Modulating serine palmitoyl transferase (SPT) expression and activity unveils a crucial role in lipid-induced insulin resistance in rat skeletal muscle cells

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INTRODUCTION

Hyperlipidaemia is one of a cluster of abnormalities associated with the metabolic syndrome and one that is considered important for both development and progression of insulin resistance in skeletal muscle, a tissue that makes a major contribution to whole-body glucose homeostasis. Sustained over supply of NEFAs (non-esterified fatty acids; ‘free’ fatty acids) to skeletal muscle has been shown to result in the accumulation of IMTGs (intramuscular triacylglycerols) and that of fatty-acid-derived metabolites, such as DAG (diacylglycerol) and ceramide. Although accumulation of IMTG has been positively correlated with a loss in skeletal muscle insulin sensitivity, the finding that muscle of endurance-trained athletes exhibit improved insulin sensitivity in the face of an increase in IMTG implies that increases in intramuscular lipid alone may not in itself be the primary driver of lipid-induced insulin resistance [1]. Recent work from our laboratory and work of others has shown that increased availability of palmitate, the most prevalent circulating saturated fatty acid, impairs the insulin-dependent regulation of glucose transport and glycogen synthesis in skeletal muscle cells [2,3]. This impairment is due, in large part, to an attendant accumulation in muscle cells of the sphingolipid ceramide, for which the fatty acid is a key precursor. Increases in intramyocellular ceramide have previously been linked to loss of insulin sensitivity in cultured muscle cells [4,5] and in skeletal muscle of both rodents [6,7] and humans [8]. However, it is only recently that the mechanistic basis of ceramide-induced insulin resistance is beginning to be understood.

We [2,4,9], and others [5,10,11], have shown that increases in intracellular ceramide promote a targeted loss in the insulin-dependent activation of the serine/threonine kinase PKB (protein kinase B; also known as Akt). This kinase plays a pivotal role in the hormonal activation of glucose transport and glycogen synthesis, and consequently its dysregulation is likely to impact significantly upon the homeostatic control of blood glucose. The loss of PKB activation by ceramide has been proposed to occur by one of two mechanisms involving either dephosphorylation of its two regulatory sites (Thr308 and Ser473) by a type 2A-like protein phosphatase activity [10] or alternatively, as we have shown, inhibition in the cell-surface recruitment and phosphorylation of PKB by a process dependent upon activation of atypical PKC (protein kinase C) isoforms (λ/ζ) [9,11]. Increasing intracellular ceramide [by provision of short-chain (C16) ceramide or stimulating endogenous palmitic (C16)-ceramide synthesis from fatty acids such as palmitate] results in potent activation of PKCζ (for a review, see [13]) and, as such, this has important implications for PKB activation. In unstimulated cells, PKCζ has been shown to directly interact with and repress PKB in numerous

Saturated fatty acids, such as palmitate, promote accumulation of ceramide, which impairs activation and signalling of PKB (protein kinase B; also known as Akt) to important end points such as glucose transport. SPT (serine palmitoyl transferase) is a key enzyme regulating ceramide synthesis from palmitate and represents a potential molecular target in curbing lipid-induced insulin resistance. In the present study we explore the effects of palmitate upon insulin action in L6 muscle cells in which SPT expression/activity has been decreased by shRNA (small-hairpin RNA) or sustained incubation with myriocin, an SPT inhibitor. Incubation of L6 myotubes with palmitate (for 16 h) increases intramyocellular ceramide and reduces insulin-stimulated PKB activation and glucose uptake. PKB inhibition was not associated with impaired IRS (insulin receptor substrate) signalling and was ameliorated by short-term treatment with myriocin. Silencing SPT expression (~90%) by shRNA or chronic cell incubation with myriocin (for 7 days) markedly suppressed SPT activity and palmitate-driven ceramide synthesis; however, challenging these muscle cells with palmitate still inhibited the hormonal activation of PKB. This inhibition was associated with reduced IRS1/p85-PI3K (phosphoinositide 3-kinase) coupling that arises from diverting palmitate towards greater DAG (diacylglycerol) synthesis, which elevates IRS1 serine phosphorylation via activation of DAG-sensitive PKCs (protein kinase Cs). Treatment of SPT-shRNA cells or those treated chronically with myriocin with PKC inhibitors antagonized palmitate-induced loss in insulin signalling. The findings of the present study indicate that SPT plays a crucial role in desensitizing muscle cells to insulin in response to incubation with palmitate. While short-term inhibition of SPT ameliorates palmitate/ceramide-induced insulin resistance, sustained loss/reduction in SPT expression/activity promotes greater partitioning of palmitate towards DAG synthesis, which impacts negatively upon IRS1-directed insulin signalling.

Key words: ceramide, diacylglycerol, insulin receptor substrate 1 (IRS1), myriocin, palmitate, protein kinase B (PKB), protein kinase C (PKC).

Abbreviations used: DAG, diacylglycerol; DTT, dithiothreitol; FBS, foetal bovine serum; IMTG, intramuscular triacylglycerol; IRS, insulin receptor substrate; LCB, long-chain base; αMEM, α-minimal essential medium; NEFA, non-esterified fatty acid; NF-κB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; shRNA, small-hairpin RNA; SPT, serine palmitoyl transferase; TLR, Toll-like receptor.

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cell types [9,11,14–17], but dissociates upon cell treatment with growth factors [14] and insulin [9], thus allowing PKB to become activated. In contrast, ceramide stabilizes the interaction between the two kinases and, moreover, ablates the ability of insulin to not only dissociate the kinase complex, but to also activate PKB [9].

It follows that limiting intramuscular ceramide production and/or accumulation would confer an insulin-sensitizing effect and that enzymes participating in ceramide synthesis may potentially serve as therapeutic targets for promoting insulin sensitivity of tissues such as skeletal muscle. Indeed, recent in vivo work suggests that attenuating endogenous ceramide synthesis ameliorates the loss in skeletal muscle insulin sensitivity induced in response to saturated fat, glucocorticoids and obesity [18].

The synthesis of ceramide from saturated fatty acids, such as palmitate, is crucially dependent upon the activity of SPT (serine palmitoyl transferase), which catalyses the first and rate-limiting step of sphingolipid biosynthesis involving the condensation of the amino acid L-serine with palmitoyl-CoA to form 3-oxodihydrosphingosine [19]. Mammalian SPT belongs to the family of pyridoxal 5′-phosphate-dependent enzymes and is composed of two distinct protein subunits known as LCB1 (long-chain base-1; 53 kDa) and LCB2 (long-chain base-2; 63 kDa), with stability of the latter being critically dependent upon expression of the former. More recently, a third SPT subunit (LCB3) had been identified, but given that its tissue expression is highly variable, being expressed predominantly in the placenta and cells of trophoblastic origin [20], it is likely to be dispensable with regard to SPT function in other cell and tissue types. SPT activity is susceptible to inhibition by serine analogues such as cycloserine and by molecules that structurally resemble transition-reactive intermediates such as the anti-fungal agent myriocin [19]. In the present study we have modulated SPT expression and activity in cultured rat skeletal muscle cells by either silencing the expression of the LCB1 gene or subjecting muscle cells to sustained incubation with myriocin in an attempt to assess the impact of these interventions on palmitate-induced insulin resistance. We have shown previously that ceramide generated de novo from palmitate is a major factor promoting insulin resistance in muscle cells and that short-term inhibition of SPT has an ameliorative effect on insulin signalling [2]. However, under circumstances when expression of SPT has been repressed or is chronically inhibited, we show that utilization of palmitate is diverted towards greater synthesis of DAG, which subsequently activates DAG-sensitive PKCs that in turn promote a reduction in IRS (insulin receptor substrate)-directed insulin signalling.

EXPERIMENTAL

Materials

α-MEM (α-minimal essential medium), FBS (foetal bovine serum) and antibiotic/antimycotic solution were from Life Technologies. Most other reagent grade chemicals, myriocin, insulin, palmitate, palmitoyl-CoA, DAG, sn-1,2-DAG kinase and antibodies against actin were purchased from Sigma–Aldrich. Ro 31.8220 and GF109203X were from Calbiochem–Merck. C2-ceramide was from Tocris and C6-ceramide was purchased from Avanti. Radioactive chemicals, 2-deoxy [14C]glucose and [3H]serine were purchased from PerkinElmer. [γ-32P]ATP and protein-G-Sepharose beads were from Amersham Biosciences. Antibodies to PKB/Akt, phospho-PKB(Ser473), PKCθ and phospho-IRS1(Thr1115) were purchased from Upstate–Millipore. Anti-LCB2 antibody was from Abcam and antibodies against LCB1 were synthesized by AstraZeneca. Anti-α1 Na+/K-ATPase (α6F) antibody was obtained from the University of Iowa Hybridoma Bank and the anti-phosphosine antibody was from Chemicon–Millipore. BSA-V (Fraction V, fatty-acid free) for conjugating with palmitate, and complete protein phosphatase inhibitor tablets were purchased from Boehringer–Roche Diagnostics.

Cell culture and treatments

L6 skeletal muscle cells were maintained in α-MEM and differentiated in culture to the stage of myotubes as previously described [21]. L6 myotubes were incubated for the time periods and with the appropriate effectors as indicated in the Figure legends. Plates were washed twice with ice-cold PBS and lysis buffer was added [50 mM Tris/HCl (pH 7.4), 0.27 M sucrose, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 % (v/v) Triton-X-100, 0.1 % 2-mercaptoethanol and protease inhibitors]. Whole-cell lysates were centrifuged at 4000 g at 4 °C for 10 min and were stored at −20 °C after being snap–frozen in liquid nitrogen. In some experiments, L6 myotubes were sub-fractionated to isolate total membranes as previously described [21]. Briefly, cells were scraped off plates using a rubber policeman in PBS and centrifuged at 4000 g at 4 °C for 10 min. The resulting cell pellet was resuspended in buffer 1 [20 mM Hepes (pH 7.4), 250 mM sucrose, 2 mM EGTA, 3 mM sodium azide and protease inhibitors] and homogenized with a Dounce glass-to-glass homogenizer. The homogenates were centrifuged at 750 g for 5 min to remove the nuclear pellet. The supernatant was centrifuged at 67000 rev./min (Beckman rotor TLA 100.3) for 60 min to pellet total cell membranes.

SPT silencing using shRNA (short-hairpin RNA)

A short RNA sequence (5′-GGAGCGGTTCGATCTTTACA-3′) complimentary to the LCB1 subunit of SPT, was chosen from sequences obtained from web tools on the Dharmacon (http://www.dharmacon.com), Promega (http://www.promega.com) and Eurogentec (http://www.eurogentec.com) websites. Once a suitable sequence was identified, a BLAST search was performed to check and exclude those sequences that generated non-specific hits. The sequence was then cloned into the siSTRIKE vector (Promega) to create a hairpin loop sequence (5′-AAC-CGGAGCGGTTCGATCTTTACAATTCAGAGATGTAAGATC-TGACGCTTTTTT-3′). The vector was transfected (1 μg of DNA) into L6 myoblasts using FuGENETM reagent (Boehringer–Roche). Myoblasts were washed free of transfection medium 5 h later and were incubated for a further 2 days in α-MEM containing 2 % (v/v) FBS and 1 % (v/v) antibiotic/antimycotic solution. Stable transformants were subsequently selected by addition of 800 μg/ml G418 (Geneticin) disulfate to the culture medium. Transformed L6 myoblasts were selected using cloning discs (Sigma–Aldrich) and propagated to generate a working cell line. Silencing of SPT was confirmed by using antibodies against the LCB1 and LCB2 subunits and by analysis of cellular SPT activity.

SDS/PAGE and immunoblotting

Cell lysates and total membrane fractions (15–50 μg of protein) were reconstituted in Laemmli buffer and subjected to SDS/PAGE on 10 % or 7.5 % resolving gels and transferred on to Immobilon-P membranes as described previously [21]. Membranes were probed with primary antibodies of interest and subsequently detected using either HRP (horseradish
peroxidase)-conjugated anti-rabbit IgG or anti-mouse-IgG (New England Biolabs). Membranes were visualized using ECL® (enhanced chemiluminescence; Pierce) on Konica Minolta X-ray medical film.

Hexose uptake

2-Deoxyglucose uptake was assayed as previously described [21]. Briefly, after relevant incubations, L6 myotubes were washed twice with HBS [Hepes-buffered saline; 140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO4 and 1 mM CaCl2 (pH 7.4)] and incubated for 10 min in HBS containing 10 μM [3H]2-deoxy-d-glucose (1 μCi/ml) at room temperature (20°C). Non-specific binding was determined by quantifying cell-associated radioactivity in the presence of 10 μM cytochalasin B. Medium was aspirated and adherent cells were washed twice with 0.9% (w/v) ice-cold NaCl. Cells were subsequently lysed in 50 mM NaOH and cell-associated radioactivity was quantified using a Beckman LS 6000IC scintillation counter. The protein concentration in cell lysates was determined using Bradford reagent [22].

Analysis of cellular ceramide and DAG content

After appropriate cell treatments, myotubes were extracted from plates using a rubber policeman in ice-cold PBS for analysis of ceramide and DAG using the DAG kinase method, which relies on the kinase catalysing the formation of ceramide 1-phosphate and phosphatidic acid respectively. Briefly, protein content in the harvested cells was assayed using the Bradford method [22] and lipids were extracted from a cell aliquot containing 400 μg of protein by the addition of chloroform/methanol [1:2 (v/v)] with the phases being broken by the addition of chloroform and 1 M NaCl. The organic phase was separated, dried and used for ceramide and DAG measurements as previously described [2]. The lipids were incubated at room temperature for 30 min in a reagent mix containing sn-1,2-DAG kinase in diluted enzyme buffer [10 mM imidazole/HC1 and 1 mM diethylenetriaminepenta-acetic acid (pH 6.6)], assay buffer [0.1 M imidazole/HC1, 0.1 M NaCl, 25 mM MgCl2, 2 mM EGTA (pH 6.6) and 20 mM DTT (dithiothreitol), and the reaction was initiated by the addition of 5 mM ATP (mixed with [γ-32P]ATP; 1 μCi/reaction) and 1 μl of DAG kinase, which equates to an activity of at least 1.3 nmol/min. The reaction was terminated by the addition of 20 μl of 1% (v/v) perchloric acid and 450 μl of chloroform/methanol [1:2 (v/v)]. The organic phase was isolated and washed twice with 1% (v/v) perchloric acid. The samples were dried and reconstituted in chloroform/methanol [95:5 (v/v)] and spotted on to TLC plates. Reaction products were separated by placing the plate into a chamber equilibrated with a solution of chloroform/methanol/acetone (65:15:5, by vol.). Radioactive products were visualized on Konica Minolta X-ray medical film using an X-ograph imaging system. Changes in ceramide and DAG content were expressed as a fold change relative to that measured in untreated cells.

Analysis of SPT activity

SPT activity was analysed using the Merrill method as described by Hollera et al. [23]. Briefly, 80–100 μg of total membrane protein from L6 myotubes was incubated at 37°C for 10 min in a reaction buffer (100 mM Hepes, 2.5 mM EDTA, 5 mM DTT, 50 μM pyridoxal phosphate and 1 mM serine mixed with 1 μCi/100 μl [3H]serine). The reaction was initiated by the addition of 20 μl of 1 M palmitoyl-CoA and terminated by the addition of 200 μl 0.5 M NH4OH and cooled on ice. The lipid phases were broken by the addition of 1.5 ml of chloroform/methanol [1:2 (v/v)], 25 μl of sphingonine (1 mg/ml, as a carrier) and 2 ml of NH4OH. Samples were vortexed and centrifuged (3000 g at 4°C for 5 min) to separate the phases. The aqueous phase was removed and the organic phase was washed twice with deionized water. The organic phase (300 μl) was then dried under vacuum and reconstituted in chloroform/methanol/acetone (6.5:1:1, by vol.) and the radioactivity was quantified using a Beckman LS 6000IC scintillation counter. Non-specific activity was assessed by assaying activity in wild-type cells in which the SPT substrate, palmitoyl-CoA, was excluded from the assay.

IRS1 immunoprecipitation

Following appropriate treatments, L6 myotubes were lysed in lysis buffer and IRS1 was immunoprecipitated using an antibody against the C-terminal domain of IRS1. Immunocomplexes were incubated with protein-G-Sepharose beads overnight at 4°C and solubilized in Laemmli sample buffer prior to immunoblotting as described above.

Statistical analyses

Data analysis was performed using GraphPad Prism software and results were considered statistically significant at P values less than 0.05.

RESULTS

Cellular silencing of SPT using shRNA

Previous work from our laboratory has shown that incubation of L6 myotubes for 16 h with 0.75 mM palmitate induces a greater than 2-fold increase in intracellular ceramide, which promotes a significant decrease in the hormonal (insulin) activation of PKB and glucose uptake [2]. Attenuating ceramide synthesis from palmitate by co-incubation of cells with myriocin (an SPT inhibitor) significantly curtails the ceramide synthesis from palmitate and the associated decrease in PKB activation [2] and see Supplementary Figure S1 at http://www.BiochemJ.org/bj/417/bj4170791add.htm). On the basis of the above observations we hypothesized that a sustained suppression in SPT expression and/or activity may protect muscle cells against the insulin-desensitizing effects of palmitate. To test this hypothesis, we generated a stable L6 muscle cell line in which cellular SPT expression was decreased substantially through silencing the expression of the LCB1-SPT subunit using shRNA. Nine positive L6 transformants were identified in which expression of LCB1 had been reduced by between 60 and 90% compared with wild-type clones. Of these, clone 6 (Figure 1A) exhibited the greatest reduction in LCB1 expression and was subsequently used for further experimental analysis. Given that the stability of the LCB2-SPT subunit is inherently dependent on the expression of LCB1 and both subunits associate with a 1:1 molar stoichiometry [19], silencing of LCB1 also led to an attendant reduction in the expression of LCB2 (Figure 1A). Consistent with the loss in cellular SPT expression observed in shRNA-treated cells, analysis of SPT activity revealed that this was suppressed significantly (by ~70%) in cells transfected with the shRNA-LCB1 sequence (SPT-shRNA) compared with wild-type L6 cells or those that had been transfected with the scrambled (SCR-shRNA) shRNA-LCB1 sequence (Figure 1B). An apparent consequence of silencing SPT expression in L6 muscle cells was increased cellular sensitivity towards palmitate, which manifests itself as an increase in palmitate-induced cell death. Wild-type L6 myotubes or SCR-shRNA cells tolerate...
prolonged incubation (for 16 h) with 0.75 mM palmitate, whereas those in which SPT expression had been reduced displayed an increased propensity to detach from plates and subsequently wither. To overcome this problem, SPT-shRNA cells were incubated with a lower palmitate concentration (0.5 mM), which was tolerated by the cells much better on the basis of the analysis of adherence, Trypan Blue exclusion and total cellular protein. It should be stressed that we did not observe any adverse changes in cellular morphology in response to chronic (7 day) treatment with myriocin and that, in the absence of palmitate, these cells exhibit a hormonal activation of PKB comparable with that observed in control cells (Figure 3A).

Effects of insulin and palmitate on PKB activation and glucose uptake in shRNA-LCB1-transfected muscle cells

Having established that palmitate does not induce any detectable increase in intracellular ceramide in muscle cells in which SPT expression had been suppressed, we subsequently investigated the effects of the fatty acid on insulin-induced PKB phosphorylation and glucose uptake. Interestingly, despite the significant loss in SPT expression and activity that prevails in SPT-shRNA cells, we found that incubation of muscle cells with 0.5 mM palmitate (for 16 h) still induced a significant loss in the insulin-dependent activation of both PKB and glucose uptake in a manner similar to that observed in wild-type control cells or SCR-shRNA cells (Figure 2). Very similar responses to palmitate were also observed in two other SPT-shRNA clonal isolates (depicted as clones 5 and 8 in Figure 1A), indicating that the results obtained did not simply reflect heightened sensitivity of a single clone to palmitate. It is also noteworthy that, as a consequence of lowering the palmitate concentration from 0.75 mM to 0.5 mM, inhibition of PKB activity in wild-type cells was not as marked as reported previously ([2], and see Supplementary Figure S1).

Effects of long-term SPT inhibition upon palmitate-induced insulin resistance

Sustained SPT inhibition using myriocin should mimic the cellular scenario created by silencing SPT expression. Figure 3 shows that this indeed appears to be the case. In contrast with the ameliorative effect associated with short-term (24 h) inhibition of SPT, more sustained inhibition over a period of 7 days did not antagonize the loss in insulin sensitivity associated with palmitate treatment, as judged by analysis of PKB(Ser473) phosphorylation (Figure 3A) and glucose uptake (Figure 3B). It should be stressed that we did not observe any adverse changes in cellular morphology in response to chronic (7 day) treatment with myriocin and that, in the absence of palmitate, these cells exhibit a hormonal activation of PKB comparable with that observed in control cells (Figure 3A).

Effects of chronic SPT inhibition and SPT silencing in L6 muscle cells upon palmitate-induced DAG synthesis

Treatment of muscle cells with myriocin for 24 h or 7 days had no discernable effect upon SPT expression as judged by analysis of SPT-LCB1 abundance (Figure 4A). Nevertheless, intramyocellular SPT activity was virtually ablated in response to palmitate-induced DAG synthesis in shRNA-LCB1-transfected cells we found, compared with wild-type cells, a virtual loss in palmitate-driven ceramide synthesis (Figure 1C).
Figure 2  Effects of insulin and palmitate on PKB activation and glucose uptake in L6 muscle cells in which SPT expression has been stably repressed by shRNA

L6 myotubes were incubated in the absence or presence of 0.5 mM palmitate for 16 h and 100 nM insulin during the penultimate 10 min (A) or 30 min (B) period prior to either cell lysis and immunoblotting of lysates with antibodies against phospho-PKB(Ser473), PKB or the two SPT subunits (LCB1 and LCB2), or (B) for assay of glucose uptake. Results are means ± S.E.M. for three to five separate experiments. Asterisks signify a significant difference from the appropriate control value (P < 0.05). WT, wild-type.

with myriocin. Under similar incubation conditions we have previously observed much larger increases in palmitate-derived DAG accumulation [2]. One potential explanation that may help account for the relative differences in DAG content is that our previous work utilized a commercial (Amersham) radiometric DAG kinase kit and we assessed the in vitro conversion of DAG into phosphatidic acid using a very sensitive electronic autoradiographic InstantImager. The Amersham kit is no longer available and so DAG was assayed using a protocol based on that described in the commercial kit, but using bacterially expressed DAG kinase from another commercial vendor. In addition, the generation of phosphatidic acid was assessed by quantifying spot intensities on autoradiographic film attributable to the radioactive phosphatidic acid once it had been resolved by TLC and the TLC plate had been exposed to film. Although the slight changes in methodology may help account for differences in the relative changes in DAG measured in the present study compared with that published previously [2], it is apparent that the method used here was able to detect a significant increase in DAG when muscle cells were subjected to a 7 day treatment with myriocin compared with those incubated with the inhibitor for 24 h (Figure 4C).

On the basis of the results above we subsequently assessed whether enhanced intracellular DAG synthesis also prevails in SPT-shRNA cells following 16 h incubation with 0.5 mM palmitate. Figure 5(A) shows that, compared with cells transfected with the SCR-shRNA-containing vector, those in which SPT expression had been silenced harbour a significantly greater accrual of DAG in response to treatment with palmitate. A significant increase in DAG would be expected to activate DAG-sensitive PKCs which can promote serine phosphorylation of IRS1, and subsequently reduce recruitment of p85-PI3K to IRS1 after insulin stimulation. Consistent with this notion, Figure 5(B) shows that, unlike wild-type L6 cells or those transfected with the SCR-shRNA sequence, SPT-shRNA cells fail to recruit the p85 subunit of PI3K to IRS1 in response to insulin following prior incubation with 0.5 mM palmitate, whereas they remain competent to do so if not exposed to the fatty acid. It is noteworthy that silencing SPT expression did not result in any significant shifts in the expression of the...
Figure 4  Effects of short- and long-term myriocin incubation on SPT-LCB1 expression, SPT activity, intracellular ceramide and DAG

L6 muscle cells were cultured for a period of 7 days in the absence or presence of 10 μM myriocin. In some experiments cells were grown in culture for 6 days and then subjected to incubation with 10 μM myriocin for 24 h. During the last 16 h incubation with myriocin, cells were also incubated in the absence or presence of 0.75 mM palmitate where indicated. Following these treatments, cells were processed for the analysis of (A) LCB1-SPT abundance by immunoblotting using the α1 subunit of the Na/K-ATPase as a membrane-loading control and for analysis of SPT activity, (B) intracellular ceramide or (C) intracellular DAG content. The blots show results from two experiments run in side-by-side lanes for the conditions shown. Results are means ± S.E.M. for three experiments. Asterisks signify a significant difference compared with the untreated control or between the indicated values (P < 0.05). No PCoA, no palmitoyl-CoA.

p85-PI3K subunit or that of IRS1 based on assessing the abundance of both proteins in whole-cell lysates (Figure 5B).

IRS1 serine phosphorylation and PKCθ recruitment to the plasma membrane are increased in cells with reduced SPT expression and activity

To assess whether the reduced interaction between p85-PI3K and IRS1 was associated with an increase in IRS1 serine phosphorylation, we immunoprecipitated IRS1 from wild-type L6 cells, cells in which SPT had been silenced or those that had been chronically treated with myriocin for 7 days following a 16 h incubation in the absence or presence of 0.5 mM palmitate.

Figure 5  Intramyocellular DAG content and IRS1/p85-PI3K association in L6 muscle cells in which SPT expression has been stably repressed by shRNA

Muscle cells with a stable repression of SPT or those transfected with the scrambled LCB1-shRNA sequence (SCR-shRNA) were incubated in the absence or presence of 0.5 mM palmitate for 16 h prior to analysis of intramyocellular DAG content (A). (B) Wild-type (WT) L6 myotubes or those transfected with the LCB1-SPT shRNA or the scrambled LCB1-shRNA sequence (SCR-shRNA) were incubated with 0.5 mM palmitate for 16 h and incubated with 100 nM insulin for the penultimate 15 min incubation period as indicated. Muscle cells were lysed and either immunoblotted (whole-cell lysates, 50 μg of protein) or used for immunoprecipitation of IRS1 prior to resolving the immunoprecipitate by SDS/PAGE and immunoblotting with antibodies against IRS1 or the p85 subunit of PI3K. Immunoblots were quantified and data were normalized to the untreated wild-type control value. Results are means ± S.E.M. for three experiments. Asterisks signify a significant difference between the indicated values (P < 0.05).

IB, immunoblot.

Figure 6(A) shows that, in the absence of any pre-incubation with palmitate, there was no significant IRS1 serine phosphorylation. Based on the analysis of three separate experiments, incubation of wild-type cells with 0.5 mM palmitate induced a modest enhancement in serine phosphorylation of immunoprecipitated IRS1, although this failed to achieve statistical significance. By contrast, IRS1 serine phosphorylation was elevated 3–4-fold in cells in which SPT had either been silenced or chronically inhibited with 7 days of myriocin treatment. Although numerous kinases have been suggested to serine phosphorylate IRS1, activation of novel and conventional DAG-sensitive PKCs have been strongly implicated in this event in response to fatty-acid overload [24]. To investigate the possibility of PKC involvement, we assessed activation of PKCθ, a PKC isoform that is abundant...
in muscle cells. Results from three separate experiments revealed that treatment of SPT-shRNA-transfected cells or those incubated with myriocin led to a significant activation of PKC\(\theta\) based on its increased association with the plasma membrane. This activation was not observed in wild-type L6 cells subjected to palmitate (16 h) treatment, but was evident upon incubation of these cells with 100 \(\mu\)M PMA, a known PKC activator, which was used as a positive control for the membrane recruitment assay (Figure 6B). Activated PKC\(\theta\) has been shown to promote phosphorylation of IRS1 Ser\(^{1101}\) [25] and, as shown in Figure 6(C), a phospho-antibody directed against this site was able to detect increased phosphorylation of this site in response to palmitate treatment of SPT-shRNA-transfected cells or those incubated with myriocin. This palmitate-induced phosphorylation was not apparent in muscle cells that had also been co-incubated with Ro 31.8220, a compound that selectively targets novel and conventional forms of PKC when used at submicromolar concentrations [26].

**DISCUSSION**

SPT occupies a critical position with regard to intracellular ceramide metabolism, catalysing the committed step in the de novo synthesis of ceramide from palmitoyl-CoA and serine [19]. Under physiological circumstances the intracellular concentration of lipid intermediates generated downstream of SPT activity such as 3-oxosphinganine, sphinganine and ceramide are normally maintained at relatively low levels [27]. However, the increased availability of circulating NEFAs, in particular palmitate, serves to drive ceramide synthesis, whose cellular accumulation not only promotes apoptosis [28,29] but impairs insulin action in tissues such as skeletal muscle and adipose tissue [30]. It follows that preventing excessive intracellular synthesis of ceramide may have potentially beneficial consequences with respect to maintaining cellular integrity and insulin sensitivity. Indeed, recent in vivo work from Summers and co-workers [18] strongly suggests that SPT is likely to be an appropriate molecular target for anti-diabetic therapies based on observations showing that myriocin administration to rodents ameliorates some of the metabolic disturbances associated with glucocorticoid-, saturated fat- and obesity-induced insulin resistance.

In an attempt to further understand the role played by SPT in fatty-acid-induced insulin resistance and to assess its value as a potential therapeutic target, the present study investigated the effects of palmitate on insulin action in muscle cells in which SPT expression/activity was chronically suppressed. While short-term (24 h) inhibition of SPT with myriocin was found to ameliorate the loss in insulin-stimulated PKB phosphorylation induced by palmitate-driven ceramide synthesis ([2] and Supplementary Figure S1), our findings indicate that sustained inhibition of SPT or shRNA-mediated silencing of the enzyme fails to confer a protective effect against the insulin-desensitizing effects of palmitate. We postulated that, in the absence of being able to utilize palmitoyl-CoA for ceramide synthesis via the SPT pathway, palmitate may have been channelled into other pathways, such as those responsible for DAG synthesis. Consistent with this notion, the present study demonstrates that muscle cells harbouring a stable loss in SPT expression exhibit...
Figure 7 Palmitate-induced inhibition of PKB activation in L6 cells with reduced SPT expression/activity is antagonized by PKC inhibition

L6 myotubes in which SPT had been stably repressed by shRNA or chronically inhibited by sustained incubation with myriocin for 7 days were incubated with 0.5 mM or 0.75 mM palmitate respectively for 16 h. Ro 31.8220 (0.5 μM) or GF109203X (1 μM) were added to the incubation medium during the last 2 h with the fatty acid. Where indicated, muscle cells were treated with insulin (100 nM) for the penultimate 10 min of the incubation. Cells were lysed and lysates were immunoblotted to assess PKB activation using an anti-phospho-PKB(Ser473) antibody. Blots in (A and B) show lysates from two separate experiments run in side-by-side lanes. Blots from at least three experiments were quantified and changes expressed relative to the untreated ‘basal’ sample. Asterisks signify a significant difference between the indicated values (P < 0.05).

Greater partitioning of palmitate into DAG (~2.3-fold) compared with those transfected with the control SCR-shRNA sequence (~1.3-fold). In the absence of any significant ceramide synthesis it is likely that the increase in intramyocellular DAG becomes an important determinant of insulin sensitivity in cells with reduced SPT expression/activity (Figure 8B). Although we are unable to fully exclude the possibility that myriocin may exert additional pharmacological effects when incubated with muscle cells over a 7 day period, the observation that SPT-shRNA cells give broadly similar results, in terms of insulin sensitivity and changes in cellular DAG, when challenged with palmitate, implies that the effects of the inhibitor are likely to be largely restricted to inhibition of SPT.

How do our cell-based observations compare with recent in vivo studies in which myriocin was been administered to rodents? In line with our work, Holland et al. [18] found that infusing rats for 6 h with lard oil (a source of saturated fat) elevated intramuscular ceramide and DAG, and promoted a reduction in PKB activation, which, with the exception of the increase in DAG, could be ameliorated by co-infusion of myriocin [18]. More...
long-term administration of myriocin was found to improve whole-body insulin sensitivity in rodent models of glucocorticoid- and obesity-induced insulin resistance. Although the authors noted that myriocin treatment markedly reduced tissue ceramide, they did not directly assess the chronic effects of myriocin administration on intramuscular DAG or insulin action in skeletal muscle. Therefore it is not implausible that the improved whole-body glucose tolerance observed by these authors in response to long-term myriocin treatment may partly reflect a greater efficacy of the drug in tissues other than skeletal muscle. Indeed, the observation that pre-treatment of rats with myriocin completely prevents glucocorticoid-induced increases in hepatic glucose output, while only being partially effective in blocking dexamethasone-induced insulin resistance in skeletal muscle, is fully consistent with this proposition [18]. Several kinases, including members of the PKC family, have been implicated in serine phosphorylation of IRS1 on multiple sites including, for example, Ser^{1101}, Ser^{1122}, Ser^{656/659} and Ser^{1101}, which are widely accepted to negatively regulate IRS1-directed insulin signalling [31]. Since DAG accrual has been linked to activation of novel and conventional PKCs, this family of proteins is likely to be an important effector of lipid-induced insulin resistance [31]. Indeed, the expression of PKCθ, a novel PKC isoform that is very abundant in skeletal muscle, has been shown to not only be elevated in response to an increase in circulating NEFAs, but activated by increases in fatty acyl-CoA and DAG [24,32–34]. The full array of serine sites that may be targeted by PKCs on IRS proteins still remain poorly defined, but PKCθ has been suggested to directly promote phosphorylation of Ser^{1101} [25] and indirectly that of Ser^{1101} via activation of JNK (c-Jun N-terminal kinase) and IKKβ (IkBα [inhibitor of NF-κB (nuclear factor κB)] kinase β) [35,36]. The notion that a PKC-mediated increase in IRS1 serine phosphorylation may underpin palmitate-induced insulin resistance in cells in which SPT expression/activity has been reduced is supported by the following studies. First, an anti-phosphoserine antibody detects increased serine phosphorylation within IRS1 immunoprecipitates prepared from palmitate-treated SPT-shRNA-transfected L6 myotubes and from those chronically treated with myriocin. Secondly, palmitate treatment of these cells led to activation of PKCθ and enhanced phosphorylation of IRS1 Ser^{1101}; a reported PKCθ target site [25]. It is noteworthy that neither PKCθ activation nor increased IRS1 Ser^{1101} were evident in wild-type cells following palmitate treatment, consistent with the idea that palmitate does not disrupt IRS1/PI3K association in such cells (Figure 5). Thirdly, palmitate-induced phosphorylation of Ser^{1101} was sensitive to Ro 31.8220, a bisindolylmaleimide that selectively targets novel and conventional PKCs at submicromolar concentrations [37] and, as such, this inhibitor had a restorative effect on insulin-stimulated PKB phosphorylation. It is important to stress, however, that since muscle cells also express other DAG-sensitive PKCs, such as PKCε and PKCθ [38,39], these are likely to act in concert with PKCθ to promote a net increase in IRS1 serine phosphorylation [40,41]. Since bisindolylmaleimides such as Ro 31.8220 and GF109203X do not discriminate between novel and conventional PKC isoforms, the ability of these compounds to antagonize palmitate-induced inhibition of PKB in SPT-shRNA-transfected cells and those treated with myriocin should, therefore, be viewed in the context of an overall reduction in the activation of DAG-sensitive PKCs.

In addition to ceramide and DAG there is growing evidence linking components of the innate immune system to lipid-induced insulin resistance. Saturated fatty acids can serve as ligands for TLR (Toll-like receptor)-2 and TLR-4, which, when activated, stimulate the pro-inflammatory NF-κB pathway [42,43]. Activation of NF-κB promotes increased expression of pro-inflammatory cytokines, such as IL-6 (interleukin-6) and TNF-α (tumour necrosis factor-α), which negatively regulate skeletal muscle insulin sensitivity, as seen in rodents placed on a high-fat diet for periods of up to 16 weeks [43,44]. In separate studies we have observed that palmitate can also induce NF-κB activation in L6 myotubes, but our findings suggest that insulin resistance induced by the fatty acid is not, at least over the 16 h palmitate incubation used in the present studies, dependent on NF-κB, given that inhibition of PKCs ameliorate the loss in PKB activation without any significant reduction in NF-κB activation (C. Lipina and H. Hundal, unpublished work). Nevertheless, given that suppression of TLR-4 and NF-κB signalling [44–46] has been suggested to antagonize diet-induced insulin resistance, it is likely that the loss in insulin sensitivity associated with fatty-acid overload involves contribution from diverse pathways, which exert both spatial and temporal effects upon cellular responses influencing the fidelity of insulin action within tissues such as skeletal muscle [47]. For example, the finding that the insulin-dependent activation of PKB can be acutely down-regulated...
within 10 min of raising intracellular ceramide, using a cell-permeant analogue of this sphingolipid [4], supports the idea that ceramide synthesis from palmitate may represent a key early event initiating fatty-acid-induced insulin resistance. However, insulin sensitivity will also be strongly influenced by responses that may temporally cause a lag in the effects associated with an increase in cell ceramide and DAG, such as changes in gene expression induced in response to pro-inflammatory (NF-kB and cytokine) signalling that will affect important cellular functions such as insulin signalling, nutrient uptake and mitochondrial function [47].

In summary, the findings of the present study demonstrate that ceramide synthesis from palmitate is critically dependent upon SPT and that increased flux through this enzyme makes an important contribution to the insulin-desensitizing effects of palmitate. Ceramide generated via this pathway induces a targeted inhibition of PKB by a mechanism that has been shown previously to involve activation of atypical PKCs [2,9,48]. The importance of ceramide generated via this pathway is underscored by the finding that short-term inhibition of SPT helps alleviate the insulin-desensitizing effects of palmitate. However, a sustained loss or reduction in SPT expression/activity, although reducing the capacity for generating ceramide from palmitate, has the effect of diverting use of the fatty acid towards greater synthesis of DAG, which then becomes a more important determinant of insulin sensitivity through its ability to activate DAG-sensitive PKCs that suppress IRS-directed insulin signalling. Consequently, although targeting SPT with inhibitors improves insulin sensitivity both in vitro (the present study and [2,49]) and in vivo [18] in the short-term, the perceived long-term benefits may need to be carefully assessed based on the impact that SPT inhibition has upon accumulation of other fatty-acid-derived metabolites with the potential to promote insulin resistance within skeletal muscle.

ACKNOWLEDGEMENTS

We thank Dr Nikolaos Dimopoulos, Emma Cwiklinski and Charlotte Green for assistance with some of the experimental work reported and Dr Chris Lipina for providing helpful comments on the manuscript prior to submission.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council/Industrial (AstraZeneca) CASE Quota Award [grant number BB/S02005/12337]; the European Commission [contract LSHM-CT-2004-005272]; Diabetes Research & Wellness Foundation [open funding programme]; and Diabetes UK [grant number BDA RD07/0003405].

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SUPPLEMENTARY ONLINE DATA
Modulating serine palmitoyl transferase (SPT) expression and activity unveils a crucial role in lipid-induced insulin resistance in rat skeletal muscle cells

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Figure S1 Effects of ceramide and palmitate on PKB activation and glucose uptake in L6 skeletal muscle cells

(A) L6 myotubes were incubated in the absence or presence of 0.75 mM palmitate for 16 h after which period intracellular ceramide was determined using the DAG kinase assay. (B and C) L6 myotubes were incubated with 0.75 mM palmitate for 16 h or 100 μM ceramide for 2 h prior to serum starvation and insulin (100 nM) treatment for (B) 10 min for the analysis of PKB activation or for (C) 30 min for assaying glucose uptake. (D) SPT activity in L6 muscle cell lysates was assayed in the absence or presence of 10 μM myriocin in vitro (left-hand panel) or following in vivo incubation of muscle cells with 10 μM myriocin for 24 h (right-hand panel). (E) L6 myotubes were incubated in the absence or presence of 10 μM myriocin for 24 h, and with 0.75 mM palmitate for the last 16 h period of the myriocin incubation. Cells were incubated with insulin for the penultimate 10 min prior to lysis. Cell lysates were immunoblotted for total PKB and phospho-PKB(Ser473) as described in the Materials and methods section of the main text. The immunoblots shown are representative of three experiments. Results are means ± S.E.M. for a minimum of three experiments and the asterisks signify a significant difference from the untreated control or basal value (P < 0.05).

Received 5 June 2008/30 September 2008; accepted 16 October 2008
Published as BJ Immediate Publication 16 October 2008, doi:10.1042/BJ20081149

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