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ARTICLE

Oleate prevents saturated-fatty-acid-induced ER stress, inflammation and insulin resistance in skeletal muscle cells through an AMPK-dependent mechanism

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Abstract
Aims/hypothesis Although the substitution of saturated fatty acids with oleate has been recommended in the management of type 2 diabetes mellitus, the mechanisms by which oleate improves insulin resistance in skeletal muscle cells are not completely known. Here, we examined whether oleate, through activation of AMP-activated protein kinase (AMPK), prevented palmitate-induced endoplasmic reticulum (ER) stress, which is involved in the link between lipid-induced inflammation and insulin resistance.
Methods Studies were conducted in mouse C2C12 myotubes and in the human myogenic cell line LHCN-M2. To analyse the involvement of AMPK, activators and inhibitors of this kinase and overexpression of a dominant negative AMPK construct (K45R) were used.
Results Palmitate increased the levels of ER stress markers, whereas oleate did not. In palmitate-exposed cells incubated with a lower concentration of oleate, the effects of palmitate were prevented. The induction of ER stress markers by palmitate was prevented by the presence of the AMPK activators AICAR and A-769662. Moreover, the ability of oleate to prevent palmitate-induced ER stress and inflammation (nuclear factor-kappa B [NF-κB] DNA-binding activity and expression and secretion of *IL6*) as well as insulin-stimulated Akt phosphorylation and 2-deoxyglucose uptake was reversed in the presence of the AMPK inhibitor compound C or by overexpression of a dominant negative AMPK construct. Finally, palmitate reduced phospho-AMPK levels, whereas this was not observed in oleate-exposed cells or in palmitate-exposed cells supplemented with oleate.
Conclusions/interpretation Overall, these findings indicate that oleate prevents ER stress, inflammation and insulin resistance in palmitate-exposed skeletal muscle cells by activating AMPK.

Keywords AMPK · ATF3 · CHOP · ER stress · IL-6 · NF-κB · Oleate · Palmitate · PPAR · XBP1

Abbreviations

ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ATF3	Activating transcription factor 3
ATF6	Activating transcription factor 6
CHOP	CCAAT/enhancer-binding protein homologous protein
DAG	Diacylglycerol
eIF2	Eukaryotic initiation factor 2

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EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
GRP78	Glucose-regulated protein 78 (BIP)
HSP70	Heat shock protein 70
IκB	Inhibitor of κB
IRE-1α	Inositol-requiring 1 transmembrane kinase/endonuclease-1
LKB1	Liver kinase B1
NF-κB	Nuclear factor-κB
PBA	Phenylbutyric acid
PERK	Eukaryotic translation initiation factor-2α kinase 3
PKA	Protein kinase A
PKCθ	Protein kinase Cθ
PP2A	Ceramide-dependent phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
SIRT1	Silent information regulator T1
TRAF2	TNF-α-receptor-associated factor 2
UPR	Unfolded protein response

Introduction

It has long been recognised that elevated plasma NEFA cause insulin resistance in humans [1]. However, saturated and monounsaturated NEFA differ significantly in their contribution to insulin resistance [2]. It is generally accepted that saturated NEFA induce insulin resistance [2, 3], whereas monounsaturated NEFA increase insulin sensitivity in diabetic patients [4, 5] and healthy individuals [2]. The mechanisms underlying the association between elevated NEFA and insulin resistance are currently unclear but accumulating evidence points to a link between enhanced NEFA levels and activation of a chronic low-level inflammatory process [6]. Elevated saturated NEFA can induce inflammation and, thus, insulin resistance, through several mechanisms including diacylglycerol (DAG)-mediated activation of protein kinase Cθ (PKCθ) [7] and activation of Toll-like receptors [8]. Both mechanisms lead to activation of the pro-inflammatory transcription factor nuclear factor-kappaB (NF-κB), which has been linked to fatty acid-induced impairment of insulin action in skeletal muscle [9]. Once activated, NF-κB regulates the expression of multiple inflammatory mediators, including IL-6. This cytokine correlates strongly with insulin resistance and type 2 diabetes [10] and its plasma levels are increased two- to threefold in patients with obesity and type 2 diabetes compared with lean control individuals [10].

Recently, endoplasmic reticulum (ER) stress has become a new potential mechanism involved in the association between saturated NEFA-induced inflammation and insulin resistance [11, 12], and it is now well accepted that limitation of the former will affect the latter [12]. In fact, patients with type 2 diabetes [13] and diet-induced and genetic *ob*/*ob* obese mice [14] have elevated levels of key ER stress markers, and elevated levels of NEFA have been proposed to induce insulin resistance by causing ER stress [15].

In conjunction with its central role in lipid synthesis, protein folding and transportation, the ER serves as a major signal transduction organelle that integrates cellular responses to stress. The accumulation of misfolded proteins and other stresses lead to the activation of an adaptive programme by the ER, known as the unfolded protein response (UPR), to re-establish equilibrium [11]. Initiation of the canonical UPR involves activation of three key signalling proteins: inositol-requiring 1 transmembrane kinase/endonuclease-1 (IRE-1α), activating transcription factor 6 (ATF6), and eukaryotic translation initiation factor-2α kinase 3 (PERK). The endoribonuclease activity of IRE-1α cleaves a 26-base-pair segment from *XBP1* mRNA, creating an alternative message that is translated into the active (or spliced) form of the transcription factor X-box binding protein 1 (XBP1) (sXBP1). ATF6 translocates to the nucleus in which it acts as a transcription factor and PERK phosphorylates and inhibits an essential initiator of translation, eukaryotic initiation factor 2 (eIF2). Together these pathways work to decrease translation and increase protein folding [16]. The three branches of the canonical UPR intersect with a variety of inflammatory and stress signalling systems, including the NF-κB pathway [11]. Thus, phosphorylation of eIF2α by PERK results in a general repression of mRNA translation. Since inhibitor of κB (IκB), which inhibits NF-κB, has a shorter half-life than NF-κB, UPR activation shifts the ratio of IκB to NF-κB, thereby releasing NF-κB, which translocates to the nucleus and increases the expression of its target genes, such as *IL6* [12]. In addition, in response to ER stress, the cytoplasmatic domain of phosphorylated IRE1α can recruit TNF-α-receptor-associated factor 2 (TRAF2), forming a complex that interacts and activates IκB kinase (IKK), leading to NF-κB activation [12]. Of note, activation of AMP-activated protein kinase (AMPK) exhibits multiple protective effects, including inhibition of inflammation, oxidative stress and insulin resistance and reduces the risk for developing obesity and type 2 diabetes [17]. Recently it has been reported that AMPK activation protects against hypoxic injury [18], atherosclerosis [19, 20] and lipid-induced hepatic disorders [21] by reducing ER stress.

Saturated NEFA-induced insulin resistance affects mainly skeletal muscle since this tissue accounts for most insulin-stimulated glucose use [22]. The present study was designed to investigate, in skeletal muscle cells, the cellular mechanisms by which the two most common fatty acids, the saturated fatty acid palmitate and the monounsaturated

oleate [23], exert their differential effects on ER stress and whether AMPK activation contributes to their differences in fatty acid-induced inflammation and impairment of insulin signalling. In addition, we aimed to discover whether oleate prevents the deleterious effects of palmitate. Our findings demonstrate that oleate prevents palmitate-induced ER stress in mouse and in human skeletal muscle cells through an AMPK-dependent mechanism, and contributes to the explanation of how the monounsaturated fatty acid prevents palmitate-induced inflammation and insulin resistance.

Methods

Cell culture and transfection studies Mouse C2C12 myoblasts (ATCC) were maintained and cultured as previously described [7].

For in vitro overexpression studies, cells were transfected with Lipofectamine 2000 in OPTI-MEM reduced-serum medium, following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). The constructs used were pcDNA3/pAMPKα2 K45R (Addgene plasmid 15992, Cambridge, MA, USA) and the corresponding LacZ-carrying plasmid as a control [24]. Transfection time and the DNA-to-Lipofectamine ratio for overexpression studies were set after optimisation with the corresponding LacZ-carrying plasmid and using a β-galactosidase reporter gene staining kit (Sigma-Aldrich Química, Madrid, Spain).

The human myogenic cell line LHCN-M2 [25] was grown as previously reported [26].

RNA preparation and quantitative RT-PCR The relative levels of specific mRNAs were assessed by real-time RT-PCR, as previously described [27] (see electronic supplementary material [ESM] Table 1 for primers used). *XBP1* splicing (sXBP1) was examined by gel electrophoresis as previously reported [28].

2-Deoxy-D-[^{14}C]glucose uptake experiments Determination of 2-deoxy-D-[^{14}C]glucose (2-DG) uptake was performed as reported elsewhere [7].

Immunoblotting Total proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). For IRE-1α a phos-tag gel was used as previously described [29]. Western blot analysis was performed using antibodies against total (catalogue number 9272) and phospho-Akt (Ser473) (9271), total (2532) and phospho-AMPK (Thr172) (2531), total (3662) and phospho-ACC (Ser79) (3661), BiP/GRP78 (3183), IRE-1α (3294) (Cell Signaling Technology, Danvers, MA, USA), PP2A catalytic subunit (52F8) (sc-6110), IκBα (sc-371), liver kinase B1 (LKB1) (sc-5638) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (A5441) (Sigma-Aldrich Química). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel). The equal loading of proteins was assessed by Ponceau S staining. The size of the proteins detected was estimated using protein molecular-mass standards (Invitrogen).

Isolation of nuclear extracts and electrophoretic mobility shift assay The isolation of nuclear extracts and electrophoretic mobility shift assay (EMSA) were performed as described elsewhere [27].

High performance liquid chromatography measurement of AMP Adenine nucleotides were separated by high performance liquid chromatography using an X-Bridge column with a 3.5 μm outer diameter (100×4.6 cm). Elution was done with 0.1 mmol/l potassium dihydrogen phosphate, pH 6, containing 4 mmmol/l tetrabutylammonium hydrogen sulfate and 15% (vol./vol.) methanol. The conditions were as follows: 20 μl sample injection, column at room temperature, flow rate of 0.6 ml/min and UV monitoring at 260 nm.

Statistical analyses Results are expressed as means ± SD of six separate experiments. Significant differences were established by one-way ANOVA using the GraphPad Prism 4.03 program (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p<0.05$.

Results

Oleate prevents palmitate-induced ER stress in mouse and human skeletal muscle cells To evaluate the differential effects of palmitate and oleate on ER stress in skeletal muscle cells we examined their effects on the expression of the ER stress markers *sXbp1*, *Atf3*, *Chop* (also known as *Ddit3*), *Hsp70* (also known as *Hspa4*) and *Grp78*/*Bip* (also known as *Hspa5*). Mouse C2C12 myotubes exposed to 0.5 mmol/l palmitate showed an increase in *sXbp1* mRNA levels compared with cells exposed only to BSA (Fig. 1a). The increase in the *sXbp1* mRNA levels caused by palmitate was of lower intensity than that induced by thapsigargin, a potent inducer of ER stress [30]. In contrast to palmitate, 0.5 mmol/l oleate did not increase *sXbp1* mRNA levels (Fig. 1b). Interestingly, co-incubation of cells with palmitate (0.5 mmol/l) and oleate (0.3 mmol/l) completely abolished the increase in *sXbp1* expression caused by the saturated fatty acid. Consistent with the changes in *sXbp1*, cells exposed to palmitate showed a marked increase in the expression of *Atf3*, *Chop*, *Hsp70* and *Bip* (Fig. 1c–f). These changes were not observed in cells incubated with oleate

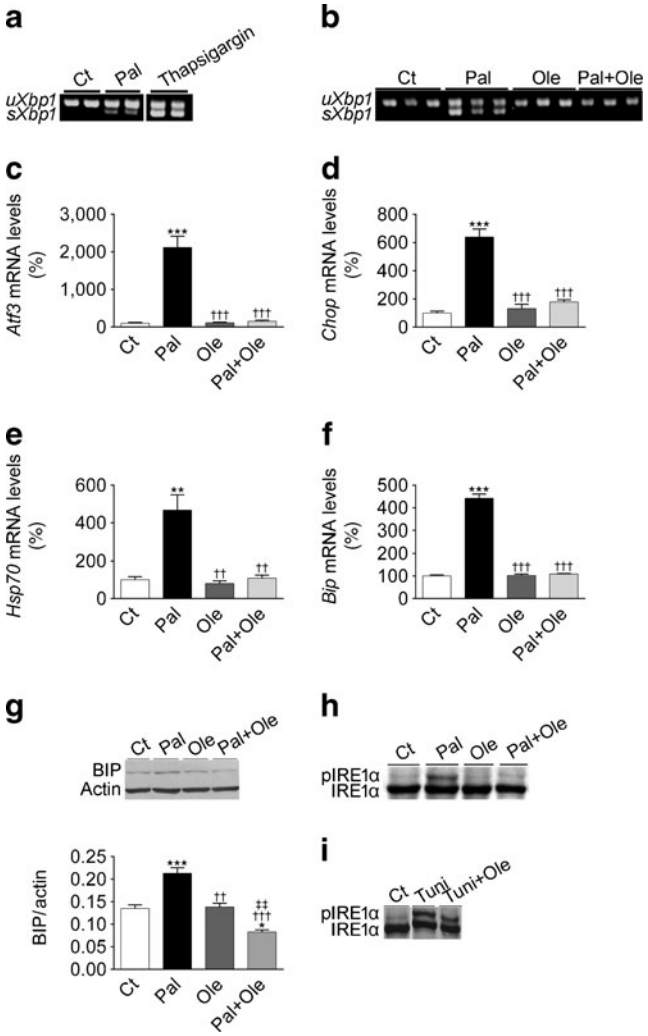

Fig. 1 Oleate prevents palmitate-induced ER stress in mouse skeletal muscle cells. C2C12 myotubes were incubated for 16 h in the presence or absence (Ct, control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Indicated cells were incubated with 1 μmol/l thapsigargin or 5 μg/ml tunicamycin (Tuni) for 16 h. *sXbp1* (**a**, **b**), *Atf3* (**c**), *Chop* (**d**), *Hsp70* (**e**) and *Bip* (**f**) mRNA levels. The graphs represent the quantification of the *Aprt*-normalised mRNA levels, expressed as a percentage of control samples ± SD of six independent experiments. Cell lysates were analysed by western blot using antibodies against BIP (**g**) and IRE1α (**h**, **i**). Immunoblots from three separate experiments were quantified and presented in the corresponding bar graphs. ***$p<0.001$ and **$p<0.01$ vs Ct; $^{\dagger\dagger\dagger}p<0.001$ and $^{\dagger\dagger}p<0.01$ vs Pal; $^{\ddagger\ddagger}p<0.01$ vs Ole

and co-incubation of palmitate-exposed cells with oleate completely blocked the effects of the saturated fatty acid. In agreement with the changes in *Bip* mRNA levels, glucose-regulated protein 78 (GRP78/BIP) protein levels were only increased in cells exposed to palmitate (Fig. 1g). Activation of IRE1α promotes the splicing of *Xbp1*. Thus, we then evaluated whether fatty acids activated IRE1α, by using phos-tag reagent, which selectively binds to phosphorylated amino acid residues [29]. Palmitate induced an increase in the phosphorylated forms of IRE1α, detected by slower migration (Fig. 1h). In contrast, this increase was observed neither in cells exposed to oleate nor in those co-incubated with palmitate and oleate. The potent pharmacological ER-stress inducer tunicamycin elicited a large increase in IRE1α phosphorylation, which was reduced by co-incubation with oleate (Fig. 1i).

In the human myogenic cell line LHCN-M2 exposure to palmitate caused a marked increase in the expression levels of *sXBP1*, *ATF3* and *CHOP* (Fig. 2). In contrast, oleate did not and supplementation of palmitate-exposed cells with oleate completely abolished the effect of the saturated fatty acid. Overall, these findings indicate that oleate also prevents palmitate-induced ER stress in human skeletal muscle cells.

The preventive effect of oleate on palmitate-induced ER stress is peroxisome proliferator-activated receptor-α -β/δ independent Since we have previously reported that oleate prevents palmitate-mediated activation of the DAG-PKCθ–NF-κB pathway by activating peroxisome proliferator-activated receptor (PPAR)-α [7], we then assessed the potential involvement of PPARs in the effects of oleate on ER stress. Co-incubation of cells with palmitate and oleate and the PPAR antagonists did not reverse the effects of the monounsaturated fatty acid on the expression of the ER stress markers in C2C12 myotubes (ESM Fig. 1 a–f). In addition, since oleate activates protein kinase A (PKA) [31] and activation of this kinase can inhibit ER stress [32], we evaluated whether this mechanism was involved in the effects of oleate. In the presence of the PKA inhibitor H89, the effect of oleate on *sXbp1* in palmitate-exposed cells was not modified (ESM Fig. 2a). Further, we have previously reported that inhibition of fatty-acid oxidation by etomoxir reversed the effects of oleate on palmitate-

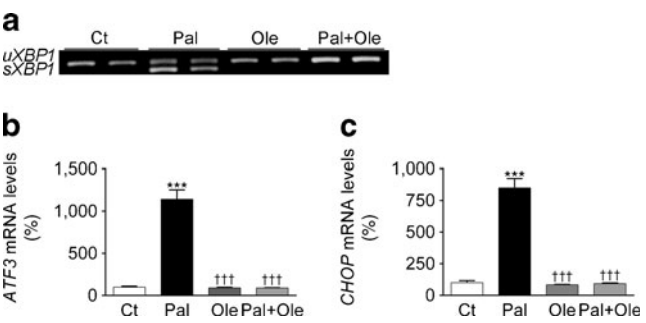

Fig. 2 Oleate prevents palmitate-induced ER stress in human skeletal muscle cells. LHCN-M2 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). *sXBP1* (**a**), *ATF3* (**b**) and *CHOP* (**c**) mRNA levels. The graphs represent the quantification of the 18S-normalised mRNA levels, expressed as a percentage of control samples ± SD of six independent experiments. ***$p<0.001$ vs Ct; $^{\dagger\dagger\dagger}p<0.001$ vs Pal

mediated activation of the DAG-PKCθ–NF-κB pathway [7]. However, etomoxir failed to reverse the effects of oleate on palmitate-induced ER stress (ESM Fig. 2b). Since depletion of ER Ca^{2+} may contribute to palmitate-induced ER stress [33], we next assessed the effects of calcimycin, a Ca^{2+} mobiliser, and BAPTA-AM, a $[Ca^{2+}]_i$ chelator. Calcimycin increased *sXbp1* and *Chop* mRNA levels (ESM Fig. 2c, d), confirming that Ca^{2+} mobilisation is involved in ER stress in mouse skeletal muscle cells, as previously described [19]. However, co-incubation of cells with palmitate plus BAPTA-AM reduced neither *sXbp1* nor *Chop* expression, indicating that under our conditions Ca^{2+} mobilisation does not contribute to palmitate-induced ER stress. Finally, a recent study demonstrated that silent information regulator T1 (SIRT1) serves as a negative regulator of UPR in type 2 diabetes mellitus [34]. However, the SIRT1 inhibitor EX527 did not reverse the effect of oleate on palmitate-induced ER stress (ESM Fig. 2e, f), indicating that the effects of the monounsaturated fatty acid on palmitate-induced ER stress did not involve SIRT1.

Oleate prevents palmitate-induced ER stress through an AMPK-dependent mechanism It is worth noting that activation of AMPK, which is considered a pharmacological target for insulin resistance and type 2 diabetes mellitus [35], inhibits ER stress [18, 19, 21] whereas reduction of this kinase promotes ER stress [20]. To confirm a role for AMPK in the preventive effects of oleate on palmitate-induced ER stress in C2C12 myotubes, we used the AMPK activators AICAR and A-769662 and the AMPK inhibitor compound C. The increase in the expression of *sXbp1*, *Atf3*, *Chop* and *Bip* caused by palmitate was reduced in cells co-incubated with palmitate plus either AICAR or A-769662 (Fig. 3), suggesting that AMPK activation prevents palmitate-induced ER stress. In addition, when we co-incubated C2C12 and LHCN-M2 cells exposed to palmitate plus oleate with the AMPK inhibitor compound C we observed that the effect of oleate on the expression of *sXbp1*/*sXBP1*, *Atf3*/*AFT3* and *Chop* was abolished (Fig. 4). Given the association of ER stress with the activation of the inflammatory process and insulin resistance [14], next we tried to link the inhibitory effects of oleate on palmitate-induced ER stress with oleate's reported ability to prevent palmitate-induced inflammation and insulin resistance [7]. We evaluated the effect of fatty acids on the expression of *Il6* and its secretion, a cytokine under the transcriptional control of NF-κB, which is involved in insulin resistance [10, 36]. Palmitate caused a 5.5-fold induction ($p<0.01$) in *Il6* mRNA levels, which was of lower intensity to the induction observed with potent ER-stress inducer tunicamycin (Fig. 5a). In contrast to palmitate, oleate exposure did not affect *Il6* expression (Fig. 5b), whereas co-incubation of palmitate-exposed cells with oleate prevented the increase in *Il6*

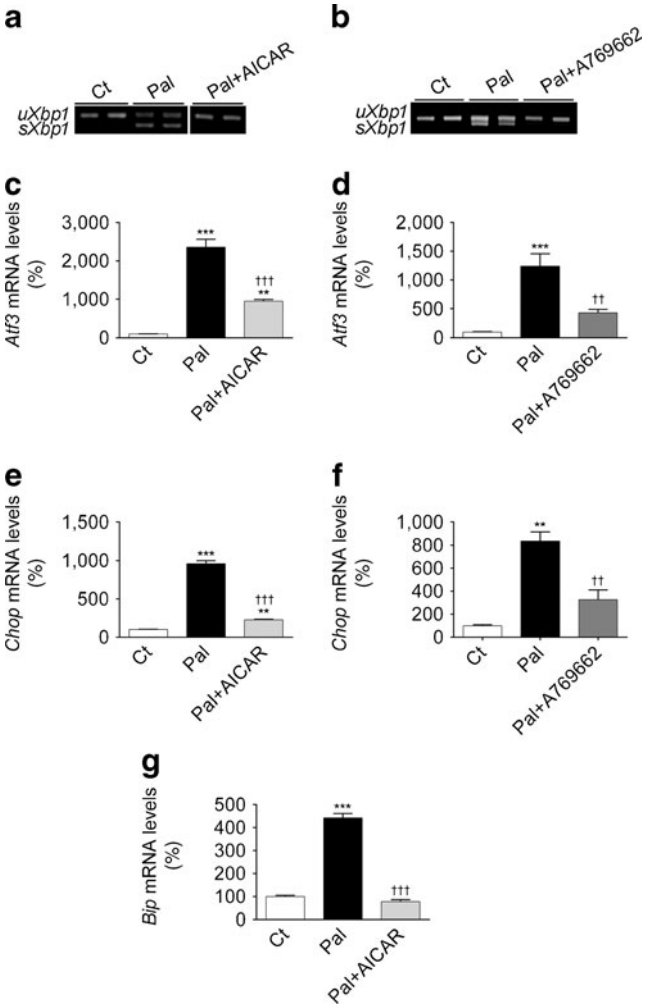

Fig. 3 AMPK activators prevent palmitate-induced ER stress in mouse skeletal muscle cells. C2C12 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of 0.5 mmol/l palmitate (Pal) and the AMPK activators AICAR (2 mmol/l) or A-769662 (60 μmol/l). *sXbp1* (**a**, **b**), *Atf3* (**c**, **d**), *Chop* (**e**, **f**) and *Bip* (**g**) mRNA levels. The graphs represent the quantification of the *aprt*-normalised mRNA levels, expressed as a percentage of control samples ± SD of six independent experiments. ***$p<0.001$ and **$p<0.01$ vs Ct; †††$p<0.001$ and ††$p<0.01$ vs Pal

mRNA levels. Interestingly, in the presence of compound C, the effect of oleate in preventing the increase in *Il6* mRNA in palmitate-exposed cells was partially reversed. A similar pattern was observed when we determined the secretion of IL-6 into the culture medium (Fig. 5c). Of note, human skeletal muscle cells showed a similar behaviour to that reported for C2C12 myotubes (Fig. 5d). Since palmitate induces *IL6* mRNA expression and IL-6 secretion through NF-κB activation driven by the reduction in IκBα levels [7, 8], we next evaluated the effects of fatty acids on the protein levels of this NF-κB inhibitor. Consistent with the changes in *Il6*, palmitate exposure reduced IκBα protein levels in C2C12 myotubes (Fig. 5e). In contrast, oleate did not and co-incubation of palmitate-exposed

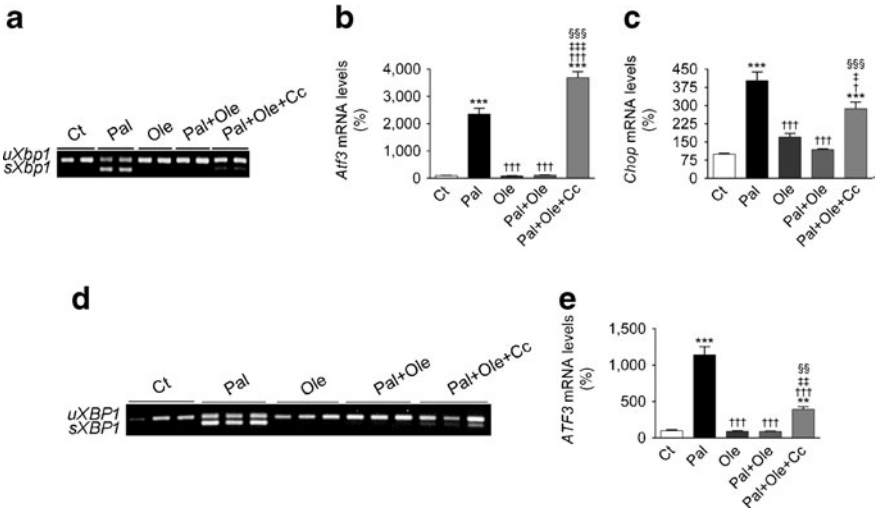

Fig. 4 Oleate prevents palmitate-induced ER stress in skeletal muscle cells through an AMPK-dependent mechanism. C2C12 or LHCN-M2 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Indicated cells were pre-incubated with 30 μmol/l of the AMPK inhibitor compound C (Cc) 8 h before the exposure to fatty acids. *sXbp1* (**a**), *Atf3* (**b**), *Chop* (**c**), human *sXBP1* (**d**) and human *ATF3* (**e**) mRNA levels. The graphs represent the quantification of the 18S- (LHCN-M2 human cells) or *aprt*-normalised mRNA levels, expressed as a percentage of control samples ± SD of six independent experiments. ***$p<0.001$ and **$p<0.01$ vs Ct; $^{\dagger\dagger\dagger}p<0.001$ and $^{\dagger}p<0.05$ vs Pal; $^{\ddagger\ddagger\ddagger}p<0.001$, $^{\ddagger\ddagger}p<0.01$ and $^{\ddagger}p<0.05$ vs Ole; $^{\S\S\S}p<0.001$ and $^{\S\S}p<0.01$ vs Pal + Ole

cells with the monounsaturated fatty acid prevented this reduction. Moreover, co-incubation of palmitate-exposed cells with oleate plus compound C reversed the effect of oleate. Measurement of NF-κB DNA-binding activity by EMSA confirmed these results. NF-κB formed one main complex with nuclear proteins (Fig. 5f) and specificity of this DNA-binding complex was assessed in competition experiments by adding an excess of unlabelled NF-κB oligonucleotide. Palmitate-exposed cells showed increased NF-κB DNA-binding activity compared with control cells, whereas cells exposed to oleate did not; co-incubation of palmitate-exposed cells with oleate prevented this increase. Interestingly, in the presence of compound C the effect of oleate on palmitate-exposed cells was blunted (Fig. 5f).

Then we evaluated whether AMPK activation by oleate contributed to the prevention of the reduction in insulin-stimulated Akt phosphorylation caused by palmitate exposure in C2C12 myotubes. Palmitate reduced insulin-stimulated Akt phosphorylation and this effect was not observed in cells exposed to oleate or in palmitate-exposed cells supplemented with oleate (Fig. 6a). In addition, the AMPK activators AICAR and A-769662 prevented this effect of palmitate (Fig. 6a). Likewise, the ER stress inducer thapsigargin reduced insulin-stimulated Akt phosphorylation. Of note, the effect of oleate on palmitate-exposed cells was reversed in the presence of compound C, indicating that the ability of oleate to prevent palmitate-induced insulin resistance requires AMPK activation. To further underpin the importance of ER stress in palmitate-induced insulin resistance in our conditions we incubated cells with palmitate in the presence of phenylbutyric acid (PBA), a pharmacological chaperone that reduces cellular ER stress and improves insulin sensitivity [37]. PBA prevented the reduction in insulin-stimulated Akt phosphorylation caused by palmitate (Fig. 6b). A similar trend to that reported for Akt was observed when we assessed 2-DG uptake (Fig. 6c). Interestingly, the ER stress inducer tunicamycin caused a similar reduction in insulin-stimulated 2-DG to that attained by palmitate.

To clearly confirm the involvement of AMPK in the effects of oleate, we manipulated AMPK activity in C2C12 cells by a molecular approach, involving overproduction of an AMPKα subunit with a point mutation that causes the enzyme to function as a dominant negative suppressor of endogenous AMPK activity (K45R). Overproduction of this construct in C2C12 cells leads to the displacement of endogenous subunits, followed by degradation of free alpha subunit [24]. This resulted in a reduction in total protein AMPK levels (Fig. 7a) and in its activity, determined by the decrease in acetyl-CoA carboxylase (ACC) phosphorylation (Fig. 7b). ACC is a substrate for AMPK [38] and serves as an indicator of AMPK activity. Inhibition of AMPK activity attenuated the effect of oleate in palmitate-exposed cells on IRE1α activation (Fig. 7c), IκBα protein levels (Fig. 7d) and insulin-stimulated Akt phosphorylation (Fig. 7e), suggesting that oleate prevents palmitate-induced inflammation and insulin resistance through an AMPK-dependent mechanism.

Oleate prevents the reduction in phospho-AMPK induced by palmitate Finally, we evaluated the potential mechanism

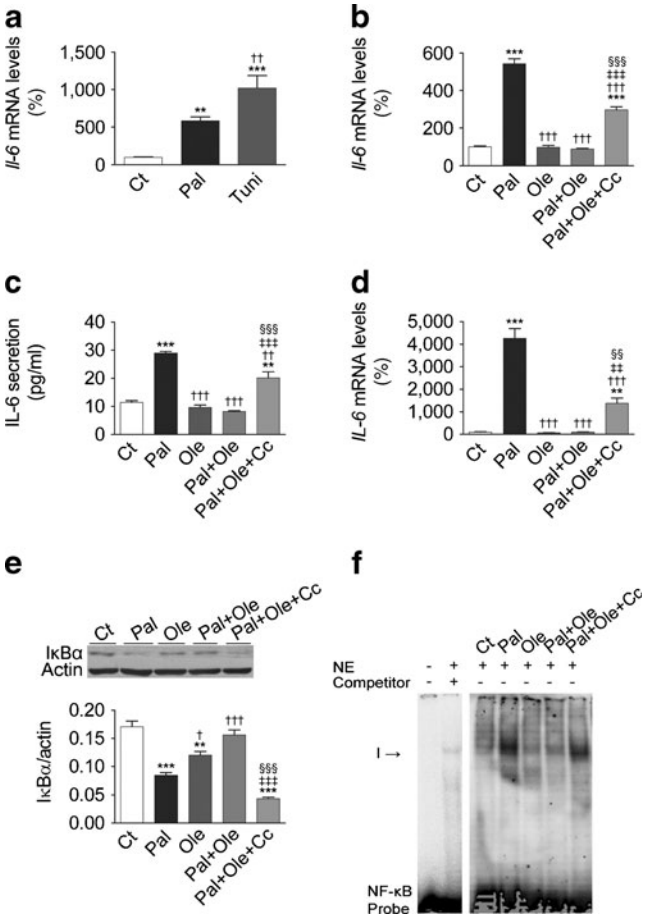

Fig. 5 Oleate prevents palmitate-induced inflammation in skeletal muscle cells through an AMPK-dependent mechanism. C2C12 or LHCN-M2 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Indicated cells were pre-incubated with 30 μmol/l of the AMPK inhibitor compound C (Cc) before the exposure to fatty acids. Relative quantification of mouse *Il6* (**a**, **b**) and human *IL6* (**d**) mRNA levels assessed by real-time RT-PCR. The graphs represent the quantification of the housekeeping-gene-normalised mRNA levels, expressed as a percentage of control samples ± SD of six independent experiments. Mouse IL-6 secretion levels (**c**) were analysed by ELISA. C2C12 cell lysates were analysed by western blot using antibodies against IκBα (**e**). Immunoblots from three separate experiments were quantified and presented in the corresponding bar graphs. (**f**) Autoradiograph of EMSA performed with ^{32}P-labelled NF-κB nucleotide and nuclear protein extracts (NE). One main specific complex (I), based on competition with a molar excess of unlabelled probe, was formed. The autoradiograph is representative of three separate experiments. ***$p<0.001$ and **$p<0.01$ vs Ct; $^{\dagger\dagger\dagger}p<0.001$, $^{\dagger\dagger}p<0.01$ and $^{\dagger}p<0.05$ vs Pal; $^{\ddagger\ddagger\ddagger}p<0.001$ and $^{\ddagger\ddagger}p<0.01$ vs Ole; $^{\S\S\S}p<0.001$ and $^{\S\S}p<0.01$ vs Pal + Ole

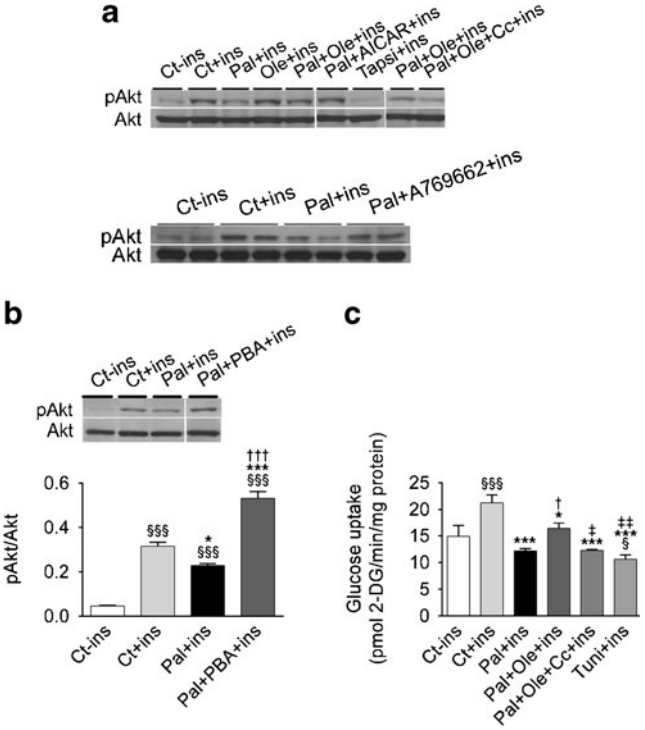

Fig. 6 Oleate prevents palmitate-induced insulin resistance in mouse skeletal muscle cells through an AMPK-dependent mechanism. C2C12 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Indicated cells were pre-incubated with 30 μmol/l of the AMPK inhibitor compound C, 2 mmol/l of AICAR, 60 μmol/l of A-769662, 2 mmol/l of the ER stress inhibitor PBA, 1 μmol/l of thapsigargin (Tapsi) or 5 μg/ml tunicamycin (Tuni). (**a**, **b**) C2C12 cell lysates were analysed by western blot using antibodies against total and phospho-Akt (Ser^{473}). Indicated cells were incubated with 100 nmol/l insulin (Ins) for the last 10 min. Immunoblots from three separate experiments were quantified and presented in the corresponding bar graphs. (**c**) 2-DG uptake was assessed without or with insulin. Values are means ± SD of six independent experiments. $^{\S\S\S}p<0.001$ and $^{\S}p<0.05$ vs Ct cells not exposed to insulin (Ct-Ins); ***$p<0.001$ and *$p<0.05$ vs Ct cells exposed to insulin (Ct + Ins); $^{\dagger\dagger\dagger}p<0.001$ and $^{\dagger}p<0.05$ vs Pal; $^{\ddagger\ddagger}p<0.01$ and $^{\ddagger}p<0.05$ vs Pal + Ole

responsible for the effect of fatty acids on AMPK activity. AMPK activation was monitored by western blot by incubating with a specific antibody against phosphorylated Thr^{172} of AMPK, which is essential for its activity [39]. When we examined the total and phospho-AMPK protein levels in C2C12 skeletal muscle cells exposed to fatty acids we observed that palmitate caused a reduction in phospho-AMPK levels compared with control cells (Fig. 8a). Unlike palmitate, oleate did not affect phospho-AMPK levels. Further, oleate supplementation prevented the reduction in phospho-AMPK levels caused by palmitate. Consistent with the phospho-AMPK levels, ACC-Ser^{79} was reduced in palmitate-exposed cells, whereas this reduction was not observed in cells exposed to oleate or in palmitate-exposed cells supplemented with oleate (Fig. 8b). The control of AMPK is complex and its phosphorylation status is regulated by both phosphatases and kinases [35]. Since a previous study reported that palmitate inhibited AMPK phosphorylation via ceramide-dependent phosphatase 2A (PP2A) activation in endothelial cells [40], we first examined the abundance of the PP2A/AC catalytic subunit. Fatty acids did not affect the protein levels of this phosphatase (Fig. 8c),

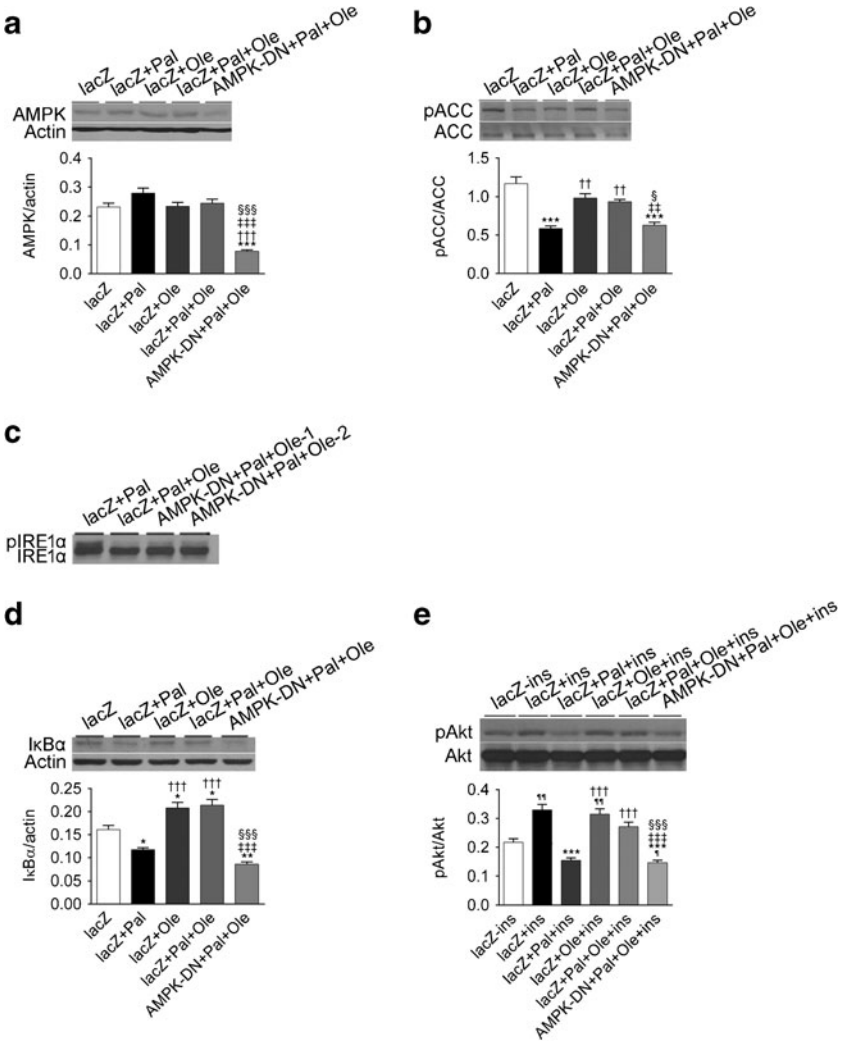

Fig. 7 The effects of oleate on ER stress, inflammation and insulin sensitivity in palmitate-exposed mouse skeletal muscle cells are AMPK dependent. C2C12 myotubes transfected with LacZ- or pAMPKα2 K45R-carrying plasmids were incubated for 16 h in the presence or absence of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Cell lysates were analysed by western blot using antibodies against total AMPK and actin (**a**), total and phospho-ACC (Ser^{79}) (**b**), IRE-1α (**c**) IκBα (**d**) and total and phospho-Akt (Ser^{473}) (**e**). Indicated cells were incubated with 100 nmol/l insulin (Ins) for the last 10 min. Data are expressed as mean ± SD of four experiments. ¶¶$p<0.01$ and ¶$p<0.05$ vs LacZ −Ins; ***$p<0.001$, **$p<0.01$ and *$p<0.05$ vs LacZ + Ins; †††$p<0.001$ and ††$p<0.01$ vs Pal; ‡‡‡$p<0.001$ and ‡‡$p<0.01$ vs Ole; §§§$p<0.001$ and §$p<0.05$ vs Pal + Ole; DN, dominant negative

making its contribution to the changes observed unlikely. Although in skeletal muscle cells LKB1 is the main upstream kinase regulating AMPK activity [41], we did not observe changes in the protein levels of LKB1 (Fig. 8d). Moreover, AMPK is activated allosterically by an increase in the intracellular AMP levels [35]. Even with minimal reduction in cellular ATP, changes in the concentration of AMP can activate AMPK [42]. Thus, we measured the AMP levels by HPLC to determine whether fatty acids affected its concentrations. Interestingly, palmitate reduced AMP levels compared with control cells or cells exposed to oleate, whereas in palmitate-exposed cells supplemented with oleate a significant increase was observed (Fig. 8e).

Discussion

High-fat diets are known to cause insulin resistance and type 2 diabetes mellitus, mainly due to their fatty-acid content. However, whereas saturated fatty acids promote insulin resistance [2, 3], the monounsaturated oleic acid improves insulin sensitivity [4, 5]. This has led to the suggestion that dietary intake of oleic acid should be used as a substitute for saturated fatty acids in the management of type 2 diabetes mellitus [43]. There is increasing evidence that the Mediterranean diet has a protective effect on both obesity and diabetes. This diet is characterised by a specific fatty acid pattern; it is low in saturated fatty acids (7–8% of energy) and high in monounsaturated fatty acids (over 20% of total energy), because the fat source consists primarily of olive oil [44]. However, the mechanisms by which oleate may improve insulin resistance are not completely known.

This study provides the first evidence that the monounsaturated fatty acid oleate prevents saturated-fatty-acid-induced ER stress, inflammation and insulin resistance through AMPK activation. First, we show that oleate, in contrast to palmitate, does not increase the levels of ER stress markers. In addition, oleate supplementation at a low concentration provides a marked protection against ER stress and reduces the levels of these markers to those present in control cells. It is worth noting that the changes

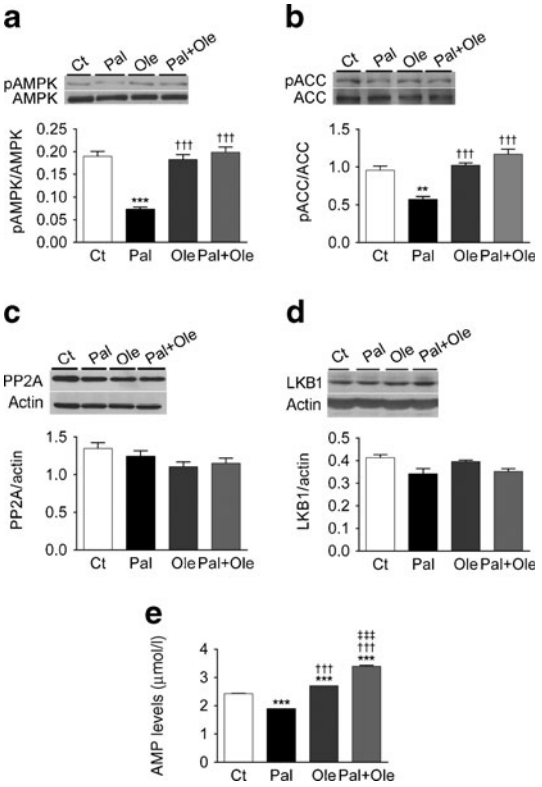

Fig. 8 Oleate prevents the palmitate-mediated reduction in phospho-AMPK levels in mouse skeletal muscle cells. C2C12 myotubes were incubated for 16 h in the presence or absence (Ct, Control) of different fatty acids: 0.5 mmol/l palmitate (Pal), 0.5 mmol/l oleate (Ole) or 0.5 mmol/l palmitate supplemented with 0.3 mmol/l oleate (Pal + Ole). Cell lysates were analysed by western blot using antibodies against total and phospho-AMPK (Thr^{172}) (**a**), total and phospho-ACC (Ser^{79}) (**b**), PP2A (**c**) and LKB1 (**d**). (**e**) AMP levels in C2C12 myotubes exposed to fatty acids. Data are expressed as mean ± SD of four experiments. ***$p<0.001$ and **$p<0.01$ vs Ct; $^{\dagger\dagger\dagger}p<0.001$ vs Pal; $^{\ddagger\ddagger\ddagger}p<0.001$ vs Ole

observed in the mouse cell line C2C12 were confirmed in human skeletal muscle cells, indicating that the effects of oleate are not species specific. Our findings discard the involvement of PPARs, Ca^{2+}, SIRT1, PKA or increased fatty acid oxidation in the effects attained by oleate. Likewise, even though extracellular-signal-regulated kinase (ERK)1/2 has been involved in palmitate-induced NF-κB activation [45], no changes were observed in ER stress markers in cells co-incubated with palmitate plus U0126 (a MEK1/2 [mitogen-activated protein kinase]–ERK1/2 inhibitor) compared with cells incubated with only the saturated fatty acid, whereas *IL6* mRNA levels were reduced (data not shown). Furthermore, oleate exposure did not affect palmitate uptake since we have previously reported that the total content of intracellular lipids is similar in palmitate- and palmitate-plus-oleate-exposed cells [7]. However, AMPK activation prevented the increase in ER stress markers in palmitate-exposed skeletal muscle cells. This is consistent with previous studies reporting that AMPK activation protects against hypoxic injury [18], atherosclerosis [19] and liver damage [21] by reducing ER stress. By using the AMPK inhibitor compound C and overproduction of a dominant negative AMPK construct we demonstrated that activation of this kinase was responsible for the reduction in ER stress attained by oleate in palmitate-exposed cells. Since ER stress is emerging as a potential site for the intersection of inflammation and insulin resistance [11], we assessed in our conditions the contribution of ER stress and AMPK activation to these processes. Interestingly, ER stress can activate NF-κB via translational suppression of IκBα, resulting in the upregulation of mediators of inflammation and insulin resistance, such as IL-6 [46]. Consistent with this, the ER stress inducers tunicamycin and thapsigargin increased the expression of *IL6* and reduced insulin-stimulated Akt phosphorylation, respectively. Likewise, PBA, which reduces cellular ER stress, prevented the reduction in insulin-stimulated Akt phosphorylation caused by palmitate. These findings are in agreement with those of a previous study [15] but contrast with those reported in a recent study indicating that ER stress does not mediate palmitate-induced insulin resistance [47]. We do not know the reasons for this discrepancy, but differences in the fatty acid–BSA conjugation could be involved.

Overall, these findings confirm the role of ER stress in inflammation and insulin resistance in skeletal muscle cells. Moreover, the ability of oleate to prevent the reduction in IκBα protein levels in palmitate-exposed cells was blocked by the AMPK inhibitor compound C and by overproduction of the dominant negative AMPK construct, suggesting that AMPK activation by oleate contributes to the prevention of palmitate-induced inflammation. In agreement with the changes in markers of inflammation, AICAR, A-769662 and oleate improved the reduction in insulin-stimulated Akt phosphorylation that had been caused by palmitate. The improvement achieved by oleate was prevented in cells supplemented with oleate plus compound C or cells in which the dominant negative AMPK was overproduced. These data confirm that oleate improves palmitate-induced insulin resistance through AMPK activation.

As a whole, the findings of this study show that AMPK activation by oleate contributes to the prevention of palmitate-induced inflammation and insulin resistance. In addition, our data also show that AMPK activation by oleate also prevents palmitate-induced NF-κB activation in human skeletal muscle cells. This is an interesting point, since NF-κB is activated in myocytes from obese individuals with type 2 diabetes compared with non-obese control individuals, whereas AMPK activation attenuates NF-κB activation [48].

Interestingly, it has been reported that reduction in AMPK levels promotes ER stress, suggesting that AMPK functions as a physiological suppressor of ER stress [20]. When we examined the potential mechanisms responsible for the increase in ER stress following palmitate exposure and the protective

effects of oleate, we observed that the saturated fatty acid reduced phospho-AMPK levels. In contrast, neither oleate- nor palmitate-exposed cells supplemented with oleate showed changes in the levels of this kinase. Since it has been reported that high-fat-diet feeding significantly decreases phospho-AMPK in the liver and muscles of rodents [49], these findings suggest that the saturated fatty acids in these diets contribute to this reduction.

The regulation of AMPK is complex, involving allosteric control by an increase in the cellular content of AMP and covalent regulation through phosphorylation of Thr^{172} within the catalytic subunit by upstream kinase LKB1 and dephosphorylation by PP2A. Our data show that palmitate reduces AMP levels, whereas no changes were observed in cells exposed to oleate; in those exposed to palmitate and supplemented with oleate, AMP levels were higher than in control cells. These findings indicate that palmitate reduces AMPK activity through a reduction in AMP levels, whereas oleate supplementation can prevent the reduction caused by palmitate in the concentration of this nucleotide.

In summary, on the basis of our findings we propose that oleate prevents palmitate-induced ER stress, inflammation and insulin resistance in skeletal muscle cells through AMPK activation. These findings offer a new mechanistic approach to the beneficial effects of oleate vs the saturated fatty acid palmitate in skeletal muscle insulin resistance.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement LS, TC, ES, EB, AMG-F, XP and MV-C processed the samples, analysed and prepared the data and were involved in drafting the article. LS, ES, AMG-F and XP contributed to the interpretation of the data and revised the article. MV-C designed the experiments, analysed and interpreted the data and wrote the manuscript. All authors approved the final version of the manuscript.

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