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SUMMARY

An increase in circulating levels of specific non-esterified fatty acids (NEFAs) has been implicated in the pathogenesis of insulin resistance and impaired glucose disposal in skeletal muscle. In particular, elevation of saturated fatty acids (SFAs), such as palmitate, has been correlated with reduced insulin sensitivity, whereas an increase in certain mono- and polyunsaturated fatty acids (MUFAs and PUFAs) has been suggested to improve glycemic control, although the underlying mechanisms remain unclear. Here, we compare the effects of palmitoleate (a MUFA) and palmitate (a SFA) on insulin action and glucose utilisation in L6 skeletal muscle cells. Basal glucose uptake was enhanced by ~2-fold following treatment of cells with palmitoleate. The MUFA-induced increase in glucose transport led to an associated rise in glucose oxidation and glycogen synthesis, which could not be attributed to activation of signaling proteins normally modulated by stimuli such as insulin, nutrients or cell stress. Moreover, whilst the MUFA-induced increase in glucose uptake was slow in onset, it was not dependent upon protein synthesis, but did, nevertheless, involve an increase in the plasma membrane abundance of GLUT1 and GLUT4. In contrast, palmitate caused a substantial reduction in insulin signaling and insulin-stimulated glucose transport, but was unable to antagonise the increase in transport elicited by palmitoleate. Our findings indicate that SFAs and MUFAs exert distinct effects upon insulin signaling and glucose uptake in L6 muscle cells and suggest that a diet enriched with MUFAs may facilitate uptake and utilisation of glucose in normal and insulin resistant skeletal muscle.

Abbreviations: non-esterified fatty acids (NEFAs), saturated fatty acids (SFAs), mono- and polyunsaturated fatty acids (MUFAs and PUFAs), protein kinase B (PKB), glycogen synthase kinase 3 (GSK3), glycogen synthase (GS), AMP-activated protein kinase (AMPK), glucose transporter (GLUT), phosphatidylinositol 3-kinase (PI3K), serine palmitoyltransferase (SPT), α-(methylamino)-isobutyric acid (Me-AIB)
INTRODUCTION

Upon binding to its receptor, insulin regulates numerous cellular responses including, for example, stimulation of glucose uptake, glycogen synthesis and lipogenesis in key target tissues such as skeletal muscle and white fat, whilst simultaneously suppressing hepatic gluconeogenesis. However, impaired insulin responsiveness (i.e. insulin resistance) of the above mentioned tissues leads to dysregulation in the hormonal control of these important physiological responses and contributes to the development of type 2 diabetes mellitus. The mechanisms that contribute to the pathogenesis of insulin resistance are poorly understood but non-esterified fatty acids (NEFAs) have been implicated strongly in its development in tissues such as skeletal muscle [1,2]. In particular, there is considerable evidence showing that increased circulating saturated fatty acids (SFAs) promote insulin resistance and reduce skeletal muscle glucose utilisation [3,4].

One potential mechanism that may help explain lipid-induced insulin resistance is the reciprocal relationship that exists with respect to skeletal muscle metabolism of glucose and fatty acids as proposed by Randle in the early 1960s [3]. The Randle hypothesis or the “glucose-fatty acid cycle”, as it is also known, proposes that increased availability of free fatty acids to muscle would lead to elevated mitochondrial acetyl CoA/CoA and NADH/NAD ratios, which, in turn, would promote inhibition of pyruvate dehydrogenase, resulting in increased citrate levels and an attendant inhibition in phosphofructokinase activity. The effect of inhibiting this latter enzyme would be an overall reduction in glycolytic flux and accumulation of glucose-6-phosphate, which would inhibit hexokinase and thereby decrease glucose uptake. However, the finding that an increase in circulating fatty acids can impair glycogen synthesis in human muscle prior to any significant rise in intramuscular glucose-6-phosphate [4] implies the existence of additional regulatory mechanisms to the Randle cycle.

Indeed, recent work from our laboratory and that of others has shown that palmitate, the most prevalent circulating SFA, can impair the insulin-dependent activation of protein kinase B (PKB, also known as Akt) [5], which has been implicated strongly in the
hormonal regulation of glucose transport and glycogen synthesis [6]. This inhibition appears to rely upon de novo synthesis of ceramide from palmitate, since inhibitors of serine palmitoyltransferase (SPT), the enzyme that commits palmitoyl-CoA to ceramide synthesis, antagonise the inhibitory effects of palmitate on PKB [5]. Indeed, short chain cell permeant analogues of ceramide mimic the inhibitory effect of palmitate on PKB activation and such studies have revealed that this inhibition, depending on the experimental system used, involves the ceramide-mediated activation of either a type 2A-like phosphatase, which dephosphorylates PKB on its regulatory phosphorylation sites, or of an atypical protein kinase C (PKC) isoform, which physically interacts with and negatively regulates PKB activation [7-12].

Intriguingly, while SFAs have been implicated in the development of insulin resistance, some mono- and poly-unsaturated fatty acids (MUFAs/PUFAs) appear to either have no adverse effects or positively enhance insulin action [13,14]. Many studies carried out in whole animals have reported contradictory results with respect to the effects of MUFAs and PUFAs on insulin action. This inconsistency may, in part, be explained by the fact that dietary fat is often administered to animals as a mixture of several different fatty acids and there is good evidence that insulin sensitivity is influenced by the dietary fatty acid profile (for review see [15]). However, distinct effects of individual saturated and unsaturated fatty acids have been documented in vitro on cell proliferation (for review see [16]), and perhaps the best characterized example seems to be the ability of MUFAs to protect against β-cell apoptosis induced by SFAs [17-19]. In addition, oleate has been shown to stimulate basal glucose uptake in rat adipocytes, probably by mediating changes in the intrinsic activity of glucose transporters [20]. A previous attempt to characterise the effects of fatty acids in murine C2C12 myotubes focused mainly on the effect of SFAs on glycogen synthesis [21], as these cells do not particularly serve as a good model for investigating GLUT4-mediated glucose uptake. Recent work from our laboratory has characterised the inhibitory effects of palmitate on insulin-stimulated glucose uptake in L6 myotubes [5]. In the present study we have investigated the effect of MUFAs (in particular the effect of palmitoleate) and PUFAs on insulin signaling and glucose metabolism. We show that, unlike SFAs, palmitoleate increases basal glucose uptake by
inducing an increase in GLUT1 and GLUT4 abundance in the plasma membrane. Although the precise mechanism underlying this change in carrier abundance remains currently unknown, it appears that exposing muscle cells to palmitoleate results in enhanced glucose oxidation and glycogen synthesis and over-rides the inhibitory effect of palmitate on insulin-stimulated glucose uptake.

EXPERIMENTAL

Materials - α-Minimal essential medium (α-MEM), foetal bovine serum (FBS), and antibiotic/antimycotic solution were from Life Technologies (Paisley, Scotland, U.K.). All other reagent-grade chemicals including insulin, BSA, palmitate, palmitoleate, oleate, linoleate, linolenate were obtained from Sigma-Aldrich (Poole, U.K.). Wortmannin, LY-294002, rapamycin, SB-203580, PD-98059 and Ro 31-8220 were purchased from Calbiochem-Novabiochem Ltd. (Nottingham, U.K.), while genistein and SB-415286 from Tocris (Bristol, U.K.). Antibodies against total PKB and GS, phospho-PKB-Ser473, phospho-p70S6K-Thr389, phospho-GSK3β–Ser9 were from New England Biolabs (Hitchin, Herts, U.K.). Anti-GLUT1 antibody was purchased from Chemicon International (Hampshire, U.K.), anti-GLUT4 monoclonal antibody (clone 1F8) was obtained from Genzyme Diagnostics (Cambridge, MA, U.S.A.), the polyclonal antibody against the SNAT2 System A transporter was generated in house as described previously [22], while a monoclonal antibody against the α1 subunit of the Na+/K+-ATPase was a kind gift from Dr K Sweadner (Massachusetts, U.S.A.). Antibody against phosphoglycogen synthase-Ser641-645 (residues 635-650 of murine GS, RYPRPVpSVpSPSLSR) was raised in house by the MRC Protein Phosphorylation Unit in conjunction with the Division of Signal Transduction and Therapy at the University of Dundee. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from New England Biolabs as well (Hitchin, Herts, U.K.). Complete protein phosphatase inhibitor tablets were purchased from Boehringer-Roche Diagnostics (Basel, Switzerland). U-[14C]-D-glucose and 1-[14C]-oleic acid were purchased from Amersham Biosciences,
while \([^{14}\text{C}]\)-Me-AIB and 2-deoxy-[\(^3\text{H}\)]-d-glucose from PerkinElmer Life Sciences (Cambridge, U.K.).

**Cell culture** - L6 muscle cells were cultured to myotubes as described previously [23] in \(\alpha\)-MEM containing 2\% (v/v) FBS and 1\% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin, 250 ng/ml amphotericin B) at 37 °C with 5\% CO\(_2\).

**Fatty acid treatment** - L6 myotubes were maintained in medium containing 2\% FBS (v/v), 1\% (v/v) antibiotic/antimycotic and 2\% BSA (w/v) for up to 24 h, and serum deprived during the last 2 h prior to any treatment with insulin. L6 muscle cells were exposed to fatty acids that had been conjugated to BSA (fraction V) for times and at concentrations indicated in the figure legends (controls were incubated with vehicle containing BSA but lacking the fatty acid) and incubated with insulin (100 nM) in the penultimate 15 min incubation period for immunoblotting analysis or 30 min for glucose uptake assays.

**Cell lysis and cellular fractionation** - L6 myotubes were incubated for the time and with the appropriate amount of effectors described in the figure legends. Following appropriate treatment, cells were washed three times by aspiration with 0.9\% (w/v) ice-cold saline and then lysed using lysis buffer [50 mM Tris, pH 7.4, 0.27 M sucrose, 1 mM Na-orthovanadate pH 10, 1 mM EDTA, 1 mM EGTA, 10 mM Na-\(\beta\)-glycerophosphate, 50 mM NaF, 5 mM Na pyrophosphate, 1\% (w/v) Triton X-100, 0.1\% (v/v) 2-mercaptoethanol, 0.1 mM microcystin-LR and protease inhibitors]. Whole cell lysates were centrifuged (15,000 \(g\), 4 °C for 10 min) and stored at -20 °C. In some experiments, confluent L6 myotubes were sub-fractionated following pre-treatment with NEFAs and/or insulin. Subcellular membranes from L6 myotubes were isolated as described previously [22,24]. Following treatment, cells from five (15 cm) dishes were harvested, pooled, and gently pelleted. The cell pellet was homogenized (250 mM sucrose, 20 mM HEPES, 5 mM Na\(_3\), 2 mM EGTA, pH 7.4, plus one protease inhibitor tablet per 50 ml) and subjected to a series of differential centrifugation steps to isolate crude cell
membranes that were subsequently fractionated on a discontinuous sucrose gradient (32, 40, and 50% sucrose by mass) at 210,000 g for 2.5 h. Membranes from top of the 32% sucrose cushion (plasma membrane fraction) were recovered and subsequently used for immunoblotting. The protein content of membrane samples was determined using the Bradford assay [25].

**SDS/PAGE and immunoblotting** - Cell lysates (50 µg protein) and plasma membrane fractions from L6 myotubes (20 µg protein) were subjected to SDS/PAGE on 10% resolving gels and transferred on Immobilon-P or Hybond-C membranes (Millipore, Harts, U.K.), as described previously [23]. Membranes were probed with primary antibodies against proteins of interest. Primary antibody detection was performed using either horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG and visualized using enhanced chemiluminescence (Pierce-Perbio Biotechnology, Tattenhall, Cheshire, U.K.) on Kodak X-OMAT film (Eastman-Kodak, Rochester, Kent, U.K.).

**Glucose and amino acid uptake** - L6 myotubes were incubated with insulin and/or fatty acids in the absence or presence of various kinase inhibitors at times and concentrations indicated in figure legends. The inhibitors were added 15 min prior to fatty acid treatment. Cells were washed three times with warm HEPES-buffered saline (HBS; 140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, pH 7.4). Glucose uptake was assayed by incubation with 10 µM 2-deoxy-[³H]-D-glucose (1 µCi/ml) for 10 min as described previously [23,26], while for amino acid transport activity cells were incubated for 10 min with 10µM [¹⁴C]-Me-AIB (47.6 kBq/ml). Nonspecific binding was determined by quantifying cell-associated radioactivity either in the presence of 10 µM cytochalasin B or in the presence of a saturating dose of unlabeled Me-AIB. Radioactive medium was aspirated prior to washing adherent cells three times with 0.9% (w/v) ice-cold saline. Cells were subsequently lysed in 50 mM NaOH and radioactivity quantified using a Beckman LS 6000IC scintillation counter. Protein concentration in cell lysates was determined using the Bradford reagent as described previously [25].
[1-14C] Oleic acid uptake – L6 myotubes cultured in six-well plates were supplemented with medium containing 0.1 µCi/ml [1-14C]-oleic acid for the times indicated in the figure legend. At specified time points the incubation medium was carefully removed and set aside. Muscle cells were then washed three times by aspiration with PBS prior to being solubilized in 50 mM NaOH. Radioactivity in the reserved media and solubilized cell lysate was then determined by liquid scintillation counting.

Glycogen synthesis and glucose oxidation assay – Following treatment of muscle cells with insulin or palmitoleate as indicated in the figure legends, muscle cells were incubated with HEPES-Buffered Saline (HBS; 140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO4, 1 mM CaCl2, pH 7.4) containing 5 mM glucose and 2 µCi/ml [U-14C-D-glucose] for 1 h at 37 °C. Following this incubation period, the culture medium was carefully removed and set aside in a culture flask for analysis of liberated [14C]-CO2, whilst the cells were used for determination of label into cellular glycogen. Briefly, a glass fibre filter (Whatman) was dipped in 1 M KOH and suspended over the medium in the culture flask. The medium was then acidified by injection of 6% (by volume) perchloric acid and the flasks were placed at 37°C with mild agitation for 2 h. The amount of labeled CO2 released from the medium and captured on the filters was then measured by liquid scintillation counting. For analysis of glycogen synthesis, cells were lysed in 10 M KOH and boiled for 30 min. To assist precipitation, 5 mg / ml glycogen was added together with 66 % ethanol and lysates held overnight at 4 °C. Following this period samples were centrifuged, the supernatant was removed by aspiration and ethanol (66%) was added again. Samples were kept at -20 °C for 4 h prior to being centrifuged at 13,000 g for 20 min. The glycogen-containing pellet was dissolved in water and radioactivity associated with the pellet measured by scintillation counting.

Statistical analyses - Data analysis was performed using GraphPad Prism software and considered statistically significant at values of p<0.05. Bands from immunoblots were quantified using ImageJ software.
RESULTS

In an attempt to assess the effects of MUFAs (Fig 1) on basal glucose uptake, we initially performed time and dose response studies. Fig 1A shows that glucose uptake was progressively stimulated with increasing exposure time to 0.75 mM palmitoleate (16:1). Maximal stimulation was achieved by 16 h as there were no significant increases in uptake beyond this incubation period. The increase in glucose uptake induced by the MUFA was also dose-dependent being maximally stimulated at palmitoleate concentrations of 0.75 mM (Fig 1B). Given that in previous work we have shown that 0.75 mM palmitate (16:0) for 16 h induces a maximal suppression in the insulin-dependent regulation of key signaling intermediates such as PKB and GSK3 and that of glucose transport [5], subsequent incubation of muscle cells with fatty acids were conducted for 16 h using 0.75 mM. The increase in glucose uptake was not restricted to palmitoleate, but was also observed in response to a 16 h incubation of L6 myotubes with equivalent concentrations of oleate (18:1), linoleate (18:2) and α-linoleneate (18:3) (Fig 1C). As shown in Fig 1D, insulin increased glucose uptake by ~50% and, in line with our previous work [5], this hormonal stimulation was abolished when cells had been preincubated for 16 h with 0.75 mM palmitate. In contrast, irrespective of whether insulin was present or not, palmitate was unable to suppress the increase in glucose uptake elicited by palmitoleate (Fig 1D). It is noteworthy, that maximal concentrations of both insulin and palmitoleate did not seem to exert any additive effect, suggesting that both stimuli either activate the same post-receptor signaling molecules or that signals initiated by each stimulus may ultimately converge upon a common end-point that promotes an increase in hexose uptake (e.g. recruitment and/or activation of glucose transporters).

Since half maximal stimulation of glucose uptake with MUFAs was observed between 4-8 h of incubation with the fatty acid, it was important to establish whether there was significant fatty acid uptake into the cells during this period. As shown in Fig 2A, within 8 h of incubation with 0.75 mM [1-14C]-oleic acid, muscle cells had accumulated a significant proportion of the labeled MUFA presented to them, which was associated with a corresponding loss in fatty acid-associated radioactivity from the culture medium. After 16 h, ~60% of the initial oleic acid presented remains in the culture media (Fig 2A).
Intriguingly, in parallel experiments, the impact of replacing the fatty acid containing medium every 4 h with fresh media supplemented with 0.75 mM oleate on glucose uptake was tested. This experimental manipulation led to a greater increase in glucose uptake (by ~50%) than that observed when the MUFA was not replenished in the incubation medium (Fig 2B). This finding implies that by maintaining the fatty acid concentration in the medium by regular renewal of the culture medium is likely to enhance the intracellular accumulation of the fatty acid and thereby raise its effect on glucose uptake in this cell system.

Given the increase in glucose uptake elicited by palmitoleate we next examined the fate of glucose taken up by muscle cells by monitoring glucose oxidation and glycogen synthesis. Palmitoleate treatment of muscle cells increased glucose oxidation by ~50% and was comparable to that seen in response to insulin, which was used as a positive control (Fig 3A). The MUFA also stimulated incorporation of glucose into glycogen by ~2-fold, but the increase was significantly lower than that induced by insulin (~4-fold, Fig 3B). The ability of insulin to stimulate glycogen synthesis is associated with a significant phosphorylation (inactivation) of GSK3 and an associated dephosphorylation of glycogen synthase on Ser\(^{641}\) and Ser\(^{645}\), two of the residues phosphorylated by GSK3 that play a critical role in regulating its activity. However, unlike insulin, palmitoleate did not promote GSK3 phosphorylation nor did it induce dephosphorylation of these GS residues (Fig 3C).

To assess the possibility that MUFAs may either activate molecules implicated in insulin signaling or antagonise the suppressive effect of SFAs on molecules mediating the insulin signal to processes regulating fuel use, we immunoblotted lysates from muscle cells following incubation with maximally effective concentrations of insulin, palmitate and/or palmitoleate with phospho-specific antibodies to PKB, GSK3 or p70S6K. Fig 4 shows that an acute (15 min) insulin incubation induced a robust phosphorylation of all three kinases (lane 2), but that cell treatment with palmitate or palmitoleate alone for 16 h (lanes 3 and 5, respectively) had no effect on the phosphorylation of these kinases. However, when cells were incubated with palmitate for 16 h prior to an acute insulin
challenge, the insulin-dependent phosphorylation of PKB-Ser\textsuperscript{473}, GSK3β-Ser\textsuperscript{9}, and p70S6K-Thr\textsuperscript{389} was reduced substantially (lane 4). In contrast, the ability of insulin to induce phosphorylation of all three proteins was unaffected following incubation of myotubes with 0.75 mM palmitoleate (lane 6) and, as such, this MUFA was unable to antagonise the inhibitory effects of palmitate when the two fatty acids were presented to muscle cells simultaneously (lane 8, Fig 4). Another kinase implicated in the regulation of glucose uptake in response to stress stimuli (e.g. hypoxia, exercise) is the AMP-activated protein kinase (AMPK) [27]. However, analysis of AMPK phosphorylation on Thr\textsuperscript{172}, a residue considered to reflect the activation status of the kinase, revealed that there was no significant induction in phosphorylation of this site in response to cell treatment with palmitoleate compared with the activation induced by energy depletion using high concentrations of 2-deoxyglucose (data not shown).

There is evidence in the literature that certain fatty acids can induce activation of phosphatidylinositol 3-kinase (PI3K) [28], MAP kinase signaling [29] or modify, via palmitoylation, members of the Src-family of tyrosine kinases [30]. To assess whether the stimulatory effect of palmitoleate on glucose uptake involves activation of specific signaling pathways we investigated the effect of various inhibitors known to target the activation of select protein kinase cascades. Since it was not desirable to have these inhibitors present in the culture media for 16 h their ability to suppress the stimulatory effect of palmitoleate was monitored over 4 h, a period over which glucose uptake was stimulated significantly by the MUFA (Fig 1A). It is important to stress that the efficacy of each of the inhibitors used was confirmed in parallel control experiments (data not shown). None of the inhibitors tested, with the exception of rapamycin, was able to suppress the increase in glucose uptake elicited by palmitoleate (Fig 5A). Rapamycin caused a modest, but significant, reduction in the stimulation of glucose uptake by palmitoleate (the net increase in hexose uptake falling from 67% to 40% in the presence of the inhibitor). While this latter finding implies that mTOR may feature in the stimulatory action of palmitoleate, it is inconsistent with the lack of any stimulation in p70S6K (a downstream mTOR target) by the MUFA (Fig 4A, lane 5). PKC isoforms have also been implicated in the action of certain free fatty acids, and so in separate
experiments we tested whether Ro 31.8220, a bisindolemaleimide, that inhibits conventional and novel PKCs in the submicromolar range [31,32] and atypical PKCs in the micromolar range [32], could antagonize the stimulation in hexose uptake elicited by palmitoleate. Although Ro 31.8220 induced a dose-dependent inhibition in basal glucose transport, it was unable to prevent the net increase in hexose uptake upon cell incubation with palmitoleate (Fig 5B).

The finding that cycloheximide fails to prevent the increase in glucose uptake elicited by palmitoleate, suggests that de novo protein synthesis is not required to support the MUFAs stimulatory effect. By extension this implies that the fatty acid either alters the activity of resident transporters in the plasma membrane or affects their abundance. To test this possibility we assayed glucose transport over a range of extracellular glucose concentrations following cell treatment with or without palmitoleate. Fig 6 shows that glucose uptake displayed saturable Michaelis-Menten type kinetics and that following a 16 h incubation of muscle cells with 0.75 mM palmitoleate the capacity to transport glucose was significantly greater than that seen in non-treated myotubes. The data shown in Fig 6 was subsequently subjected to non-linear regression curve fit analysis using GraphPad Prism software to determine $V_{\text{Max}}$ and $K_M$. This analysis revealed that palmitoleate induced a significant (2.4-fold) increase in maximal transport from $924 \pm 153$ picomoles/min/mg protein to $2193 \pm 142$ picomoles/min/mg protein, whereas the concentration at which transport was half-maximal ($K_M$) was not altered significantly from $470 \pm 90 \, \mu M$ (values are mean $\pm$ SEM from three separate experiments conducted in triplicate at each hexose concentration).

To assess whether palmitoleate may have modified the cellular abundance or distribution of GLUT1 and GLUT4 we immunoblotted total cell membranes, as well as isolated plasma membranes from L6 cells following treatment with the MUFA. Consistent with the cycloheximide data shown in Fig 5A, we could not detect any increase in the total amounts of either GLUT1 or GLUT4 (Fig 7A). However, analysis of plasma membranes isolated by subcellular fractionation revealed that the abundance of both transporters was noticeably elevated following palmitoleate treatment (Fig 7B, about 3.5-fold for GLUT1.
and 1.7-fold for GLUT4). In line with the well documented insulin-dependent translocation of both these transporters in this particular muscle cell line [23,33,34], acute treatment of L6 myotubes with insulin induced a comparable increase in the cell surface abundance of both transporters (Fig 7A and 7B). In separate experiments, exploring the effects of palmitate on palmitoleate-induced transporter recruitment, we could not detect any inhibitory effect of the SFA (data not shown) consistent with the uptake data shown in Fig 1D. It is also noteworthy that the ability of palmitoleate to induce an increase in cell surface GLUT1/GLUT4 content with an associated enhancement in glucose uptake does not form part of a generalized cell response to increase nutrient uptake. Like GLUT1 and GLUT4, the SNAT2 (System A) amino acid transporter is also recruited to the plasma membrane in response to insulin [24], but unlike the glucose transporters it does not undergo an increase in cell surface abundance in response to palmitoleate (Fig 8A and 8B). This finding is fully consistent with the lack of any stimulation in functional System A transport over the 16 h incubation period with the MUFA (Fig 8C). The abundance of the α1 subunit of the Na⁺/K⁺-ATPase was used as a gel loading control to ensure that changes in immunoreactive content of the three carrier proteins were not due to aberrant loading of protein on SDS-gels.

DISCUSSION

Accumulation of intramuscular triglyceride and increased synthesis of fatty acid-derived metabolites such as ceramide are features commonly associated with an elevation in circulating NEFAs and are thought to play an important role in the pathogenesis of insulin resistance in skeletal muscle [5,12,21,35]. The composition of accumulated lipid in skeletal muscle has been reported as being highly reflective of dietary fat [36] and there is general acceptance that intake and deposition of saturated fat in particular, reduces the sensitivity of skeletal muscle to insulin [15]. Indeed, studies in rodents and human subjects have revealed that changing from a diet rich in SFA to one with a high proportion of monounsaturated fat improves glucose tolerance and insulin sensitivity [37,38]. Whilst the molecular basis of this beneficial shift in glucose utilisation and insulin sensitivity remains poorly understood it is possible that it may, in part, arise via
effects of unsaturated fatty acids on skeletal muscle, a primary target for insulin action and a major site of whole body glucose disposal [39]. To assess whether this may be the case the present work compared the effects of different NEFAs on insulin signaling and glucose transport in cultured L6 skeletal muscle cells. We show that in line with previous work [5], incubation of muscle cells with palmitate (16:0) induces a state of insulin resistance as judged by the reduction in PKB-directed insulin signaling and the associated loss in insulin-stimulated glucose transport. In contrast, incubation of muscle cells with MUFAs (16:1, palmitoleate; 18:1, oleate) or PUFAs (18:2, linoleate; 18:3, linoleneate) led to a significant increase in basal glucose uptake. Some of the glucose taken up under these circumstances is oxidised, but a significant proportion is also channeled into synthesis of glycogen. Interestingly, the increase in glycogen synthesis induced by the MUFA is not dependent on dephosphorylation of GS on GSK3 target residues, which represent the principal sites of phosphate loss in response to insulin and which underpin the hormonal activation of GS [40]. Whilst we cannot exclude the possibility that activation of GS might be mediated by dephosphorylation of other sites, a more likely mechanism by which the enzyme is stimulated is via an increase in cytosolic glucose-6-phosphate [41]. The concentration of this allosteric activator of GS will increase as a direct consequence of the greater influx of glucose into cells exposed to the MUFA. The finding that insulin induces a much greater activation of glycogen synthesis than palmitoleate is fully in keeping with the fact that the hormone not only elevates glucose-6-phosphate via stimulation of glucose uptake, but will also simultaneously promote the dephosphorylation and thereby greater activation of GS.

We hypothesised that the increase in glucose uptake elicited by palmitoleate may involve activation of signaling molecules regulating glucose uptake in response to stimuli such as insulin and various stress-inducing agents [6,27]. This possibility is supported by the observation that oleate, but not palmitate, activates PI3-kinase in human breast cancer cells and that this underlies the increase in cell proliferation induced by this MUFA [28]. However, our findings indicate that it is highly unlikely that palmitoleate stimulates PI3-kinase in L6 muscle cells given that the fatty acid does not activate PKB (a molecule whose activation is dependent on PIP3, a PI3-kinase reaction product) and its stimulatory
effect upon glucose uptake was insensitive to PI3-kinase inhibitors (wortmannin and LY294002). Moreover, palmitate, which ablates insulin-stimulated glucose uptake and causes a significant reduction in the hormonal activation of PKB via a ceramide and PKCζ-dependent mechanism [5], was unable to antagonise the MUFA-induced stimulation in glucose uptake, most likely because palmitoleate acts at a point downstream of PKB. We currently do not know the precise signaling proteins involved in the MUFA response, but our data does not support the involvement of p38 MAP kinase, mTOR and AMPK, which can be activated by changes in nutrient availability (including that of certain NEFAs) [27,42,43], nor, based on using a variety of kinase inhibitors, does it support the participation of tyrosine kinases, classical MAP kinases or members of the PKC family.

Unlike insulin, which induces a rapid (within minutes) stimulation of glucose uptake, the increase observed in response to palmitoleate was slow and protracted suggesting that the effects of this MUFA may depend upon increased synthesis of glucose transporters. However, this possibility is negated by the lack of any sensitivity to cycloheximide and the observation that there were no detectable changes in cellular GLUT1 or GLUT4 content following 16 h of incubation with the MUFA. The increase in maximal glucose transport activity (~2.4 fold) elicited by palmitoleate does rely upon a rise in the plasma membrane abundance of both GLUT1 and GLUT4. This suggests that palmitoleate must either induce recruitment of these transporters from a subcellular compartment in a manner similar to that seen in response to insulin and/or promote retention of surface carriers by reducing their endocytic recycling. An elevation in plasma membrane GLUT1 and GLUT4 in response to unsaturated fatty acids is not unprecedented and has been reported previously in 3T3-L1 adipocytes following exposure to arachidonic acid [44]. Whilst the mechanism by which this PUFA increases cell surface GLUTs in fat cells remains unknown the associated increase in basal glucose uptake was partially sensitive to inhibitors of lipoxygenase and, in part, reliant upon PPARγ [44]. It is plausible that prolonged exposure of muscle cells to NEFAs may lead to changes in the phospholipid composition of the plasma membrane influencing membrane fluidity with potential knock-on consequences for the activity of solute transporters resident in the plasma
membrane. However, neither the work of Nugent et al. [44] nor pilot studies conducted in our laboratory were able to detect any significant changes in membrane fluidity (data not shown). Moreover, it is noteworthy, that unlike GLUT1 and GLUT4, the SNAT2 amino acid transporter, which is recruited to the plasma membrane in response to insulin [24] and whose insulin-stimulated activity is suppressed by palmitate [45], does not undergo changes in cell surface abundance or activity following cell treatment with palmitoleate. These latter findings lend credence to the idea that the increase in glucose transport elicited by palmitoleate is unlikely to be part of a generalized response to the MUFA arising from non-specific effects on membrane fluidity.

Taken together, our results suggest that prolonged exposure of L6 skeletal muscle cells to increased concentrations of palmitoleate stimulates basal glucose uptake by promoting an increase in plasma membrane GLUT1 and GLUT4 abundance. Although the signaling processes that underlie this effect remain poorly understood, our studies exclude a number of potential candidates that have been implicated in the regulation of glucose transport by insulin, nutrients and stress-inducing agents. Moreover, whilst evidence exists in the literature showing that SFAs and MUFAs exert differential effects upon cell proliferation and apoptosis [18,28,42], our study shows, for the first time, that saturated and unsaturated fatty acids exert distinct effects upon glucose uptake in skeletal muscle cells. Taking into account that the average dietary intake of MUFAs (of which ~90% is oleate) accounts for ~14% of total energy intake, an amount that is comparable to the intake of SFA (PUFAs contribute less, ~7% of energy intake), our findings suggest that a change in dietary fatty acid composition to one weighted in favour of MUFAs and PUFAs may enhance glucose uptake and disposal in both normal and insulin resistant skeletal muscle. This proposition is consistent with studies showing that diets rich in MUFAs protect against the risk of cardiovascular disease and improve glucose homeostasis in type 2 diabetes [46,47].
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LEGENDS

Figure 1 Effect of MUFAs/PUFAs on basal and insulin-stimulated glucose uptake in L6 myotubes

Uptake of 2-deoxyglucose was assayed in L6 myotubes following (A) incubation with 0.75 mM palmitoleate for the times indicated, (B) incubation of muscle cells with palmitoleate for 16 h at the concentrations indicated, (C) incubation with 0.75 mM of either oleate, palmitoleate, linoleate or linolenate for 16 h, (D) incubation with 0.75 mM palmitate and/or 0.75 mM palmitoleate for 16 h followed by incubation in the absence and presence of insulin (100 nM) during the last 30 min of incubation with fatty acids. Controls were treated with vehicle solution alone. Values are expressed as a fold change relative to untreated control (values are means ± SEM of three separate experiments each performed in triplicate). *p<0.05, compared with the control (vehicle only), N.S., non-significant change.

Figure 2 Uptake of oleate in L6 myotubes

(A) L6 myotubes were treated with 0.75 mM oleate containing 0.1 µCi/ml [1-14C] for the indicated times. Radioactivity in media and lysates was measured as described in Methods. Data are mean dpm/well ± SEM from six experimental determinations. *p<0.05, compared with the 2h group (cells), # p<0.05 compared with the 2h group (supernatant). (B) Cells were treated either with 0.75 mM oleate for 16 h or medium was refreshed with 0.75 mM oleate every 4 h over a period of 16 h. Cells were then assayed for glucose uptake. *p<0.05, compared with the control (vehicle only).

Figure 3 Effects of palmitoleate on glucose oxidation, glycogen synthesis and phosphorylation of glycogen synthase in L6 myotubes

L6 myotubes were pre-incubated in the absence or presence of 0.75 mM palmitoleate and then medium was replaced with HBS buffer containing 5 mM glucose (2 µCi/ml [U-14C]) in the absence or presence of insulin. Subsequently, culture medium was processed for glucose oxidation (A) and cells were lysed for determination of glycogen synthesis (B), as described in Methods. In (C) L6 cells were treated either with 100 nM insulin for 15 min or with 0.75 mM palmitoleate for 16 h, then lysed and immunoblotted with phospo-
specific antibodies against GSK3β-Ser⁹, GS-Ser⁶⁴¹-⁶⁴⁵ or total GS. Immunoblots are representative of three separate experiments. *p<0.05, compared with the control (vehicle only).

**Figure 4** Effects of palmitate and palmitoleate on the phosphorylation/activation status of PKB, GSK3, p70S6K in L6 myotubes

L6 myotubes were pre-incubated with 0.75 mM palmitoleate and/or palmitate for 16 h prior to incubation with either 100 nM insulin for 15 min. Whole cell lysates were prepared and immunoblotted with phospho-specific antibodies against PKB-Ser⁴⁷³, GSK3β-Ser⁹, p70S6K-Thr³⁸⁹ and total PKB. Immunoblots from three separate experiments were quantified and presented in the corresponding bar graphs (values are mean ± SEM).

**Figure 5** Effect of protein kinase and protein synthesis inhibitors on palmitoleate-induced glucose uptake in L6 myotubes

(A) L6 myotubes were pre-incubated in the absence or presence of one of the following inhibitors: 100 nM wortmannin (wort); 10 µM LY-294002 (LY); 100 nM rapamycin (rap); 50 µM SB-415286 (SB4); 10 µM SB-203580 (SB2); 10 µM PD-98059 (PD); 5 µM cycloheximide (ChX); 100 µM genistein (gen) or (B) with either 0.1 µM or 1 µM Ro 31.8220 for 15 min prior to incubation with 0.75 mM palmitoleate for a further 4 h prior to assaying 2-deoxyglucose uptake. Values are means ± SEM of at least three separate experiments each performed in triplicate. Asterisks signify a significant (p<0.05) increase compared with the appropriate untreated control, whereas the hash symbol signifies a significant inhibition (p<0.05) compared to palmitoleate treatment alone.

**Figure 6** Effects of palmitoleate upon the kinetics of 2-deoxyglucose uptake in L6 myotubes

2-deoxyglucose transport was assayed over a range of extracellular glucose concentrations following cell treatment with (filled circles) or without (open circles) palmitoleate (0.75 mM, 16 h). The data were subsequently subjected to non-linear regression curve fit analysis using GraphPad Prism software to determine V₉₉₉₉ and Kₐₐ₉ₐ.
Palmitoleate induced a significant (2.4-fold) increase in \( V_{\text{Max}} \) from 924±153 picomoles/min/mg protein to 2193±142 picomoles/min/mg protein, whereas the concentration at which transport was half-maximal (\( K_M \)) was not altered significantly from 470±90 µM (values are mean ± SEM from three separate experiments conducted in triplicate at each hexose concentration). *\( p<0.05 \) compared with the appropriate untreated control for each hexose concentration.

**Figure 7 Effect of palmitoleate on GLUT1 and GLUT4 plasma membrane abundance**

(A) L6 myotubes were incubated with either 0.75 mM palmitoleate for 16 h or with 100 nM insulin for 30 min (control cells were treated with vehicle alone). Following this incubation period cells were harvested and total cell or plasma membrane fractions isolated as described in methods. Membrane fractions were loaded onto SDS gels and immunoblotted with antibodies against GLUT1, GLUT4 and Na\(^+\)/K\(^+\)-ATPase. Immunoblots are representative of three separate experiments. (B) GLUT1 and GLUT4 immunoreactive bands from plasma membrane fractions were quantified and abundance quantified relative to \( \alpha_1\)-Na\(^+\)/K\(^+\)-ATPase, a plasma membrane protein not affected by fatty acid treatment. The data (expressed as a ratio) represent means ± SEM of three separate experiments. The asterisks represent a significant change from the untreated control (\( p<0.05 \)).

**Figure 8 Effect of palmitoleate on System A transporter (SNAT2) and Me-AIB uptake**

(A) L6 myotubes were incubated with either 0.75 mM palmitoleate for 16h or with 100 nM insulin for 30 min (control cells were treated with vehicle alone). Following this incubation period cells were harvested and total cell or plasma membrane fractions isolated as described in methods. Membrane fractions were loaded onto SDS gels and immunoblotted with antibodies against SNAT2 and Na\(^+\)/K\(^+\)-ATPase. (B) SNAT2 immunoreactive bands from plasma membrane fractions were quantified and abundance quantified relative to \( \alpha_1\)-Na\(^+\)/K\(^+\)-ATPase, a plasma membrane protein not affected by fatty acid treatment. The data (expressed as a ratio) represent means ± SEM from two
experiments. (C) L6 myotubes were incubated with 0.75 mM palmitoleate for various times and then assayed for Me-AIB uptake as described in the methods section. Values are means ± SEM of three separate experiments each performed in triplicate. The asterisk represents a significant change from the untreated control ($p<0.05$). N.S., non-significant change.
Figure 1

(A) Palmitoleate (0.75mM) vs. 2-deoxyglucose uptake (fold change over basal)

(B) Palmitoleate (mM) vs. 2-deoxyglucose uptake (fold change over basal)

(C) 2-deoxyglucose uptake (fold change over basal) with various fatty acids and insulin

(D) 2-deoxyglucose uptake (fold change over basal) with palmitate and palmitoleate

Figure 1
Figure 2
Figure 3

(A) Glucose oxidation (nmol/h)

(B) Glycogen synthesis (nmol glucose/min/mg protein)

(C) Western blot images of p-GSK3β Ser9, p-GS Ser641-645, and GS total

Figure 3
Figure 5

(A) 2-deoxyglucose uptake (pmol/min/mg of protein)

- Wort  LY  Rap  SB4  SB2  PD  ChX  Gen

(B) 2-deoxyglucose uptake (pmol/min/mg of protein)

Ro 31.8220 - 0.1µM 1µM
Figure 6

- palmitoleate
+ palmitoleate

2-deoxyglucose uptake (pmol/min/mg of protein)

2-deoxyglucose (µM)
Figure 7

(A) Total membranes

GLUT1

GLUT4

Na⁺/K⁺-ATPase

(B) Fold change in PM content relative to Na⁺,K⁺-ATPase

GLUT1

GLUT4

Figure 7
Figure 8

(A) Total membranes

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(B) Plasma membranes

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Fold change in PM content relative to Na⁺,K⁺-ATPase

(C) Me-AIB Uptake (pmol/min/mg of protein)

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N.S.

Figure 8