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Counter modulation of fatty acid-induced proinflammatory NFκB signalling in rat skeletal muscle cells by AMPK

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Running title: Palmitate-induced proinflammatory signalling

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SYNOPSIS

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. Here we show that exposure of L6 myotubes to palmitate, a saturated fatty acid, induces activation of the NFκB pathway (based on increased IKK phosphorylation, IκBα loss and elevated IL-6 mRNA expression) and that this was associated with enhanced phosphorylation/activation of p38 MAPK, JNK and ERK as well as impaired insulin-dependent activation of PKB/Akt and glucose transport. NFκB activation by palmitate was unaffected by pharmacological inhibition of p38 MAPK or JNK, but was suppressed significantly by inhibition of MEK/ERK signalling. The importance of ERK with respect to downstream NFκB signalling was underscored by the finding that PMA, a potent ERK activator, enhanced IKK phosphorylation. Strikingly, both palmitate- and PMA-induced activation of IKK/NFκB were antagonised by AMPK activators because of reduced ERK signalling. Whilst palmitate-induced activation of NFκB was repressed by AMPK activation and by cellular over-expression of a mutated IκBα (S32A/S36A) super-repressor, this did not ameliorate the loss in insulin-stimulated PKB activation or glucose transport. Our data indicates that ERK plays a pivotal role in palmitate-induced activation of the IKK/NFκB signalling axis and that AMPK can restrain the activity of this proinflammatory pathway. The finding that insulin resistance persists in myotubes in which NFκB 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.

Abbreviations: PKB, Protein kinase B; AMPK, AMP-activated protein kinase; ACC, Acetyl Co-A carboxylase; NFκB, Nuclear factor kappa-B; IKK, Inhibitor of nuclear factor kappa-B kinase; IκB, Inhibitor of nuclear factor kappa-B; MAPK, Mitogen-activated protein kinase; MEK, Mitogen/ERK activated protein kinase; ERK, Extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; IL-6, Interleukin 6; CINC-1, Cytokine-Induced Neutrophil Chemoattractant-1; TNF, Tumour necrosis factor; TLR, Toll-like receptor; FFA, free fatty acid; PMA, phorbol 12-myristate 13-acetate;

INTRODUCTION

There is mounting evidence that chronic activation of proinflammatory 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 II diabetes [1,2]. This inflammatory response is triggered by circulating proinflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor- α (TNF α) and by sustained increases in the concentration of free fatty acids (FFAs), such as palmitate. The actions of these stimuli serve not only to further induce tissue expression and secretion of IL-6 and TNF α (*via* the NF κ B pathway [3,4]), but impair control of numerous signalling pathways regulating skeletal muscle insulin signalling, glucose uptake and cellular oxidative capacity thereby impacting on energy balance [2,5-7]. The precise mechanism that governs activation of proinflammatory signalling by FFAs is, however, poorly understood. Whilst evidence exists linking Toll-like receptors (TLRs) of the innate immune system, in particular TLR2 and TLR4 (for which FFAs may be ligands), to activation of NF κ B [8,9] the extent of their involvement remains unclear. This uncertainty stems from the observation that the effect of FFAs 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 typically are initiated within minutes of the ligand-receptor binding event. Such observations imply that palmitate may induce NF κ B 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 and ceramide) to regulate proteins promoting proinflammatory signalling [5].

In unstimulated cells, cytoplasmic NF κ B exists in an inactive state complexed with its accessory inhibitory protein, I κ B α [11]. Activation of NF κ B involves phosphorylation and degradation of I κ B α , which permits nuclear translocation of NF κ B and subsequent regulation of its target genes. Phosphorylation of I κ B α is directed by a heteromeric kinase (IKK) complex consisting of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, NEMO/IKK γ [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 involved in lipid-induced IKK activation in skeletal muscle still remain poorly defined the NF κ B pathway can serve as a target for mitogen-activated protein kinases (MAP kinases) [14]. Three subfamilies of MAP kinase whose members respectively include the extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal kinases (JNKs) and p38 MAPK have thus far been characterised. All three subfamilies have been implicated in the generation of proinflammatory 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]. Recent work has implicated the AMP-activated protein kinase (AMPK) as a modulator of inflammatory responses based on observations that its activation by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) suppresses LPS-induced nuclear translocation and DNA binding of NF κ B as well as expression of proinflammatory cytokines such as IFN- γ , TNF α , 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

antagonising 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 MAP kinases in promoting NF κ B activation in response to palmitate in L6 skeletal muscle cells and explored what effect manipulating AMPK activity has upon palmitate's proinflammatory and insulin-desensitising potential. We show here that whilst 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 κ B signalling. We also demonstrate that activation of AMPK antagonises the fatty acid-induced activation of NF κ B 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 PROCEDURES

Materials - α -MEM (α -Minimal Essential Medium), DMEM (Dulbecco's Modified Eagle's Medium), FBS (Foetal Bovine Serum), DCS (Donor Calf Serum) and antibiotic/antimycotic solution were from Life Technologies (Paisley, UK). All other reagent-grade chemicals, including insulin and Bovine Serum Albumin (BSA), were obtained from Sigma-Aldrich (Poole, UK) or BDH (Poole, UK) unless otherwise stated. Fraction V fatty acid free BSA was from Roche (Basel, Switzerland), phorbol 12-myristate 13-acetate was from Ascent Scientific (Bristol, UK), U0126, SB202190 and SP600125 were purchased from Calbiochem-Novabiochem (CA, U.S.A.), PD184352 and BIRB 796 were sourced in house from the Division of Signal Transduction and Therapy (DSTT), University of Dundee. A769662 was synthesised in house as described previously [27]. Complete protein phosphatase inhibitor tablets were purchased from Boehringer-Roche Diagnostics (Basel, Switzerland). 2-deoxy-D-[3 H]-glucose was from PerkinElmer Life Sciences (Boston, MA, U.S.A.). Phospho-PKB (Thr³⁰⁸), phospho-PKB (Ser⁴⁷³), phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), phospho-IKK α / β (Ser^{180/181}), phospho-AMPK (Thr¹⁷²), phospho-ACC (Ser⁷⁹), phospho-MEK1/2 (Ser^{217/221}), phospho-SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵), phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), anti- β -actin, anti-PKB, and anti-HA antibodies were from Cell Signaling Technology (Beverly, MA). Anti-IkBa antibody was from Santa Cruz. Anti total pan-AMPK α 1/ α 2 were generated as described previously [28].

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, 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 times and at concentrations indicated in the figure legends (controls were incubated with vehicle containing BSA but lacking the fatty acid) and incubated with insulin (100 nM) in the penultimate 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 adenovirus harbouring dominant negative-AMPK D157A (DN-AMPK) were generated as described previously [30,31]. Adenovirus harbouring IkBaS32A/S36A was kindly provided by Dr Harry Heimberg (Vrije Universiteit Brussel, Belgium). Adenoviruses were propagated in HEK293 cells and stored at -80°C. The viral titre was determined by standard plaque assay in

HEK293 cells. Confluent mononucleated L6 myoblasts were infected with adenovirus at 20 pfu/cell for DN-AMPK or 5.5 pfu/cell for I κ B α S32A/S36A in serum free α -MEM for 2 h at 37°C. Cells were subsequently maintained in fresh α -MEM containing 2% FBS at 37°C and allowed to differentiate into myotubes prior to experimental analysis.

Cell Lysis - L6 myotubes were incubated for times and with the appropriate amount of effectors described in the figure legends. After treatment, cells were rinsed twice with ice-cold phosphate-buffered saline (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, 10mM sodium β -glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and protease inhibitor (1 tablet/50 ml)). Whole-cell lysates were centrifuged (15,000 g at 4 °C for 10 min), and supernatants removed for storage at -80°C until required.

Immunoblotting - Cell lysates were subjected to SDS-PAGE and immunoblotted as previously reported [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 visualized using enhanced chemiluminescence by exposure to Kodak autoradiographic film. Quantification of immunoblots was done using Image J software.

Immunoprecipitation - Protein G-Sepharose beads were washed 3 times in PBS and incubated with anti-AMPK α 1/ α 2 for 1 h at 4 °C on an orbital platform shaker. Bead-antibody mix was then incubated with 500 μ g 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 0.5M NaCl and twice with Hepes assay buffer (50 mM Na Hepes, pH 7.0, 1 mM DTT, 0.02% Brij-35).

AMPK activity assay - AMPK were immunoprecipitated from 500 μ g of muscle cell lysates using the pan-AMPK α 1/ α 2 antibody and kinase activity towards SAMS peptide (HMRSAMSGLVKRR) was measured as described previously [32].

RNA extraction and PCR - Total RNA was extracted from L6 myotubes using TRI reagent according to the manufacturer's instructions (Sigma-Aldrich, Dorset, U.K.). Quantitative real-time PCR was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA), SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and primers targeting IL-6, CINC-1 and GAPDH as a control. The sequences for these primers are as follows: IL-6 forward 5'-GACTGATGTTGTTGACAGCCA-3', IL-6 reverse 5'-ATGCTTAGGCATAACGCACTAGGTT - 3', CINC-1 forward 5' - ACCCGCTCGCTTCTCTGTGC - 3', CINC-1 reverse 5' - CAGCGCAGCTCATTGGCGAC - 3', GAPDH forward 5'-TGGAAAGCTGTGGCGTGAT-3' and GAPDH reverse 5'-GCTTCACCACCTTCTTGAT-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 to GAPDH 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 AGCCACTGCCTTCCCTACTT and the 3'-primer GCCATTGCACAACCTCTTTCTC, designed and synthesised 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

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 times and at concentrations indicated in the figure legends. Cells were washed 2 times with Hepes-buffered saline (140 mM NaCl, 20 mM Hepes, 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-[³H]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 2 times with 0.9% (w/v) 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 analysis of variance (ANOVA). For individual comparisons statistical analysis was performed using a t-test. Data analysis was performed using GraphPad Prism software and considered statistically significant at P values < 0.05.

RESULTS

Effects of palmitate upon NFκB and MAP kinase 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 NFκB 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 MAP kinases towards activation of the NFκB pathway we initially assessed the activation/phosphorylation status of ERK1/2, JNK and p38 MAPK. Figure 1 shows that all three MAP kinases 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 NFκB in L6 myotubes – To establish what role, if any, activation of the MAP kinase subfamilies play with respect to regulation of the NFκB 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 MAP kinase subfamilies were all activated following 6-8 h of palmitate treatment (Fig 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 whilst neither prevents the stimulus-induced phosphorylation of p38 MAPK by its upstream kinase (Fig 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 [35,36]. The inhibition of MAPKAPK2 with BIRB 796 was verified as part of the present study (see supplementary Figure S1). 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 (Fig 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 IKK α/β complex or the associated loss in I κ B α (Fig 2A and 2B). In contrast, PD184532 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 I κ B α (Fig 2C). This latter finding implies that in L6 myotubes ERK activation is likely to be an important upstream component of the NF κ B proinflammatory response triggered by fatty acid excess.

A role for AMPK in modulating the proinflammatory potential of palmitate – Several reports have suggested that AMPK is able to antagonise the effect of numerous proinflammatory cytokines in various cell types [23-26]. We were therefore keen to assess whether AMPK could also oppose the proinflammatory potential of palmitate. Whilst 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 data already presented, also induced an associated loss in the abundance of I κ B α (Fig 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 increase in I κ B α abundance. On the basis of these observations we hypothesised that activation of AMPK may protect the muscle cells against the proinflammatory effects of palmitate.

In order to test this hypothesis L6 muscle cells were treated with palmitate for 16 h in the absence or presence of AMPK activating drugs, phenformin or A769662. Fig 3B shows that when presented alone to muscle cells both drugs induced activation of AMPK based on the increased phosphorylation of ACC (Ser79), an AMPK target and thus a downstream readout for the kinase. Both phenformin and A769662 antagonised the palmitate-induced activation of IKK (Fig 3B), and consistent with this effect, both compounds ameliorated the fatty acid-induced loss in I κ B α (Fig. 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 α 1 and α 2 subunits of AMPK (Fig 3B). Our observations thus imply that AMPK activation exerts a suppressive effect upon palmitate-induced NF κ B 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 κ B-regulated muscle genes [38,39], was assessed using real-time quantitative PCR following cell incubation with palmitate. Fig 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 incubation with the fatty acid significantly antagonised the increase in both IL-6 and CINC-1 expression. Given that AMPK activation opposes the palmitate-induced phosphorylation of ERK1/2 in a time dependent manner that correlates tightly with reduced phosphorylation of IKK (Fig 3B) we postulated that AMPK may suppress the proinflammatory potential of palmitate *via* inhibition of ERK1/2 signalling.

Since AMPK antagonises the palmitate-induced phosphorylation of ERK1/2 (Fig 3B) we assessed whether AMPK activation in combination with MEK inhibition exerted an additive effect with respect to suppression of IKK phosphorylation/activation and I κ B α protein loss. Fig 5A 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 I κ B α .

abundance. Of interest, in parallel experiments we assessed whether AMPK activation would also antagonise the palmitate-induced activation of p38 MAPK and JNK. The immunoblot data presented in Fig 5B shows that unlike the ERK pathway, activation of the two other MAP kinase sub-families 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. Fig 5C shows 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 antagonised the PMA-induced phosphorylation of MEK, ERK and IKK.

Effects of expressing a dominant negative AMPK on palmitate-induced I κ B α degradation - To further validate that AMPK acts a negative modulator of NF κ B signalling, a myc-tagged dominant negative AMPK adenoviral construct (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 to an alanine in α 1 AMPK as previously described [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 virus lacking the AMPK construct (Fig 6A). Confirmation that the construct behaved in a dominant negative manner was established by demonstrating that whilst A769662 induced ACC Ser79 phosphorylation in wild type muscle cells, those expressing the AdAMPK-DN construct lacked this stimulus-induced phosphorylation of ACC (Fig 6A). Consistent with other data presented in this paper, AMPK activation antagonised the fatty-acid induced loss in I κ B α in wild-type L6 muscle cells. However, this antagonism was not observed in cells expressing the AMPK-DN construct (Fig 6A and 6B). Furthermore, the ability of A769662 to counter the palmitate-induced activation of ERK was lost in cells expressing the AMPK-DN construct (Fig 6A).

Effects of ceramide on PKB and NF κ B signalling - We have shown previously that ceramide generated from palmitate or added exogenously to cells in a cell-permeable form promotes a targeted loss in the insulin-dependent activation of PKB by a mechanism involving atypical PKCs (λ/ζ) [6,40,41]. However, there is also evidence in the literature suggesting that in some cell types ceramide promotes activation of NF κ B 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 κ B activation. Consistent with our previous work [6], Fig 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 (2h, 100 μ M) or more chronic low grade (16h, 10 μ M) 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 I κ B α .

Effects of suppressing NF κ B activation on insulin sensitivity of L6 muscle cells - Whilst the data presented in Fig 7 suggests that the insulin-desensitising effect of ceramide in L6 muscle cells is unlikely to involve NF κ B activation, our findings do not exclude the possibility that palmitate may act independently of ceramide to suppress insulin signalling via the NF κ B pathway. To test this we investigated the effect of suppressing NF κ B signalling by two separate approaches and monitored palmitate's effect upon the insulin-dependent activation

of PKB. Fig 8A shows that palmitate blunted the insulin-dependent phosphorylation of PKB. Under these circumstances palmitate induces IKK phosphorylation and a loss of I κ B α and, in line with data presented in earlier figures (Fig 5A), this was antagonised by co-incubation of cells with A769662. However, despite this antagonism in NF κ B signalling, A769662 was unable to halt the loss in the insulin activation of PKB elicited by the fatty acid (Fig 8A). Consistent with this latter finding, AMPK activation with A769662 also failed to antagonise the palmitate-induced loss in insulin-stimulated glucose uptake, which is widely accepted as being a key PKB-regulated process (Fig 8B). To further substantiate these findings L6 cells were infected with an adenoviral construct harbouring a mutant form of I κ B α (S32A/S36A) that acts as a potent repressor of NF κ B activity. Mutation of Ser32 and Ser36 to alanine renders I κ B α resistant to IKK phosphorylation, thereby protecting it from proteosomal degradation and retaining its capacity to hold NF κ B in an inhibited state. Fig.8C shows that palmitate induces IKK activation, I κ B α 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 I κ B α S32A/S36A protein do not exhibit a stimulus-induced increase in IL-6 expression despite the attendant increase in IKK activation (Fig 8C). Moreover, despite suppressing palmitate-induced NF κ B signalling using the super repressor, this experimental approach did not antagonise the loss in insulin-stimulated PKB activation caused by palmitate (Fig 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 free fatty acids that prevail during this condition [43]. In skeletal muscle, fatty acid overload has been shown to enhance proinflammatory NF κ B 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, promote an increase in inflammatory tone is not well understood and, although there is mounting interest of the role played by TLRs in this response, there is a large body of evidence that suggests that 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 here was to (i) gain an insight into how palmitate induces proinflammatory NF κ B signalling in cultured myotubes, with particular focus on the role played by members of the MAP kinase family and ceramide as potential protagonists of the inflammatory response, (ii) establish whether AMPK activation opposes the proinflammatory drive initiated by palmitate and (iii) assess whether activation of NF κ B signalling underpins the loss in insulin sensitivity invoked by palmitate in L6 myotubes.

Our findings indicate that whilst sustained incubation of muscle cells with palmitate induces activation of all three MAP kinase subfamilies, only the classical ERK pathway appears to be important with respect to supporting activation of NF κ B signalling in L6 myotubes (Fig 9A). We suggest that activation of ERK not only precedes that of IKK, but also lies upstream of the IKK/NF κ B 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 I κ B α 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. PD184532 and U0126) significantly

reduce palmitate's capacity to stimulate NF κ B signalling (Fig 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 proinflammatory stimuli (e.g. lipopolysaccharide (LPS), TNF α , IL-8) promote ERK-dependent NF κ B activation, presumably *via* signalling initiated from binding to their respective cell surface receptors. Precisely how palmitate initiates proinflammatory signalling in L6 myotubes is not entirely clear, but is unlikely, in our view, to be initiated *via* TLRs based on 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 TLR2/4 agonist, fails to promote any detectable activation of IKK/NF κ B signalling when incubated with L6 myotubes for periods of up to 12 h yet, under identical incubation conditions, induces robust IKK activation and I κ B α loss in mouse dendritic cells within 30 min (Macrae, Lipina and Hundal, unpublished data). 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 deployed with the muscle cells used in the current 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 κ B signalling under circumstances when TLR2/4 and their adaptor proteins are either stably and moderately over-expressed or silenced in L6 muscle cells. Such studies may provide a more definitive assessment of whether TLRs are involved or not in the proinflammatory response triggered by palmitate in our experimental model.

If not TLRs, what might initiate the fatty acid-induced proinflammatory 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 data indicate that ceramide is unlikely to contribute to activation of the ERK/NF κ B 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, PKC θ , has been implicated in palmitate-induced NF κ B signalling in murine C2C12 myotubes [47]. Whether PKC θ acts directly to activate the IKK/NF κ B 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 κ B 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 κ B activity and expression of NF κ B-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 antagonise the palmitate-induced degradation of I κ B α [57]. Although the latter study did not establish the mechanism by which AICAR spares the fatty acid-induced loss of I κ B α a very recent study has reported that the ribonucleoside impairs

binding of NF κ B to DNA without affecting nuclear translocation of NF κ B or turnover of I κ B α protein in endothelial cells [24]. In contrast, our data suggests 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]) antagonises the palmitate- and PMA-induced activation of ERK, and that this results in a concomitant reduction in IKK/NF κ B signalling and restrained expression of IL-6 and CINC-1 (two known NF κ B 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 serine 621 *in vitro* [58]. It is currently unknown if 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 IKK β or inhibiting nuclear translocation of NF κ B has been shown to protect skeletal muscle against lipid-induced insulin resistance in rodent and cell based studies [22,44]. However, our work indicates that inhibiting the NF κ B pathway either *via* activation of AMPK or by expression of the I κ B α (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 κ B did not prove to be protective in our experimental model. We believe that it is highly unlikely that inhibition of NF κ B 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 current study, occurs in the absence of any detectable activation of NF κ B 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, whilst 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 to ceramide induces a dynamic shift in palmitate metabolism towards greater synthesis of DAG [46]. This increase in DAG exerts an insulin-desensitising effect upon IRS-directed insulin signalling *via* activation of DAG-sensitive PKCs [46]. Consequently, if palmitate-driven synthesis of DAG and ceramide remains unabated under circumstances when NF κ B 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 κ B did not prevent palmitate's capacity to activate p38 MAPK and JNK in L6 myotubes. We cannot exclude the possibility that activation of these MAP kinases 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 work demonstrates that sustained exposure of skeletal muscle cells to palmitate induces NF κ B signalling in an ERK-dependent manner and that activation of AMPK serves to counter-regulate this proinflammatory pathway (Fig 9). Whilst activation of

NFκB appears not to contribute significantly to the insulin-desensitising 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 proinflammatory 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 over looked.

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Figure Legends

Figure 1. Effect of palmitate on NF κ B and MAP kinase signalling. L6 myotubes were treated with (A) BSA-conjugated palmitate or BSA (control) for 16 h at indicated concentrations or (B) palmitate (750 μ M) for the indicated periods. (A and B) Lysates were immunoblotted to assess the phosphorylation status of IKK α/β , ERK1/2, p38 MAPK and JNK and total protein abundance of I κ B α . Equal gel loading was ascertained by immunoblotting with an antibody against β -actin. (B) Effects of palmitate upon I κ B α protein (filled bars) was quantified and expressed as a percentage change from untreated control cells (open bar). Values shown are the mean \pm S.E.M from at least 3 separate experiments.

Figure 2. Effects of p38 MAPK, JNK and ERK inhibition on palmitate-induced NF κ B signalling in L6 myotubes. L6 myotubes were treated with palmitate (750 μ M) for 16 h in the absence or presence of (A) p38 MAPK inhibitors; BIRB 796 (0.1 μ M) or SB202190 (1 μ M) for indicated times, (B) JNK inhibitor; SP600125 (30 μ M) for 8 h or (C) MEK inhibitors; PD184532 (1 μ M) or U0126 (10 μ M). Cell lysates were immunoblotted to assay the phosphorylation status of IKK α/β , p38 MAPK, JNK or ERK1/2 and total protein abundance of I κ B α . Analysis of β -actin was used to assess protein loading in gel lanes.

Figure 3. Effects of AMPK activators on palmitate-induced NF κ B and MAP kinase signalling in L6 myotubes. (A) L6 myotubes were treated with palmitate (750 μ M) for 16 h or with A769662 (100 μ M) for 2 h. Lysates were either immunoprecipitated with a pan-AMPK α 1/ α 2 antibody and used to assay AMPK activity or immunoblotted to assess the phosphorylation status of AMPK and ACC and total protein abundance of I κ B α . The asterisk signifies a significant difference from the control (untreated) value ($P < 0.05$). (B) L6 myotubes were treated with palmitate for 16 h (750 μ M) in the absence or presence of either A769662 (100 μ M) or phenformin (200 μ M) for the indicated periods. Cell lysates were immunoblotted to assess the phosphorylation status of IKK α/β , ERK1/2 and ACC as well as total protein abundance of I κ B α , 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 I κ B α protein levels from (B) were quantified and expressed as a percentage change from untreated control. Values shown are the mean \pm S.E.M from three separate experiments. The asterisks in A signify significant differences ($P < 0.05$) from the untreated control, whereas in C the significance is relative to the single palmitate-treated value.

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 μ M) for 16 h in the absence or presence of A769662 (100 μ M) for the penultimate 8 h of the fatty acid incubation. RNA was extracted from cells and IL-6 and CINC-1 mRNA determined by real time qPCR as described in methods. Values shown are the mean \pm S.E.M from three separate determinations. The asterisks signify significant differences ($P < 0.05$) between the indicated bars.

Figure 5. Effects of AMPK activation on palmitate and PMA-induced NF κ B and MAP kinase signalling in L6 myotubes. Muscle cells were treated with (A and B) palmitate (750 μ M) for 16 h or (C) PMA (100 nM) for 2 h in the absence or presence of A769662 (100 μ M) for 8 h (A and B) or 2 h (C). In some experiments, L6 myotubes were also treated with U0126 (10 μ M) for 8 h. Cell lysates were immunoblotted to assess the phosphorylation status

of IKK α / β ACC, ERK1/2, p38 MAPK, JNK and MEK1/2 as well as total protein abundance of I κ B α . Analysis of β -actin was used to assess protein loading in gel lanes.

Figure 6. Expression of a dominant negative AMPK in L6 myotubes antagonises the repressive effect of A769662 on palmitate-induced NF κ B 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 μ M) for 16 h in the absence or presence of A769662 (100 μ M) for the final 8 h of treatment. (A) Cell lysates were immunoblotted to assess the phosphorylation status of ACC and ERK1/2 and cellular levels of I κ B α , AMPK α 1/ α 2 and myc. Immunoblotting for β -actin provided a means for assessing protein loading in gel lanes. (B) Quantification of I κ B α abundance and ERK1/2 phosphorylation, values shown are the mean \pm S.E.M for between 3-5 separate experiments. Asterisks signify significant differences from the appropriate mock-infected untreated control, the hash signifies a significant difference from the palmitate treatment value ($P < 0.05$).

Figure 7. Effects of C2-ceramide on activation of the NF- κ B proinflammatory and MAP kinase pathways. Representative immunoblots showing the effect of incubating L6 myotubes with either palmitate (Pal, 750 μ M) for 16 h or C2-ceramide for 2 h (Cer, 100 μ M) or 16 h (Cer, 10 μ M) 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, IKK and total protein amount of I κ B α . Equal loading was ascertained by immunoblotting with an antibody against total PKB.

Figure 8. Effects of antagonising NF κ B signalling on palmitate-induced inhibition of insulin-stimulated PKB activation and glucose uptake in L6 myotubes. L6 myotubes were treated with palmitate (750 μ M) for 16 h in the absence or presence of (A) A769662 (100 μ M) for the final 8 h or (A, C and D) insulin (100 nM) for 10 min or (B) insulin (100 nM) 30 min. (A) Cell lysates were immunoblotted for assessing the phosphorylation status of ACC, PKB and IKK α / β and total protein abundance of I κ B α . 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 mean \pm SEM 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 I κ B α (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 data is expressed as mean \pm SEM from three separate experiments. The lower panel shows immunoblot analysis of cell lysates assessing the phosphorylation status of IKK α / β and PKB and total protein abundance of I κ B α (endogenous and HA-tagged). Gel loading was ascertained by immunoblotting with an antibody against total PKB. The asterisk signifies a significant difference from appropriate control values ($P < 0.05$).

Figure 9. Schematic illustrating the effects of palmitate and AMPK activation upon the IKK/NF κ B signalling axis in L6 muscle cells.

(A) Sustained exposure of L6 myotubes to palmitate induces phosphorylation and activation of MAP kinase family members. Activation of ERK promotes downstream phosphorylation/activation of IKK causing a concomitant phosphorylation and proteosomal mediated loss of I κ B α thereby promoting an increase in the expression of NF κ B target genes.

(B) MEK inhibition or AMPK activation antagonises palmitate-induced activation of ERK thus curtailing activation of downstream proinflammatory NF κ B signalling. Red arrow head lines indicate a stimulatory action, whereas those that are black indicate an inhibitory effect.

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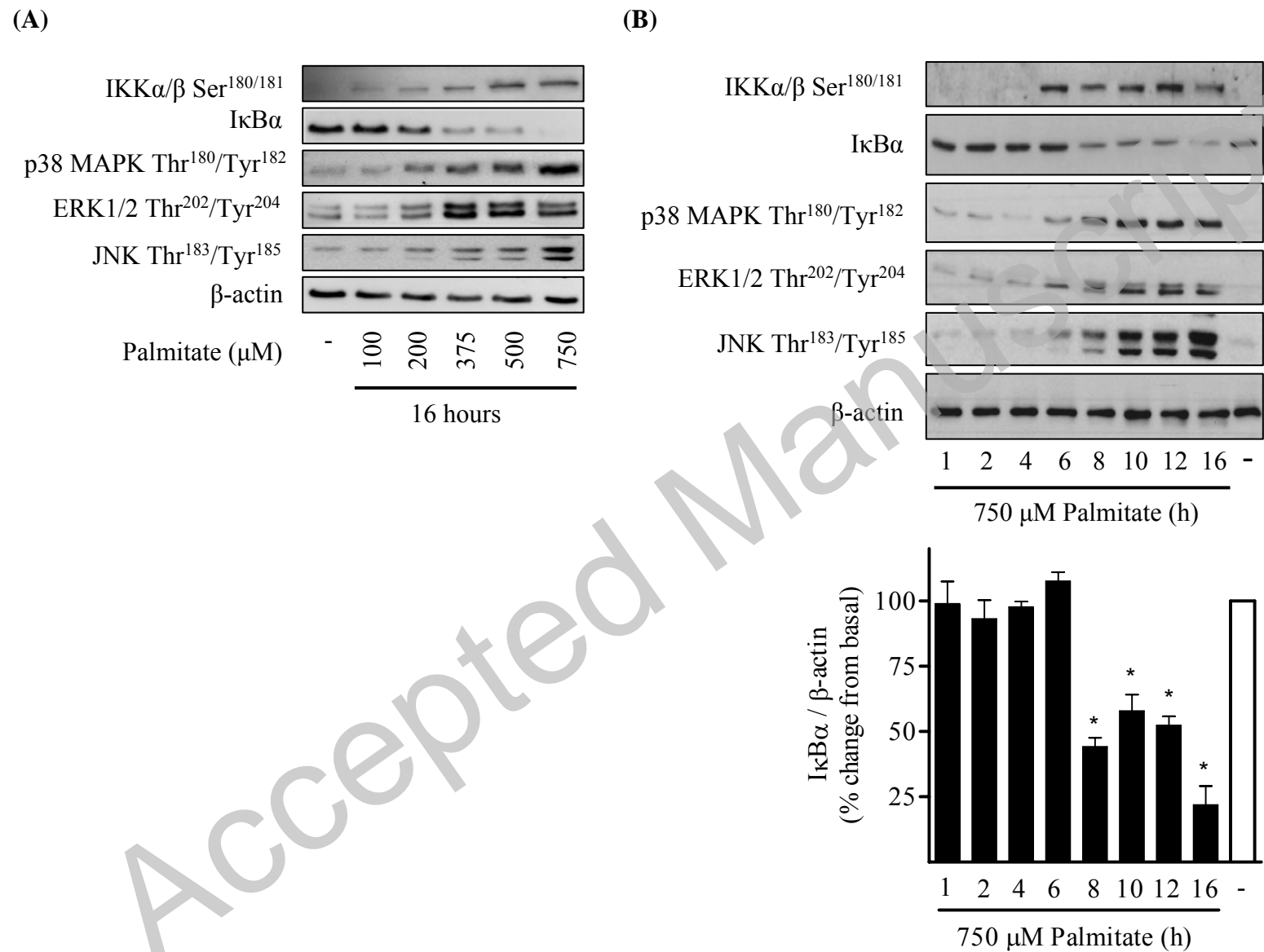


Figure 1

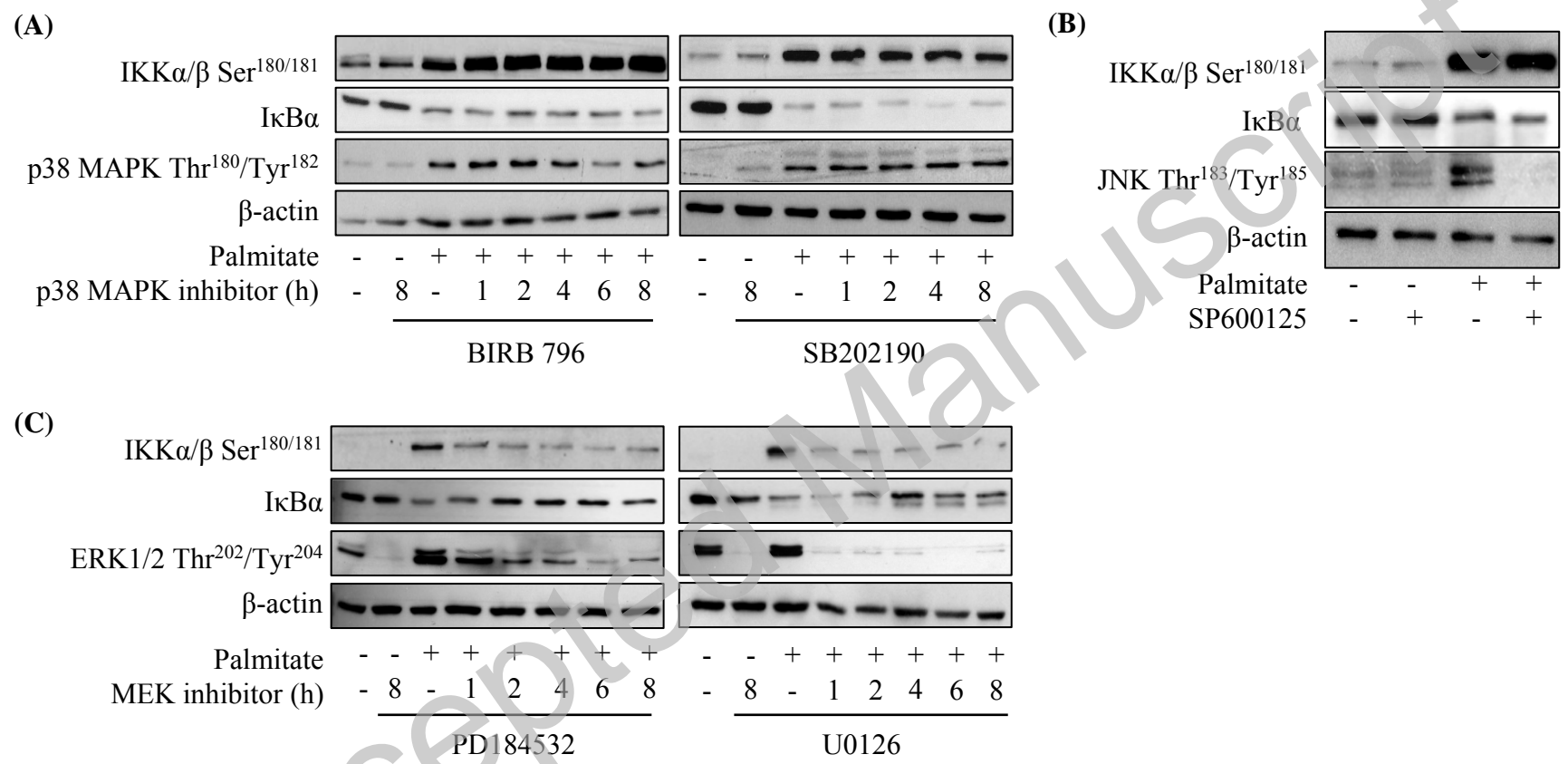


Figure 2

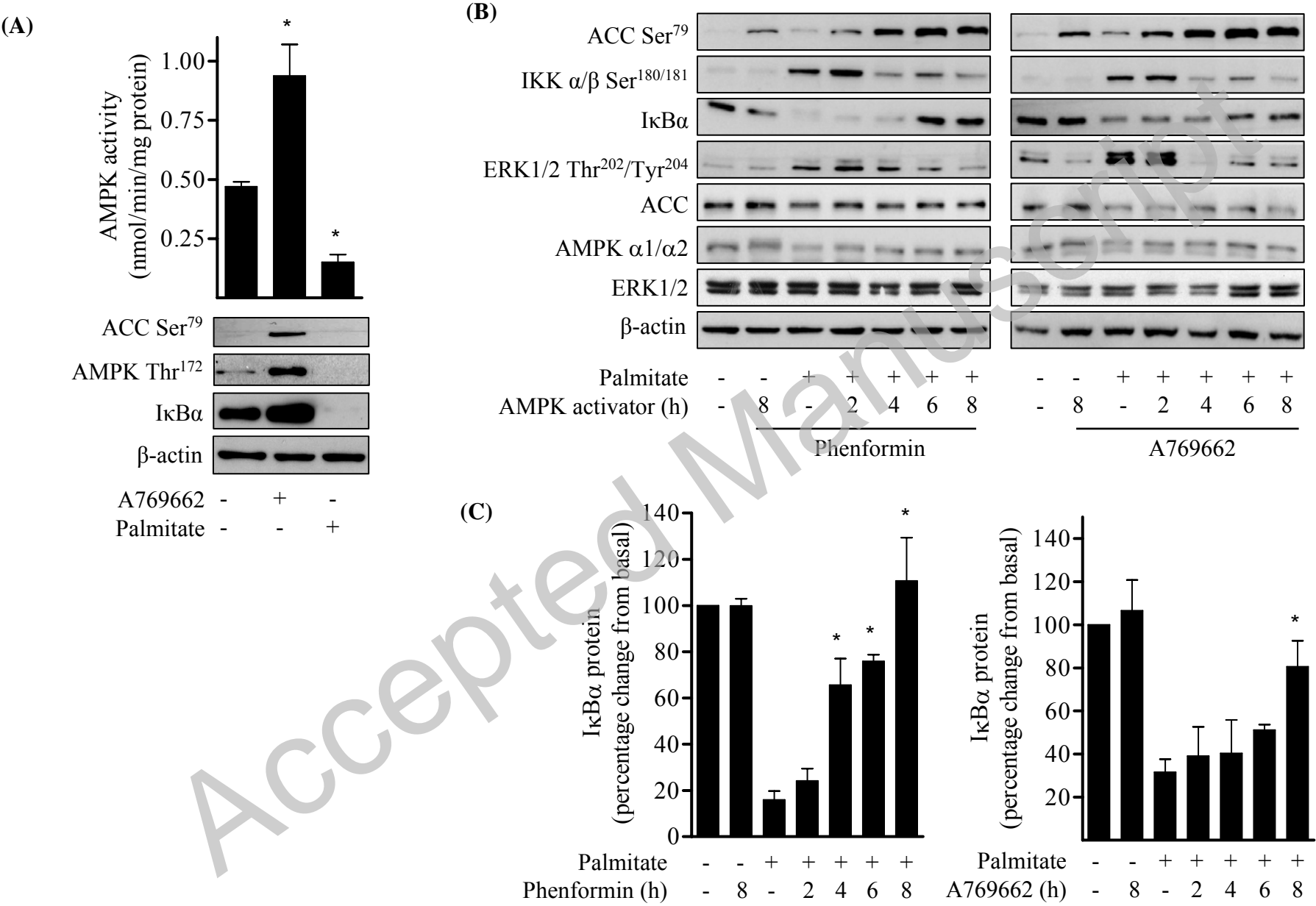


Figure 3

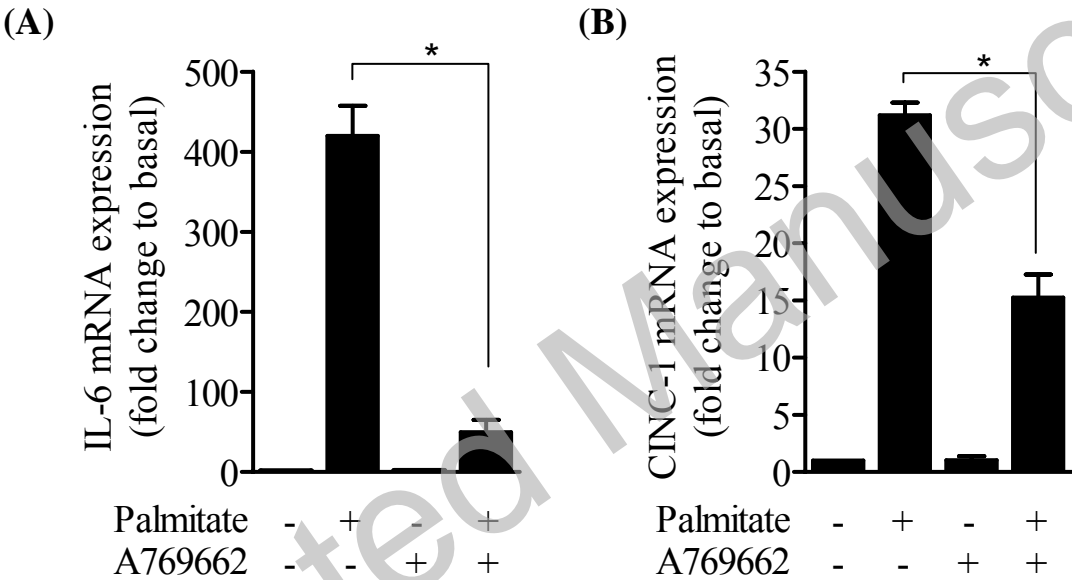


Figure 4

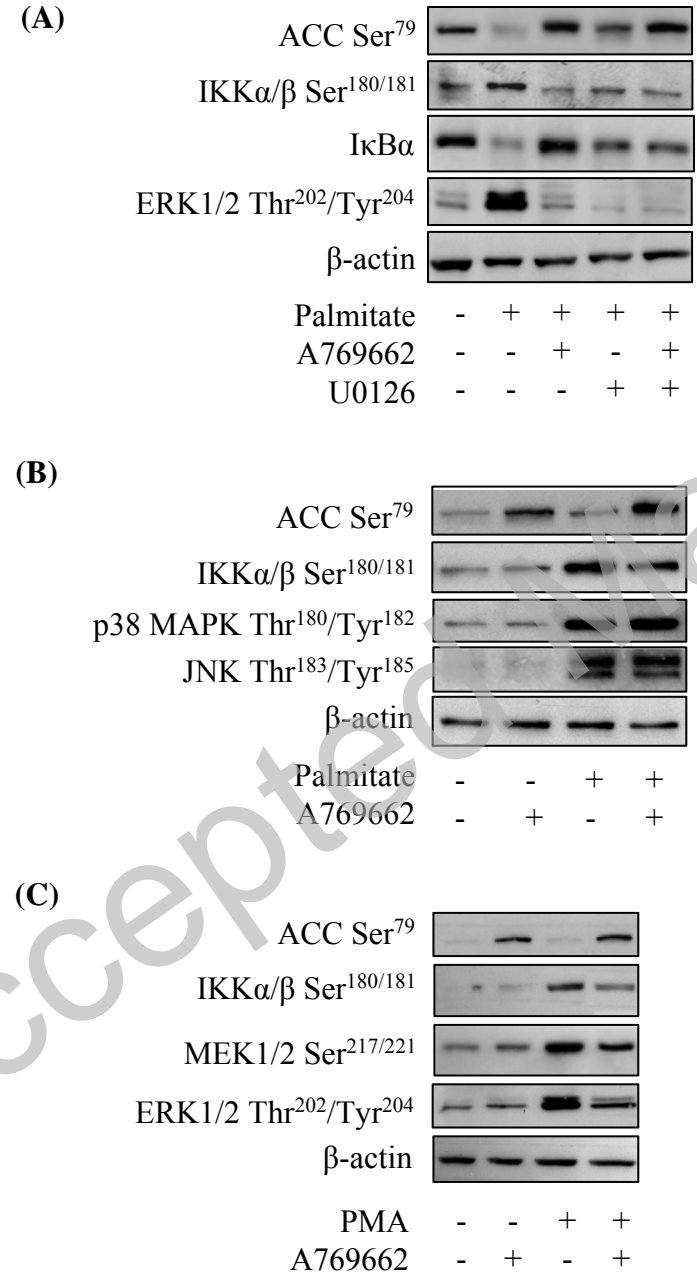
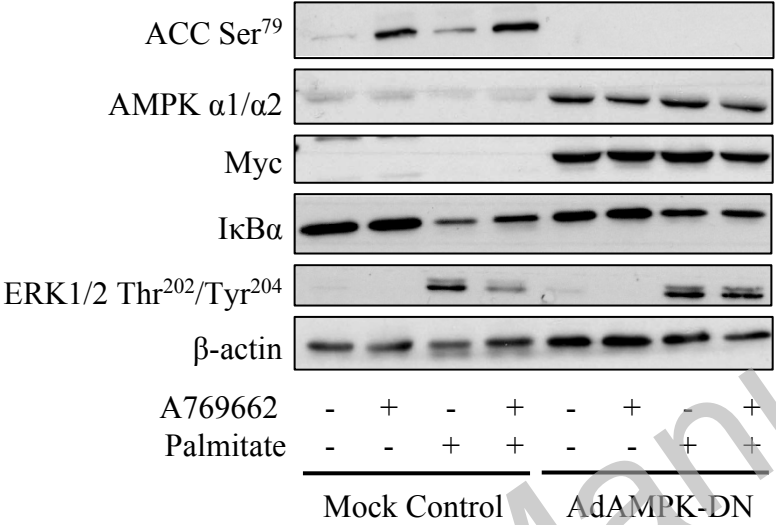


Figure 5

(A)



(B)

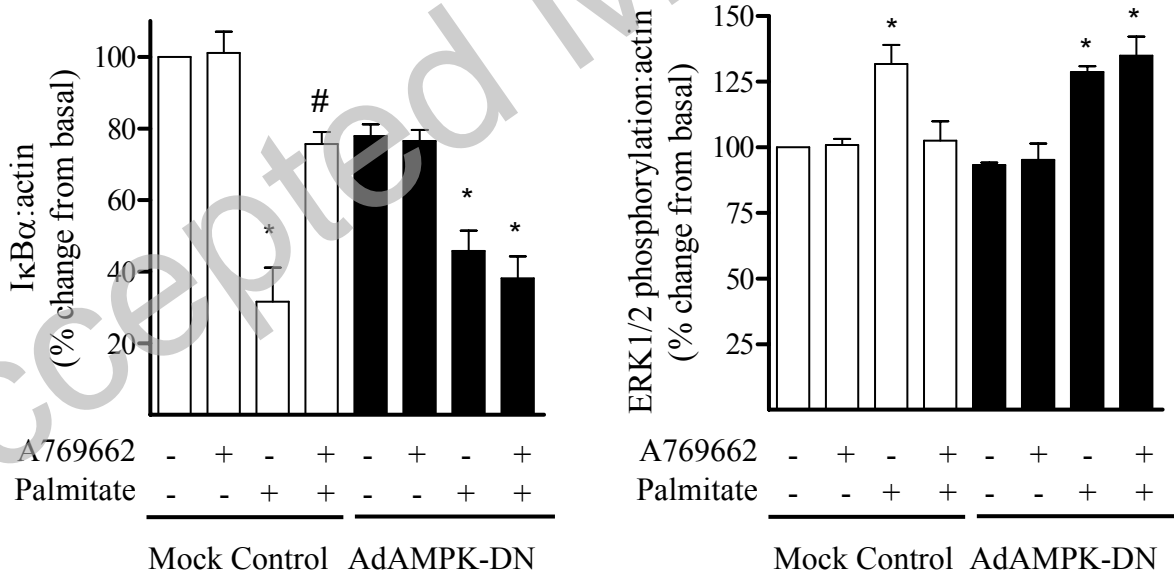


Figure 6

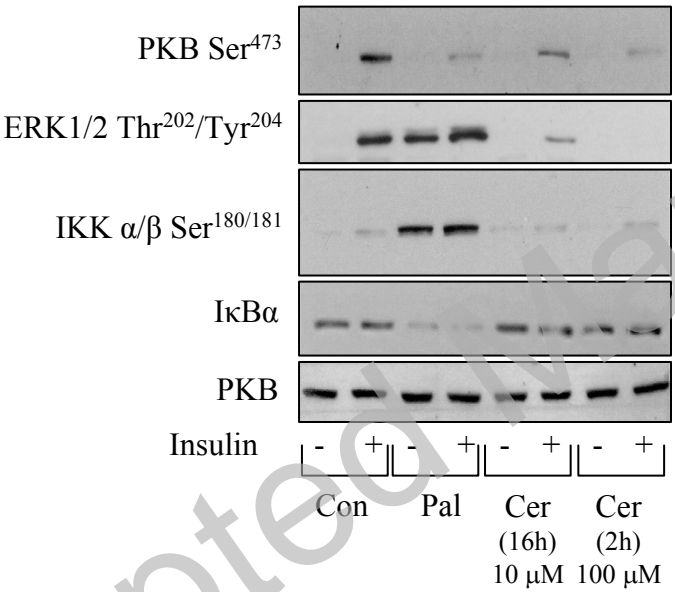


Figure 7

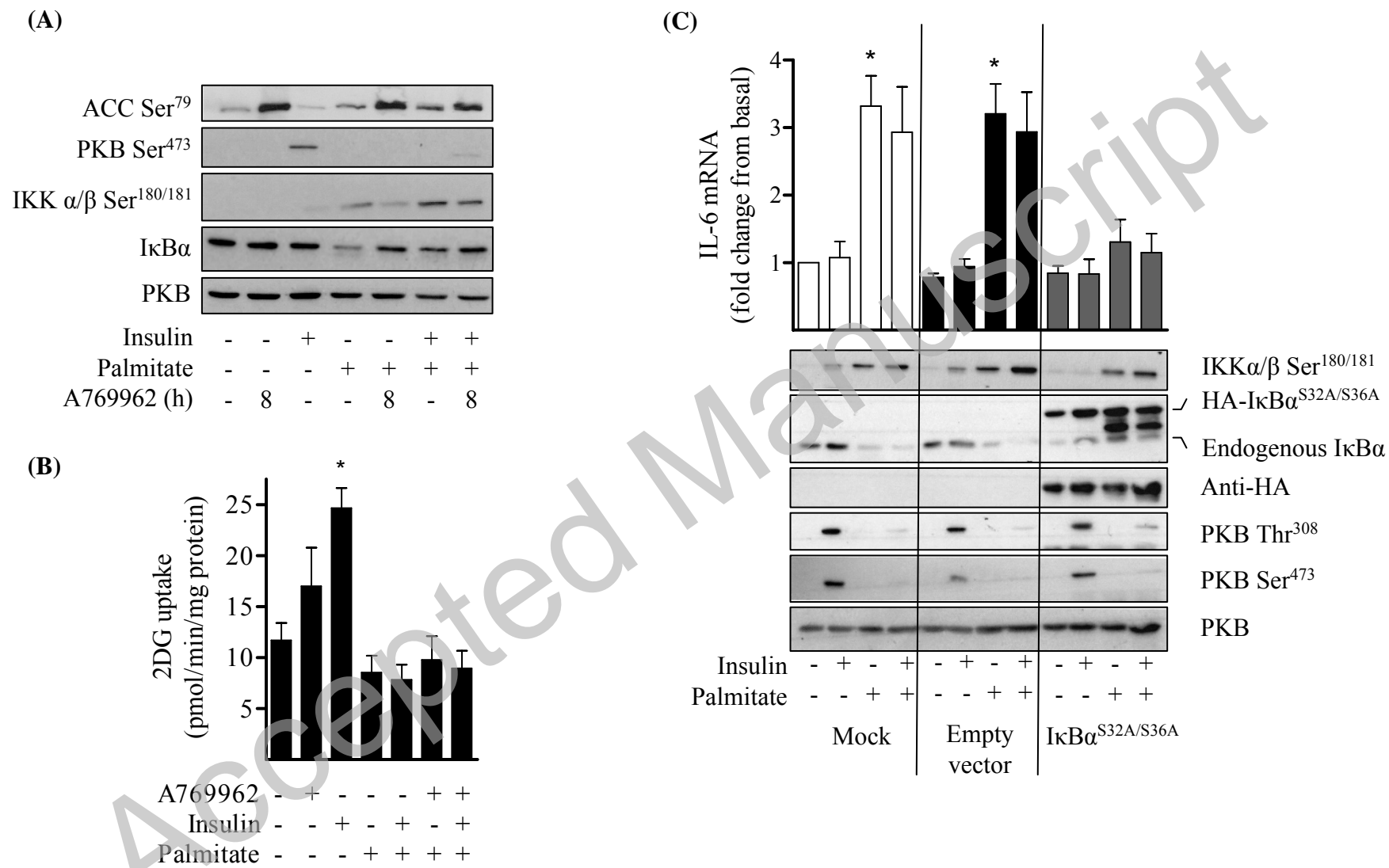
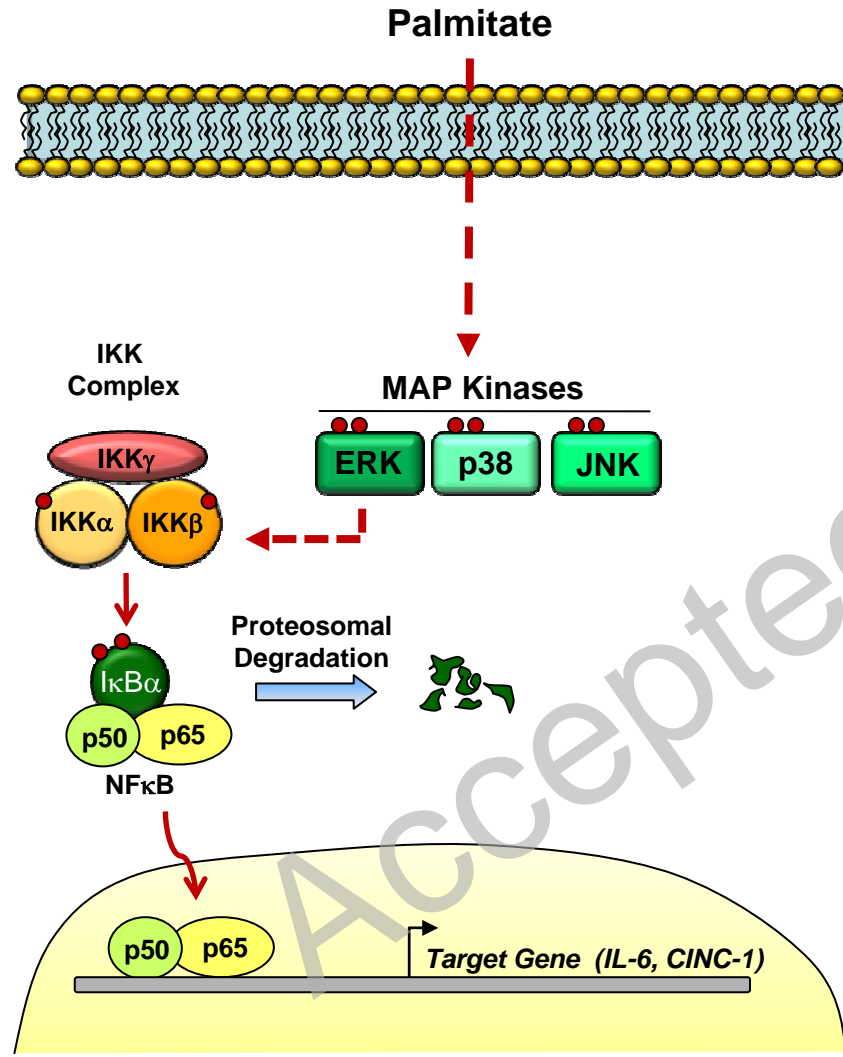


Figure 8

(A) Palmitate effects



(B) Effect of MEK inhibition or AMPK activation

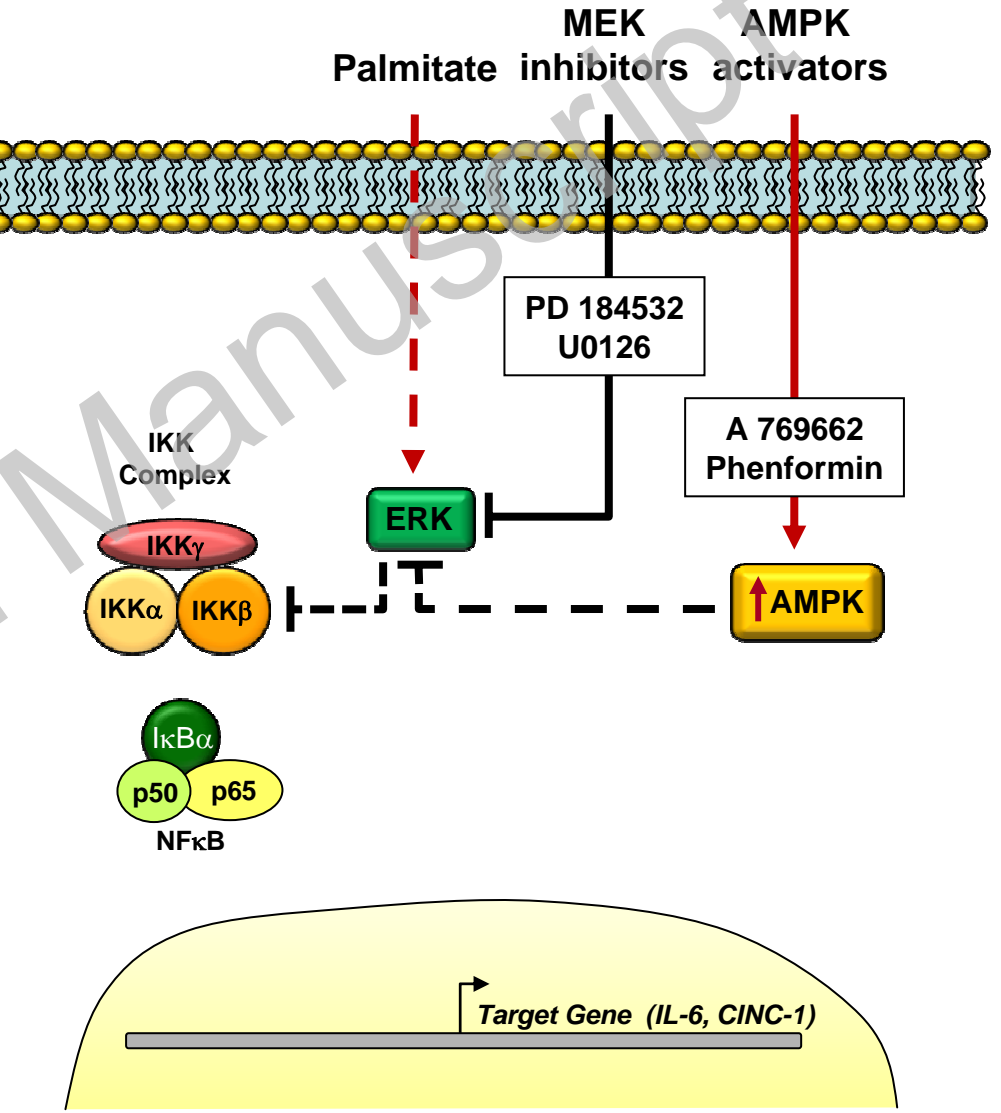


Figure 9