Endocrine CONNECTIONS

Palmitate and oleate exert differential effects on insulin signalling and glucose uptake in human skeletal muscle cells

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Abstract

Saturated fatty acids are implicated in the development of insulin resistance, whereas unsaturated fatty acids may have a protective effect on metabolism. We tested in primary human myotubes if insulin resistance induced by saturated fatty acid palmitate can be ameliorated by concomitant exposure to unsaturated fatty acid oleate. Primary human myotubes were pretreated with palmitate, oleate or their combination for 12 h. Glucose uptake was determined by intracellular accumulation of [^{3}H]-2-deoxy-D-glucose, insulin signalling and activation of endoplasmic reticulum (ER) stress by Western blotting, and mitochondrial reactive oxygen species (ROS) production by fluorescent dye MitoSOX. Exposure of primary human myotubes to palmitate impaired insulin-stimulated Akt-Ser^{473}, AS160 and GSK-3β phosphorylation, induced ER stress signalling target PERK and stress kinase JNK 54 kDa isoform. These effects were virtually abolished by concomitant exposure of palmitate-treated myotubes to oleate. However, an exposure to palmitate, oleate or their combination reduced insulin-stimulated glucose uptake. This was associated with increased mitochondrial ROS production in palmitate-treated myotubes co-incubated with oleate, and was alleviated by antioxidants MitoTempo and Tempol. Thus, metabolic and intracellular signalling events diverge in myotubes treated with palmitate and oleate. Exposure of human myotubes to excess fatty acids increases ROS production and induces insulin resistance.

Key Words
- ER stress
- fatty acid
- glucose uptake
- human
- insulin signalling
- skeletal muscle

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Introduction

Excess dietary fat and sedentary lifestyle predispose to insulin resistance and type 2 diabetes. As skeletal muscle accounts for 80% of glucose disposal under insulin-stimulated conditions (1, 2), it is an important target tissue to study insulin action. Increased fat availability and reduced fatty acid oxidation in skeletal muscle from obese type 2 diabetic people favour ectopic storage of fat in muscle (3). This has been suggested to induce insulin resistance via generation of active fatty acid-derived metabolites, such as diacylglycerol (DAG), long-chain fatty acyl-CoA:s and ceramides. These metabolites can interfere with insulin signal transduction (4). Mitochondrial fat overload and incomplete oxidation of fatty acids have also emerged as players in pathogenesis

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of muscle insulin resistance (5). However, the molecular mechanisms whereby excess fatty acids lead to reduced insulin action are still incompletely understood.

Increased intake of saturated fatty acids leads to insulin resistance (6, 7). Conversely, diet high in monounsaturated fatty acids has been associated with improved insulin sensitivity (7, 8). Mediterranean diet is rich in monounsaturated fatty acids and low in saturated fats, and it has been shown to protect against major cardiovascular events (9) and T2D (10). However, the beneficial effect of monounsaturated fat on insulin sensitivity has been lost if the overall fat intake has been high (7). Taken together, these data indicate that both quality and quantity of dietary fat play a role in regulating insulin sensitivity.

Recent cell-based studies have provided evidence that co-incubation with monounsaturated fatty acid oleate protects against saturated fatty acid palmitate-induced insulin resistance (11, 12, 13). Since data on human skeletal muscle are limited, the current study was initiated to explore whether there is a similar interaction with different fatty acids in primary human muscle cells. We tested if adding oleate to palmitate would protect muscle cells from palmitate-induced insulin resistance.

Materials and methods

Participants

The study was reviewed and approved by the Ethical Committee of Department of Medicine, Helsinki University Central Hospital, and written informed consent was obtained from all subjects before participation. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. We studied 8 healthy nonsmoking men (Table 1). None of the subjects was taking any medications, and all had normal glucose tolerance in standard (75 g glucose) oral glucose tolerance test (WHO criteria). Subjects were studied after an overnight fast and were instructed to abstain from physical exercise for at least 72 h before the studies.

Table 1 Clinical characteristics of the healthy male volunteers studied.

N	8
Age (years)	55 ± 3
BMI (kg/m^2)	23.2 ± 0.7
Waist (cm)	90 ± 4
Hip (cm)	96 ± 1
Waist-to-hip ratio	0.94 ± 0.03
Systolic blood pressure (mmHg)	141 ± 6
Diastolic blood pressure (mmHg)	87 ± 4
Fasting plasma glucose (mmol/L)	5.5 ± 0.2
HbA1c (% (mmol/mol))	5.4 ± 0.1 (36 ± 1)
Fasting cholesterol (mmol/L)	5.4 ± 0.4
Fasting HDL cholesterol (mmol/L)	1.5 ± 0.2
Fasting LDL cholesterol (mmol/L)	3.3 ± 0.5
Fasting triacylglycerols (mmol/L)	1.4 ± 0.2

Data are presented as mean ± S.E.M.

Muscle cell preparation

Satellite cells were isolated, and primary human muscle cell cultures were established as described (14). In brief, after obtaining a biopsy from *vastus lateralis* muscle under local anaesthesia (10 mg/mL lidocain hydrochloride), the muscle tissue was dissected into small pieces, followed by trypsinization in a shaking water bath at +37°C for 1 h. Satellite cells were isolated and maintained in DMEM-F12 culture medium (Gibco/Thermo Fisher Scientific) containing 20% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 1% fungizone. Primary myoblasts were separated from non-myogenic cells with CD56-coupled magnetic beads (Miltenyi Biotec, Gologne, Germany). To obtain myotubes, myoblasts at 80% confluence were switched to differentiation medium containing 2% FBS for 5–7 days. The cells were serum starved for 2 h before any treatment in low-glucose DMEM supplemented with 0.5% fatty acid-free BSA and 4 mM L-glutamine. All chemical treatments were prepared in starvation medium supplemented with 2 mM L-carnitine (5).

Pre-treatment with BSA-conjugated fatty acids

Fatty acids conjugated with BSA and vehicle control–BSA-NaOH were prepared before each experiment based on previously reported procedure (15). Primary human myotubes were pretreated with 0.4 mM palmitate or 0.2 mM oleate, or with combination of 0.4 mM palmitate and 0.2 mM oleate, at 37°C for 12 h. In some experiments, myotubes were pretreated with antioxidants MitoTempo (50 µM) or Tempol (0.5 mM) for 12 h with or without the combination of palmitate (0.4 mM) and oleate (0.2 mM).

Glucose uptake

Glucose uptake was measured in triplicate, as described (14, 16). In brief, myotubes pretreated with or without fatty acids were stimulated with or without 100 nM insulin (Actrapid, Novo Nordisk) for 1 h at +37°C in the pre-treatment medium, washed from glucose and

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other compounds followed by 15 min incubation in glucose-free DMEM medium containing 0.5% fatty acid-free BSA, 10 µM 2-DG and [^{3}H]-2-deoxy-D-glucose (100 mCi/mmol). Myotubes were washed with ice-cold PBS and lysed in 0.4 M NaOH for 3 h on a rotating platform. Radioactivity of the cell lysate was measured in a scintillation counter.

Western blot analysis

Myotubes pretreated with or without fatty acids were stimulated with or without 100 nM insulin for 10 min at +37°C. Total and phosphorylated proteins were detected by Western blotting in PVDF membranes with primary antibodies from Cell Signaling: p-Akt^{Ser473} (#9271), total Akt (#9272), pAS160^{Thr642} (#4288), total AS160 (#2447), p-GSK-3β^{Ser9} (#9336), total GSK-3β (#9315), total PERK (#3192), p-JNK$^{Thr183/Tyr185}$ (#9251), total JNK (#9252), total AMPK (#2532), from Santa Cruz: p-PERK^{Thr981} (#sc-32577) and from Millipore: p-AMPK (#07-626). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody, visualized by enchanced chemiluminescence (Pierce ECL 2 Western Blotting Substrate, Thermo Scientific) and quantified using ImageJ software (NIH, http://rsbweb.nih.gov/ij/).

Measurement of mitochondrial ROS production

Mitochondrial ROS levels were measured using fluorescent dye MitoSOX (Molecular Probes, Invitrogen). Cells were incubated with dye at +37°C in pre-treatment medium. After 45 min, cells were washed from the dye, trypsinized and resuspended in normal Tyrode's solution (140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM D-glucose, pH 7.4) for measurement of fluorescent intensity in a BD Accuri C6 (BD Biosciences, San Jose, CA, USA). The dye was excited at 488 nm, and fluorescent emission was collected through FL3 channel.

Reagents

Trypsin, FBS, penicillin/streptomycin, low-glucose DMEM, L-glutamine, palmitic acid, oleic acid, Tempol, 2-DG, L-carnitine and sodium pyruvate were from Sigma. DMEM-F12, glucose-free DMEM, MitoTempo and fungizone were from Gibco. [^{3}H]-2-deoxy-D-glucose was from PerkinElmer. CD56-coupled magnetic beads were from Miltenyi Biotec (Gologna, Germany), and Actrapid insulin was from Novo Nordisk.

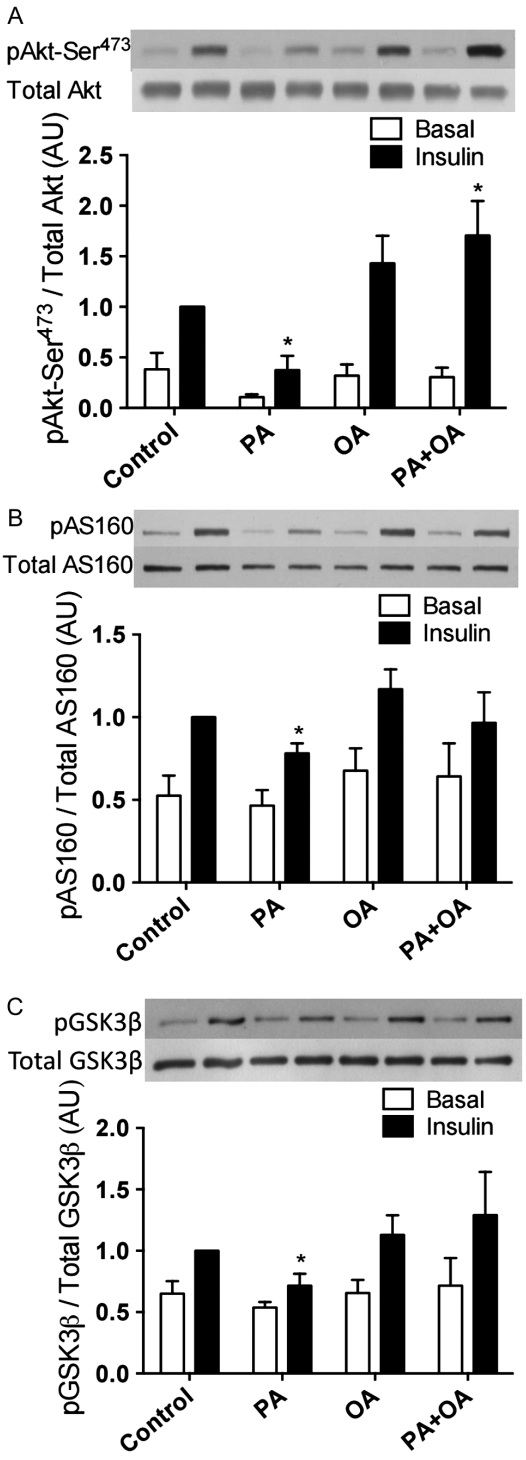

Figure 1
Phosphorylation of Akt-Ser^{473}, AS160 and GSK-3β. Human primary myotubes were incubated with 0.4 mM palmitate (PA), 0.2 mM oleate (OA) or their combination (PA + OA), for 12 h, and stimulated with 100 nM insulin for 10 min. Phosphorylation of Akt-Ser^{473} (A), AS160 (B) and GSK-3β (C) was assessed. Data are expressed as mean ± S.E.M. from 5 men. *$P < 0.05$ compared to insulin-stimulated control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test (A and B), Student's paired *t*-test (C). AU, arbitrary units.

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Statistical analysis

Data are presented as mean ± S.E.M. Statistical analyses were performed using GraphPad Prism statistical software (version 6). One- or two-way ANOVA with repeated measurements followed by Sidak's *post hoc* test for multiple comparisons or paired Student's *t*-test was used. *P* value ≤0.05 was considered statistically significant.

Results

Insulin signalling

Akt-Ser^{473} Primary human myotubes were incubated with palmitate, oleate or their combination for 12 h. Palmitate caused a profound reduction in insulin action on Akt-Ser^{473} signalling, whereas oleate did not have an effect. Co-incubation with palmitate and oleate completely restored and even enhanced insulin action on Akt-Ser^{473} phosphorylation (Fig. 1A).

AS160 Incubation with palmitate inhibited insulin-stimulated AS160 phosphorylation, and this was rescued by co-incubation with oleate (Fig. 1B).

GSK-3β Palmitate led to a decrease in insulin-stimulated GSK3β phosphorylation, which was recovered by co-incubation with oleate (Fig. 1C).

ER stress and AMPK signalling

PERK Incubation with palmitate for 12 h induced ER stress as observed by increased PERK phosphorylation. This was restored by co-incubation with oleate (Fig. 2A).

JNK Activation of stress kinase JNK signalling is implicated in response to ER stress (17). Palmitate increased the phosphorylation of JNK isoform of 54 kDa in muscle cells. Co-incubation with palmitate and oleate prevented JNK activation (Fig. 2B).

AMPK Phosphorylation of AMP-activated protein kinase was increased in myotubes pretreated with palmitate and oleate for 12 h (Fig. 2C).

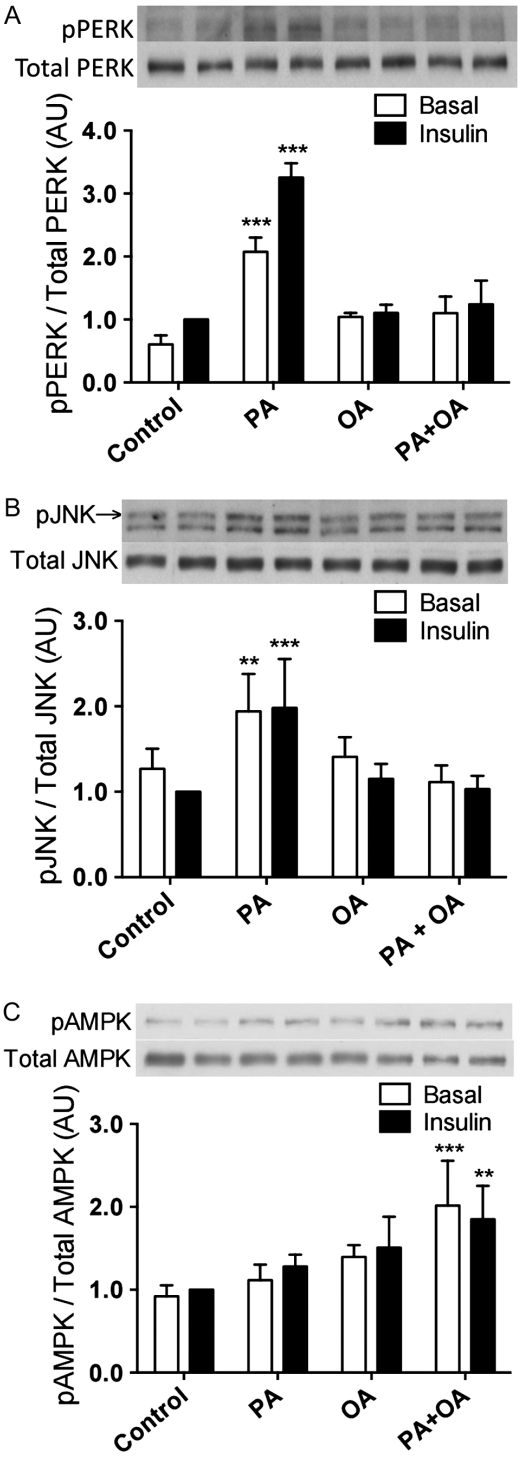

Figure 2
Phosphorylation of PERK, JNK54 and AMPK. Human primary myotubes were incubated with 0.4 mM palmitate (PA), 0.2 mM oleate (OA) or their combination (PA + OA), for 12 h, and stimulated with 100 nM insulin for 10 min. Phosphorylation of PERK (A), JNK54 (B) and AMPK (C) was assessed. Data are expressed as mean ± S.E.M. from 4 (A, B) and 5 (C) men. $^{**}P<0.01$ and $^{***}P<0.001$ compared to respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test. AU, arbitrary units.

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Glucose metabolism

Glucose uptake To analyse if the observed changes in signalling events are reflected at glucose metabolism, human primary myotubes were pre-exposed to palmitate, oleate or their combination for 12 h before analysis of insulin-stimulated glucose uptake. Exposure to either palmitate or oleate or their combination reduced basal or insulin-stimulated glucose uptake (Fig. 3).

Mitochondrial ROS production

Since metabolism of fatty acids leads to ROS production (18), we next analysed if development of insulin resistance of glucose uptake is related to ROS. Human myotubes were exposed for 12 h to palmitate, oleate or their combination. Mitochondrial ROS production was significantly increased in cells incubated with both palmitate and oleate, but not with palmitate or oleate alone (Fig. 4A).

Antioxidants We next tested if insulin responsiveness in myotubes exposed to fatty acids can be restored by using antioxidants. Concomitant exposure of myotubes to mitochondrial-targeted antioxidant MitoTempo restored and even enhanced basal and insulin-stimulated glucose uptake in myotubes treated with both palmitate and oleate (Fig. 4B). Exposure to MitoTempo alone increased

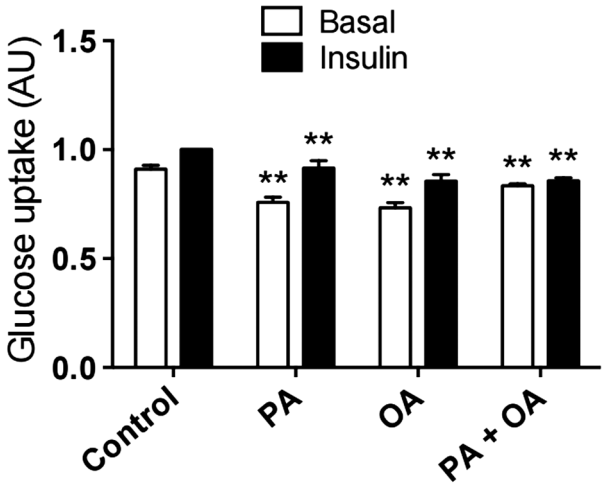

Figure 3
Glucose uptake. Human primary myotubes were pre-exposed to 0.4 mM palmitate (PA), 0.2 mM oleate (OA) or their combination for 12 h before analysis of glucose uptake with or without 100 nM insulin. Data (in pmol/mg protein/min) were normalized to insulin-stimulated glucose uptake (control) of each subject. Data are expressed as mean ± S.E.M. from 5 men $^{**}P<0.01$ vs respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test. AU, arbitrary units.

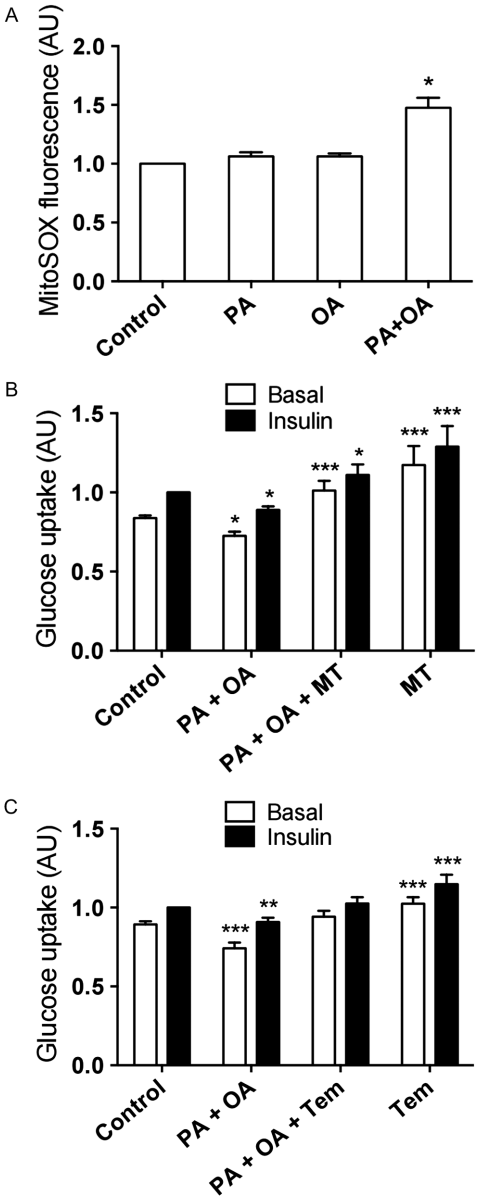

Figure 4
(A) Mitochondrial ROS. Human primary myotubes were pre-exposed to 0.4 mM palmitate (PA), 0.2 mM oleate (OA), or their combination for 12 h. Mitochondrial ROS production was determined as described in the 'Materials and methods' section. Data are expressed as mean ± S.E.M. from 5 men. $^{*}P<0.05$ compared to basal, one-way ANOVA with repeated measurements, Sidak's *post hoc* test. (B) Glucose uptake with MitoTempo. Human primary myotubes were pre-exposed for 12 h to a combination of 0.4 mM palmitate (PA) and 0.2 mM oleate (OA), with or without 50 µM MitoTempo (MT), before analysis of glucose uptake with or without 100 nM insulin. Data are expressed as mean ± S.E.M. from 6 men $^{*}P<0.05$ and $^{***}P<0.001$ vs respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test. (C) Glucose uptake with Tempol. Human primary myotubes were pre-exposed for 12 h to a combination of 0.4 mM palmitate (PA) and 0.2 mM oleate (OA), with or without 0.5 mM Tempol (Tem), before analysis of glucose uptake with or without 100 nM insulin. Data are expressed as mean ± S.E.M. from 6 men $^{**}P<0.01$ and $^{***}P<0.001$ vs respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test.

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basal and insulin-stimulated glucose uptake. Tempol, a small molecule cell-permeable SOD mimetic, also restored basal and insulin-stimulated glucose uptake in myotubes treated with fatty acids, and enhanced basal and insulin-stimulated glucose uptake in untreated cells (Fig. 4C).

Discussion

Here, we show that exposure of primary human myotubes from healthy donors to saturated fatty acid palmitate impairs insulin signalling, increases ER stress and activates stress kinase JNK signalling. Co-incubation of primary human myotubes with monounsaturated fatty acid oleate has a protective effect by restoring defects at insulin signalling, and preventing activation of ER stress and JNK. However, the changes at the level of signalling are not translated into metabolic events, since provision of myotubes with both palmitate and oleate results in impairment of insulin action on glucose uptake. This is related to increased mitochondrial ROS production, and can be ameliorated with antioxidants.

The strength of our study is that we used carefully controlled conditions to study effects of saturated fatty acid palmitate and unsaturated fatty acid oleate in myotubes from clinically well-characterized healthy donors. The weaknesses of our study are that we only studied men and the sample size was limited. Moreover, we used only one concentration of palmitate (0.4 mM) and oleate (0.2 mM). However, we performed an initial dose-response and time-course experiment using palmitate at different concentrations (0.1, 0.2 and 0.4 mM) for a duration of 4, 8 or 12 h, and we observed a time- and dose-dependent inhibition of insulin-stimulated Akt-Ser^{473} phosphorylation, with the maximum inhibition at 12 h by 0.4 mM palmitate (data not shown here). Therefore, we chose to expose the cells to 0.4 mM palmitate for 12 h in the subsequent experiments. Oleate concentration was chosen based on the study by Peng and coworkers where the negative effects of 0.5 mM palmitate could be combated by concomitant exposure to lower (0.1–0.2 mM or 0.3 mM) oleate concentrations (12). In the future, it would be important to compare the effects of different fatty acid concentrations, and the effects of different fatty acids with varying chain lengths and degrees of saturation, on metabolism in human skeletal muscle. It would also be interesting to compare the effects of exposing muscles to different proportions of unsaturated to saturated fats.

However, our observations on the effects of different fatty acids were robust. Interestingly, several *in vivo* studies have provided compelling evidence that substituting saturated fat for mono- and polyunsaturated fatty acids improves metabolic health and protects against coronary artery disease (7, 8, 9, 10, 19). In contrast, diet enriched in saturated fatty acids, a typical Western diet, aggravates insulin resistance (6, 7, 20). These data suggest that different fatty acids are indeed not alike, which was also apparent in our study where the effects of palmitate and oleate on intracellular signalling in human myotubes diverged. It is possible that differential routing of fatty acids contributes to variable impact of fatty acids. Clinically, this is evident, for example, in an overfeeding experiment, where overconsumption of saturated fatty acids leads to more increased hepatic and visceral fat storage than overconsumption of polyunsaturated fatty acids, despite similar weight gain (21). Moreover, an isocaloric diet rich in polyunsaturated fatty acids diminishes hepatic fat content and may improve insulin resistance in viscerally obese people (22).

Our data on human myotubes are also in agreement with other *in vitro* studies. The beneficial effect of adding monounsaturated oleate to cells exposed to saturated fatty acid palmitate has been convincingly demonstrated (11, 12, 13, 23, 24, 25, 26). Several mechanisms seem to be operative. In agreement to our observations, palmitate reduces insulin-stimulated phosphorylation of Akt. This is rescued by concomitant exposure to oleate (12, 25), an effect suggested to involve inhibition of protein phosphatase 2A (13). Palmitate activates diacylglycerol/PKC-θ/NF-κB pathway in mouse C2C12 myotubes, which results in increased interleukin-6 secretion and downregulation of PGC-1α and DGAT2. Co-incubation with oleate prevents these negative sequelae, which is suggested to be mediated by enhanced mitochondrial β-oxidation and by channelling palmitate into triglycerides (11). In another experiment, palmitate inhibited insulin signalling and increased mitochondrial ROS production. Addition of oleate to palmitate restored insulin signalling, increased ATP levels and cell viability (25). Similarly, palmitoleate, a monounsaturated fatty acid, protected from the negative effects of palmitate on glucose uptake (27).

ER stress has been suggested to play a role in the pathogenesis of insulin resistance (28). Here, we observed an activation of ER stress signalling, as reflected by increased PERK. This was associated with the activation of stress kinase JNK. Protein kinase JNK is activated via IRE1 of the unfolded protein response (UPR) signalling

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pathway (17), and it phosphorylates IRS at inhibitory serine residues and thus contributes to insulin resistance by blocking PI3K pathway (29, 30). We demonstrate that co-incubation with oleate protects human myotubes from palmitate-induced activation of ER stress and JNK. This is in agreement with a study by Peng and coworkers where co-incubation of L6 and C2C12 muscle cells with palmitate and oleate protected cells from ER stress (12). These observations have also been confirmed by others. Studies using mouse C2C12 myotubes and human myogenic cell line LHCN-M2 have revealed that co-incubation with oleate prevents palmitate-induced increase in ER stress markers (31). One possible mechanism relates to partitioning of intracellular lipids, as oleate directs palmitate into triglyceride pool. This reduces cellular active lipid metabolites such as phospholipids, DAG or ceramide (11, 12, 23). Palmitate-induced activation of ER stress markers is also prevented by AICAR and A-769662, compounds both of which activate AMPK. Expression of dominant-negative AMPK or AMPK inhibitor compound C prevents the beneficial effects of oleate on palmitate-induced ER stress, inflammation and insulin resistance (31). These data suggest that the protective effects of oleate may be mediated via AMPK activation. In agreement, we observed increased AMPK phosphorylation in myotubes co-incubated with palmitate and oleate, although this was not within the scope of our study.

ROS are formed in mitochondria during aerobic metabolism. Oxidative stress is present in many physiological as well as pathophysiological states such as exercise and insulin resistance (32, 33). Chronic hyperinsulinemia and exposure to cytokine TNFα, dexamethasone or palmitate induce insulin resistance in 3T3-L1 adipocytes and in L6 myotubes. Each of these insults increases mitochondrial superoxide production, and insulin resistance can be reversed using interventions such as mitochondrial antioxidants or MnSOD expression. Thus, mitochondrial superoxide production is a common denominator in multiple insults that contribute to insulin resistance (33). Exposure of skeletal muscle mitochondria to even low concentrations of fatty acids results in enhanced ROS production (18). High-fat diet increases H_2O_2 emitting potential of muscle mitochondria and leads to changes in redox balance and redox buffering capacity. Moreover, insulin resistance following a diet with high-fat content can be overcome by mitochondrial-targeted antioxidant or overexpression of mitochondrial-targeted catalase which catalyzes conversion of H_2O_2 to H_2O (34). Overall, our data are in agreement with these observations. When we exposed primary human myotubes to fatty acids, insulin action on glucose uptake was reduced, and an exposure to a combination of palmitate and oleate increased mitochondrial ROS production. When we treated the fatty acid-exposed cells with antioxidants, there was an amelioration of insulin resistance. These data support the concept that energy excess leads to enhanced mitochondrial ROS production and insulin resistance.

In conclusion, while monounsaturated fatty acid oleate protected human muscle cells from palmitate-induced alterations in intracellular signalling, this did not result in protection against insulin resistance – insulin action on glucose uptake remained impaired. Overall, our data fit with the emerging concept that in a situation when energy is provided in excess of energy need, cells respond with development of insulin resistance (35). Therefore, calorie restriction and energy expenditure-increasing interventions, such as exercise, remain the cornerstones of treatment of people with insulin resistance.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
S M, Y H N and H A K designed the study; S M, Y H N, P S and H A K acquired the data. S M, Y H N and H A K analysed and interpreted the data. S M and H A K drafted the article, which was critically revised by Y H N and P S. All authors have approved the manuscript.

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