) is promoted by diacylglycerol, which is generated from palmitate or by inhibitor \(\kappa\)B kinase \(\beta\), a serine kinase that normally prevents activation of NF-\(\kappa\)B\(^{(23)}\). Oleate has been reported to inhibit the activation of NF-\(\kappa\)B in endothelial cells\(^{(36)}\). Therefore, the protective effect of oleate against palmitate-induced insulin resistance may result from the channelling of diacylglycerol into neutral TAG which does not induce insulin resistance\(^{(13–15,37–39)}\). It remains to be investigated whether ceramide generation is involved in the protection by oleate against palmitate-induced insulin resistance.

Another detrimental effect of palmitate observed in the present study was the loss of spindle shape of L6 myotubes. Normal cell morphology was restored by co-incubation with 50 \(\mu\)M-oleate, and this was associated with a small increase in membrane integrity. However, the reduction in membrane integrity was much smaller than the reduction in insulin-stimulated glucose uptake caused by palmitate, suggesting that the small loss in cell membrane integrity is not the primary contributor to the palmitate-induced insulin resistance in L6 myotubes. Mitochondrial dysfunction has been implicated in the development of insulin resistance\(^{(460)}\), and palmitate diminished the mitochondrial-reducing capacity of L6 cells. Nevertheless, the inability of 50 \(\mu\)M-oleate to restore mitochondrial function while reversing 300 \(\mu\)M-palmitate-induced insulin resistance suggests that improved mitochondrial function is not crucial for the protective effect of oleate against palmitate-induced insulin resistance in muscle. Addition of 150 \(\mu\)M- and 300 \(\mu\)M-oleate partially prevented the palmitate-induced decrease in mitochondrial activity and this protective effect is similar to the addition of increasing concentrations of BSA. This suggests that the protection by the higher concentrations of oleate may partly reflect the increased amount of BSA. There was no effect of palmitate or oleate on apoptosis under the same conditions as assessed by caspase-3 assay on the adherent cells. However, apoptotic cells can lose adherence, so additional measures of cell death are required to exclude an effect on apoptosis.

In summary, the present study has demonstrated a protective effect of the MUFA oleate against insulin resistance induced by the SFA palmitate in L6 muscle cells. This protective effect is associated with the ability of oleate to preserve insulin-stimulated PI3-kinase activity in palmitate-treated cells. The protective effect of oleate has potentially important implications for the balance of dietary monounsaturated and saturated fats in the development of insulin resistance and these data suggest possible benefits of increasing plasma oleate \textit{in vivo}; an early meta-analysis of dietary fat intake supported the hypothesis that monounsaturated fat may improve diabetic control and this has been substantiated by more recent studies\(^{(41–43)}\). Whether dietary oleate offers a strategy to improve glucose homeostasis in type 2 diabetes mellitus requires further investigation through well-designed, appropriately powered dietary intervention studies.

**Acknowledgements**

The authors gratefully acknowledge financial support for the present study from Aston University and for D. G. from an Overseas Research Students Awards Scheme (ORSAS) scholarship award.

D. G. undertook all experiments and drafted the manuscript.

H. R. G. was principal investigator and supervisor to D. G. and C. J. B. was associate supervisor.

The authors declare that there is no conflict of interest.

**References**


Fatty acids and muscle insulin sensitivity


