Tumor necrosis factor α-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling

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Summary

Elevated levels of tumor necrosis factor (TNFα) are implicated in the development of insulin resistance, but the mechanisms mediating these chronic effects are not completely understood. We demonstrate that TNFα signaling through TNF receptor (TNFR) 1 suppresses AMPK activity via transcriptional upregulation of protein phosphatase 2C (PP2C). This in turn reduces ACC phosphorylation, suppressing fatty-acid oxidation, increasing intramuscular diacylglycerol accumulation, and causing insulin resistance in skeletal muscle, effects observed both in vitro and in vivo. Importantly even at pathologically elevated levels of TNFα observed in obesity, the suppressive effects of TNFα on AMPK signaling are reversed in mice null for both TNFR1 and 2 or following treatment with a TNFα neutralizing antibody. Our data demonstrate that AMPK is an important TNFα signaling target and is a contributing factor to the suppression of fatty-acid oxidation and the development of lipid-induced insulin resistance in obesity.

Introduction

Obesity and type 2 diabetes are associated with a chronic inflammatory response that is characterized by abnormal cytokine production and systemic insulin resistance (Wellen and Hotamisligil, 2005). It is becoming increasingly apparent that increased lipid metabolites contribute to insulin resistance in skeletal muscle (reviewed in Shulman, 2000). Recent work has demonstrated that obesity and excess lipid accumulation within skeletal muscle could trigger the activation of a serine/threonine kinase cascade involving activation of protein kinase C (PKC) (Li et al., 2004; Yu et al., 2002), IK kinase-β (IKK-β) (Arkan et al., 2005; Yuan et al., 2001), and c-*jun* terminal amino kinase (JNK) (Hirosumi et al., 2002). Of the proinflammatory cytokines, tumor necrosis factor-α (TNFα) has been implicated in the pathogenesis of insulin resistance because it is elevated in the circulation (Zinman et al., 1999), skeletal muscle (Saghizadeh et al., 1996), and adipose tissue (Hotamisligil et al., 1995) of patients with type 2 diabetes. TNFα expression is correlated with reduced insulin-stimulated glucose disposal (Kern et al., 2001; Saghizadeh et al., 1996; Zinman et al., 1999), and a recent study has demonstrated that an acute infusion inhibits insulin-stimulated glucose disposal (Plomgaard et al., 2005). The link between inflammatory cytokines and insulin action in obesity and type 2 diabetes was first recognized by the discovery of adipose tissue TNFα overexpression in obesity (Hotamisligil et al., 1993, 1994a, 1994b, 1995, 1996) and its impact on systemic insulin sensitivity (Uysal et al., 1997). These studies have clearly shown that TNFα causes metabolic dysregulation at multiple sites including altered lipid homeostasis and insulin signaling (Wellen and Hotamisligil, 2005). However, the precise molecular signals underlying TNFα-induced metabolic dysregulation are still largely unknown.

AMP-activated protein kinase (AMPK) is pivotal in the regulation of skeletal muscle fatty-acid metabolism (Steinberg et al., 2006). AMPK activation results in increasing rates of skeletal muscle fatty-acid oxidation by phosphorylating acetyl-CoA carboxylase (ACC), leading to reduced malonyl-CoA and increased long-chain fatty acyl CoA flux into the mitochondria via carnitine palmitoyl transferase-1 (Ruderman et al., 1999). The regulation of AMPK is complex involving allosteric control by an increase in the cellular AMP:ATP ratio (Kemp et al., 2003) and covalent regulation through phosphorylation of Thr172 within the α catalytic subunit by the upstream kinase LKB1 (Hawley et al., 2003; Woods et al., 2003) and dephosphorylation by protein phosphatase 2C (PP2C) (Davies et al., 1995). AMPK is activated in skeletal muscle by hormones including leptin (Minokoshi et al., 2002), adiponectin (Yamauchi et al., 2002), interleukin-6 (Carey et al., 2006; Kelly et al., 2004), and ciliary neurotrophic factor (Watt et al., 2006), while AMPK phosphorylation is suppressed by resistin (Satoh et al., 2004). Defective skeletal muscle fatty-acid metabolism in obesity involves suppressed rates of fatty-acid oxidation (Gaster et al., 2004; Hulver et al., 2003; Kelley and Simoneau, 1994) effects that may be related to reduced skeletal muscle AMPK activity (Bandyopadhyay et al., 2006). As TNFα is elevated in obesity and insulin resistance we tested the hypothesis that suppressed rates of fatty-acid oxidation are mediated via TNFα-induced inhibition of AMPK signaling.

Results and discussion

TNFα inhibits AMPK signaling via PP2C

To investigate whether TNFα altered AMPK signaling, initial studies were conducted in cultured L6 muscle cells. TNFα reduced AMPK activity after 8 hr of treatment, an effect that was maintained for 16 hr but was not significant at earlier time points (Figure 1A). This effect was observed following 16 hr at concentrations of 10 and 100 ng/ml (Figure 1B). TNFα treatment (10 ng/ml) for 16 hr suppressed basal and AICAR (5-aminoimidazole-4-carboxamide) stimulated AMPK Thr172 phosphorylation and ACCβ phosphorylation (Figures 1C and 1D, respectively). AMPK and ACC protein expression were unaltered (Figures 1C and 1D).

LKB1 is the principal upstream kinase of AMPK in skeletal muscle (Sakamoto et al., 2004). Consistent with previous studies, which have demonstrated that LKB1 is constitutively active (Hawley et al., 2003; Woods et al., 2003), TNFα treatment for 16 hr did not alter LKB1 activity in L6 myotubes (data not shown). The covalent regulation of AMPK is also regulated by the activity of PP2C (Davies et al., 1995). Consistent with the time course of changes in AMPK activity, *PP2C* (also known as *Ppm1a*) mRNA (Figure 1E) and PP2C protein expression (Figure 1F) were elevated after 16 hr of TNFα treatment. These data indicated that increased PP2C levels may account for the reduced AMPK activity and phosphorylation observed following TNFα treatment. To test this hypothesis directly we attempted to inhibit PP2C transcription using short interfering RNA (siRNA) in L6 myoblasts. We utilized low titers of PP2C siRNA which tended to modestly reduce basal PP2C expression (−25%, p= 0.061, Figure 1G) but importantly prevented TNFα-mediated regulation. This experimental set-up was critical so that the impact of PP2C expression on AMPK activity could be examined without increasing basal phosphorylation of AMPK (Figure 1H), hence avoiding complications of elevated AMPK in PP2C siRNA-treated myotubes. In this paradigm, TNFα treatment for 16 hr increased PP2C expression in Con siRNA but not PP2C siRNA myotubes (Figure 1G). Importantly, AMPK phosphorylation was maintained in PP2C siRNA-treated cells following TNFα treatment (Figure 1H).

Since AMPK is an important regulator of fatty-acid oxidation we next examined whether the suppressive effects of TNFα on AMPK activity altered ACC phosphorylation and fatty-acid oxidation. In preliminary experiments TNFα suppressed ACC phosphorylation and fatty-acid oxidation (−27% and −17%, $p < 0.05$, respectively). We therefore examined whether the maintenance of AMPK signaling through inhibition of PP2C transcription using siRNA as described above or a constitutively active AMPK (α1T172D) adenovirus (Ad-CA) (Woods et al., 2000) would reverse these downstream events. TNFα treatment suppressed ACC phosphorylation and fatty-acid oxidation in control siRNA-treated cells, but this effect was reversed following PP2C siRNA (Figures 2A and 2B). Similarly, in mock-infected cells (Ad null), TNFα treatment reduced ACC phosphorylation (Figure 2A) and palmitate oxidation (Figure 2B), but Ad-CA prevented the suppressive effects of TNFα on ACC phosphorylation and palmitate oxidation.

PP2C siRNA reverses TNFα-induced insulin resistance

TNFα-induced insulin resistance involves the inhibition of insulin signaling by JNK (Hirosumi et al., 2002) and IκKβ/NFκB (Arkan et al., 2005; Cai et al., 2005). To determine whether the

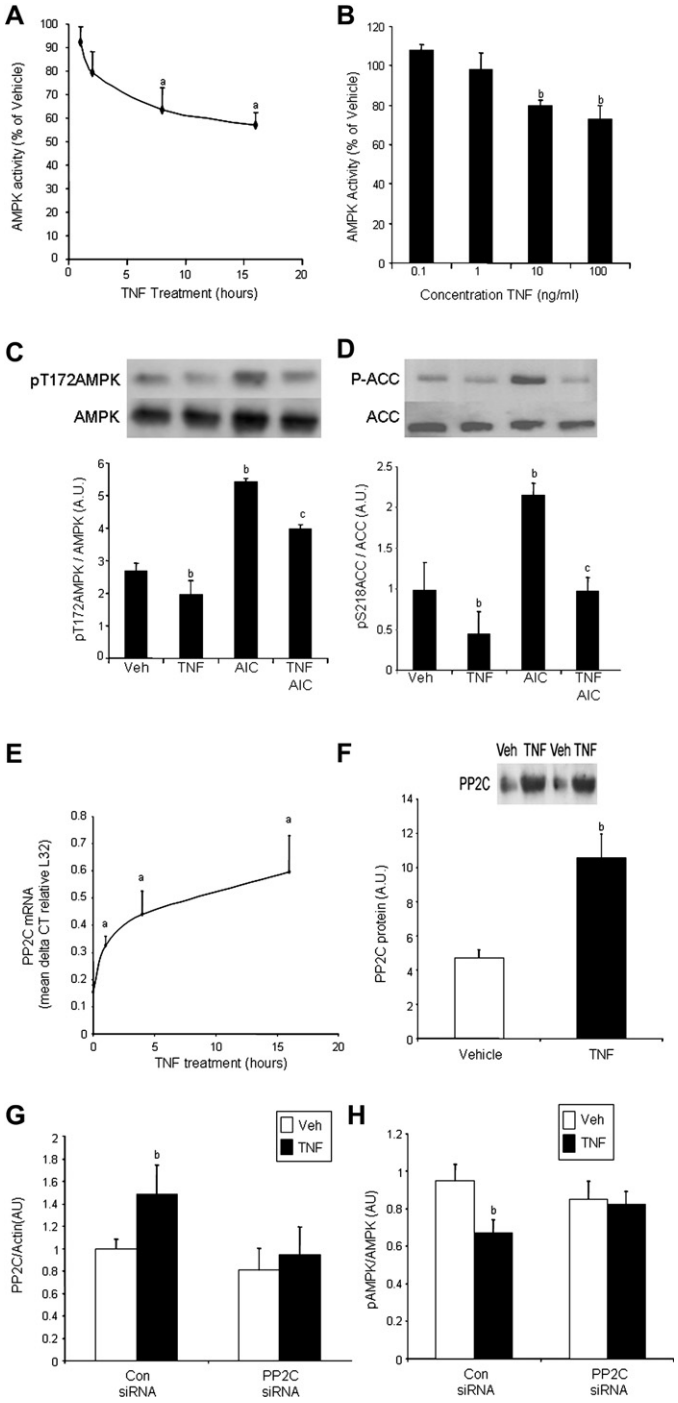

Figure 1. TNF reduces AMPK activity in cultured skeletal muscle via protein phosphatase 2C

A) TNFα reduced AMPK activity after 8 hr of treatment, an effect that was maintained for 16 hr. **B)** Reduced AMPK activity was observed following 16 hr at concentrations as low as 10 ng/ml. TNFα treatment (10 ng/ml) for 16 hr suppressed basal and AICAR-stimulated (**C**) AMPK pThr172 and (**D**) ACC pS218. Consistent with the time course of changes in AMPK activity, *PP2C* mRNA (**E**) and PP2C protein (**F**) were elevated following 16 hr of TNFα treatment. Short interfering RNA directed toward PP2C (PP2C siRNA) inhibited increases in PP2C protein expression (**G**) and the suppression of AMPK pT172 (**H**) by TNFα. Values are means ± SEM (n = 6–12 for 2–3 separate experiments). [a]$p < 0.05$ versus time 0, [b]$p < 0.05$ versus vehicle for that treatment, [c]$p < 0.05$ versus AICAR.

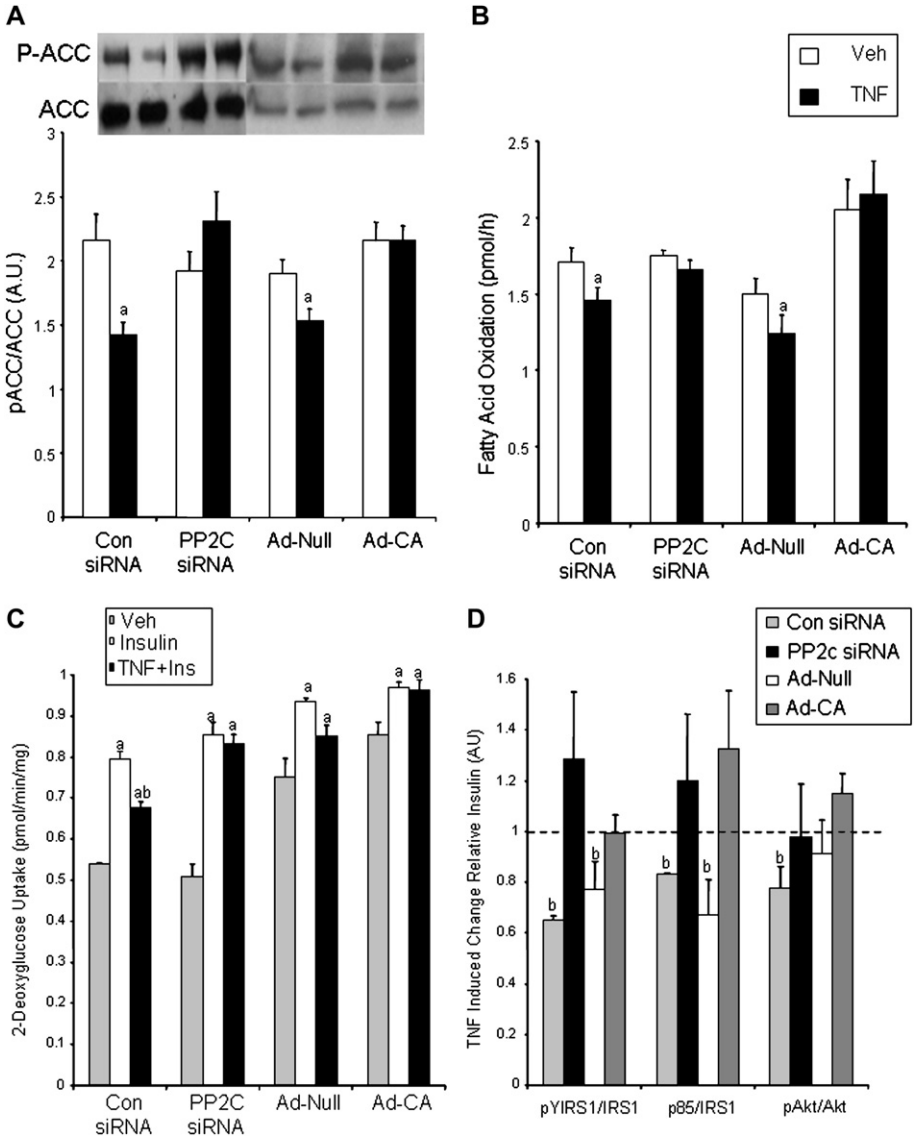

Figure 2. PP2C siRNA or a constitutively active AMPK reverses TNFα effects on fatty-acid oxidation and glucose uptake

TNFα suppressed (**A**) ACC pS218 and (**B**) palmitate oxidation effects that were reversed in cells treated with PP2C siRNA or infected with Ad-CA. (**C**) For 2-deoxyglucose uptake experiments cells were treated with vehicle (PBS) or insulin (50 nM) for 20 min following treatment with or without TNFα for 16 hr. (**D**) For insulin signaling experiments cells were treated with or without TNFα for 16 hr followed by vehicle (PBS) or insulin (50 nM) for 5 min. Data are presented as changes relative to insulin. Values are means ± SEM (n = 6–8 for 2 separate experiments). $^{a}p < 0.05$ versus vehicle. $^{b}p < 0.05$ versus insulin from the same condition.

suppressive effects of TNFα on AMPK signaling also contributed to the development of insulin resistance 2-DG uptake was determined in L6 myotubes treated with PP2C siRNA or CA-AMPK adenovirus as described above. Insulin increased glucose uptake by ~50% in both Con and PP2C siRNA-L6 myotubes. As anticipated, a 16 hr treatment with TNFα partially suppressed insulin-stimulated glucose uptake, but this effect was reversed in PP2C siRNA-treated cells (Figure 2C). In Ad null infected cells insulin increased glucose uptake by ~25%. Reduced insulin-stimulated glucose uptake relative to siRNA-treated cells appeared to be due primarily to a higher rate of basal glucose uptake. TNFα modestly inhibited glucose uptake in Ad null treated cells but Ad-CA-infected cells were protected from TNFα-induced suppression of insulin-stimulated glucose uptake (Figure 2C).

To examine the potential mechanism(s) by which PP2C siRNA or Ad-CA may rescue TNFα-induced insulin resistance, we examined proximal and distal aspects of the insulin signaling pathway including tyrosine phosphorylation of IRS1, p85 subunit of PI3-kinase IRS1 association, and Akt phosphorylation. In this experiment we compared insulin relative to TNFα plus insulin, and because there were no significant differences in insulin stimulation relative to basal controls between treatments (i.e., Con siRNA, PP2C siRNA, Ad null or Ad-CA) insulin conditions were standardized across all groups. TNFα treatment suppressed all aspects of insulin signaling in Con siRNA-treated cells, an effect that was also observed in Ad null infected cells with the exception of pAkt/Akt, which was not suppressed following TNFα treatment (Figure 2D). Consistent with improved 2-DG uptake, cells treated with PP2C siRNA or Ad-CA displayed improved insulin signaling following TNFα treatment relative to TNFα-treated Con siRNA and Ad null myotubes (Figure 2D). The mechanism by which PP2C siRNA or Ad-CA rescues TNFα-induced insulin resistance is not clear but may involve AMPK directly inhibiting JNK or NFκB, as has been demonstrated in cultured endothelial cells (Cacicedo et al., 2004).

TNFα infusion inhibits AMPK signaling

To investigate if the effects of TNFα treatment on AMPK signaling, fatty-acid oxidation, and insulin sensitivity observed in cultured skeletal muscle also occurred in vivo, wild-type (WT) or mice that lack the TNFR1 (p55) receptor (*TNFR*$^{-/-}$) were treated for 24 hr with TNFα at a dose previously demonstrated

to induce skeletal muscle insulin resistance (Miles et al., 1997; Ruan et al., 2002). TNFα treatment did not alter body mass or food intake in WT controls or TNFR$^{-/-}$ littermates (data not shown). In WT but not *TNFR*$^{-/-}$ mice TNFα suppressed AMPKα1 and α2 activities (Figure 3A). Reduced AMPK activity was due to reduced AMPK-Thr172 phosphorylation (Figure 3A) as protein expression was unaltered following TNFα treatment (Figure 3A). Consistent with our observations in cultured skeletal muscle, reductions in AMPKThr172 phosphorylation were associated with elevated *PP2C* mRNA (Figure 3B) and PP2C activity (Figure 3C) following TNFα treatment in WT but not *TNFR*$^{-/-}$ mice. The activities of protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) were unaltered as was LKB1 expression and activity (Table S1). Reduced AMPK activity following TNFα treatment was associated with reduced phosphorylation of ACCβ in WT but not *TNFR*$^{-/-}$ mice (Figure 3D).

AMPK activity is also regulated allosterically by alterations in the AMP:ATP ratio via the AMPKγ subunit (Adams et al., 2004; Cheung et al., 2000). We found that TNFα did not alter skeletal muscle nucleotide levels (Table S1). Reduced activation of AMPK by AICAR (Aschenbach et al., 2002; Wojtaszewski et al., 2002) or contraction (Derave et al., 2000; Kawanaka et al., 2000) have been correlated with high glycogen levels, although the mechanisms by which this occurs are not clear. Glycogen levels tended to be reduced following TNF treatment in WT and TNFR$^{-/-}$ mice (Table S1, p = 0.11).

TNFα infusion increases skeletal muscle lipid

Consistent with reduced AMPK and ACCβ phosphorylation, TNFα suppressed fatty-acid oxidation (Figure 3E). Suppressed AMPK activity and fatty-acid oxidation in WT mice treated with TNFα was associated with increased triacylglycerol (TAG) and diacylglycerol (DAG) (Figure 3F) accumulation in skeletal muscle. TNFα had no effect on ceramide accumulation in WT or *TNFR*$^{-/-}$ mice (WT: saline 54 ± 10, TNF 55 ± 6; *TNFR*$^{-/-}$: saline 53 ± 10, TNF 63 ± 5 nmol/g dm). These data demonstrate that TNFα signaling through the p55/TNFR1 is critical for mediating the effects of TNFα on AMPK signaling and fatty-acid oxidation.

Several studies have implicated elevated DAG in the development of skeletal muscle insulin resistance by direct inhibition of insulin signaling through the activation of the novel PKC isoforms ε and θ (Kim et al., 2004; Yu et al., 2002). We therefore sought to examine whether the TNFα-mediated increases in DAG content were associated with enhanced PKC activation. Consistent with this hypothesis, TNFα treatment increased the expression of membrane-associated/activated DAG-sensitive PKC isoforms θ and ε in skeletal muscle of WT but not *TNFR*$^{-/-}$ mice (Figure 4A). PKC α/β1/β2 were not different between groups or treatments (data not shown). In separate experiments, we assessed the effect of TNFα treatment on insulin sensitivity using the hyperinsulinemic-euglycaemic clamp technique. Treatment with TNFα in WT mice suppressed rates of whole-body glucose disposal/Rd (Figure 4B), reduced 2-deoxyglucose uptake in the red *gastrocnemius* (Figure 4C), red and white *quadriceps*, brown adipose tissue, and diaphragm (Table S2), and tended to reduce 2-deoxyglucose uptake in heart and white adipose tissue (Table S2). TNFα-induced insulin resistance in WT animals was associated with reduced IRS1 tyrosine phosphorylation, reduced association of IRS1 with the p85 catalytic subunit of PI3-K, and reduced phosphorylation of Akt (Ser473) in WT mice (Figure 4D). Skeletal muscle insulin sensitivity and insulin signaling were conserved in *TNFR*$^{-/-}$ mice (Figures 4B–4D). Taken together these observations indicate that suppressed AMPK signaling by TNFα may contribute to the development of TNFα-mediated skeletal muscle insulin resistance in vivo. While we cannot eliminate the possibility that the effects of TNFα in vivo were secondary to reductions in leptin, adiponectin, or other cytokines that signal via AMPK, our findings are consistent with the direct

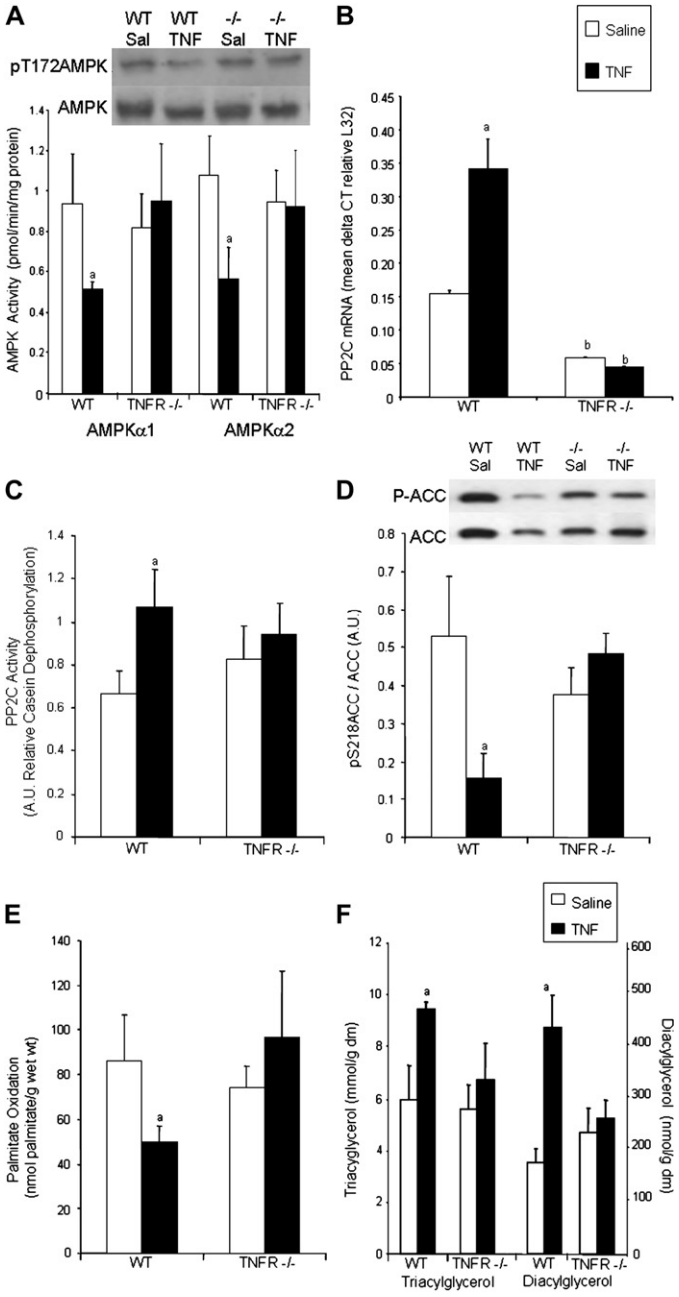

Figure 3. TNF suppresses skeletal muscle AMPK signaling via PP2C in vivo

Wild-type (WT) and *TNFR*$^{-/-}$ mice were treated with vehicle or TNFα for 24 hr. (**A**) AMPKα1 and α2 activities were measured from *gastrocnemius* muscle lysates in immunocomplexes (representative Western blot of pT172AMPK relative to total AMPKα1 and α2 expression above), (**B**) *PP2C* mRNA, (**C**) PP2C activity, (**D**) ACCβ phosphorylation relative total ACC expression (representative Western blot [above] and quantification [below]). (**E**) Fatty-acid oxidation in isolated *soleus* muscle. (**F**) Mixed *gastrocnemius* triacylglycerol and diacylglycerol lipid. Values are means ± SEM (n = 6–8). $^{a}p < 0.05$ versus saline. $^{b}p < 0.05$ versus wild-type.

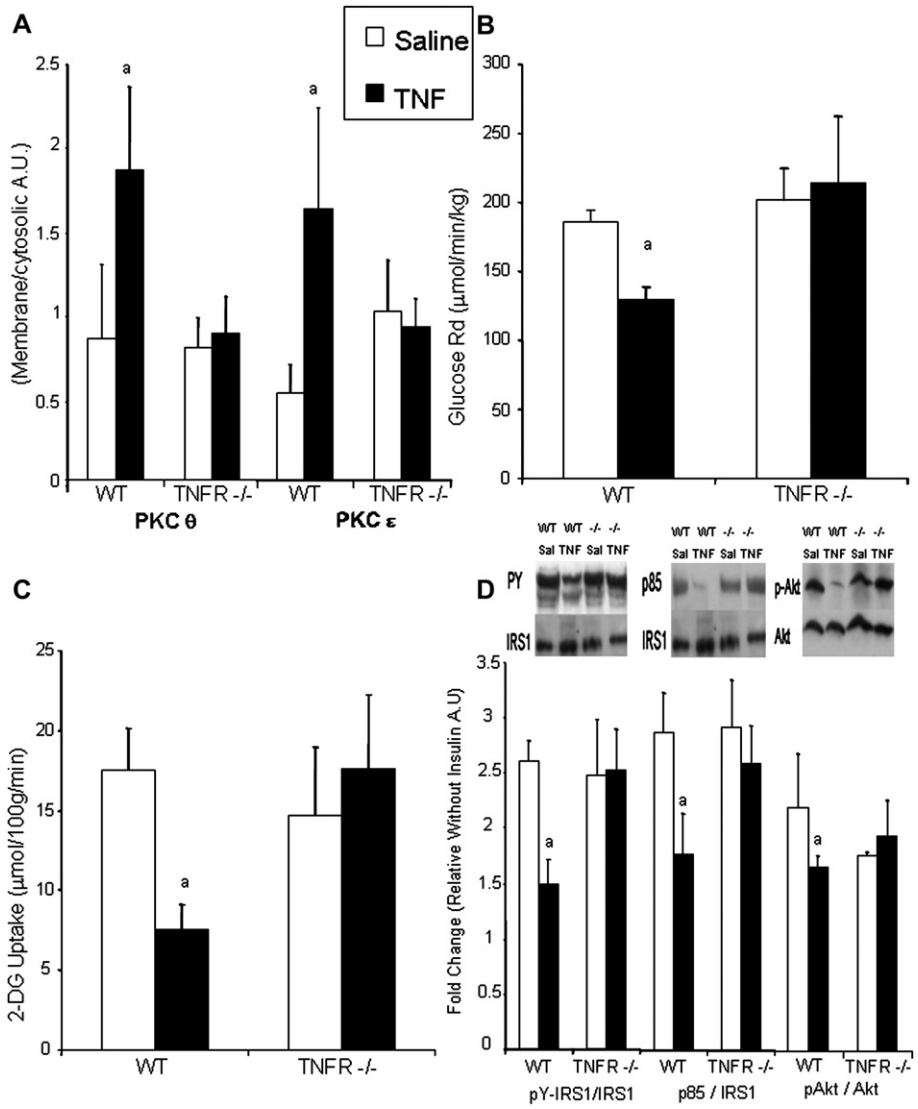

Figure 4. TNF suppression of AMPK signaling is associated with insulin resistance in vivo

Wild-type (WT) and *TNFR*$^{-/-}$ mice were treated with vehicle or TNFα for 24 hr and fasted overnight before euglycemic-hyperinsulinemic clamping studies. (**A**) Protein kinase C (PKC) expression was measured in cytosolic and membrane fractions isolated from 60 mg of insulin-stimulated *gastrocnemius* muscle collected following the clamp and immunoblotted using phosphospecific antibodies to PKC ε and θ. Values are means ± SEM (n = 5) and are expressed as a ratio of membrane versus cytosolic densitometry scanning units. TNFα treatment induced peripheral insulin resistance as indicated by (**B**) suppressed whole-body glucose rate of disappearance (Rd) and (**C**) 2-deoxyglucose uptake into *gastrocnemius* muscle following TNFα treatment in WT but not *TNFR*$^{-/-}$ animals. (**D**) To examine components of the insulin signaling pathway, insulin-stimulated *gastrocnemius* muscle were collected following the euglycemic-hyperinsulinemic clamp and homogenized. Wild-type and *TNFR*$^{-/-}$ mice that were treated with vehicle or TNFα for 24 hr and fasted overnight but not clamped were used as basal controls. IRS1 and associated p85 PI3 kinase were immunoprecipitated from muscle homogenates (1 mg protein) using anti-IRS1 antibody bound protein A beads, then immunoblotted for tyrosine phosphorylation and p85 PI3 kinase. Muscle homogenates were also immunoblotted for phosphorylated and total Akt and p85. Values are means ± SEM (n = 5) corrected for total IRS1, p85, and Akt relative to basal unstimulated muscle for that group. [a]$p < 0.05$ versus saline.

effects of TNFα on PP2C expression, AMPK signaling, and fatty-acid oxidation in skeletal muscle cells.

Inhibition of TNFα signaling in obesity rescues AMPK signaling and impaired lipid metabolism

Several reports have demonstrated reduced skeletal muscle AMPK activity with rodent obesity (Barnes et al., 2002; Yu et al., 2004). A recent study has suggested that in cardiac muscle PP2C may mediate this effect, as elevated PP2C expression was associated with reduced AMPK phosphorylation in *ob*/*ob* mice and Obese Zucker rats, although mechanisms mediating this association were not determined (Wang and Unger, 2005). These findings together with those of our own raise the possibility that TNFα neutralization may prevent the defective AMPK signaling observed in obesity. To test this hypothesis, untreated male lean mice (body mass, 21 ± 0.3 g, n = 6) were compared with obese *ob*/*ob* littermates that were treated with either a control (body mass, 48.2 ± 2.8 g, n = 6) or TNFα (body mass, 46.8 ± 2.2 g, n = 6) neutralizing monoclonal antibody for 48 hr. TNFα neutralization did not restore *extensor digitorium longus* (EDL) insulin-stimulated glucose uptake (Table S3), indicating that skeletal muscle insulin resistance was not reversed following 48 hr of treatment. As anticipated control *ob*/*ob* mice had significantly lower AMPKα2 activity (Figure 5A) and phosphorylation (Figure 5B) than lean controls, an effect that was associated with modestly elevated PP2C expression (Figure 5C). TNF neutralization in *ob*/*ob* mice restored AMPKα2 activity and phosphorylation and reduced PP2C expression to that of lean controls. Obese mice displayed reduced ACCβ phosphorylation (−29%, p = 0.062, Figure 5D) and fatty-acid oxidation (p = 0.02, Figure 5E) and increased DAG (p = 0.01, Figure 5F), defects that were not observed following TNF neutralization. These data demonstrate that elevated levels of TNFα are an important contributing factor to the suppressed rates of fatty-acid oxidation and the development of lipid-induced insulin resistance in obesity.

Interestingly, while 48 hr of TNFα neutralization restored AMPK activity and phosphorylation to that of lean controls, this treatment resulted in no noticeable improvement in skeletal muscle insulin sensitivity of *ob*/*ob* mice. Obesity, insulin resistance, and the accumulation of intramuscular lipid correlate with mitochondrial dysfunction (Petersen et al., 2004), and *ob*/*ob* mice have reduced mitochondrial density (Tanaka et al., 2003). It seems reasonable that these defects may be due to

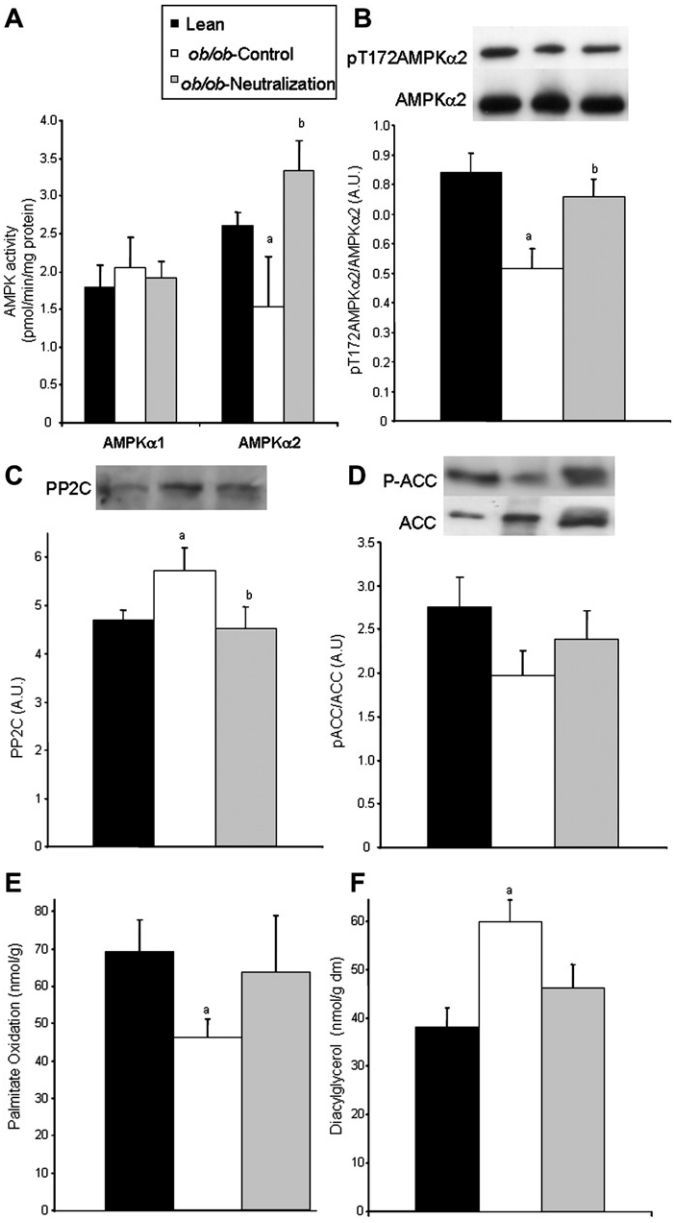

Figure 5. TNF neutralization in *ob/ob* mice reverses defects in AMPK signaling and fatty-acid oxidation

Untreated lean mice and obese *ob/ob* mice were treated with either a control or TNF-neutralizing monoclonal antibody for 48 hr. (**A**) AMPKα1 and α2 activities, (**B**) AMPKα2 phosphorylation, (**C**) PP2C protein expression, (**D**) ACCβ phosphorylation, (**E**) *soleus* palmitate oxidation, and (**F**) mixed *gastrocnemius* DAG. Values are means ± SEM (n = 5–6) [a]p < 0.05 versus lean. [b]p < 0.05 versus *ob/ob*-control.

reduced AMPK activity, as AMPK is important for mitochondrial biogenesis (Zong et al., 2002), suggesting that a more prolonged neutralization period may have been effective in reversing these defects. Supporting this idea are findings that chronic treatment of *ob/ob* mice with rosiglitazone, which is an AMPK activator (Fryer et al., 2002) and suppressor of TNFα production (Jiang et al., 1998), reverses defects in mitochondrial density in adipose tissue (Wilson-Fritch et al., 2004). Our data are also in agreement with findings in cardiac muscle in which reduced AMPK phosphorylation in *ob/ob* mice was associated with elevated PP2C expression effects that were reversed with troglitazone (Wang and Unger, 2005). Taken together these data indicate that a principal mechanism by which PPARγ agonists reverse defects in fatty-acid metabolism and therefore insulin sensitivity may be through the suppression of TNFα production.

Uysal et al. (Uysal et al., 1997) demonstrated that *ob/ob* mice that lack both the p55 and p75 isoforms of the TNFR (*ob/ob* $TNFR^{-/-}$) display enhanced insulin sensitivity relative to *ob/ob* control mice despite similar degrees of obesity. We therefore examined whether improved insulin sensitivity in *ob/ob* $TNFR^{-/-}$ mice involved enhanced AMPK signaling. AMPKα1 and α2 activities were higher (+49 and 76%, p = 0.026 and p = 0.027 respectively) in *ob/ob* $TNFR^{-/-}$ relative *ob/ob* controls (Figure 6A). Increased AMPK activity was associated with reduced PP2C expression (Figure 6B) and elevated ACCβ phosphorylation (Figure 6C), suggesting that increases in fatty-acid oxidation may contribute to the enhanced insulin sensitivity observed in *ob/ob* $TNFR^{-/-}$ mice.

Conclusions

Studies in rodents and humans have established a causative role for TNFα in the development of skeletal muscle insulin resistance; however the signaling mechanisms by which chronically elevated levels of TNFα inhibit insulin signaling have not been fully elucidated. Defective skeletal muscle fatty-acid metabolism in obesity involves suppressed rates of fatty-acid oxidation (Gaster et al., 2004; Hulver et al., 2003; Kelley and Simoneau, 1994), effects which may be related to reduced skeletal muscle AMPK activity (Bandyopadhyay et al., 2006; Barnes et al., 2002; Yu et al., 2004). In the present study we demonstrate a pathway mediated by TNFα involving the upregulation of PP2C and subsequent suppression of AMPK phosphorylation/activity and fatty-acid oxidation (Figure 7). Importantly, we demonstrate that even at physiologically elevated levels of TNFα, as observed in *ob/ob* mice, the effects of TNFα on PP2C and AMPK activity persist. As such these data demonstrate that TNFα suppression of AMPK signaling contributes to defective fatty-acid metabolism in obesity and is an important contributing factor to the development of insulin resistance.

Experimental procedures

Animals

All experimental protocols were approved by St. Vincent's Hospital's Animal Ethics Committee. *TNFR1* null mice were derived from 129/OlaHsd (E14 ES cells) and backcrossed to C57BL/6 for two generations and then backcrossed into NOD/Lt for seven generations before being intercrossed to generate homozygous mice. At backcross seven, the NOD genes around the *TNFR1* locus were mapped and NOD DNA was found at D6Mit135 (51.4cM) and D6Mit14 (63.4cM). The *TNFR1* locus is at about 57 cM. Micro osmotic pumps (Model 1003D, Alzet, Palo Alto, California) were implanted into both WT and *TNFR1* null littermates as previously described (Steinberg et al., 2002), and TNF (1 μg/kg/day) or saline was infused for 24 hr. This concentration of TNFα was selected as it has previously demonstrated to induce skeletal muscle insulin resistance (Ruan et al., 2002) and increase plasma levels to ~630 ng/ml (Miles et al., 1997). Following the prescribed treatment fatty-acid oxidation was determined in soleus muscle as previously described (Steinberg and Dyck, 2000) and *gastrocnemius* were removed and snap frozen for analysis of AMPK activity, phosphorylation of ACCβ, protein phosphatase activity and expression, and muscle metabolites and substrates (see below). In separate experiments to assess insulin sensitivity via hyper-insulinemic-euglycaemic clamps, animals were fasted overnight (see below). For TNF antibody treatment experiments male *ob/ob* (n = 6) mice (Monash Mouseworks, Clayton, Victoria Australia) 10–12 weeks of age were injected at 0 and 24 hr with 500 μg of control or TNF-neutralizing

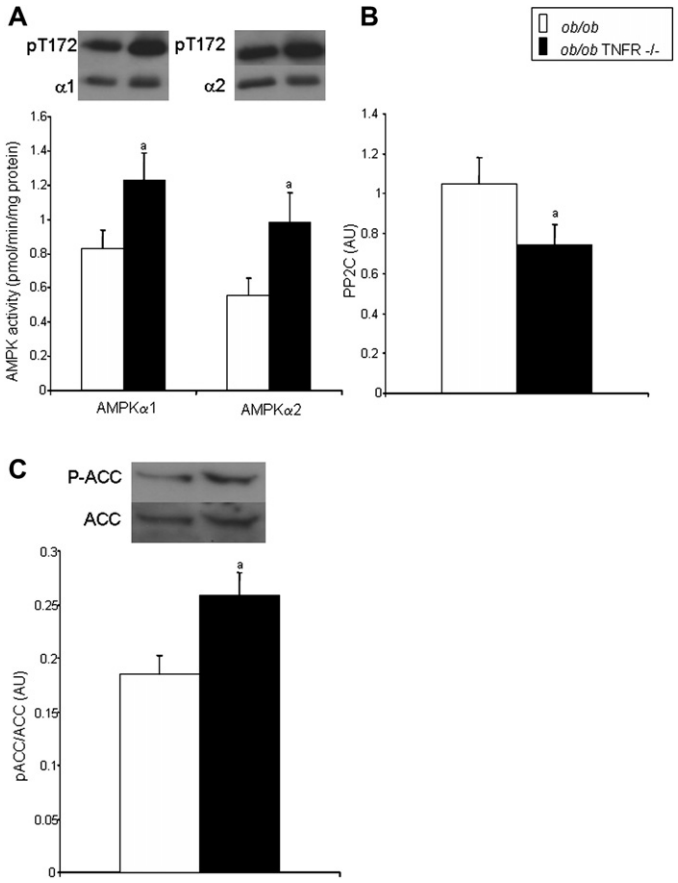

Figure 6. Enhanced whole-body insulin sensitivity of *ob/ob TNFR*$^{-/-}$ mice is associated with increased AMPK signaling and reduced PP2C expression

Skeletal muscle from obese *ob/ob TNFR*$^{-/-}$ mice and *ob/ob* controls. (**A**) Representative pThr172 phosphorylation relative AMPK (above) and AMPKα1 and α2 activities (below), (**B**) PP2C protein expression, and (**C**) ACCβ phosphorylation. Values are means ± SEM (n = 8). [a]p < 0.05 versus *ob/ob*.

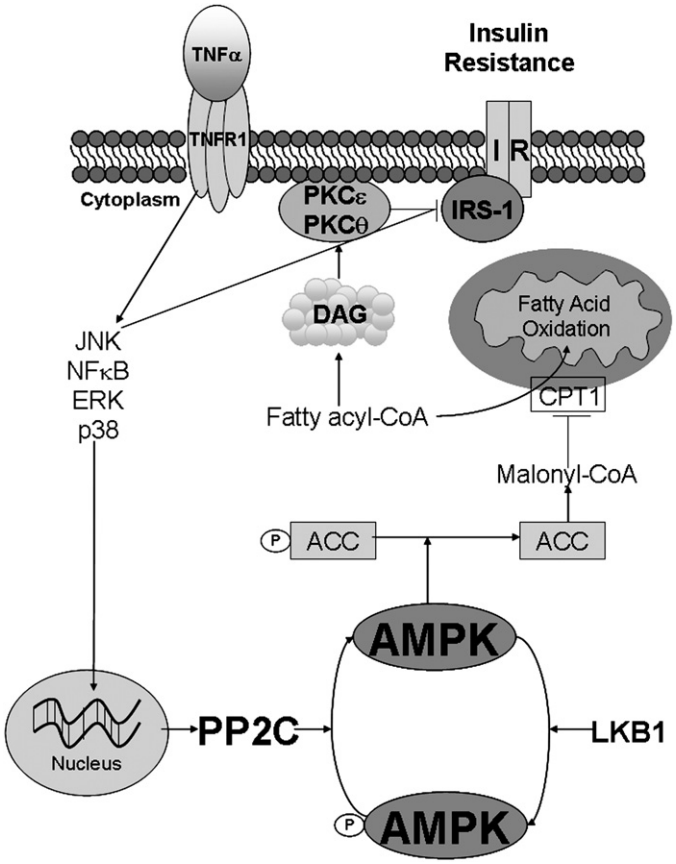

Figure 7. Proposed schematic outlining mechanism by which elevated TNFα may induce insulin resistance

antibody (clone XT3) (Korner et al., 2000) and tissues collected for determination of fatty-acid metabolism and AMPK signaling as described above. Lean littermates (+/*ob*) served as controls. Obese TNFR-deficient mice (*ob/ob TNFR*$^{-/-}$) were generated as previously described (Uysal et al., 1997).

Cell culture transfection, infection, and lysis

L6 myoblasts were maintained on 10 cm plates in α-MEM with 10% fetal bovine serum (FBS) culture media at 37°C in 5% CO_2 humidified air. Differentiation was induced by switching to medium containing 2% FBS when the myoblasts were ~90% confluent. Experimental treatments were started after 7 days, by which time nearly all of the myoblasts had fused to form myotubes. Myotubes were serum starved for 16 hr in α-MEM + 0.1% FBS and treated with or without TNFα (R&D systems) at the indicated concentrations and times, which have been used previously to induce insulin resistance in skeletal muscle myotubes (De Alvaro et al., 2004). In separate experiments cells were treated as described above followed by incubation for 30 min with AICAR (2 mM, 5-aminoimidazole-4-carboxamide, Toronto, Research Chemicals Inc. Toronto, Canada). For experiments involving adenovirus infection, L6 myotubes were infected with adenovirus containing control vector (Ad null) or the constitutively active AMPK mutant (Ad-CA) as previously described (Woods et al., 2000). Adenoviral expression levels of both Ad null and Ad-CA infected cells were determined by visual examination of green fluorescent protein under ultraviolet light. Seventy-two hours post-infection ~85% of all myotubes were infected in both Ad null and Ad-CA infected cells (data not shown). Cells were then serum starved and treated with vehicle or TNFα (10 ng/ml) for 16 hr before cells were harvested or treated with vehicle or insulin for insulin signaling or glucose uptake experiments. For PP2C siRNA experiments, myoblasts were grown to ~90% confluence. Media were then changed to serum and antibiotic-free low-glucose DMEM and Con (scramble) and PP2C oligonucleotides were transfected as described (Yoshizaki et al., 2004). After 24 hr, media were changed to αMEM with 1% FBS containing vehicle or TNFα for 16 hr before cells were harvested or treated with insulin for insulin signaling or glucose uptake experiments.

Analytical methods

AMPKα1 and α2 activities and ACCβ phosphorylation

Isoform-specific AMPK activities and phosphorylation were measured in muscle and cell lysates using rabbit polyclonal AMPK antibodies for immunoprecipitation and immunoblotting as previously described (Chen et al., 1999).

LKB1 activity

LKB1 assays were performed following immunoprecipitation with LKB1 antibody (Upstate) on the beads using a two-step reaction with full-length, bacterially expressed human AMPK (α1,β1,γ1), as previously described (Chen et al., 2005).

Protein phosphatase assays

Protein phosphatase activity in muscle homogenates was determined by measuring the generation of γ ^{32}Pi from ^{32}P-labeled casein prepared using a modification of the procedure described previously (Mackintosh and Moorhead, 1999). Casein was phosphorylated by protein kinase A incubated with 375 μM [γ $^{-32}$P] ATP (2500 cpm/pmole) in 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT buffer at 20°C for 16 hr. The reaction was terminated by the addition of 100 mM NaF, 10 mM $Na_4P_2O_7$ (pH 7). The free γ ^{32}P was removed by G-25 chromatography using PD-10 columns (Amersham Biosciences, United Kingdom). Approximately 0.1 mole Pi/mole casein was achieved yielding approximately 40 μM ^{32}P-labeled casein. Muscle samples (~100 mg) were homogenized in 50 mM Hepes, pH 7.5, 2 mM EDTA, 1 mM DTT, 1% NP40, 10 μg/ml aprotinin, 10 μg/ml soyabean trypsin inhibitor, 1 mM PMSF (7 μl/ mg) then cleared by centrifugation at 14,000 rpm for 10 min. To detect phosphatase activities the muscle extracts

(10 μl × 50 μg/ml) were incubated with 5 nM okadaic acid (OA) to inhibit PP2A exclusively, 100 nM OA to inhibit both PP1 and PP2A, or 20 mM $MgCl_2$ (± 100 nM OA) to activate PP2C (and inhibit PP1 and PP2A), with 5 μM ^{32}P-casein in 50 mM Hepes, pH 7.5, 1 mM DTT, 0.1% Triton X-100 for 15 min at 30°C in 25 μl. Twenty percent TCA (100 μl) was added to stop the reaction and precipitate casein. The free ^{32}P in the supernatant fraction was measured by liquid scintillation counting.

Total RNA was isolated from skeletal muscle samples using Trizol reagent (Invitrogen) according to the manufacturer's protocol. For each sample 3 μg RNA was reverse transcribed using the SuperScript II first strand synthesis kit (Invitrogen) and oligo dT primers, in a final volume of 20 μl. Samples were diluted 1:100 in water and 1 μl used for quantitation. Real-time quantitative RT-PCR was performed on an ABI Prism 5700 Sequence Detection system (Perkin-Elmer Applied Biosystems, Australia), as previously reported (Steinberg et al., 2004). Reactions were performed in triplicate and the results were normalized against the *RPL32* "housekeeping" gene. Relative gene expression was determined using the comparative Ct method. Amplification of *PP2C* mRNA was performed using forward (TGTCAATGGCTCTCTGGCTG TATC) and reverse (ACACTCATGTTGTCTCGACTTCCC) oligonucleotides that amplify all three *PP2C* transcript variants (variant 1 NM_021003, variant 2 NM_177951, and variant 3 NM_177952). The use of primer pairs specific for each of the transcript variants revealed negligible expression of transcript variants 2 and 3 (data not shown). PP2C protein expression was determined via immunoblot of cell and muscle lysates from 100 μg of protein using a PP2Cαβ antibody (Abcam, Cambridge, United Kingdom).

Measurement of glycogen, nucleotides, and lipid species

Muscle glycogen, nucleotides, and lipids were analyzed as previously described (Watt et al., 2006).

Euglycemic-hyperinsulinemic clamps

A primed (2 min, 3 μCi.min^{-1}) continuous infusion (0.15 μCi.min^{-1}) of [6-^{3}H]-glucose was administered during basal and euglycaemic-hyperinsulinaemic clamp experiments to measure whole-body glucose turnover, as we have previously described (Jamieson et al., 2005) with minor modifications. Blood glucose concentration was maintained at basal levels by the infusion of a 5% glucose solution. Blood samples were collected during steady-state conditions at 90, 100, and 110 min. Following the collection of blood samples for measurement of whole-body glucose turnover, a bolus of 2-[1-^{14}C]-deoxyglucose (10 μCi) was injected intravenously and blood samples were collected at 2, 5, 10, 15, 30, and 45 min. Immediately following the collection of the last blood sample at 45 min, animals were sacrificed by a lethal overdose of pentobarbitone sodium and tissues including heart, diaphragm, quadriceps (red and white), soleus, gastrocnemius (red and white), brown adipose, and white adipose tissue were rapidly removed, frozen in liquid nitrogen, and stored at −70°C for subsequent analysis. The time course of 2-[1-^{14}C]-deoxyglucose disappearance from plasma and the levels of phosphorylated 2-deoxyglucose accumulation in individual tissues were determined as previously described (Kraegen et al., 1985).

Fatty-acid oxidation and glucose uptake/insulin signaling experiments in L6 myotubes

Fatty-acid oxidation in L6 myotubes was determined as previously described (Chen et al., 2005). For 2-deoxyglucose assays, L6 myotubes were incubated in the presence or absence of insulin (50 nM) for 30 min followed by incubation with 10 μM 2-deoxyglucose (Sigma) and 1 μCi/ml 2-[1-^{14}C]-2-deoxyglucose (Amersham, Life Sciences) for 20 min as described (Watt et al., 2006). For insulin signaling L6 myotubes were treated with vehicle or insulin (50 nM) for 5 min and harvested in ice-cold lysis buffer. Clarified lysates were immunoprecipiated with an IRS1 antibody (Santa Cruz) prebound to protein-A agarose beads and immunoblotted with phosphotyrosine (4G10, Upstate) and PI3 Kinase p85 (Upstate) antibodies. Phosphorylated Akt (Ser473), total Akt, and p85 (Cell Signaling) were measured via immunoblot from 100 μg of protein as described (Carey et al., 2006).

PKC translocation and IRS1/Akt signaling

Insulin-stimulated *gastrocnemius* skeletal muscle extracts and cell lysates (1 mg) were immunoprecipitated using antibodies and procedures as previously described (Jamieson et al., 2005). PKC expression was measured in cytosolic and membrane fractions isolated from 60 mg of *gastrocnemius* muscle as described (Michell et al., 2001) and immunoblotted using phosphospecific antibodies (Cell Signaling).

Statistical analysis

Results are expressed as the mean ± SEM. Data were analyzed for differences by one-way analysis of variance with specific differences located with a Student Newman-Keuls post-hoc test or a Student's t test for unpaired samples where appropriate. $p < 0.05$ was considered to be statistically significant.

Supplemental data

Supplemental data include three tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/6/465/DC1/.

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