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Ceramide-Activated Protein Phosphatase (CAPP) involvement in insulin resistance via Akt, SRp40, and RNA splicing in L6 skeletal muscle cells

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Abstract

Elevated TNFα levels are associated with insulin resistance, but the molecular mechanisms linking cytokine signaling to impaired insulin function remain elusive. We previously demonstrated a role for Akt in insulin regulation of PKCβII alternative splicing through phosphorylation of SRp40, a required mechanism for insulin-stimulated glucose uptake. We hypothesized that TNFα attenuated insulin signaling by dephosphorylating Akt and its targets via ceramide-activated protein phosphatase (CAPP). Western blot analysis of L6 cell lysates demonstrated impaired insulin–stimulated phosphorylation of Akt, SRp40, and GSK3β in response to TNFα and the short chain C6 ceramide analog. TNFα increased serine/threonine phosphatase activity of PP1 in response to C6, but not insulin, suggesting a ceramide-specific effect. Myriocin, an inhibitor of *de novo* ceramide synthesis, blocked stimulation of the PP1 activity. Ceramide species measurement by LC-MS showed consistent increases in C24:1 and C16 ceramides. Effects of TNFα and C6 on insulin–stimulated phosphorylation of GSK3β were prevented by myriocin and tautomycin, a PP1 inhibitor, further implicating a *de novo* ceramide-PP1 pathway. Alternative splicing assays demonstrated that TNFα abolished insulin-mediated inclusion of the $PKC\beta_{II}$ exon. Collectively, our work demonstrates a role for PP1-like CAPP in mediating TNFα effects blocking insulin phosphorylation cascades involved in glycogen metabolism and alternative splicing.

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Keywords

TNFα; Ceramide activated protein phosphatase (CAPP); Akt; SR proteins; PKCβII; alternative splicing

Introduction

Several lines of evidence indicate that tumor necrosis factor alpha (TNFα[1]), a pro-inflammatory cytokine, plays a role in insulin resistance. TNFα expression is increased in insulin resistant individuals and infusing humans with TNFα induces insulin resistance (1–3). Most animal models of insulin resistance produce significantly higher levels of TNFα, and the TNFα knockout mouse demonstrated increased insulin sensitivity and improved lipid metabolism (2,4). Furthermore, cells treated with TNFα also present with impaired insulin signaling: decreased insulin receptor tyrosine kinase activity, increased serine phosphorylation of insulin receptor substrate 1, decreased GLUT4 translocation and abolishment of insulin mediated Akt phosphorylation (4–7).

Ceramide, a sphingolipid second messenger, is also linked to insulin resistance (2). Ceramide is generated via hydrolysis of sphingomyelin by sphingomyelinase or by the *de novo* pathway via condensation of palmitoyl CoA and serine (8). Ceramide is elevated in insulin responsive tissues of diabetic animals (9) and inhibits a number of kinases that are stimulated by insulin, including protein kinase B (PKBβ/Akt) (10–13) and protein kinase C (7,14,15). It is also known to activate atypical PKCs (16,17). TNFα activates both *de novo* and hydrolysis pathways of ceramide generation (18), and ceramide mimics the effects of TNFα on insulin signaling (19–22) and apoptosis (23). Furthermore, when ceramide production is suppressed, TNFα-induced insulin resistance is reversed (24). However, the molecular mechanisms defining ceramide-induced alterations in insulin signaling remain unclear. Ceramide-activated protein phosphatases (CAPPs), including PP1 and PP2A, are allosterically activated by ceramide (25,26). CAPPs have been shown to inhibit Akt by dephosphorylation of serines (27) and to modulate key cellular processes including exocytosis, alternative pre-mRNA splicing and glycogen metabolism (28–30). Hence, we investigated whether a ceramide-activated protein phosphatase could act on Akt and subsequent downstream substrates including SR proteins involved in alternative splicing of PKCβII mRNA in skeletal muscle cells.

PKCβ is a component of the serine/threonine kinase superfamily with roles in insulin signaling related to glucose transport and glycogen metabolism (31–33). It is a conventional PKC with two splice variants, βI and βII, that have distinct cellular functions. Insulin regulates alternative splicing of the protein kinase CβII (PKCβ) pre-mRNA (34–36). The regulation occurs by the phosphorylation of serine/arginine “SR” rich proteins under the control of Akt (37). The regulation is impaired in insulin resistant states and diabetes (35).

This study was designed to define the molecular link between acute inflammatory cytokines, the activation of CAPPs, the ceramide species involved, phosphorylation status of Akt and SR proteins and TNFα effects on insulin stimulated PKCβII splicing. To investigate this, L6 rat myotubes were treated with TNFα and C6 ceramide, a short chain ceramide analogue, and examined for insulin dependent phosphorylation of Akt, SRp40, and GSK3β. Inhibition of *de novo* ceramide generation blocked PP1, but not PP2A activation by TNFα. C16 and C24:1 ceramides were the major species increased by TNFα, and there was a significant reduction in

[1]Abbreviations Used: CAPP, ceramide activated protein phosphatase; GSK3β, glycogen synthase kinase 3β; PI3 Kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; SR, serine/arginine rich; SRp40, serine/arginine rich protein 40; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A, TNFα, tumor necrosis factor α.

insulin-mediated PKCβ_{II} exon inclusion in cells treated with the cytokine. Collectively, the results suggested that TNFα caused alterations in insulin signaling in a ceramide-dependent mechanism by activating CAPPs, which resulted in Akt and SRp40 dephosphorylation and decreased PKCβ_{II} splicing.

Materials and Methods

Cell culture

L6 rat skeletal muscle cells (obtained from Dr. Amira Klip, Hospital for Sick Children, Toronto, Canada) were grown in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) (Cellgro) to 50–60% confluency. Penicillin G and streptomycin sulfate (100U/100 μg) were added and the cells were incubated at 5% CO_2, 37°C, and 95% humidity. Cells were induced to differentiate by changing medium to α-MEM supplemented with 2% FBS for approximately 8 days. The extent of cellular differentiation was established by observation of multi-nucleation of >70% of cells. For phosphatase assays, cells were placed in HEPES buffered saline (HeBS) with 0.1% bovine serum albumin (BSA) prior to treatments. For western blot analysis, cells remained in α-MEM. TNFα was added to a final concentration of 15–150 ng/ml for 30 minutes. C6 ceramide (20 μM) was added for 2 hours. Insulin was added at a final concentration of 10 nM for 30 minutes. Myriocin (5 nM), an inhibitor of *de novo* ceramide synthesis, was added for 1 hour prior to TNFα treatment. Tautomycin (10 nM), an inhibitor of PP1, was added 1 hour prior to TNFα treatment.

Immunoprecipitation

Culture dishes with differentiated cells (100 mm) were washed with Ca^{2+}/Mg^{2+}-free PBS and cells mechanically detached in buffer (Tris HCl pH 7.4 50mM; NaCl, 150 mM; EDTA 1 mM; NaF, 10 mM; Triton X 100, 1% SDS, 0.1%; Na deoxycholate, 1%) with a cocktail of antiproteases (Leupeptin, 20 μg/ml; aprotinin, 10 μg/ml; PMSF, 0.1 mM; DTT, 1 mM). The lysate was centrifuged at 17,000 x g for 20 min at 4°C. The supernatant (0.5 ml) was used for immunoprecipitation by adding 25 μl A/G sepharose, rotating the suspension 30 min at 4°C. SRp40 antibody was then added and samples rotated overnight at 4°C. The suspension was centrifuged at 2000 x g for 1 min and the pellet washed 2x in Tris-buffered saline with Tween (TBST). After addition of Laemmli buffer, samples were separated on SDS-PAGE (38). Proteins were detected using anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase and enhanced chemiluminescense reagents. Controls include protein A and IgG (pre-immune sera).

Western blotting

Culture dishes with differentiated cells were washed with Ca^{2+}/Mg^{2+}-free PBS and cells mechanically detached in buffer (Tris HCL pH 7.4 50 mM; NaCl, 150 mM; EDTA 1 mM; NaF, 10 mM; Triton X 100, 1%; SDS, 0.1%; Na deoxycholate, 1%) with a coctail of antiproteases (leupeptin, 20 μg/ml; aprotinin, 10 μg/ml; PMSF, 0.1 mM; DTT, 1mM). The lysate was centrifuged at 17,000 x g for 20 min at 4°C. Cell lysates in Laemmli buffer (50 μg) were separated via 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes and blocked with phosphate buffered saline with 0.1% Tween 20 and 4% non-fat dried milk. Membranes were washed and incubated with the following antibodies: phospho-Akt (serine 473, Cell Signaling); phospho-GSK3β (serine 9, Cell Signaling); Mab104, a monoclonal antibody against the phosphoepitope (RS domain) of SR proteins; phospho-Akt substrate, detects phosphorylation of Akt substrates at their Akt motifs (R-X-R-X-X-S/T), SRp40, phospho-IRS-1 (serine 616, Cell Signaling) or IRS-1 (Cell Signaling). Equal loading or identification of SR proteins was verified by stripping and reprobing membranes with β-actin or SRp40. Following incubation with secondary anti-

rabbit or anti-mouse IgG-HRP, detection was performed using enhanced chemiluminesence (Pierce). Results were verified in 2–6 separate experiments.

Phosphatase assays

Cells washed 3 times in ice-cold HeBS were scraped into 1ml lysis buffer (50 mmol/l Tris, pH7.4, 150 mmol/l NaCl, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and sonicated. Lysates were centrifuged at 400,000 × g for 10 min at 4°C. Up to 250 μl of supernatants were passed through 10 ml Sephadex G-25 columns to eliminate ATP and free phosphate. Serine/threonine phosphatase activities in these fractions were determined by measuring generation of free phosphate from phosphopeptide RRA(pT)VA using the molybdate-malachite green-phosphate complex assay (Promega). The peptide substrate was used in the absence of Ca^{2+} and Mg^{2+}, which are required for the activity of PP2B and 2C. Assays were carried out using 10 μl of lysate in 10 μl of a Ca^{2+} and Mg^{2+}-deficient reaction buffer (final concentration 50 mmol/l imidazole [pH 7.2], 0.2 mmol/l EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml BSA, and 100 μmol/l phosphopeptide substrate), 5 μl phosphopeptide and 25 ul phosphate free water with the addition of either okadaic acid (20 nm or 0.2 nm) to distinguish between PP1 and PP2A activities. Reactions were carried out for 15 minutes in a 37°C shaker water bath. To stop the reaction, molybdate dye was added and free phosphate measured by OD at 630 nm. A standard curve was generated to determine free phosphate concentrations. Results were corrected for total protein in cell fractions by the Bradford method using a BSA standard curve and specific activity was calculated. Experiments were repeated a minimum of 2 times and reactions were carried out in duplicate or triplicate. Buffer plus peptide was used as a blank control.

Ceramide mass spectrometry

Cells were treated, and collected for phosphatase assay and approximately ½ of the total cell pellet was reserved for ceramide mass-spec analysis. The pellet was stored at −80°C. Ceramide molecular species analyses were performed by the Lipidomics Core Facility at the Medical University of South Carolina. Briefly, cell pellets were extracted into a one-phase neutral organic solvent system and analyzed by a Surveyor/TSQ 7000 LC/MS system. Qualitative analysis of ceramides was performed by a Parent Ion scan of a common fragment ion characteristic for a particular class of ceramides (39). The results are presented as mean ± SD of picomoles of ceramide species per μg of protein in the sample.

Alternative splicing of PKCβII

L6 cells were plated at 40–50% confluency in 6-well plates and differentiation to myotubes was initiated for 24 hrs prior to transfection of the PKCβII reporter minigene (2μg) mixed with 10μl of Lipofectamine™ (Invitrogen) reagent for 4 hours (35). After 36 hrs, fused myotubes (>70%) were pre-treated for 30 min with TNFα or inhibitors then insulin was added for 30 min. The minigene was evaluated for exon inclusion as follows. Total RNA was obtained using Trizol™ reagent (Invitrogen). One μg (total RNA) was reverse transcribed using oligo dT and the Superscript II™ preamplification kit. 1μl of the RT reaction was amplified using SD and SA specific primers and Platinum Taq™ DNA polymerase. After 25 cycles (95° 30 sec melt and 68°C 1 min anneal/extension), the PCR reaction is examined on PAGE followed by silver staining. As a control, β-actin was amplified (35).

Results

TNFα and Ceramide Altered Insulin-Dependent Phosphorylation Cascades

The activation of Akt via PI3-kinase is a central step in insulin action. Data from our lab has demonstrated that SRp40 is a direct target for phosphorylation and activation by Akt (37).

SRp40 phosphorylation by PI3-kinase dependent pathways is required for the splicing of PKCβII in insulin-dependent cells (35,36). It has not been demonstrated whether TNFα via *de novo* ceramide synthesis activates a serine/threonine protein phosphatase activity that targets Akt and SRp40. This could represent another mechanism for TNFα to attenuate insulin action in skeletal muscle in addition to other known pathways that inhibit IRS-1 function (40). To examine the potential role of the ceramide pathway, L6 myotubes were pre-treated for 30 minutes with TNFα or the short chain ceramide analogue, D-e-C_6 ceramide, and the phosphorylation states of Akt and SRp40 were analyzed by Western immunoblot. Insulin treatment increased Akt phosphorylation on Serine 473 while TNFα pre-treatment attenuated the action of insulin and Akt was less phosphorylated. C6 ceramide mimicked the effects of TNFα but had a much more potent effect in attenuating insulin effects (Figure 1a). Similar results were obtained when mAb104 was used to detect phosphorylation at the RS domain of SRp40 immunoprecipitated from L6 myotubes (Figure 1b). Since SRp40 is also an Akt substrate (37), insulin regulated phosphorylation patterns at the Akt substrate motifs of SRp40 were also analyzed in L6 myotubes (Figure 1c). TNFα and C6 ceramide reduced insulin stimulated phosphorylation of SRp40 at its Akt motifs. This effect was not observed with dihydro-C6 ceramide, an inactive ceramide analog, suggesting that the ceramide effects are not due to non-specific effects of lipid treatment. Therefore, both ceramide and TNFα treatment resulted in the decreased phosphorylation of Akt and SRp40.

TNFα Blocked PKCβII Exon Inclusion

Previous work in our lab has demonstrated that phosphorylated SRp40 is required for PKCβII exon inclusion (36), and insulin is responsible for this phosphorylation via Akt activity. To test the functional relevance of TNFα-mediated decrease in phosphorylation of Akt and SRp40, alterations in insulin induced PKCβII exon inclusion were assessed utilizing a heterologous splicing minigene. Our data demonstrate that insulin promoted inclusion of the PKCβII exon. Two splice sites were activated. Activation of splice site II (SSII) was blocked by TNFα and the PI3Kinase inhibitors. Splice site I usage was partially blocked which may reflect the stronger role of SRp40 on splice site II selection (35). Importantly, TNFα pre-treatment blocked insulin effects on PKCβII SSII activation to the same extent as blocking Akt activation via the PI3K/PDK pathway with LY294002 or wortmannin (Figure 2).

TNFα Increased Ceramide Activated Phosphatase Activity in Skeletal Muscle Cells

TNFα increases ceramide production via the *de novo* and sphingomyelinase pathways (2,18). PP1 and PP2A are ceramide-activated protein phosphatases (CAPPs) (25). Although increases in ceramides are associated with insulin resistance (10,19–21,24), the activation of serine/threonine protein phosphatases in skeletal muscle by cytokines has not been examined. To further determine if the aberrant phosphorylation patterns noted in the immunoblot analyses above were consistent with ceramide-activated protein phosphatase activity, we examined L6 myotubes for protein phosphatase activity following TNFα, insulin, and C6 ceramide treatment along with an inhibitor of *de novo* ceramide synthesis. Short term TNFα treatment increased protein phosphatase specific activity approximately 2-fold ($p < 0.05$, Student's t-test). C6-ceramide treatment mimicked this within 2 hours, while insulin treatment showed no increase in protein phosphatase activity. To distinguish between PP1-type and PP2A-type activities, okadaic acid was added to the phosphatase assay reaction with concentrations that inhibit both (20 nM) or PP2A specifically (0.2 nM). In L6 cells, high dose okadaic acid led to nearly complete abolishment of phosphatase activity, while the lower dose led to little or no inhibition indicating that the predominant phosphatase activated by TNFα and C6 ceramide was PP1 ($p < 0.05$) (Figure 3). Exposure of L6 cells to myriocin, an inhibitor of serine palmitoyltransferase in the *de novo* pathway (41), significantly suppressed activation of the phosphatase activity induced by TNFα to levels comparable to controls in L6 cells suggesting that TNFα activated *de novo* ceramide synthesis which, in turn activated PP1 activity in L6 cells.

TNFα increased ceramide levels via the de novo sphingolipid pathway in L6 cells

To determine if intracellular ceramide levels were increased as a result of treatment with TNFα, cell pellets were assayed for ceramide content by liquid chromatography-mass spectrometery. In L6 cells, C16 and C24:1 ceramides were the predominant species detected (Figure 4A). Both species were increased by TNFα treatment, but only elevations of C24:1 ceramide (35–40%) were noted in the presence of insulin. This increase was blunted by pre-treatment with myriocin. Insulin treatment alone did not significantly increase C24:1 ceramide levels (Figure 4B). The levels of ceramide noted correspond to the decrease in protein phosphorylation noted in Figure 1 and increase in protein phosphatase activity.

Ceramide Impaired Phosphorylation of Glycogen Synthase Kinase 3-β and Akt

In order to further characterize the mechanisms of TNFα-induced insulin resistance, we examined L6 cells for phosphorylation/dephosphorylation of glycogen synthase kinase 3-β (GSK3β). GSK3β is also an Akt substrate, which would make it a potential target of CAPPS activated by TNFα. As expected, insulin increased the phosphorylation of GSK3β and C6 ceramide attenuated this (Figure 5A). The attunation was not as great as noted with Akt in Figure 1, and this may be due to the subcellular partitioning of C6 and its ability to interact with phosphatases. The phosphorylation of GSK3β is an inactivating phosphorylation that leads to downstream glycogen production. When phosphoGSK3β was evaluated in the presence of TNFα, insulin's effect on phosphorylation was blunted. When *de novo* ceramide synthesis was blocked by myriocin or when PP1 was inhibited by tautomycin, TNFα inhibition of insulin stimulated GSK3β phosphorylation was largely reversed (Figure 5B). The effect of C6 ceramide was more robust than TNFα in this case and may reflect the short time course used in these experiments to demonstrate activation of CAPPs. A similar reversal of TNFα activity was also noted on pAkt by myriocin and tautomycin (Figure 5B). IRS-1 phosphorylation was not significantly increased by TNFα and neither myriocin nor tautomycin treatment had any effect suggesting IRS-1 phosphorylation associated with insulin resistance was not occurring in this scenario (42, 43).

Discussion

There is an abundance of correlative evidence indicating that TNFα causes insulin resistance. The molecular mechanisms are not well defined since TNFα has multiple effects on the insulin receptor and IRS-1 phosphorylation (4,6,43,44). It also alters the IKK/NF-kB/I-kB complex (45). There are also reports of impaired PP1, glycogen synthase activity, and insulin-stimulated glucose uptake in L6 cells following TNFα treatment (46,47). The molecular details of these pathways have not been demonstrated. The current studies expand the pathways involved in TNFα signaling to CAPPs, which, in this case, are PP1-like in nature.

We first investigated the effects of TNFα and ceramide on the insulin-induced phosphorylation state of Akt, a central controller of insulin action, and SRp40, an Akt2 target important for insulin regulation of alternative splicing of the PKCβ gene. Ceramide analogues have been shown to diminish Akt phosphorylation in brown adipocytes and skeletal muscle (12,48). However, whether this was due to a serine/threonine protein phosphatase activity was not shown, nor were the increases in endogenous ceramide species. The effects of C6 ceramide treatment on Akt phosphorylation strongly suggest that its change in phosphorylation might be due to a protein phosphatase activity. To evaluate this, the substrate system was extended to other potential targets in the insulin signaling cascade, SRp40, also a substrate for Akt. Its phosphorylation was evaluated using the monoclonal antibody, mAb 104, which recognizes phosphoepitopes in "RS", arginine-serine domains (49) as well as with the Akt substrate antibody. TNFα and C6 ceramide modulated insulin effects on SRp40 phosphorylation. C6 ceramide pretreatment attenuated insulin's effect on Akt, SRp40, and GSK3β phosphorylation.

We linked TNFα's effects in skeletal muscle to PKCβII alternative splicing since aberrant mRNA splicing is associated with many disease states including type2 diabetes (37). PKCβII splicing is regulated by insulin via PI3-kinase/Akt phosphorylation of SRp40 (35–37). TNFα abolished insulin-stimulated $PKC\beta_{II}$ exon inclusion in L6 myotubes. This is consistent with the inhibition of Akt activation and decreased SRp40 phosphorylation anticipated by LY294002 (36).

We characterized the protein phosphatase activity that TNFα stimulated presuming that it could be modified by ceramides. Previous studies suggested that protein phosphatases could be mediators of TNFα induced insulin resistance (47), and TNFα is known to be increased in skeletal muscle of diabetic subjects (50). Earlier studies identified a PP2A activity and showed that ceramide analogues mimicked the TNFα effect on MAPK inhibition and PP2A activation (46,47). The phosphatase substrate presented to the enzyme in this study is different and thought to be more specific for PP2A, however, using differential doses of okadaic acid to distinguish between the two activities in the assay, it is clear that a PP1 activity was modified by TNFα in a manner consistent with CAPPS activation. Recently, a PP2A activity was found to dephosphorylate Akt (48,51). However, ceramides allosterically activate both PP1 and PP2A (25,28,52). In the present case, a PP1-type of activity seems most likely since no inhibitory effects on CAPPS were observed with insulin but TNFα activation was blocked with myriocin treatment, suggesting a specific *de novo* ceramide pathway distinct from other phosphatases reported. The inhibition of alternative splicing via TNFα also supports our belief that a PP1 activity is relevant since PP1, not PP2A, has been shown to be involved in alternative splicing (53,54). Nuclear inhibitor of PP1 (NIPP1) a major nuclear PP1-binding protein, co-localizes with splicing factors in the nucleus, and only PP1 activity has been shown to dephosphorylate SR proteins (28).

We were interested in determining the specific ceramide species responsible for the insulin resistant state induced by TNFα. TNFα treatment was associated with an increase in C16 and C24:1 ceramides, the major species detected in L6 myotubes, suggesting that these species regulated the PP1 activity. However, only C24:1 ceramide levels but not C16 ceramide levels remained elevated in the presence of insulin. Insulin resistance is induced via *de novo* ceramide synthesis by treating C2C12 skeletal muscle and primary rat islets cells with palmitate (10, 55), however, the species was not identified. In addition, treating 3T3-L1 adipocytes with both C2 and C6 ceramide analogs, causes increases in various markers of deficient insulin signaling such as insulin stimulated glucose uptake (56). These studies do not rule out the additional involvement of the sphingomyelinase pathway since this also increases ceramide levels and it has been demonstrated that TNFα inhibits insulin signaling in 3T3L1 adipocytes and myeloid 32D cells via activation of sphingomyelinase (19).

Previous work in L6 myotubes demonstrates that insulin increases a PP1 activity while concomitantly decreasing PP2A (57,58). The difference noted may be explained by evidence of combinatorial control among serine/threonine phosphatases that confers functional specificity (30). It is not clear specifically which PP1 heterodimer is activated by insulin and whether this PP1 is ceramide-activated (46). Furthermore, the allosteric modulation of PP1 by ceramide may change its substrate specificity, subcellular localization, and metabolic consequences.

Finally, the ability of tautomycin and myriocin to inhibit a PP1 phosphatase and ceramide synthesis, respectively, clearly reversed the phosphorylation state of GSK3β and Akt. It could be argued that TNFα induced phosphorylation of IRS-1 at Ser 312 via PKCζ activation could be the cause for inhibition of Akt phosphorylation (59), and this possibility is not ruled out. However, we saw no effect of TNFα or myriocin and tautomycin on IRS-1 Ser 616, another phosphorylation site associated with the development of peripheral insulin resistance (42)

suggesting that this sight is not a target for CAPP or TNFα phosphorylation and the effect described here is different..

In summary, this study has identified an additional molecular mechanism for cellular TNFα-induced insulin resistance which involves the *de novo* generation of ceramide that activates a PP1 activity capable of dephosphorylating both kinases and splicing factors. Although further characterization of the phosphatase binding protein are in order, this study has identified the species of ceramides increased by TNFα and an additional signaling pathway relevant to the cytokine in skeletal muscle cells which explains some of the actions of TNFα in insulin resistance..

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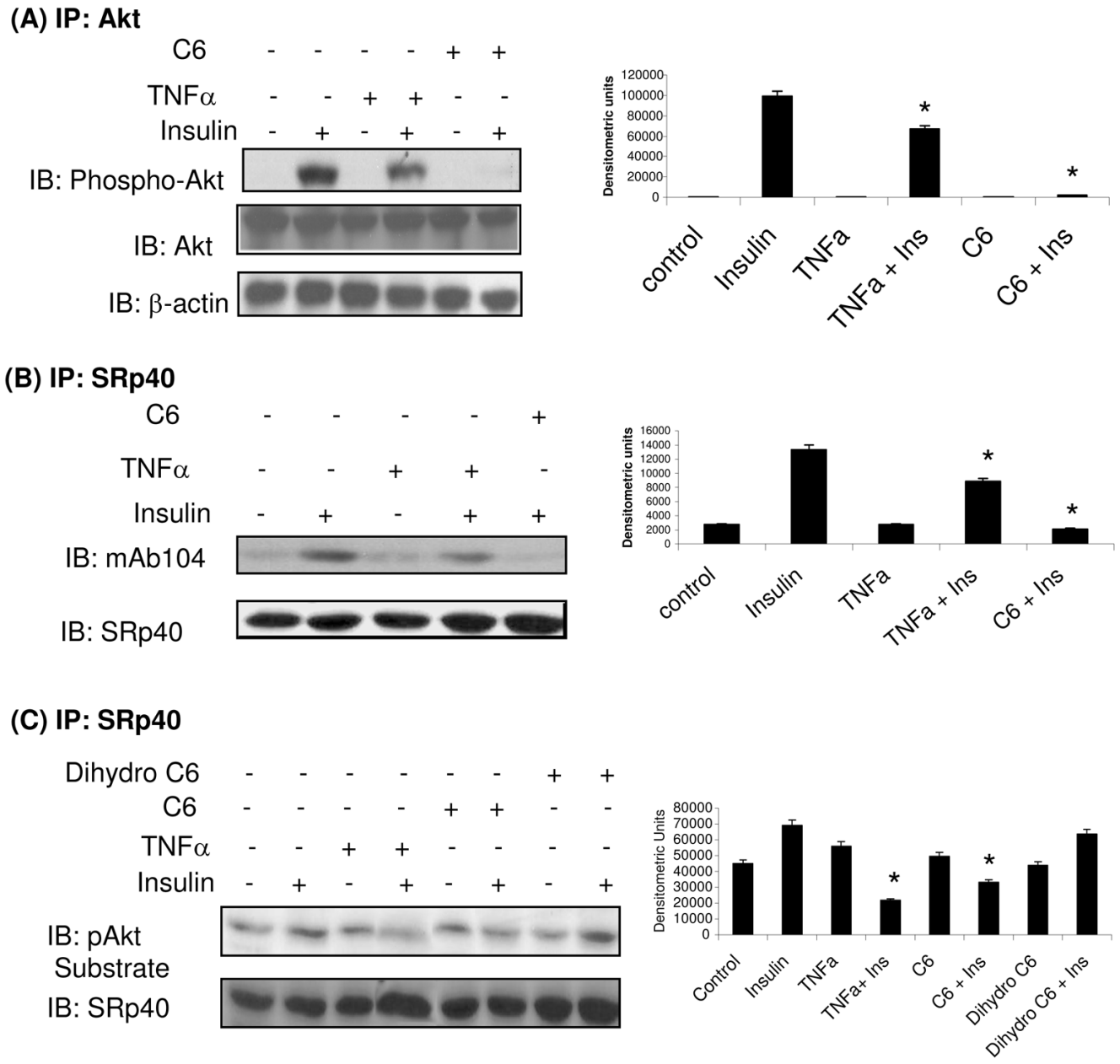

Figure 1. TNFα and C6 ceramide blocked insulin-mediated phosphorylation of Akt and SRp40

(**a**) L6 cells were pre-treated with TNFα (150ng/ml, 30 minutes) or C6 ceramide (20uM, 2 hours) prior to insulin (I) (10nM, 30 minutes) addition as indicated. Western blot analysis was conducted with phospho-Akt antibody (serine 473). The membrane was stripped and re-probed with β-actin to ensure equal protein loading. (**b**) L6 cells underwent similar treatments as above except that TNFα was 15 ng/ml. Lysates were immunoprecipitated with SRp40 antibody, proteins separated and membranes were probed first with Mab104 followed by SRp40 antibody. (**c**) L6 cells were treated as above with the addition of a dihydroC6 treatment (20uM, 2 hours) or as a pre-treatment followed by insulin as a control. Membrane was probed with phosphoAkt substrate antibody, stripped and re-probed with SRp40 antibody to confirm identity of the protein. Each experiment was repeated a minimum of three times with similar

results. The bar graphs summarize results from 3 experiments. The asterisk indicates a significant difference ($p < 0.05$, t-test compared to insulin effect.)

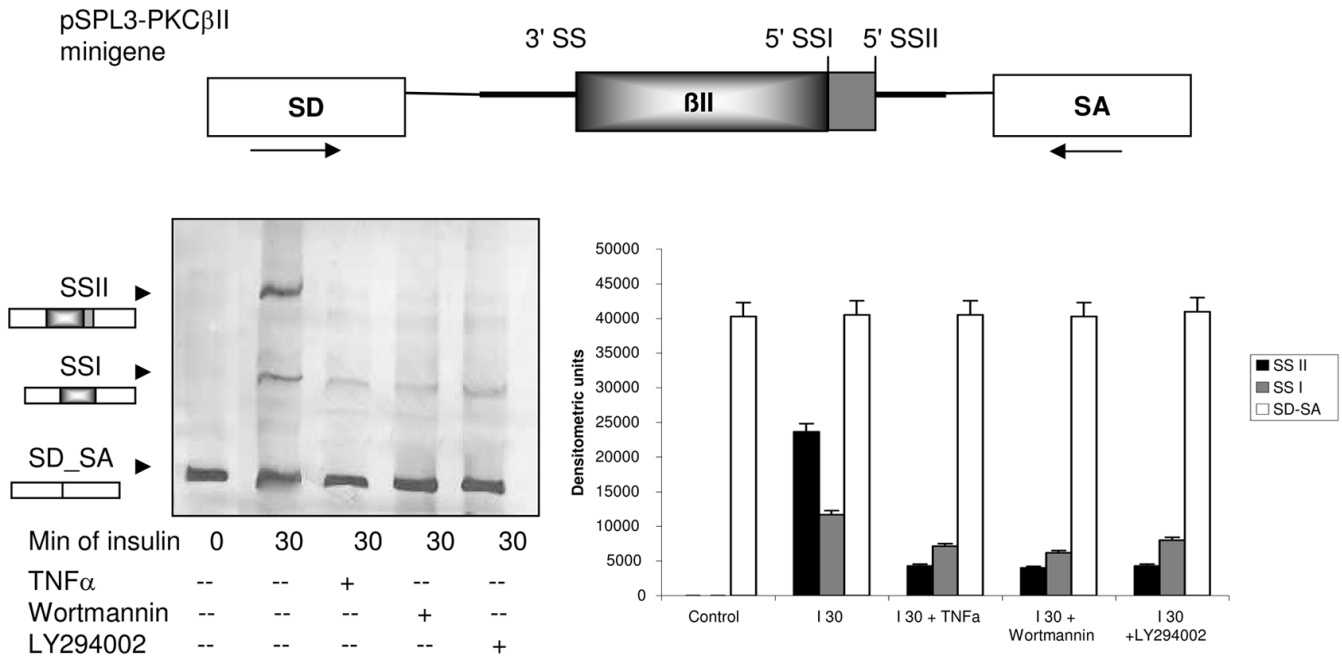

Figure 2. TNFα blocked insulin induced PKCβII exon inclusion

A heterologous minigene constructed by inserting the PKCβII genomic fragment into a multi-cloning site between the splice site donor (SD) and splice site acceptor (SA) in pSPL3 vector was used as a reporter for alternative splicing as described (35). PCR primers corresponding to the arrows in the upper diagram were used to detect insulin-mediated inclusion of the PKCβII exon at both 5′ splice sites within 30 minutes; both splice variants encode PKCβII. Insulin effects were blocked by TNFα (15 ng/ml) similar to the two PI3Kinase/Akt inhibitors: Wortmannin (100 nM) and LY294002 (10 μM). The densitometric scan summarizes of three separate experiments.

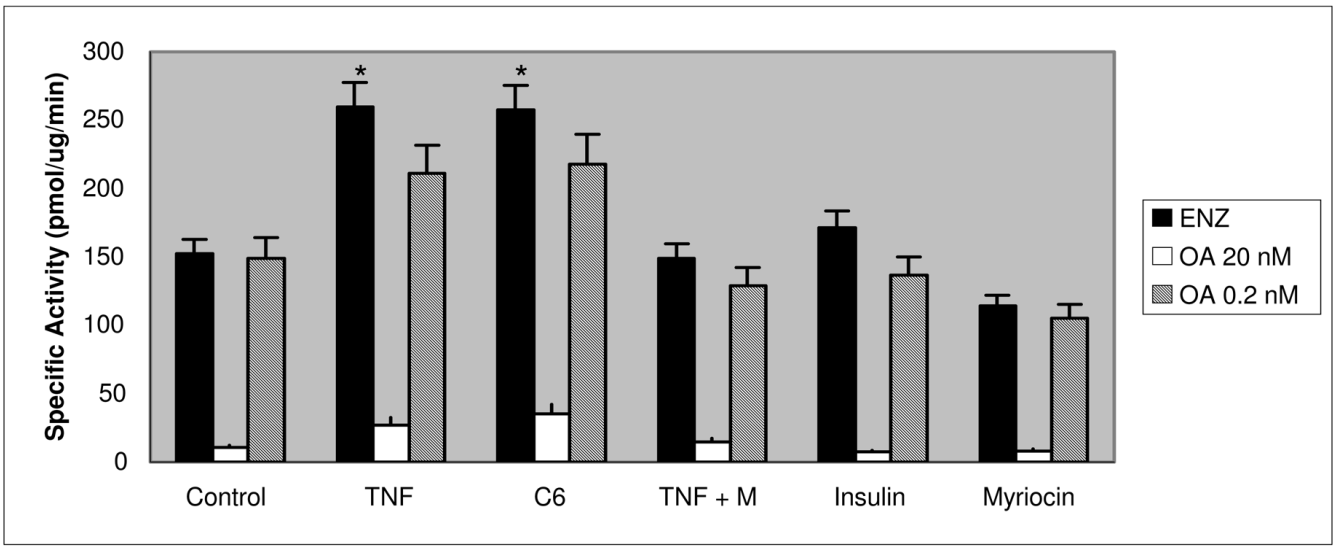

Figure 3. TNFα and C6 ceramide stimulated a PP1-like serine/threonine phosphatase activity

L6 cells were treated with TNFα (15 or 150 ng/nl) for 30 minutes, C6 ceramide (20 μM) for 2 hours insulin (10 nM) for 30 minutes or myriocin (M) (5nM) for 30 minutes. In the phosphatase assay wells, samples were treated with no inhibitor, okadaic acid 20 nM or okadaic acid 0.2 nM. Free phosphate was measured by optical density at 630 nm. Results are mean ±SEM of specific activity from 2–6 separate experiments assayed in duplicate. The asterisk indicates a significant difference from control ($p < 0.05$, t-test).

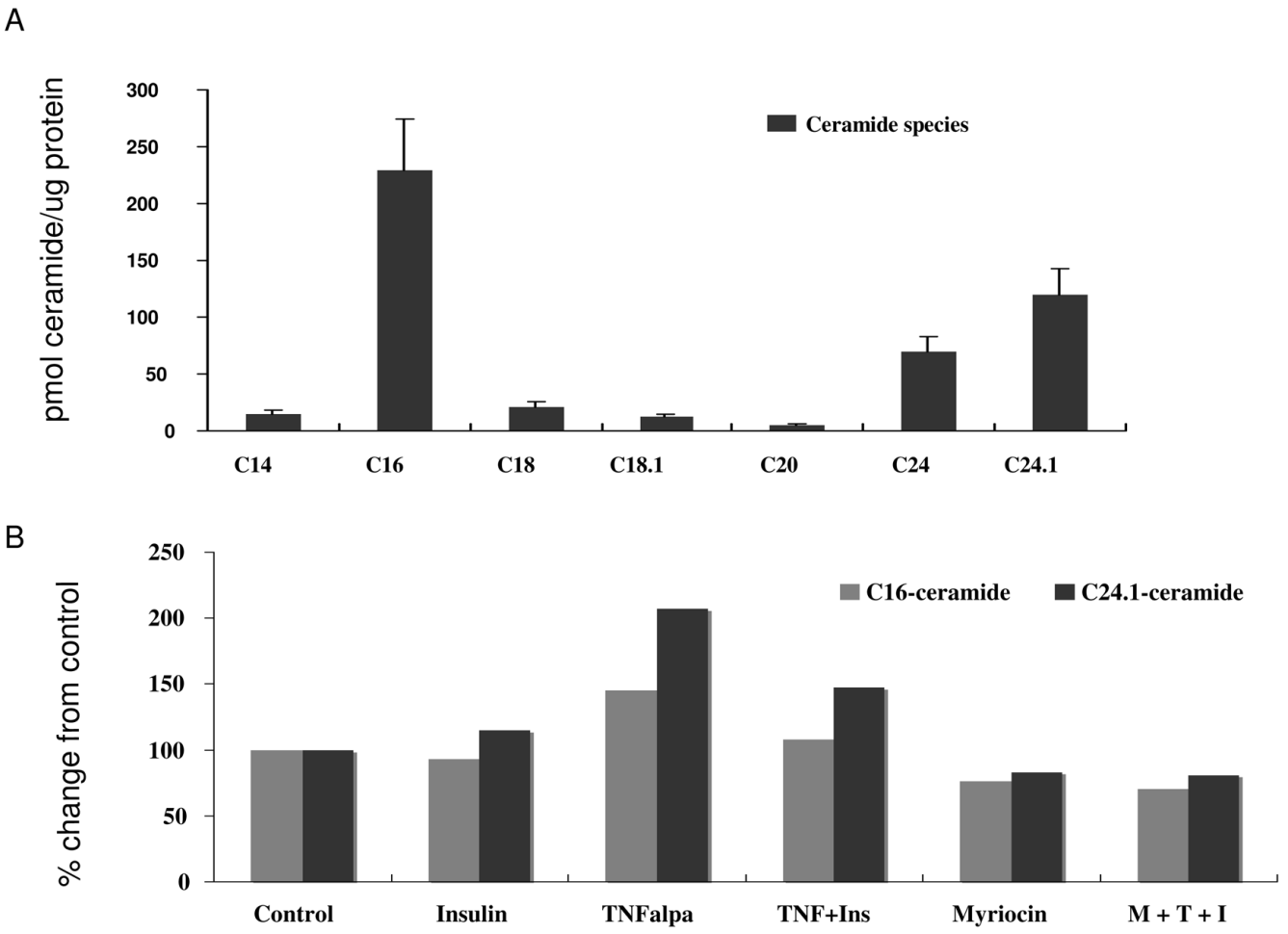

Figure 4. TNFα stimulated *de novo* synthesis of ceramide

(A) L6 cell pellets were analyzed via liquid chromatography-mass spectrometry to determine all the known cellular ceramide species. The predominant species detected in 6 different experiments were C16 and C24:1 ceramide. (B) The change from control for C16 and C24:1 ceramide following TNFα (T) pretreatment followed by insulin (I) or in the presence of myriocin (M) as determined by LC-MS. Shown is the mean of 2 representative experiments.

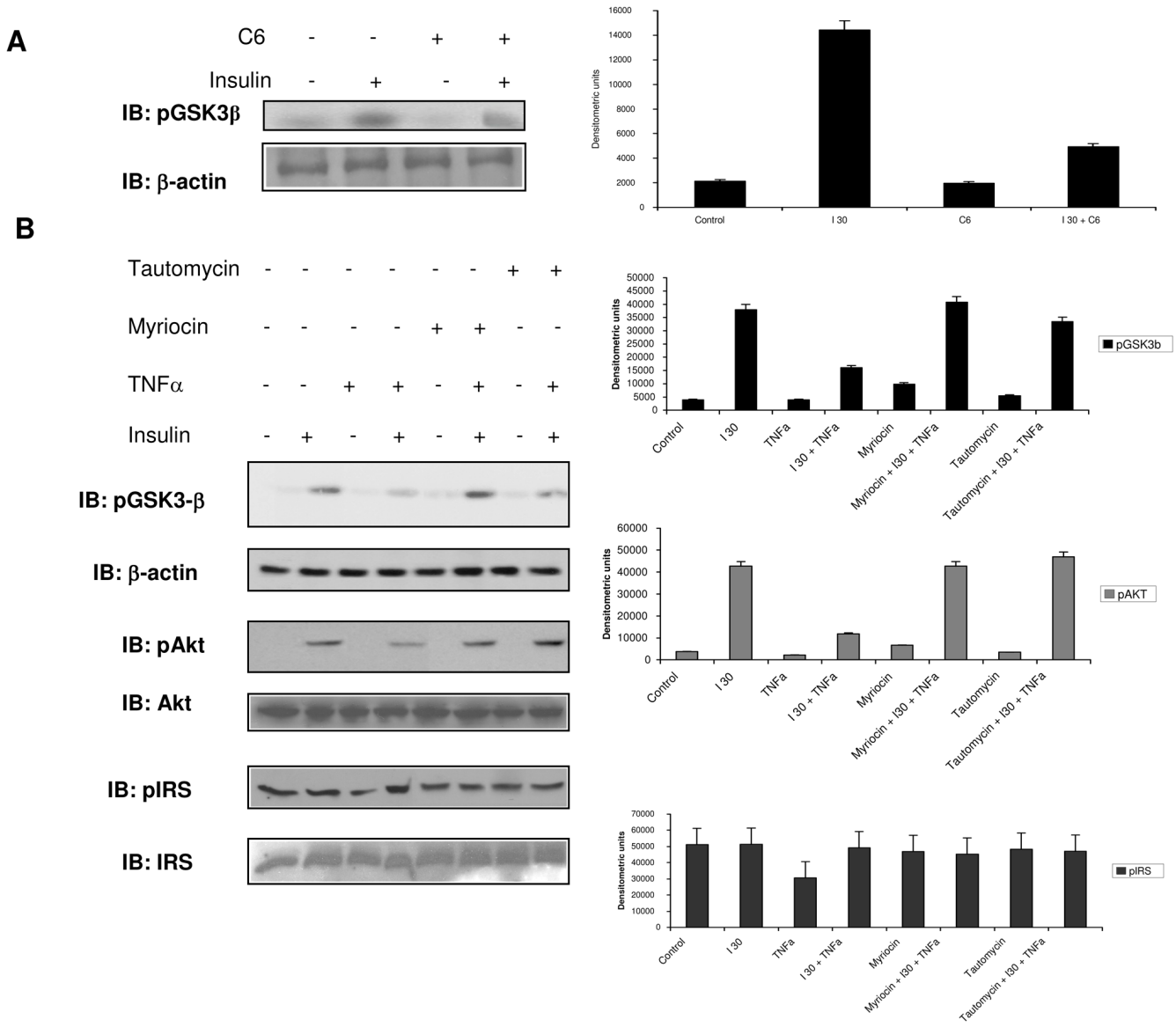

Figure 5. Ceramide Activated Protein Phosphatase inhibits GSK3β phosphorylation and behaves in a *de novo* ceramide-dependent, PP1 manner

(A) L6 cells were treated as previously described. Membrane was probed with phospho-GSK3β antibody (serine 9) and reprobed with β-actin to ensure equal loading. (B) L6 cells were treated with myriocin (50 nM) for 1 hour, myriocin 1 hour then TNFα (150 ng/ml) for 30 minutes, tautomycin (10 nM) for 1 hour, or tautomycin for 1 hour then TNFα for 30 minutes prior to insulin (10 nM) for 30 minutes. Membrane was probed with phospho-GSK3β antibody before re-probing with phospho-Akt and β actin antibodies. A separate membrane was probed or phospho-IRS and IRS. Densitometric scans summarize data from three separate experiments.