Counter-modulation of fatty acid-induced pro-inflammatory nuclear factor κB signalling in rat skeletal muscle cells by AMP-activated protein kinase

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INTRODUCTION

There is mounting evidence that chronic activation of pro-inflammatory signalling in tissues such as skeletal muscle and adipose is a significant contributing factor in the development and progression of metabolic disorders such as insulin resistance, obesity and Type 2 diabetes [1,2]. This inflammatory response is triggered by circulating pro-inflammatory cytokines, such as IL-6 (interleukin-6) and TNFα (tumour necrosis factor α), and by sustained increases in the concentration of NEFAs (non-esterified fatty acids), such as palmitate. The actions of these stimuli serve not only to further induce tissue expression and secretion of IL-6 and TNFα [via the NF-κB (nuclear factor κB) pathway [3,4]], but to impair control of numerous signalling pathways regulating skeletal muscle insulin signalling, glucose uptake and cellular oxidative capacity, thereby having an impact on energy balance [2,5–7]. The precise mechanism that governs activation of pro-inflammatory signalling by NEFAs is, however, poorly understood. Although evidence exists linking TLRs (Toll-like receptors) of the innate immune system, in particular TLR2 and TLR4 (for which NEFAs may be ligands), to activation of NF-κB [8,9] the extent of their involvement remains unclear. This uncertainty stems from the observation that the effect of NEFAs on inflammatory signalling via TLRs is not observed in a number of different cell types [10] or may be delayed for many hours following cell exposure to the fatty acid, which is considerably longer than would otherwise be expected from receptor-mediated signalling events that are typically initiated within minutes of the ligand–receptor binding event. Such observations imply that palmitate may induce NF-κB activation via alternative mechanisms that rely upon uptake and metabolism of the fatty acid and the capacity of fatty acid-derived metabolites [e.g. DAG (diacylglycerol) and ceramide] to regulate proteins promoting pro-inflammatory signalling [5].

In unstimulated cells, cytoplasmic NF-κB exists in an inactive state complexed with its accessory inhibitory protein IκBα (inhibitory κBα) [11]. Activation of NF-κB involves phosphorylation and degradation of IκBα, which permits nuclear translocation of NF-κB and subsequent regulation of its target genes. Phosphorylation of IκBα is directed by a heteromeric kinase [IκK (IκB kinase)] complex consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (NF-κB essential modulator)/IKKγ [11]. Activation of IKK itself is dependent on serine phosphorylation by upstream kinases and is considered to be crucial in obesity-induced inflammatory signalling [12,13]. Although the upstream elements

Sustained over-supply of saturated non-esterified ‘free’ fatty acids has been shown to promote skeletal muscle insulin resistance, which may be driven, in part, by an increase in inflammatory signalling within this tissue. In the present manuscript we show that exposure of L6 myotubes to palmitate, a saturated fatty acid, induces activation of the NF-κB (nuclear factor κB) pathway (based on increased IκK [IκB (inhibitory κB) kinase] phosphorylation, IκBα loss and elevated interleukin-6 mRNA expression) and that this was associated with enhanced phosphorylation/activation of p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase) and ERK (extracellular-signal-regulated kinase) as well as impaired insulin-dependent activation of PKB (protein kinase B)/Akt and glucose transport. NF-κB activation by palmitate was unaffected by pharmacological inhibition of p38 MAPK or JNK, but was suppressed significantly by inhibition of MEK (MAPK/ERK kinase)/ERK signalling. The importance of ERK with respect to downstream NF-κB signalling was underscored by the finding that PMA, a potent ERK activator, enhanced IκK phosphorylation. Strikingly, both palmitate- and PMA-induced activation of IκK/NF-κB were antagonized by AMPK (AMP-activated protein kinase) activators because of reduced ERK signalling. Although palmitate-induced activation of NF-κB was repressed by AMPK activation and by cellular overexpression of a mutated IκBα (S32A/S36A) super-repressor, this did not ameliorate the loss in insulin-stimulated PKB activation or glucose transport. Our results from the present study indicate that ERK plays a pivotal role in palmitate-induced activation of the IκK/NF-κB signalling axis and that AMPK can restrain the activity of this pro-inflammatory pathway. The finding that insulin resistance persists in myotubes in which NF-κB signalling has been repressed implies that palmitate and/or its lipid derivatives retain the capacity to impair insulin-regulated events independently of the increase in inflammatory signalling.

Key words: glucose transport, insulin, insulin resistance, fatty acid, nuclear factor κB (NF-κB), protein kinase B (PKB).

Abbreviations used: ACC, acetyl-CoA carboxylase; ADAMPK-DN, adenosine harbouring dominant-negative AMPK D157A; AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; CINC-1, cytokine-induced neutrophil chemoattractant-1; DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; IL, interleukin; IκB, inhibitory κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; α-MEM, α-minimal essential medium; NEFA, non-esterified fatty acid; NF-κB, nuclear factor κB; PKB, protein kinase B; PKC, protein kinase C; TLR, Toll-like receptor; TNFα, tumour necrosis factor α.

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involved in lipid-induced IKK activation in skeletal muscle still remain poorly defined, the NF-κB pathway can serve as a target for MAPKs (mitogen-activated protein kinases) [14]. Three subfamilies of MAPK, whose members respectively include the ERKs (extracellular-signal-regulated kinase), JNKs (c-Jun N-terminal kinases) and p38 MAPK, have thus far been characterized. All three subfamilies have been implicated in the generation of pro-inflammatory mediators in numerous cell types [15–19] and there is also strong evidence linking their activation to the pathogenesis of insulin resistance [20]. It is therefore not surprising, given the increasing recognition of a link between lipid-induced inflammatory signalling and the development of insulin resistance, that there is considerable interest in defining potential anti-inflammatory strategies that counter the loss in insulin sensitivity induced by obesity and circulating lipid overload [12,13,21,22]. Previous studies have implicated the AMPK (AMP-activated protein kinase) as a modulator of inflammatory responses based on observations that its activation by AICAR (5-amino-4-imidazolecarboxamide riboside) suppresses LPS (lipopolysaccharide)-induced nuclear translocation and DNA binding of NF-κB as well as expression of pro-inflammatory cytokines such as IFN-γ (interferon γ), TNFα, IL-6 and IL-8 in different cell types [23–26]. Precisely how AMPK promotes this anti-inflammatory response and whether it is as effective in antagonizing the associated reduction in insulin sensitivity caused by fatty acid excess in skeletal muscle cells is currently unknown. In an attempt to address this issue we have investigated the role played by MAPKs in promoting NF-κB activation in response to palmitate in L6 skeletal muscle cells and explored what effect manipulating AMPK activity has upon palmitate’s pro-inflammatory and insulin-desensitizing potential. We show in the present manuscript that, although palmitate induces activation of p38 MAPK, JNK and that of the ERKs, only inhibition of the ERK pathway results in suppression of fatty acid-induced NF-κB signalling. We also demonstrate that activation of AMPK antagonizes the fatty acid-induced activation of NF-κB and critically show that this anti-inflammatory effect involves attenuating the increase in ERK signalling. However, despite suppressing the palmitate-induced increase in inflammatory signalling, AMPK activation was unable to correct the reduction in insulin sensitivity caused by fatty acid overload.

**EXPERIMENTAL**

**Materials**

- α-MEM (α-minimal essential medium), DMEM (Dulbecco’s modified Eagle’s medium), FBS (fetal bovine serum), DCS (donor calf serum) and antibiotic/antimycotic solution were from Life Technologies. All other reagent-grade chemicals, including insulin and BSA, were obtained from Sigma–Aldrich or BDH unless otherwise stated. Fraction V fatty acid-free BSA was from Roche, PMA was from Ascent Scientific, U0126, SB202190 and SP600125 were purchased from Calbiochem–Novabiochem, PD184352 and BIRB 796 were sourced in-house from the Division of Signal Transduction and Therapy (DSTT), University of Dundee, Dundee, U.K. A769662 was synthesized in-house at DSTT, University of Dundee, Dundee, U.K. 100 mg/ml streptomycin and 250 ng/ml amphotericin B at 37°C with 5% CO2. L6 muscle cells were exposed to fatty acids that had been conjugated to BSA (fraction V) for the times and at the 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 10 min incubation period for immunoblotting analysis or 30 min for glucose uptake assays. L6 myotubes were routinely serum-deprived for 2 h prior to any treatment with insulin.

**Cell culture and fatty acid treatment**

L6 muscle cells were cultured to myotubes as described previously [29] in α-MEM containing 2% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B) at 37°C with 5% CO2. L6 muscle cells were exposed to fatty acids that had been conjugated to BSA (fraction V) for the times and at the 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 10 min incubation period for immunoblotting analysis or 30 min for glucose uptake assays. L6 myotubes were routinely serum-deprived for 2 h prior to any treatment with insulin.

**Adenoviral constructs and cell infection**

Recombinant replicative-deficient Myc-tagged AdAMPK-DN (adenovirus harbouring dominant-negative AMPK D157A) was generated as described previously [30,31]. Adenovirus harbouring IkBaS32A/S36A was kindly provided by Dr Harry Heimberg (Diabetes Research Centre, Vrije Universiteit Brussel, Brussels, Belgium). Adenoviruses were propagated in HEK (human embryonic kidney)-293 cells and stored at −80°C. The viral titre was determined by standard plaque assay in HEK-293 cells. Confluent mononucleated L6 myoblasts were infected with adenovirus at 20 pfu (plaque-forming units)/cell for AdAMPK-DN or 5.5 pfu/cell for IkBaS32A/S36A in serum-free α-MEM for 2 h at 37°C. Cells were subsequently maintained in fresh α-MEM containing 2% (v/v) FBS at 37°C and allowed to differentiate into myotubes prior to experimental analysis.

**Cell lysis**

L6 myotubes were incubated for 3 days and with the appropriate amount of effectors described in the Figure legends. After treatment, cells were rinsed twice with ice-cold PBS and then lysed using lysis buffer [50 mM Tris/HCl, pH 7.4, 0.27 M sucrose, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium 2-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 0.1% 2-mercaptoethanol and protease inhibitor (one tablet/50 ml)]. Whole-cell lysates were centrifuged (15000 g at 4°C for 10 min), and supernatants were removed for storage at −80°C until required.

**Immunoblotting**

Cell lysates were subjected to SDS/PAGE and immunoblotted as reported previously [29]. Nitrocellulose membranes were probed with primary antibodies against proteins of interest as indicated in the Figure legends. Detection of primary antibodies was performed using an appropriate peroxidase-conjugated IgG, and protein signals were visualized using enhanced chemiluminescence by exposure to Kodak autoradiographic film. Quantification of immunoblots was performed using Image J software (http://rsbweb.nih.gov/ij/).
**Immunoprecipitation**

Protein G–Sepharose beads were washed three times in PBS and incubated with anti-AMPKα1/α2 antibody for 1 h at 4°C on an orbital platform shaker. The bead–antibody mixture was then incubated with 500 μg of L6 cell lysate protein for 2 h at 4°C before washing. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl and twice with Heps assay buffer (50 mM Heps, pH 7.0, 1 mM dithiothreitol and 0.02 % Brij-35).

**AMPK activity assay**

AMPK was immunoprecipitated from 500 μg of muscle cell lysates using the pan-AMPK α1/α2 antibody and kinase activity towards SAMS peptide (HMRSAMSGLHVKR) was measured as described previously [32].

**RNA extraction and PCR**

Total RNA was extracted from L6 myotubes using TRIzol® reagent according to the manufacturer’s instructions (Sigma–Aldrich). Quantitative real-time PCR was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems), SYBR Green JumpStart Taq Ready-Mix (Sigma–Aldrich) and primers targeting IL-6 (cytokine-induced neutrophil chemoattractant-1) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a control. The sequences for these primers are as follows: IL-6 forward, 5′-GACTGTAGTGTGTGACGCA-3′; IL-6 reverse, 5′-ATGGTCAGCATAACCCTAGGGT-3′; CINC-1 forward, 5′-ACCCGTCTGTTCTCTGTGC-3′; CINC-1 reverse, 5′-CAGGCGACGTTCATTGGCGAC-3′; GAPDH forward, 5′-TGGAAGCTGTGGCGTAG-3′ and GAPDH reverse 5′-GCTCACACTCGTCTTCT-3′. PCR amplification was performed with an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 68°C for 1 min. The ratio of IL-6 and CINC-1 mRNA expression were calculated as described previously [33]. In some experiments, semi-quantitative PCR was performed for analysis of IL-6 expression. For these, cDNA was amplified using rat-specific primers for IL-6 using the 5′-primer AGCCACTGCTTTCTCCTTTT and the 3′-primer GCCATTGCAACACTTTTCTC, designed and synthesized in-house. PCR was performed using a Hybaid thermal cycler under the following conditions: initial denaturation was for 2 min at 94°C for one cycle, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C followed by a final extension for 5 min at 72°C. The products were separated and visualized by ethidium bromide staining on a 1% (w/v) agarose gel.

**Glucose uptake**

L6 myotubes were incubated with reagents for the times and at the concentrations indicated in the Figure legends. Cells were washed twice with Heps-buffered saline (140 mM NaCl, 20 mM Heps, 5 mM KCl, 2.5 mM MgSO4, and 1 mM CaCl2, pH 7.4). Glucose uptake was assayed by incubation with 10 μM 2-deoxy-

*D*-[14C]glucose (1 μCi/ml) for 10 min as described previously [29]. Non-specific binding was determined by quantifying cell-associated radioactivity in the presence of 10 μM cytochalasin B. Medium was aspirated before washing adherent cells twice with 0.9% ice-cold NaCl. Cells were subsequently lysed in 50 mM NaOH, and radioactivity was quantified using a Beckman LS 6000IC scintillation counter. Protein concentration in cell lysates was determined using the Bradford reagent [34].

**Statistical analyses**

For multiple comparisons, statistical analysis was performed using one-way ANOVA. For individual comparisons statistical analysis was performed using a Student’s *t* test. Data analysis was performed using GraphPad Prism software and considered statistically significant at *P* < 0.05.
Figure 2  Effects of p38 MAPK, JNK and ERK inhibition on palmitate-induced NF-κB signalling in L6 myotubes

L6 myotubes were treated with palmitate (750 μM) for 16 h in the absence or presence of (A) the p38 MAPK inhibitors BIRB 796 (0.1 μM) or SB202190 (1 μM) for indicated times, (B) the JNK inhibitor SP600125 (30 μM) for 8 h or (C) the MEK inhibitors PD184352 (1 μM) or U0126 (10 μM). Cell lysates were immunoblotted to assay the phosphorylation status of IKKα/β, p38 MAPK, JNK or ERK1/2 as well as the total protein abundance of IκBα. Analysis of β-actin was used to assess protein loading in gel lanes.

RESULTS

Effects of palmitate upon NF-κB and MAPK signalling in L6 skeletal muscle cells

We initially monitored the effect of palmitate on the phosphorylation of IKKα/β and the associated loss in its downstream target, IκBα, serving as a readout for NF-κB activation. Figure 1 shows that palmitate induces a dose- and time-dependent increase in IKKα/β phosphorylation which is associated with a concomitant loss in cellular IκBα protein levels. In an attempt to understand the contribution played by MAPKs towards activation of the NF-κB pathway, we initially assessed the activation/phosphorylation status of ERK1/2, JNK and p38 MAPK. Figure 1 shows that all three MAPKs were activated in response to palmitate at a dose and exposure period similar to that seen for activation of IKKα/β. Maximal activation of IKK, ERKs, p38 MAPK and JNK was observed at a dose of 750 μM palmitate and was detectable within 6–8 h of cell incubation with the fatty acid, with phosphorylation of all four kinases being sustained in muscle cells incubated with palmitate for up to 16 h.

Effects of inhibiting p38 MAPK, JNK and ERK upon palmitate-induced activation of NF-κB in L6 myotubes

To establish what role, if any, activation of the MAPK subfamilies play with respect to regulation of the NF-κB pathway, we incubated muscle cells with palmitate for 16 h in the absence or presence of small-molecule inhibitors that selectively target each kinase family. Since the three MAPK subfamilies were all activated following 6–8 h of palmitate treatment (Figure 1B), cells were exposed to kinase inhibitors for the last 8 h of the 16 h palmitate incubation period. BIRB 796 and SB202190 both target p38 MAPK and, although neither prevents the stimulus-induced phosphorylation of p38 MAPK by its upstream kinase (Figure 2A), previous work from our group and that of others has shown that each compound potently suppresses the catalytic activity of p38 MAPK towards its downstream target MAPKAPK2 (MAPK-activated protein kinase 2) [35,36]. The inhibition of MAPKAPK2 with BIRB 796 was verified as part of the present study (see Supplementary Figure S1 at www.BiochemJ.org/bj/435/bj4350463add.htm). SP600125 has been widely reported to inhibit JNK and we find that it significantly attenuates the palmitate-induced phosphorylation of JNK in our L6 muscle cells (Figure 2B). However, co-incubation of muscle cells with palmitate and BIRB 796, SB202190 or SP600125 did not suppress the fatty acid-induced activation of the IKKα/β complex or the associated loss in IκBα (Figures 2A and 2B). In contrast, PD184352 and U0126, both of which inhibit MEK [36] and thereby ERK, induced a marked reduction in the palmitate-driven activation of IKK and in the loss of IκBα (Figure 2C). This latter finding implies that, in L6 myotubes, ERK activation is likely to be an important upstream component of the NF-κB pro-inflammatory response triggered by fatty acid excess.

A role for AMPK in modulating the pro-inflammatory potential of palmitate

Several reports have suggested that AMPK is able to antagonize the effect of numerous pro-inflammatory cytokines in various cell types [23–26]. We were therefore keen to assess whether AMPK could also oppose the pro-inflammatory potential of palmitate. Although there is evidence in the literature showing that acute palmitate treatment increases AMPK activity [37], sustained overloading of muscle cells with palmitate led to a significant (3-fold) reduction in basal AMPK phosphorylation and activity, which, in line with the results already presented, also induced an associated loss in the abundance of IκBα (Figure 3A). As expected, treatment of muscle cells with the specific AMPK activator A769662 [27] significantly increased phosphorylation and activity of AMPK and, intriguingly, also promoted a modest
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Figure 3  Effects of AMPK activators on palmitate-induced NF-κB and MAPK signalling in L6 myotubes

(A) L6 myotubes were treated with palmitate (750 μM) for 16 h or with A769662 (100 μM) for 2 h. Lysates were either immunoprecipitated with a pan-AMPK α1/α2 antibody and used to assay AMPK activity or immunoblotted to assess the phosphorylation status of AMPK and ACC and total protein abundance of IκBα. *P < 0.05, a significant difference from the control (untreated) value. (B) L6 myotubes were treated with palmitate for 16 h (750 μM) in the absence or presence of either A769662 (100 μM) or phenformin (200 μM) for the indicated periods. Cell lysates were immunoblotted to assess the phosphorylation status of IKKα/β, ERK1/2 and ACC as well as total protein abundance of IκBα, ACC, AMPK and ERK1/2. Analysis of β-actin was used to assess protein loading in gel lanes. The blots are representative of three separate experiments. (C) Changes in IκBα protein levels from (B) were quantified and expressed as a percentage change from untreated control. Values shown are the means ± S.E.M from three separate experiments. *P < 0.05 compared with the single palmitate-treated value.

In order to test this hypothesis, L6 muscle cells were treated with palmitate for 16 h in the absence or presence of the AMPK-activating drugs phenformin or A769662. Figure 3(B) shows that, when presented alone to muscle cells, both drugs induced activation of AMPK on the basis of the increased phosphorylation of ACC (Ser79), an AMPK target and thus a downstream readout for the kinase. Both phenformin and A769662 antagonized the palmitate-induced activation of IKK (Figure 3B) and, consistent with this effect, both compounds ameliorated the fatty acid-induced loss in IκBα (Figures 3B and 3C). Treatment with palmitate or AMPK activators did not cause any detectable change in the cellular abundance of ACC, ERK1/2 or that of the α1 and α2 subunits of AMPK (Figure 3B). Our observations thus imply that AMPK activation exerts a suppressive effect upon palmitate-induced NF-κB signalling.

To further substantiate this possibility, the effect of AMPK activation on the expression of IL-6 and CINC-1 (an IL-8-like cytokine), two NF-κB-regulated muscle genes [38,39], was assessed using quantitative real-time PCR following cell incubation with palmitate. Figure 4 shows that palmitate induced a significant increase in both IL-6 and CINC-1 mRNA, although the magnitude with which IL-6 expression was enhanced was substantially greater than that of CINC-1. Cell treatment with A769662 for the penultimate 8 h of incubation with the fatty acid significantly antagonized the increase in both IL-6 and CINC-1 expression. Given that AMPK activation opposes the palmitate-induced phosphorylation of IKK (Figure 3B), we postulated that AMPK may suppress the pro-inflammatory potential of palmitate via inhibition of ERK1/2 signalling.

Since AMPK antagonizes the palmitate-induced phosphorylation of ERK1/2 (Figure 3B), we assessed whether AMPK activation in combination with MEK inhibition exerted an additive effect with respect to suppression of IKK phosphorylation/activation and IκBα protein loss. Figure 5(A) shows that both A769662 and U0126 inhibit the palmitate-induced phosphorylation of IKK and ERK. However, combined treatment of muscle cells with both drugs did not result in any noticeable enhancement in the antagonism of palmitate’s effect.
on IKK or IκBα abundance. Of interest, in parallel experiments we assessed whether AMPK activation would also antagonize the palmitate-induced activation of p38 MAPK and JNK. The immunoblot results presented in Figure 5(B) show that, unlike the ERK pathway, activation of the two other MAPK subfamilies was not suppressed by A769662. In order to further substantiate the notion that AMPK activation counters MEK/ERK signalling, L6 muscle cells were treated with PMA (a DAG mimetic and a potent activator of ERK signalling) in the absence or presence of A769662. Figure 5(C) shows that PMA treatment induced an increase in the phosphorylation of MEK and ERK and was associated with an increase in IKK phosphorylation. Importantly, incubation of cells with A769662 antagonized the PMA-induced phosphorylation of MEK, ERK and IKK.

**Effects of expressing a dominant-negative AMPK on palmitate-induced IκBα degradation**

To further validate that AMPK acts a negative modulator of NF-κB signalling, AdAMPK-DN was expressed in L6 muscle cells prior to incubation with palmitate and A769662. AMPK was rendered dominant-negative by mutating an aspartate residue at position 157 into an alanine residue in α1 AMPK as described previously [30]. Expression of this viral construct was validated by blotting cell lysates with an anti-Myc antibody to detect the tagged protein and also by analysis of total AMPK levels, which were considerably elevated in the viral-infected cells compared with control cells that were mock-infected with the virus lacking the AMPK construct (Figure 6A). Confirmation that the construct behaved in a dominant-negative manner was established by demonstrating that, although A769662 induced ACC Ser79 phosphorylation in wild-type muscle cells, those expressing the AdAMPK-DN construct lacked this stimulus-induced phosphorylation of ACC (Figure 6A). Consistent with other results presented in the present paper, AMPK activation antagonized the fatty acid-induced loss in IκBα in wild-type L6 muscle cells. However, this antagonism was not observed in cells expressing the AdAMPK-DN construct (Figure 6). Furthermore, the ability of A769662 to counter the palmitate-induced activation of ERK was lost in cells expressing the AdAMPK-DN construct (Figure 6A).

**Figure 4.** Quantitative real-time PCR analysis of IL-6 and CINC-1 mRNA expression in L6 myotubes

Muscle cells were incubated with palmitate (750 μM) for 16 h in the absence or presence of A769662 (100 μM) for the penultimate 8 h of the fatty acid incubation. RNA was extracted from cells and IL-6 and CINC-1 mRNA were determined by quantitative real-time PCR as described in the Experimental section. Values shown are the means ± S.E.M from three separate determinations. *P < 0.05 between the indicated bars.

**Figure 5.** Effects of AMPK activation on palmitate- and PMA-induced NF-κB and MAPK signalling in L6 myotubes

Muscle cells were treated with (A and B) palmitate (750 μM) for 16 h or (C) PMA (100 nM) for 2 h in the absence or presence of A769662 (100 μM) for 8 h (A and B) or 2 h (C). In some experiments, L6 myotubes were also treated with U0126 (10 μM) for 8 h. Cell lysates were immunoblotted to assess the phosphorylation status of IκBα/β ACC, ERK1/2, p38 MAPK, JNK and MEK1/2 as well as total protein abundance of IκBα. Analysis of β-actin was used to assess protein loading in gel lanes.

**Effects of ceramide on PKB and NF-κB signalling**

We have shown previously that ceramide generated from palmitate or added exogenously to cells in a cell-permeant form promotes a targeted loss in the insulin-dependent activation of PKB by a mechanism involving atypical PKCs (protein kinase Cs) (ζ/ζ) [6,40,41]. However, there is also evidence in the literature suggesting that in some cell types ceramide promotes activation of NF-κB signalling, which may contribute to the reduction in insulin signalling [42]. To assess whether this might be the case in our muscle cells, we investigated the effects of ceramide on both PKB and NF-κB activation. Consistent with our previous work [6], Figure 7 shows that sustained incubation of muscle cells with palmitate inhibited PKB activation in response to an acute insulin challenge. This effect was mimicked by subjecting L6 cells to either a short (2 h, 100 μM) or more chronic low grade (16 h, 10 μM) incubation with C2-ceramide. Under both circumstances C2-ceramide reduced the insulin-dependent activation of PKB. In contrast, unlike palmitate treatment, C2-ceramide did not induce phosphorylation/activation of IKK or promote loss of IκBα.
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Figure 6 Expression of a dominant-negative AMPK in L6 myotubes antagonizes the repressive effect of A769662 on palmitate-induced NF-κB and ERK signalling

L6 myoblasts were infected with a dominant-negative AMPK adenoviral construct or mock-infected with virus lacking the AMPK construct and allowed to differentiate into myotubes. Cells were treated with palmitate (750 μM) for 16 h in the absence or presence of A769662 (100 μM) for the final 8 h of treatment. (A) Cell lysates were immunoblotted to assess the phosphorylation status of ACC and ERK1/2 as well as the cellular levels of IκBα, AMPKα1/α2 and Myc. Immunoblotting for β-actin provided a means for assessing protein loading in gel lanes. (B) Quantification of IκBα abundance and ERK1/2 phosphorylation. Values shown are the means ± S.E.M for 3–5 separate experiments. *P < 0.05 compared with the appropriate mock-infected untreated control; #P < 0.05 compared with the palmitate-treated value.

Effects of suppressing NF-κB activation on insulin sensitivity of L6 muscle cells

Although the results presented in Figure 7 suggests that the insulin-desensitizing effect of ceramide in L6 muscle cells is unlikely to involve NF-κB activation, our findings do not exclude the possibility that palmitate may act independently of ceramide to suppress insulin signalling via the NF-κB pathway. To test this, we investigated the effect of suppressing NF-κB signalling by two separate approaches and monitored palmitate’s effect upon the insulin-dependent activation of PKB. Figure 8(A) shows that palmitate blunted the insulin-dependent phosphorylation of PKB. Under these circumstances, palmitate induces IKK phosphorylation and a loss of IκBα and, in line with the results shown in Figure 5(A), this was antagonized by co-incubation of cells with A769662. However, despite this antagonism in NF-κB signalling, A769662 was unable to halt the loss in the insulin activation of PKB elicited by the fatty acid (Figure 8A). Consistent with this latter finding, AMPK activation with A769662 also failed to antagonize the palmitate-induced loss in insulin-stimulated glucose uptake, which is widely accepted as being a key PKB-regulated process (Figure 8B). To further substantiate these findings, L6 cells were infected with an adenoviral construct harbouring a mutant form of IκBα (S32A/S36A) that acts as a potent repressor of NF-κB activity. Mutation of Ser32 and Ser36 to alanine renders IκBα resistant to IKK phosphorylation, thereby protecting it from proteosomal degradation and retaining its capacity to hold NF-κB in an inhibited state. Figure 8(C) shows that palmitate induces IKK activation, IκBα loss and expression of IL-6 (assessed using a semi-quantitative PCR approach) in wild-type (mock-transfected) L6 cells and those infected with the empty viral vector. However, cells expressing the stable IκBαS32A/S36A protein do not exhibit a stimulus-induced increase in IL-6 expression despite the attendant increase in IKK activation (Figure 8C). Moreover, despite suppressing palmitate-induced NF-κB signalling using the super repressor, this experimental approach did not antagonize the loss in insulin-stimulated PKB activation caused by palmitate (Figure 8C).

DISCUSSION

There is growing recognition that an increase in tissue inflammation is a key feature associated with obesity and that this may, in part, be initiated by the sustained elevation and tissue delivery of circulating NEFAs that prevail during this condition [43]. In skeletal muscle, fatty acid overload has been shown to enhance pro-inflammatory NF-κB signalling and this has been linked to the development and progression of insulin resistance in this tissue [22,44]. Precisely how fatty acids, such as palmitate,
Figure 7 Effects of C2-ceramide on activation of the NF-κB pro-inflammatory and MAPK pathways

Representative immunoblots showing the effect of incubating L6 myotubes with either palmitate (Pal, 750 μM) for 16 h or C2-ceramide for 2 h (Cer, 100 μM) or 16 h (Cer, 10 μM) in the absence or presence of insulin (100 nM) for 10 min. Lysates from control (Con) and treated cells were immunoblotted for the phosphorylation status of PKB, ERK and IKK as well as the total protein amount of IκBα. Equal loading was ascertained by immunoblotting with an antibody against total PKB.

promote an increase in inflammatory tone is not well understood and, although there is mounting interest in the role played by TLRs in this response, there is a large body of evidence that suggests that the increased accumulation of fatty acid-derived metabolites (e.g. ceramide, DAG etc.) play a crucial role in modulating the activity of numerous molecules, including those involved in insulin and inflammatory signalling [41,45–47]. The major focus of the work described in the present study was to: (i) gain an insight into how palmitate induces pro-inflammatory NF-κB signalling in cultured myotubes, with particular focus on the role played by members of the MAPK family and ceramide as potential protagonists of the inflammatory response; (ii) establish whether AMPK activation opposes the pro-inflammatory drive initiated by palmitate; and (iii) assess whether activation of NF-κB signalling underpins the loss in insulin sensitivity invoked by palmitate in L6 myotubes.

Our findings indicate that, although sustained incubation of muscle cells with palmitate induces activation of all three MAPK subfamilies, only the classical ERK pathway appears to be important with respect to supporting activation of NF-κB signalling in L6 myotubes (Figure 9A). We suggest that activation of ERK not only precedes that of IKK, but also lies upstream of the IKK/NF-κB signalling pathway. This proposition is based on the following evidence. First, activation of ERK correlates temporally with that of IKK and the associated reduction that this causes in IκBα abundance. Secondly, PMA, which potently activates ERK [48], also induces activation of IKK in L6 myotubes and, thirdly, pharmacological inhibitors that selectively target the MEK/ERK signalling axis (i.e. PD184352 and U0126) significantly reduce palmitate’s capacity to stimulate NF-κB signalling (Figure 9B). The concept that ERK activation may contribute to an increase in inflammatory tone is not unprecedented. Previous studies in macrophages [18], epithelial cells [49,50] and vascular smooth muscle cells [19] have shown that a variety of pro-inflammatory stimuli (e.g. LPS, TNFα and IL-8) promote ERK-dependent NF-κB activation, presumably via signalling initiated from binding to their respective cell-surface receptors. Precisely how palmitate initiates pro-inflammatory signalling in L6 myotubes is not entirely clear, but is unlikely, in our view, to be initiated via TLRs on the basis of the rather slow response time (6–8 h) with which the fatty acid induces IKK activation. Moreover, it is noteworthy that in separate studies we have found that bacterial LPS, a well established TLR2/TLR4 agonist, fails to promote any detectable activation of IKK/NF-κB signalling when incubated with L6 myotubes for periods of up to 12 h; however, under identical incubation conditions, it induces robust IKK activation and IκBα loss in mouse dendritic cells within 30 min (K. Macrae, C. Lipina and H.S. Hundal, unpublished work). The absence of an acute LPS- or palmitate-induced inflammatory signalling response in L6 myotubes implies that, although TLR2 and TLR4 are expressed in skeletal muscle tissue [51] and cells [8,52], they may be refractory to stimulation by LPS and palmitate, at least over the incubation periods employed with the muscle cells used in the present study. It is currently unclear whether this observation represents a cell line-specific effect, but it would be of interest to assess the effects of both LPS and palmitate upon IKK/NF-κB signalling under circumstances when TLR2/TLR4 and their adaptor proteins are either stably and moderately overexpressed or silenced in L6 muscle cells. Such studies may provide a more definitive assessment of whether TLRs are involved or not in the pro-inflammatory response triggered by palmitate in our experimental model.

If not TLRs, what might initiate the fatty acid-induced pro-inflammatory response? We have shown previously that accumulation of both DAG and ceramide is enhanced significantly in muscle cells following incubation with palmitate [6], and although our present findings indicate that ceramide is unlikely to contribute to activation of the ERK/NF-κB signalling axis we believe that DAG may do so. An important consequence of accumulating this fatty acid-derived molecule is the activation of DAG-sensitive PKC isoforms. It is noteworthy that one member of this family, PKCθ, has been implicated in palmitate-induced NF-κB signalling in murine C2C12 myotubes [47]. Whether PKCθ acts directly to activate the IKK/NF-κB pathway or mediates its effect via another route is currently unclear, but given that PMA, a DAG mimetic, is capable of inducing IKK activation in an ERK-dependent manner it seems plausible that DAG-sensitive PKCs may act via the MEK/ERK signalling pathway to promote NF-κB signalling in L6 myotubes as has been suggested in macrophages [18].

Activated AMPK can target a panoply of signalling proteins that regulate metabolic pathways affecting cellular energy balance [53]. In skeletal muscle, AMPK activation enhances glucose uptake and fatty acid oxidation [54] and it is not unsurprising therefore that the kinase is perceived as a potential drug target whose manipulation may prove beneficial in the treatment of metabolic disorders such as diabetes and obesity [55]. However, there is growing recognition that AMPK may also confer an anti-inflammatory response based on work showing that metformin and AICAR, two well established AMPK activators, inhibit NF-κB activity and expression of NF-κB-dependent gene targets in endothelial cells [24,56]. Similarly, incubation of primary human skeletal muscle cells from lean and obese individuals with AICAR has been shown to antagonize the palmitate-induced degradation of IκBα [57]. Although the latter study did not establish the mechanism by which AICAR spares the fatty acid-induced loss of IκBα, a recent study has reported that the ribonucleoside impairs binding of NF-κB to DNA without affecting nuclear translocation of NF-κB or turnover of IκBα protein in endothelial cells [24]. In contrast, our results from the present study suggest that AMPK targets a point considerably more upstream in the inflammatory signalling cascade;
specifically, at the level of, or upstream of, MEK/ERK. The finding that A769662 (which is regarded as a more specific AMPK activator than either metformin or AICAR [27]) antagonizes the palmitate- and PMA-induced activation of ERK, and that this results in a concomitant reduction in IKK/NF-κB signalling and restrained expression of IL-6 and CINC-1 (two known NF-κB target genes expressed in muscle) would support our proposition of a more proximal AMPK effect. What remains unresolved is the precise nature of this upstream AMPK target. There is no evidence in the literature to suggest that MEK or ERK1/2 serve as direct AMPK substrates, although Raf1, which lies upstream of MEK, can be phosphorylated by AMPK on Ser621 in vitro [58]. It is currently unknown whether AMPK phosphorylates Raf1 in vivo (or in intact cells) and whether it is inhibitory for the downstream activation of MEK and ERK. Alternatively, it is possible that AMPK activation enhances palmitate oxidation, which may help limit accumulation of molecules such as DAG and consequently their potential to promote inflammatory signalling via members of the PKC family. Testing these possibilities represent important investigative goals of future work.

Loss of IKKβ or inhibiting nuclear translocation of NF-κB has been shown to protect skeletal muscle against lipid-induced insulin resistance in rodent and cell-based studies [22, 44]. However, our present work indicates that inhibiting the NF-κB pathway either via activation of AMPK or by expression of the IκBα (S32A/S36A) super-repressor fails to alleviate the repressive effect that palmitate has upon insulin sensitivity of L6 myotubes. There are a number of reasons that may explain why inhibition of NF-κB did not prove to be protective in our experimental model. We believe that it is highly unlikely that inhibition of NF-κB would have any significant effect upon palmitate-driven synthesis of ceramide or DAG. Our previous work has shown that ceramide added exogenously to cells as a cell-permeant analogue or generated in vivo from palmitate causes a targeted loss in PKB-directed insulin signalling [6, 41]. This inhibition, as shown in the present study, occurs in the absence of any detectable activation of NF-κB by ceramide; a finding that indicates that, in L6 myotubes, this sphingolipid is unlikely to be a major contributor to fatty acid-induced inflammation. Moreover, although suppressing the de novo synthesis of ceramide from palmitate can be beneficial with respect to insulin action, we have found that sustained inhibition of the metabolic pathway that converts palmitate into ceramide induces a dynamic shift in palmitate metabolism towards greater synthesis of DAG [46]. This increase in DAG exerts an insulin-desensitizing effect upon IRS (insulin receptor substrates)-directed insulin signalling via activation of DAG-sensitive PKCs [46]. Consequently, if palmitate-driven synthesis

**Figure 8 Effects of antagonizing NF-κB signalling on palmitate-induced inhibition of insulin-stimulated PKB activation and glucose uptake in L6 myotubes**

L6 myotubes were treated with palmitate (750 μM) for 16 h in the absence or presence of (A) A769662 (100 μM) for the final 8 h or (A and C) insulin (100 nM) for 10 min or (B) insulin (100 nM) for 30 min. (A) Cell lysates were immunoblotted for assessing the phosphorylation status of ACC, PKB and IκBα as well as the total protein abundance of IκBα. Equal loading was ascertained by immunoblotting with an antibody against total PKB. (B) Muscle cells were assayed for 2-deoxyglucose (2DG) uptake. Values are the means ± S.E.M. from four separate experiments each performed in triplicate. (C) L6 myoblasts were infected with an empty control adenoviral vector or one expressing an HA-tagged non-phosphorylatable IκBα (S32A/S36A) construct. Infected myoblasts were allowed to differentiate into myotubes prior to treatment with palmitate and insulin as indicated. Cells were used for semi-quantitative PCR analysis of IL-6 mRNA (upper panel). The IL-6 expression results are expressed as means ± S.E.M. from three separate experiments. The lower panel shows immunoblot analysis of cell lysates assessing the phosphorylation status of IKKα/β and PKB as well as the total protein abundance of IκBα (endogenous and HA-tagged). Gel loading was ascertained by immunoblotting with an antibody against total PKB. * P < 0.05 compared with the appropriate control values.
of DAG and ceramide remains unabated under circumstances when NF-κB signalling is suppressed, it is unlikely that one might expect a significant gain in insulin sensitivity of muscle cells. In addition, AMPK-mediated antagonism of NF-κB did not prevent palmitate’s capacity to activate p38 MAPK and JNK in L6 myotubes. We cannot exclude the possibility that activation of these MAPKs may also contribute to lipid-induced insulin resistance either directly by targeting proximal components of the insulin signalling cascade or indirectly via modification of mitochondrial function/lipid oxidation as reported in some studies [59,60].

In summary, our present work demonstrates that sustained exposure of skeletal muscle cells to palmitate induces NF-κB signalling in an ERK-dependent manner and that activation of AMPK serves to counter-regulate this pro-inflammatory pathway (Figure 9). Although activation of NF-κB appears not to contribute significantly to the insulin-desensitizing effects of palmitate over the incubation periods that we have studied, we suggest that the pathogenic effects that are likely to be associated with enhanced and sustained expression of pro-inflammatory cytokines (e.g. IL-6) on, for example, gene expression, cell metabolism and cell survival over the long term, and the ability to potentially offset these via activation of AMPK, should not be discounted or overlooked.

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SUPPLEMENTARY ONLINE DATA

Counter modulation of fatty acid-induced pro-inflammatory nuclear factor κB signalling in rat skeletal muscle cells by AMP-activated protein kinase

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Figure S1 Effects of palmitate and BIRB 796 upon MAPKAPK2 activity

L6 myotubes were treated with 750 μM palmitate complexed with fatty acid-free BSA (2 % w/v) for 16 h in the absence (−) or presence (+) of BIRB796 (0.1 μM) for 8 h. MAPKAPK2 was assayed by immunoprecipitation of the kinase and analysis of 32P incorporation into a substrate peptide. Values are the means ± S.E.M. of four determinations. *P < 0.05 compared with the untreated assay sample; #P < 0.05 compared with the palmitate-treated value.

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