Phenylmethimazole blocks palmitate-mediated induction of inflammatory cytokine pathways in 3T3L1 adipocytes and RAW 264.7 macrophages

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Abstract

Visceral adipocytes and associated macrophages produce and release excessive amounts of biologically active inflammatory cytokines via the portal and systemic vascular system, which induce insulin resistance in insulin target tissues such as fat, liver, and muscle. Free fatty acids (FFAs) absorbed via the portal system or released from adipocytes also induce insulin resistance. In this report, we show that phenylmethimazole (C10) blocks basal IL6 and leptin production as well as basal *Socs-3* expression in fully differentiated 3T3L1 cells (3T3L1 adipocytes) without affecting insulin-stimulated AKT signaling. In addition, C10 inhibits palmitate-induced IL6 and *iNos* up-regulation in both 3T3L1 adipocytes and RAW 264.7 macrophages, LPS-induced NF- κ B and IFN- β activation in 3T3L1 cells, and LPS-induced *iNos*, *Ifn- β* , *Il1 β* , *Cxcl10*, and

Il6 expression in RAW 264.7 macrophages. C10 also blocks palmitate-induced *Socs-3* up-regulation and insulin receptor substrate-1 (IRS-1) serine 307 phosphorylation in 3T3L1 adipocytes. Additionally, we show for the first time that although palmitate increases IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes, AKT serine 473 phosphorylation is enhanced, not reduced, by palmitate. These results suggest that through inhibition of FFA-mediated signaling in adipocytes and associated macrophages, as well as possibly other insulin target cells/tissues (i.e. non-immune cells), C10 might be efficacious to prevent or reverse cytokine-induced insulin resistance seen in obesity-related insulin resistance and type 2 diabetes mellitus.

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Introduction

The activation of inflammatory pathways in adipose and other tissues is an important mediator of obesity-related insulin resistance (Kahn & Flier 2000, Lau *et al.* 2005). In obesity and type 2 diabetes mellitus (T2DM), visceral adipocytes and associated inflammatory cells (macrophages) produce and release excessive amounts of biologically active 'cytokines/adipokines' (TNF- α , resistin, IL6, and PAI-1, etc.), which circulate via the portal and systemic vascular system to other insulin target tissues (liver, muscle and islet cells) and induce insulin resistance (Kahn & Flier 2000, Steppan *et al.* 2001, Wellen & Hotamisligil 2003, Lau *et al.* 2005). Excess saturated free fatty acids (FFAs) such as palmitate activate inflammation and induce insulin resistance via multiple mechanisms including, but not limited to, serine phosphorylation of insulin receptor substrate-1 (IRS-1) and up-regulation of suppressors of cytokine signaling (SOCS;

Paz *et al.* 1997, Lin *et al.* 2000, 2005, Emanuelli *et al.* 2001, Perreault & Marette 2001, Aguirre *et al.* 2002, Chavez *et al.* 2003, Furukawa *et al.* 2004, Ajuwon & Spurlock 2005, Boden *et al.* 2005, Hotamisligil 2005, Jove *et al.* 2005, Khamzina *et al.* 2005, Shi *et al.* 2006, Solinas *et al.* 2006, Nakamura *et al.* 2009, Ragheb *et al.* 2009). Therefore, agents capable of blocking FFA-mediated inflammation and insulin resistance may be useful as novel therapeutics for the treatment of T2DM.

We have previously shown that phenylmethimazole (C10) is effective in blocking toll-like receptor (TLR)-mediated activation of inflammatory pathways in non-immune cells (Dagia *et al.* 2004, Harii *et al.* 2005, McCall *et al.* 2007, Schwartz *et al.* 2009). Given that adipose tissue and associated macrophages are important sources of inflammatory molecules that mediate insulin resistance, we investigated the ability of C10 to block inflammatory pathways in 3T3L1 adipocytes and RAW 264.7 macrophages.

Materials and Methods

Control solvents

Control solvents used were dimethyl sulfoxide (DMSO) and a proprietary FDA-approved cyclodextrin derivative. All experiments were conducted using C10 dissolved in both solvents for comparison of solvent effects. Both solvents produced similar results on all parameters measured.

Materials

3T3L1 cells were purchased from ATCC (Manassas, VA, USA). The IL6 ELISA kit was purchased from BioSource International (Camarillo, CA, USA). The Leptin ELISA kit was purchased from Alpha Diagnostics International (San Antonio, TX, USA). DMSO, LPS, and palmitate were obtained from Sigma–Aldrich. Phenylmethimazole (C10) and the proprietary FDA-approved cyclodextrin derivative were gifts of the Interthyr Corporation (Marietta, OH, USA).

Phenylmethimazole (C10) solutions

C10 was prepared as a fresh 200 mM stock solution in 100% (v/v) DMSO, and then diluted into medium at 37 °C to achieve the noted concentrations in individual experiments. Since DMSO itself may have effects on different bioactivities, these studies also used 50 mM C10 dissolved in 40% (v/v) cyclodextrin by sonication at 50 °C for 30 min and mixing on a rotary shaker at 37 °C overnight. Again, C10 was diluted with medium to the noted concentrations in individual experiments. Regardless of the solvent used, C10 was similarly effective in all experiments, and its effects were not duplicated by either solvent.

Cell culture

Undifferentiated 3T3L1 cells were cultured in modified DMEM (ATCC 30-2002) containing 10% (v/v) calf serum at 37 °C in a 5% CO₂ incubator. RAW 264.7 cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS) at 37 °C in a 5% CO₂ incubator.

Adipocyte differentiation

Undifferentiated 3T3L1 cells were cultured as described above until confluency was reached. Two days post confluency, cells were placed in DMEM supplemented with 10% (v/v) FBS containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone, and 1 µg/ml insulin for induction of differentiation. Two days after induction of differentiation, the induction medium was replaced with DMEM supplemented with 10% (v/v) FBS containing 1 µg/ml insulin. The medium was then replaced again with fresh DMEM supplemented with 10% (v/v) FBS every other day for the following 10–12 days.

Palmitate treatments

Palmitate solutions containing 2% FFA-free BSA were prepared fresh in DMEM containing no serum immediately before each experiment. Cells were incubated for the indicated times with 0.75 mM palmitate. A 2% BSA control was included with all palmitate treatments, and in all cases, no BSA effect was observed compared to untreated control.

Luciferase assays

The construction of the plasmid pIFN-β-Luciferase has been previously described (Harii *et al.* 2005). Briefly, to construct pIFN-β-Luciferase, the human *IFN-β* promoter sequence was amplified from human genomic DNA (Clontech) using Ex Taq Polymerase (Takara, Madison, WI, USA). The PCR fragment contained the human *IFN-β* promoter sequence from –125 to +34 relative to the transcription start site (+1) and incorporated KpnI and XhoI restriction sites at the 5' and 3' ends respectively. The primers were as follows: hIFN-β (–125) KpnI (5'-CAG GGT ACC GAG TTT TAG AAA CTA CTA AAA TG-3') and hIFN-β (+34) XhoI (5'-GTA CTC GAG CAA AGG CTT CGA AAG G-3'). The fragment was digested with KpnI and XhoI, and then ligated into a similarly digested pGL3 Basic (Promega) vector. The plasmid pNF-κB-Luciferase was purchased from Stratagene (La Jolla, CA, USA) (catalog #219078). Undifferentiated 3T3L1 pre-adipocytes were grown to roughly 70% confluency, and were then transiently transfected with 100 ng of luciferase reporter plasmid pNF-κB-Luciferase or pIFN-β-Luciferase and 2 ng of internal control plasmid pRL-TK using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Twenty four hours post transfection, cells were incubated with LPS (1 µg/ml), palmitate (0.75 mM), and/or with palmitate plus 0.5 mM C10 or 0.25% (v/v) control solvent, and/or LPS plus 0.5 mM C10 or 0.25% (v/v) control solvent. Luciferase activity was measured using the Dual Luciferase Assay System from Promega.

RT-PCR analysis

3T3L1 adipocytes and RAW 264.7 cells were treated as indicated, and total RNA was isolated using the RNeasy Lipid Tissue Mini Kit from Qiagen according to the manufacturer's instructions. DNase was used to remove any contaminating DNA using the DNA-free kit from Ambion (Austin, TX, USA). cDNA was then synthesized using the RT-for-PCR kit from BD Biosciences (San Jose, CA, USA) according to the manufacturer's protocol. Mouse *Socs-3* primers were as follows: sense primer, 5'-CCC TgC ACA gCC CTC CTT TCT CAC-3'; antisense primer, 5'-gCC CCA CCC AgC CCC ATA CC-3'. Mouse *Gapdh* primers were as follows: forward primer, 5'-ATg TCA gAT CCA CAA Cgg ATA CAT-3'; reverse primer, 5'-ACT CCC TCA AgA TTg TCA gCA AT-3'. The PCR conditions are as follows: 94 °C for 30 s, 30 cycles of 94 °C for 1 min, 60 °C for

1 min, 72 °C for 1 min, and 72 °C for 5 min. Mouse *iNOS* primers were as follows: 5' primer, 5'-CCC TTC CgA AgT TTC Tgg CgA CAg Cgg C-3'; 3' primer, 5'-ggC TgT CAG AgC CTC gTg gCT TTg g-3'. The PCR conditions are as follows: 35 cycles of 94 °C for 10 s, 58 °C for 30 s, 72 °C for 10 s, and 72 °C for 5 min. One nanogram of cDNA was used for PCR amplification of *iNOS* from RAW 264.7 macrophages. For all other PCRs, 50 ng cDNA was used.

Western blot analyses

3T3L1 adipocytes were treated as indicated, and cells were lysed using a lysis buffer which has been previously described (Carlson *et al.* 2004). For the detection of pIRS-1 (Ser³⁰⁷), 20 µg of total protein was subjected to SDS-PAGE and western blot analysis to evaluate serine 307 phosphorylation of IRS-1 using an antibody specific for the detection of IRS-1 when phosphorylated at serine 307 from Cell Signaling Technology (Danvers, MA, USA). For the detection of phosphorylated AKT, 10 µg of total protein was subjected to western blot analysis and identified using an anti-phospho-AKT serine 473 antibody from Cell Signaling Technology. Phospho-IRS-1 and phospho-AKT blots were stripped using a standard stripping buffer and re-probed using anti-IRS-1 or anti-AKT antibodies respectively from Cell Signaling Technology to detect protein levels independent of phosphorylation status. Blots were also stripped and re-probed with anti-β-actin antibody from Cell Signaling Technology as an internal loading control. The Magic Mark XP western Protein standard from Invitrogen was used for all western blots for protein molecular weight determination.

Northern blot analyses

RAW 264.7 cells were treated as indicated. RNA was extracted using Trizol (Invitrogen) and subjected to northern blot analysis in a manner similar to that described previously (Suzuki *et al.* 1999). The *Gapdh* cDNA was from Clontech. Other probe sequences were synthesized by RT-PCR (Suzuki *et al.* 1999) using the following cDNA specific primers: mouse *Cxcl10*, 5'-CCATCAGCACCATGAACCAAGTCCTGCCG-3' and 5'-GGACGTCCTCCTCATCGTCGACTACACTGG-3' (469 bp); mouse *Il1β*, 5'-CTCATCTGGGATCCTCTCCAGCCAAGCTTC-3' and 5'-CCATGGTTTCTTG TGACCCTGAGCGACCTG-3 (1006 bp); mouse *Il6*, 5'-CCAGTTGCCTTCTTGGGACTGATGCTGGTG-3' and 5'-GTCCTTAGCCACTCCTTCTGTGACTCCAGC-3' (530 bp); mouse *Ifn-β*, 5'-AAGATCATTCTCACTGCAGCC-3' and 5'-TGAAGACTTCTGCTCGGACC-3' (586 bp).

ELISAs

Cell supernatants were collected following indicated treatments. For detection of basal IL6 levels in differentiated 3T3L1 cells, cell supernatant was collected and concentrated

using YM3 centricon tubes from Millipore (Billerica, MA, USA). For all other ELISAs, cell supernatant removed directly from cells (NOT concentrated via YM3 centricon tubes) was used to determine IL6 levels via ELISA. The latter was not concentrated since inducible levels of IL6 are much higher than basal levels. For the Leptin ELISA, cell supernatants removed directly from cells were used to determine leptin levels. All ELISAs were performed according to the manufacturer's protocols.

Statistical analyses

All experiments were replicated at least three times on different groups of cells. All data are expressed as mean ± S.D. Statistical significance was evaluated using one-way ANOVA, and statistical significance for comparison of means of different groups was calculated using Bonferroni's *post hoc* analyses.

Results

C10 decreases basal IL6, Socs-3, and leptin levels in 3T3L1 adipocytes, but does not affect basal IRS-1 serine 307 phosphorylation

Since adipocytes are known to produce adipokines that diminish insulin signaling, we evaluated the basal expression of IL6, *Socs-3*, and leptin in fully differentiated 3T3L1 cells and evaluated the effects of C10 on basal expression of these molecules. First, we measured the basal expression of IL6 protein in cell culture supernatants using ELISA, after the cell culture supernatants were concentrated 4-fold using YM3 centricon tubes from Millipore. 3T3L1 adipocytes express basal IL6 as evidenced by its presence in the supernatants (Fig. 1A, column 1), and C10 treatment significantly ($P < 0.001$) reduced basal IL6 protein levels in the supernatants (Fig. 1A, column 3). Control solvent had no effect (Fig. 1A, column 2).

Since IL6 is a strong inducer of insulin resistance via its ability to up-regulate *Socs-3* gene expression (Heinrich *et al.* 1998, 2003, Fasshauer & Paschke 2003), we evaluated basal *Socs-3* expression and the effects of C10 on basal *Socs-3* expression in 3T3L1 adipocytes. *Socs-3* was expressed basally in 3T3L1 adipocytes and C10 was effective at inhibiting its expression (Fig. 1B).

Of note, and suggesting this basal activity and the C10 effect were potentially relevant to disease expression, we measured the levels of leptin in the supernatants of 3T3L1 adipocytes (Table 1). We observed that measurable leptin levels were present in the supernatants of cultured 3T3L1 adipocytes and that 0.5 mM C10 inhibited leptin production by these cells (Table 1). Although there was a solvent effect, C10 exhibited a much greater inhibitory effect than solvent alone, $P < 0.001$ (Table 1).

Figure 1 C10 decreases basal IL6, *Socs-3*, and leptin levels in 3T3L1 adipocytes, but does not affect basal IRS-1 serine 307 phosphorylation. 3T3L1 adipocytes were treated with 0.5 mM C10 or 0.25% (v/v) control solvent for 24 h. (A) Cell culture supernatant was collected and concentrated using YM3 centricon tubes, and concentrated samples were then used to determine basal IL6 levels via ELISA analysis. Error bars represent s.d. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. ****P* < 0.001 between groups as indicated. (B) Total RNA was isolated, and *Socs-3* expression was measured by RT-PCR. Images shown are representative images from ≥ 3 independent experiments. (C) 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 24 h followed by stimulation with 100 nM insulin for 10 min as indicated. Cell lysates were then prepared and subjected to SDS-PAGE and western blotting. IRS-1 serine 307 phosphorylation was detected using an anti-phospho-IRS-1 serine 307-specific antibody. Images shown are representative immunoblots from ≥ 3 independent experiments.

It has been shown that serine and threonine phosphorylation of IRS-1, including serine residue 307, by multiple kinases in response to FFAs, inflammatory cytokines, and insulin itself can impair insulin signaling in cells in culture (Zick 2005). Therefore, we evaluated the effects of C10 on basal levels of IRS-1 serine 307 phosphorylation in 3T3L1

adipocytes. Consistent with the other reports, we observed an increase in serine 307 phosphorylation of IRS-1 following insulin stimulation (Fig. 1C). C10 had no effect on either basal IRS-1 serine 307 phosphorylation or insulin-stimulated IRS-1 serine 307 phosphorylation (Fig. 1C).

C10 does not block insulin-stimulated AKT signaling in 3T3L1 adipocytes

For C10 to be a useful therapeutic agent, it is important that it blocks pathologic insulin resistance without affecting normal insulin signaling. Insulin stimulation leads to the activation of AKT via phosphorylation of multiple residues including serine residue 473 (Brozinick & Birnbaum 1998). Thus, we evaluated the effects of C10 on insulin-stimulated AKT serine 473 phosphorylation in 3T3L1 adipocytes. As can be seen in Fig. 2, C10 did not block insulin-stimulated AKT serine 473 phosphorylation in 3T3L1 adipocytes (Fig. 2).

C10 inhibits palmitate-induced IL6 production, iNos expression, and activation of NF-κB and IFN-β signaling in 3T3L1 adipocytes

Excess saturated FFAs such as palmitate can reduce insulin sensitivity and glucose uptake. Some of the ways FFAs mediate insulin resistance are by up-regulating IL6 and *iNos* and by the activation of the NF-κB and TLR4 signaling pathways (Lin *et al.* 2000, Wellen & Hotamisligil 2003, Ajuwon & Spurlock 2005, Boden *et al.* 2005, Jove *et al.* 2005, Shi *et al.* 2006, Nakamura *et al.* 2009, Ragheb *et al.* 2009). First, 3T3L1 adipocytes were treated with palmitate with or without C10 or control solvent for 24 h, and IL6 protein levels were measured. We observed a significant (*P* < 0.01) increase in IL6 production following treatment with palmitate (Fig. 3A). Both C10 and control solvent exhibited an ability to significantly decrease palmitate-induced IL6 (*P* < 0.01); however, it was clear in all experiments that C10 was much more potent at inhibiting palmitate-induced IL6 production than control solvent (Fig. 3A).

Next, we evaluated the effects of C10 on palmitate-induced *iNos* expression. 3T3L1 adipocytes were treated with palmitate plus or minus C10 for 24 h, and *iNos* expression was

Table 1 C10 inhibits basal leptin levels in 3T3L1 adipocytes

	Leptin levels (pg/ml)
Untreated	2753 ± 284.0
Control solvent	1847 ± 50.7 ^a
C10	498 ± 40.9 ^{a,b}

^aIndicates significant difference compared to untreated group.
^bIndicates significant difference compared to control solvent group.

Figure 2 C10 does not block insulin-stimulated AKT signaling in 3T3L1 adipocytes. 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 24 h followed by stimulation with 100 nM insulin for 10 min as indicated. Total cell lysates were then prepared and directly subjected to SDS-PAGE and western blotting. AKT serine 473 phosphorylation was identified using an anti-phospho-AKT serine 473-specific antibody. Images shown are representative immunoblots from ≥ 3 independent experiments.

evaluated via RT-PCR. Clearly, *iNos* was induced by palmitate, and C10 inhibited palmitate induction of *iNos* in these cells (Fig. 3B).

We also questioned whether C10 would inhibit increased NF- κ B and IFN- β signaling of palmitate in 3T3L1 cells. Undifferentiated 3T3L1 pre-adipocytes were transfected with a NF- κ B-Luciferase or an IFN- β -Luciferase reporter plasmid and treated with palmitate with or without C10, and relative luciferase activity was measured as an indicator of NF- κ B and IFN- β promoter activity. Palmitate activated both NF- κ B-Luciferase and IFN- β -Luciferase activity ($P < 0.01$ and $P < 0.05$ respectively) (Fig. 3C), and C10 inhibited both palmitate-induced NF- κ B-Luciferase and IFN- β -Luciferase activity (Fig. 3C) ($P < 0.01$ and $P < 0.05$ respectively).

C10 inhibits palmitate-induced IL6 production and *iNos* expression in RAW 264.7 macrophages

Adipose-associated macrophages are also thought to play an important role in the pathogenesis of insulin resistance and T2DM (Weisberg *et al.* 2003, Xu *et al.* 2003, Curat *et al.* 2006). We questioned whether palmitate would increase IL6 and *iNos* in macrophages similar to that observed in 3T3L1 adipocytes, and whether such induction would be inhibited by C10.

Murine macrophage RAW 264.7 cells were incubated with palmitate in the absence or presence of C10, and IL6 protein levels were measured using an IL6-specific ELISA. Palmitate significantly increased IL6 protein levels, whereas C10 inhibited this induction (Fig. 4A). Additionally, we evaluated the effects of C10 on palmitate induction of *iNos* RNA expression via RT-PCR. Palmitate significantly increased *iNos* RNA levels, and this was inhibited by C10 (Fig. 4B).

C10 blocks palmitate-mediated up-regulation of Socs-3 expression in 3T3L1 adipocytes

It is known that IL6 up-regulates *Socs-3* expression to mediate insulin resistance (Heinrich *et al.* 1998, 2003, Fasshauer & Paschke 2003); thus, we evaluated *Socs-3* gene expression following treatment with palmitate and C10. As expected,

Figure 3 C10 inhibits palmitate-induced IL6 production, *iNos* expression, and activation of NF- κ B and IFN- β signaling in 3T3L1 cells. 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 24 h. (A) Supernatant levels of IL6 were then measured using a mouse IL6-specific ELISA. Error bars represent s.d. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. The brackets and **denote significance of $P < 0.01$ between groups as indicated. (B) Total RNA was isolated, cDNA was synthesized, and *iNos* and *Gapdh* were amplified by PCR as described in Materials and Methods. Images shown are representative images from ≥ 3 independent experiments. (C) Undifferentiated 3T3L1 cells were transiently transfected with 100 ng of luciferase reporter plasmid pNF- κ B-Luciferase or pIFN- β -Luciferase and 2 ng of internal control plasmid pRL-TK. Twenty four hours post transfection, cells were incubated with palmitate (0.75 mM) alone or with 0.25% (v/v) control solvent or 0.5 mM C10 for 6 h. Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Error bars represent s.d. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. ** $P < 0.01$ and * $P < 0.05$ between groups as indicated.

Figure 4 C10 inhibits palmitate-induced IL6 and *iNos* in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with 0.75 mM palmitate alone or in combination with 0.25% (v/v) control solvent or 0.5 mM C10 for 24 h. (A) Supernatant levels of IL6 were then measured using a mouse IL6-specific ELISA. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. * $P < 0.0000001$ between groups as indicated. (B) Total RNA was isolated, and RT-PCR was then performed to evaluate *iNos* expression. Fifty nanograms of cDNA were used as a template for PCR. Images shown are representative images from ≥ 3 independent experiments.

Socs-3 gene expression increased following palmitate treatment in 3T3L1 adipocytes, and this induction was inhibited by C10 (Fig. 5). In this case, control solvent had no effect on palmitate induction of *Socs-3* gene expression (Fig. 5).

*C10 blocks LPS-induced NF- κ B and IFN- β activation in 3T3L1 cells, and LPS-induced *iNos*, *Ifn- β* , *Il1 β* , *Cxcl10*, and *Il6* gene expression, as well as *IL6* protein production in RAW 264.7 macrophages*

Since C10 has been shown to block TLR signaling in other cell types (Dagia *et al.* 2004, Harii *et al.* 2005, McCall *et al.* 2007, Schwartz *et al.* 2009), we questioned whether C10 may be blocking TLR signaling in 3T3L1 cells and RAW 264.7 macrophages. TLR4 signaling has been implicated in the development of insulin resistance (Lin *et al.* 2000, Shi *et al.* 2006); thus, we questioned whether C10 could inhibit LPS-induced inflammatory signaling in these cell lines. We first

confirmed that TLR4 is present in both undifferentiated and fully differentiated 3T3L1 cells as well as in RAW 264.7 macrophages (data not shown).

We then questioned whether C10 would inhibit LPS-induced NF- κ B and IFN- β signaling in 3T3L1 cells. Undifferentiated 3T3L1 cells were transfected with a NF- κ B-Luciferase or an IFN- β -Luciferase reporter plasmid. Transfected cells were then treated with LPS with or without C10, and then measured for relative luciferase activity as a measure of NF- κ B and IFN- β promoter activity. LPS significantly induced the activation of NF- κ B-Luciferase and IFN- β -Luciferase activity ($P < 0.01$ and $P < 0.05$ respectively), and C10 inhibited both LPS-induced NF- κ B-Luciferase and IFN- β -Luciferase activity ($P < 0.01$ and $P < 0.05$ respectively) (Fig. 6A).

Next, we questioned whether C10 had similar effects on LPS induction of other inflammatory molecules in RAW 264.7 macrophages. First, we evaluated the effects of C10 on LPS-induced *iNos* gene expression using RT-PCR. C10 largely reduced LPS induction of *iNos* (Fig. 6B). Next, we evaluated the effects of C10 on LPS induction of IL6 using ELISA. LPS greatly induced the production of IL6, and C10 significantly blocked this induction (Fig. 6C). Finally, to confirm these results, RAW 264.7 cells were treated with LPS, and northern blot analyses were conducted to evaluate the expression of *Ifn- β* , *Il1 β* , *Cxcl10*, and *Il6* over a 6 h time course. After 1 h of LPS treatment, *Ifn- β* , *Il1 β* , and *Cxcl10* were largely up-regulated (Fig. 6D). *Il6* up-regulation by LPS was evident after 3 h (Fig. 6D). C10 inhibited LPS induction of all of the genes evaluated (*Ifn- β* , *Il1 β* , *Cxcl10*, and *Il6*) (Fig. 6D).

C10 blocks palmitate-induced IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes

Since elevated FFAs have been shown to impair insulin action via serine phosphorylation of IRS-1 (Paz *et al.* 1997, Aguirre *et al.* 2002, Hotamisligil 2005), we evaluated the effects of

Figure 5 C10 inhibits palmitate-induced *Socs-3* expression in 3T3L1 adipocytes. 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 24 h. Total RNA was isolated, cDNA was synthesized, and *Socs-3* and *Gapdh* were amplified by RT-PCR as described in Materials and Methods. Images shown are representative images from ≥ 3 independent experiments.

palmitate on induction of IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes. In non-insulin-stimulated 3T3L1 adipocytes, we observed increases in IRS-1 serine 307 phosphorylation within 6 h of palmitate treatment, but by 16 h, IRS-1 serine 307 phosphorylation was diminished back to basal levels (Fig. 7A). Additionally, we observed increases in IRS-1 serine 307 phosphorylation with insulin treatment and a further enhancement of insulin-stimulated IRS-1 serine 307 phosphorylation with palmitate treatment up to 16 h (Fig. 7A); by 24 h, however, insulin-stimulated IRS-1 serine

307 phosphorylation was back to basal levels (data not shown). C10 blocked palmitate induction of IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes (Fig. 7B)

Palmitate enhances insulin-stimulated AKT serine 473 phosphorylation in 3T3L1 adipocytes

Given that palmitate increased IRS-1 serine 307 phosphorylation, we questioned whether palmitate had any influence on insulin signaling in 3T3L1 adipocytes. Insulin stimulation leads

Figure 6 C10 blocks LPS-induced signaling in 3T3L1 cells and RAW 264.7 macrophages. (A) Undifferentiated 3T3L1 cells were transiently transfected with 100 ng of luciferase reporter plasmid pNF-κB-Luciferase or pIFN-β-Luciferase and 2 ng of internal control plasmid pRL-TK. Twenty four hours post transfection, cells were incubated with LPS (1 μg/ml) alone or with 0.5 mM C10 or 0.25% (v/v) control solvent for 6 h. Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Error bars represent s.d. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. ** $P < 0.01$ and * $P < 0.05$ between groups as indicated. (B and C) RAW 264.7 macrophages were treated with 50 ng/ml LPS alone or in combination with 0.25% (v/v) control solvent or 0.5 mM C10 for 4 h. (B) RT-PCR was then performed to evaluate *iNos* expression. One nanogram of cDNA was used as a template for PCR. Images shown are representative images from ≥ 3 independent experiments. (C) Supernatant levels of IL6 were then measured using a mouse IL6-specific ELISA. Significance was determined using one-way ANOVA followed by Bonferroni's *post hoc* analysis. * $P < 0.0000001$ between groups as indicated. (D) RAW 264.7 cells were treated with 1 μg/ml LPS alone or in combination with 0.25% (v/v) control solvent or 0.5 mM C10 for 1, 3, and 6 h. Total RNA was then collected, and northern blot analysis was conducted to evaluate the expression of *Ifn-β*, *Il1β*, *Cxcl10*, and *Il6*. *Gapdh* served as a loading control. Images shown are representative images from ≥ 3 independent experiments.

Figure 7 C10 blocks palmitate-induced IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes. (A) 3T3L1 adipocytes were treated with palmitate (0.75 mM) for the designated times followed by stimulation with 100 nM insulin for 10 min as indicated. Cell lysates were prepared and subjected to SDS-PAGE and western blotting. IRS-1 serine 307 phosphorylation was detected using an anti-phospho-IRS-1 serine 307-specific antibody. (B) 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 16 h followed by stimulation with 100 nM insulin for 10 min as indicated. Total cell lysates were then prepared and directly subjected to SDS-PAGE and western blotting. IRS-1 serine 307 phosphorylation was detected using an anti-phospho-IRS-1 serine 307-specific antibody. Images shown are representative immunoblots from ≥3 independent experiments.

to the activation of AKT via phosphorylation of multiple residues including serine residue 473 (Brozinick & Birnbaum 1998). Thus, we questioned whether palmitate could block insulin-stimulated AKT phosphorylation in 3T3L1 adipocytes. Surprisingly, palmitate enhanced insulin-stimulated AKT phosphorylation in these cells (Fig. 8A). C10 had no effect on palmitate-induced AKT phosphorylation (Fig. 8B).

Discussion

There is a general consensus that visceral obesity plays a significant role in the pathogenesis of T2DM through induction of insulin resistance (Lau *et al.* 2005). The insulin resistance induced by obesity is in part secondary to the release of circulating pro-inflammatory cytokines/adipokines from adipocytes and macrophages, which, through their downstream effectors in other target tissues such as liver, muscle, etc. induce insulin resistance. Ectopic fat deposition from excessive circulating levels of FFA's within somatic cells and their consequent release of these same pro-inflammatory adipokines/cytokines within insulin target cells can also induce insulin resistance (Smith & Ravussin 2002,

Wellen & Hotamisligil 2003) commonly termed 'lipotoxicity'. The mechanisms by which excess FFAs contribute to insulin resistance are not well understood; however, recent studies show that FFAs can induce insulin resistance by activating the NF-κB, protein kinase C, c-Jun NH₂-terminal kinase, and TLR signaling pathways (Lin *et al.* 2000, Ajuwon & Spurlock 2005, Boden *et al.* 2005, Jove *et al.* 2005, Shi *et al.* 2006, Solinas *et al.* 2006, Nakamura *et al.* 2009, Ragheb *et al.* 2009). Here, we confirm that the saturated FFA palmitate is a strong inducer of the inflammatory cytokine pathways known to mediate insulin resistance in 3T3L1 adipocytes, and that palmitate is also a strong inducer of inflammatory pathways that produce cytokines/molecules that mediate insulin resistance in RAW 264.7 macrophages as well. Specifically, we show that palmitate can induce significant increases in IL6 and *iNos* production in both cell types. In 3T3L1 adipocytes, palmitate-induced IL6 production resulted in *Socs-3* up-regulation, providing additional evidence to suggest that FFAs are important in the development of insulin resistance since SOCS-3 inhibits tyrosine phosphorylation of IRS-1 and IRS-2 (Ueki *et al.* 2004). The observed palmitate-induced up-regulation of *iNos* lends even more support to the

Figure 8 Palmitate enhances insulin-stimulated AKT serine 473 phosphorylation in 3T3L1 adipocytes. (A) 3T3L1 adipocytes were treated with palmitate (0.75 mM) for the designated times followed by stimulation with 100 nM insulin for 10 min as indicated. Cell lysates were then prepared and subjected to SDS-PAGE and western blotting. AKT serine 473 phosphorylation was identified using an anti-phospho-AKT serine 473-specific antibody. (B) 3T3L1 adipocytes were treated with palmitate (0.75 mM) alone or in combination with C10 (0.5 mM) or control solvent (0.25% (v/v)) for 16 h followed by stimulation with 100 nM insulin for 10 min as indicated. Total cell lysates were then prepared and directly subjected to SDS-PAGE and western blotting. IRS-1 serine 307 phosphorylation was detected using an anti-phospho-IRS-1 serine 307-specific antibody. Images shown are representative immunoblots from ≥3 independent experiments.

importance of FFAs in the development of insulin resistance since up-regulation of *iNos* has been shown to contribute to inflammation-induced insulin resistance (Perreault & Marette 2001). Most importantly, we report the novel finding that C10 is effective at blocking palmitate and LPS-induced activation of IL6 and *iNos*-producing inflammatory pathways in both 3T3L1 adipocytes and RAW 264.7 macrophages without blocking insulin-stimulated AKT signaling, suggesting that C10 may be efficacious as a novel therapeutic agent for the treatment of obesity/inflammation-induced insulin resistance. Furthermore, since C10 inhibits LPS and palmitate induction of *iNos* expression in both adipocytes and macrophages, it might also be beneficial in correcting the stress-induced hyperglycemia observed in acute inflammatory processes such as infection/sepsis (Berstein 2005, De Souza *et al.* 2005, Elsammak *et al.* 2005). It is clear, however, that additional studies into the effects of C10 on FFA-induced insulin resistance (i.e. glucose uptake assays, etc.) in adipocytes and other insulin target tissues such as skeletal muscle and liver are warranted before clinical efficacy can be established.

Since palmitate is the major FFA released from 3T3L1 adipocytes, and palmitate induces the expression of inflammatory molecules in 3T3L1 adipocytes, it is not surprising that we observed basal expression of inflammatory pathway products such as IL6, *Socs-3*, and *iNos* as well as basal levels of IRS-1 serine 307 in 3T3L1 adipocytes. The fact that C10 effectively reduced basal levels of IL6, *Socs-3*, and leptin in addition to palmitate-induced IL6, *Socs-3*, and *iNos* levels in 3T3L1 adipocytes lends support to the notion that C10 specifically blocks palmitate-mediated inflammatory pathways.

Although insignificant compared to the effects of C10, in some assays, we observed a significant effect of control solvent on palmitate-induced IL6 production and palmitate-induced IFN- β -Luciferase activity in 3T3L1 cells. This was seen with two different solvents used herein, DMSO and cyclodextrin. It is not clear at this time why both solvents, which are unrelated chemically, give similar results, nor is the mechanism of this phenomenon understood. Despite the finding that control solvent significantly inhibited palmitate-induced IL6 production, we observed no effect of control solvent on palmitate induction of *Socs-3* gene expression in 3T3L1 adipocytes. At this time, we can only speculate that the control solvent might be modulating a different signaling pathway from that effecting *Socs-3* expression.

Given that C10 effectively reduced basal levels of IL6, *Socs-3*, and leptin in addition to palmitate-induced IL6, *Socs-3*, *iNos*, and IRS-1 serine 307 levels in 3T3L1 adipocytes, we observed no effect of C10 on basal IRS-1 serine 307 levels via western blot analysis. Perhaps the levels of palmitate secreted from 3T3L1 adipocytes in culture are insufficient to elicit a measurable change in IRS-1 serine 307 phosphorylation with C10 treatment that is detectable by conventional western blotting. We encountered a similar situation when measuring basal levels of IL6 as well; concentration of cell culture media was necessary prior to ELISA measurement, as basal IL6 levels in cell culture media

were too low to detect without centricon concentration of media prior to ELISA as described in Materials and Methods.

Recently, the 'abnormal' expression of TLR in non-immune cells and their resultant overexpression of the same immunomodulatory molecules such as IL6 and TNF- α which are known to be important in the onset/progression of T2DM, have been recognized (Lin *et al.* 2000). TLR4 has been reported in 3T3L1 adipocytes (Lin *et al.* 2000), and LPS as well as FFA treatment results in the activation of TLR4 and consequent IL6 and TNF- α production, which is not observed in *Tlr4* knockdown adipocytes (Lin *et al.* 2000); directly linking *Tlr4* expression in adipocytes and insulin resistance. We have previously shown that C10 can block TLR3-mediated expression and signaling in multiple tissues (Harui *et al.* 2005, McCall *et al.* 2007, Schwartz *et al.* 2009). In this study, we also demonstrate that C10 can block LPS and palmitate-mediated NF- κ B and IFN- β activation as well as LPS-induced *iNos*, *I β* , *Il1 β* , *Cxcl10*, and *Il6* gene expression, and IL6 protein production. Since these are all downstream products of both TLR3 and TLR4 signaling, it suggests that C10 may prevent palmitate-mediated up-regulation of IL6 and *iNos* in 3T3L1 adipocytes and RAW 264.7 macrophages, at least in part, by blocking palmitate-mediated TLR signaling. Although these data are suggestive of TLR specificity, additional studies need to be conducted to identify if this activity of C10 is specific to palmitate activation of TLR4 signaling or if C10 may be affecting other pathways that up-regulate IL6 and *iNos*.

Multiple studies have suggested that elevated FFAs impair insulin action via serine 307 phosphorylation of IRS-1 (Hotamisligil *et al.* 1996, Paz *et al.* 1997, Aguirre *et al.* 2000, 2002, Hirosumi *et al.* 2002, Yu *et al.* 2002, Um *et al.* 2004, Morino *et al.* 2005, Zick 2005, Ragheb *et al.* 2009). Thus, in an attempt to evaluate the efficacy of C10 to block palmitate-induced insulin resistance and restore insulin sensitivity, we looked at the effects of C10 on palmitate-induced up-regulation of IRS-1 serine 307 phosphorylation in 3T3L1 adipocytes. We found that C10 effectively blocked palmitate-induced serine 307 phosphorylation of IRS-1, suggesting that C10 may be effective in restoring insulin sensitivity in adipocytes.

Insulin increases glucose transport in muscle and adipose tissue by stimulating the translocation of GLUT-4 to the cell surface, a process that requires the activation of AKT (Kohn *et al.* 1996). This event predominately relies on insulin-stimulated threonine 308 and serine 473 phosphorylation of AKT (Vanhaesebroeck & Alessi 2000). FFA-induced IRS-1 serine 307 phosphorylation is postulated to induce insulin resistance by blocking interactions with the insulin receptor, which blocks the downstream phosphorylation of AKT (Paz *et al.* 1997, Aguirre *et al.* 2002). Since no correlation between palmitate-induced up-regulation of IRS-1 serine 307 phosphorylation and consequent down-regulation of AKT serine 473 phosphorylation in 3T3L1 adipocytes has been reported, we evaluated the effects of palmitate on AKT serine 473 phosphorylation in 3T3L1 adipocytes in culture. Instead of observing an expected decrease in AKT serine 473

phosphorylation with palmitate treatment, we surprisingly observed that insulin-stimulated phosphorylation of AKT serine 473 increased linearly over time with palmitate treatment; a novel finding. This finding is, however, consistent with a 2008 report by Banerjee *et al.* (2008), showing that in human hepatocytes infected with chronic hepatitis C virus (HCV) core protein, phosphorylation of IRS-1 serine 312 (the human equivalent of murine serine 307) is increased which correlated with an increase in AKT serine 473 phosphorylation, while IRS-1 threonine 308 phosphorylation was not significantly altered. This same study showed that overexpression of the HCV core protein alone or HCV infection induced insulin resistance as measured by HCV-mediated suppression of 2-deoxy-D- 3 H]glucose uptake (Banerjee *et al.* 2008). Taken together, these results suggest that perhaps palmitate-induced IRS-1 serine 307 phosphorylation mediates insulin resistance via a pathway that is independent of AKT serine 473 phosphorylation status. In the absence of additional studies, the significance of palmitate-mediated increases in AKT serine 473 phosphorylation and its relationship to insulin resistance remain unclear.

Since palmitate increased insulin-stimulated AKT serine 473 phosphorylation in 3T3L1 adipocytes, and since C10 blocked palmitate-induced increases in IRS-1 serine 307 phosphorylation, we were curious to see what effects, if any, C10 might have on palmitate-induced AKT serine 473 phosphorylation. We observed no inhibitory effect of C10 on palmitate-mediated increases in AKT serine 473 phosphorylation with 16 h of palmitate treatment. Additional studies of the effects of C10 on palmitate-induced AKT serine 473 phosphorylation over time are clearly needed before conclusions can be made; however, these studies may help to elucidate additional information about C10 mechanism of action.

Together these observations suggest that through inhibition of FFA-mediated signaling in adipocytes and possibly other insulin target cells/tissues (i.e. non-immune cells) or associated macrophages, C10 might be efficacious to prevent or reverse cytokine-induced insulin resistance seen in obesity-related insulin resistance and T2DM. Studies are currently underway to evaluate the efficacy of C10 to restore insulin sensitivity in palmitate-treated adipocytes, hepatocytes, and myocytes, as well as in animal models of obesity-related insulin resistance and T2DM.

Declaration of interest

K D M, A L S, L D K, and F L S are co-inventors on a continuation-in-part of US Pat. App. Ser. No. 11/130922 (2008). K D M and A L S have previously consulted for the Interthyr Corporation. L D K has equity interests in the Interthyr Corporation. K D M, A L S, L D K, and F L S are participants of a funded NIH grant application. E D and C S were previously employed by the Interthyr Corporation. D H, C J L, and B W have nothing to disclose.

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