Apoptosis in skeletal muscle myotubes is induced by ceramides and is positively related to insulin resistance

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Fatty acid-induced apoptosis occurs in pancreatic β-cells and contributes to the metabolic syndrome. Skeletal muscle insulin resistance is mediated by fatty acid oversupply, which also contributes to the metabolic syndrome. Therefore, we examined whether fatty acids induce apoptosis in skeletal muscle myotubes, the proapoptotic signaling involved, and the effects on insulin sensitivity. Exposure of L6 myotubes to palmitate induced apoptosis, as demonstrated by increased caspase-3 activation, phosphatidylserine exposure on the plasma membrane, and terminal deoxynucleotidyl transferase dUTP nick end labeling and DNA laddering, both markers of DNA fragmentation. Ceramide content was concomitantly increased, indicating a potential role for ceramides in palmitate-induced apoptosis. Supporting this notion, reducing stearoyl-CoA desaturase-1 (SCD-1) protein content with short interfering RNA resulted in ceramide accumulation and was associated with increased apoptosis in the absence of palmitate. Furthermore, the membrane-permeable C2-ceramide enhanced apoptosis in myotubes, whereas the ceramide synthase inhibitor, fumonisin B1, abrogated the proapoptotic effects of palmitate. Insulin-stimulated glucose uptake was inhibited by palmitate treatment, whereas the addition of effector caspase inhibitors [Ac-DEVAD-aldehyde (DEVD-CHO), Z-DQMD-FMK] independently restored >80% of the insulin-stimulated glucose uptake. These effects were observed independently from changes in the protein content of insulin signaling proteins, suggesting that proteosomal degradation is not involved in this process. We conclude that lipoapoptosis occurs in skeletal muscle myotubes, at least partially via de novo ceramide accumulation, and that inhibiting downstream apoptotic signaling improves glucose uptake in vitro.

lipoapoptosis; caspase 3; proteolysis; glucose uptake

Cardiovascular disease is a leading cause of death, and obesity is a major risk factor for cardiovascular disease. The pathogenesis of atherosclerosis is complex and involves multiple processes, including inflammation, lipid transport, and cellular injury. Recent evidence suggests that ceramides, a class of sphingolipids, play a role in the pathogenesis of atherosclerosis and may contribute to the development of insulin resistance and type 2 diabetes. Ceramides are produced by sphingomyelin degradation, and their accumulation in cells can lead to apoptosis. In this study, we investigated the role of ceramides in fatty acid-induced apoptosis in skeletal muscle myotubes, and we found that ceramides enhance apoptosis in these cells. We also demonstrated that ceramide accumulation is associated with increased apoptosis in the absence of palmitate. These findings suggest that ceramides may contribute to the development of insulin resistance and type 2 diabetes through their effects on apoptosis and lipid metabolism.
accumulate in the skeletal muscle of obese, insulin-resistant individuals (2, 44).

In the present study, we tested the hypothesis that FAs induce apoptosis in skeletal muscle myotubes. We also examined the role of ceramides in this process and whether apoptosis impacts upon insulin-stimulated glucose uptake in vitro. The results demonstrate that skeletal muscle myotubes undergo lipoapoptosis and indicate that ceramides play a critical role in this process. In addition, they also indicate that blocking downstream proapoptotic signals partially ameliorates FA-induced insulin resistance in vitro.

METHODS

Cell culture and experimental treatments. L6 rat myoblasts were maintained in 5 mM glucose and α-Modified Eagle’s Medium (αMEM; Invitrogen, Mt. Waverley, Australia) supplemented with 10% foetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (pen-strep; Invitrogen) at 37°C, with 5% CO2 and 95% air. Myoblasts at 50–60% confluence were differentiated in αMEM containing 2% horse serum (Invitrogen) and 1% pen-strep for 72–96 h prior to all experiments. Fully differentiated myotubes within passages 3–20 were used for all experiments. Differentiation was confirmed in preliminary experiments by probing for myogenin (Fig. 1A).

A stock of 100 mM palmitate (Sigma-Aldrich, Castle Hill, Australia) solution in 100% ethanol was added to αMEM supplemented with 4% FA free bovine serum albumin (BSA; Bovogen Biologicals, Essendon, Australia) with ethanol vehicle and 1% pen-strep at a final concentration of 750 μM, unless stated otherwise. This gave a FA concentration within the physiological range (i.e., 0.4–1.0 mM) and a final molar FA:BSA ratio of 1.25:1.0, similar to that observed for human serum. The palmitate solution was sterilized by ultraviolet (UV) light and was added to myotubes for the time periods indicated. Control cells were incubated in medium containing 4% BSA without the addition of palmitate. Lactate dehydrogenase in the cell medium was measured spectrophotometrically at 492 nm (no. 1644793; Roche Diagnostics) to provide a semiquantitative measure of cell death where required.

FA subtype. In initial experiments, myotubes were incubated with various saturated and unsaturated FAs alone, co-incubated with saturated and unsaturated FAs, or pre-incubated with either saturated or unsaturated FAs before addition of the other. Stock solutions of 100 mM laurate (12:0), myristate (14:0), palmitate (16:0), olate (18:1), and linoleate (18:2) (all from Sigma-Aldrich) were prepared in 100% ethanol. Aliquots were added to αMEM containing 2% FA free BSA and 1% pen-strep at a final concentration of 750 μM. Myotubes were incubated in respective FA solutions for 6 h (including coincubation trial of palmitate and linoleate, excluding the preincubated and saturated FA incubations). The preincubation conditions involved 3 h of preincubation with palmitate or linoleate and then 6 h of incubation with the opposing FAs. Following treatments, myotubes were washed with warmed phosphate-buffered saline (PBS), detached with 0.25% trypsin, and transferred to tubes for caspase-3 activity analysis.

We also assessed the effects of a short-chain ceramide analog (C2-ceramide) or a structurally related metabolically inactive control (C2-dihydroceramide) (Calbiochem, San Diego, CA) on ceramide/diacylglycerol content and caspase-3 activity. L6 myotubes were cultured with 50 μM C2-ceramide or C2-dihydroceramide for 16 h in medium without FAs, and cells were harvested for analysis (see below). In other experiments, L6 myotubes were incubated for 16 h in 750 μM palmitate with or without 50 μM fumonisin B1, an inhibitor of serine palmitoyltransferase, a key enzyme involved in de novo ceramide synthesis.

Caspase-3 activity assay. Caspase-3 activity was measured using the EnzChek Caspase-3 Assay kit that uses Z-DEVD-AMC as a substrate (Molecular Probes, Mt. Waverley, Australia). Briefly, following FA treatments, myotubes were harvested, washed with PBS, and then lysed before being subjected to a freeze-thaw cycle. After centrifugation at 2,300 g for 5 min, the supernatant was transferred to a 96-well microtitre plate. Substrate-working solution was added to each well, and the plate was incubated in the dark for 30 min at room temperature. Caspase-3 activity was fluorometrically determined at 342 ex/441 em (Victor3 1420 Multilabel Counter; PerkinElmer, Rowville, Australia). Caspase-9 activity in cell lysates was measured using a commercially available fluorometric assay (Calbiochem).

Fluorescence microscopy. Myotubes were grown directly onto sterile glass slides in 24-well plates and then treated with 750 μM palmitate before being washed with PBS. Annexin Binding Buffer (ABB; 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2) was added to adhered myotubes with annexin V-FITC (fluorescein) conjugate (Molecular Probes) and propidium iodide (PI). Myotubes were incubated in the dark for 20 min before being inverted onto clear glass microscopic slides, covered with ABB, and examined at 488 ex/525 em and 535 ex/620 em, respectively, with an Olympus BX60 fluorescence microscope. Annexin V-FITC binds to phosphatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis. PI binds to double-stranded DNA, but it can only cross the plasma membrane of nonviable cells. Cells that
have lost membrane integrity will show red PI staining throughout the nuclei with a rhodamine filter. Thus FITC staining in the absence of PI staining indicates apoptosis, FITC and PI staining indicates late-stage apoptosis or secondary necrosis, and PI staining alone indicates necrosis.

**Flow cytometry.** Following palmitate treatment, myotubes were detached using 0.25% trypsin and centrifuged at 400 g for 5 min. The supernatant was removed, and myotubes were resuspended in ice-cold PBS. This was repeated before the myotubes were resuspended in ABB containing Annexin V-FITC and PI stains. The myotubes were incubated in the dark for 20 min, with inversions every 5 min to facilitate complete binding. A second volume of ABB was added, and the myocyte suspension was passed through a 250-μm mesh via syringe to prevent clumping. Stained myotubes were analyzed on a Beckman Coulter Epics Elite ESP flow cytometer measuring fluorescence at 488 ex/525 em and 535 ex/620 em for Annexin V-FITC and PI, respectively. Data were analyzed using the flow cytometric Expo 32 version 1.2 software.

**Determination of DNA fragmentation.** After palmitate treatment myotubes were detached, isolated by centrifugation at 400 g, and extracted according to the methods of Gong et al. (17) or by use of a commercially available kit (Apoptotic DNA Ladder kit; Roche Applied Science, Mannheim, Germany). Immediately prior to electrophoresis, 1:100 SYBR Green (Applied Biosystems, Scoresby, Australia) and loading buffer (0.25% bromophenol blue, 30% glycerol) were added to each sample and mixed. Samples were electrophoresed on a 0.8 or 1.5% agarose gel, and DNA laddering was determined upon examination of the gel under UV light. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to show apoptosis in L6 myotubes according to the manufacturer’s guidelines (DeadEnd TUNEL kit; Promega, Madison, WI). Briefly, cells were rinsed in PBS, fixed in 4% paraformaldehyde, and digested with proteinase K before incubation in terminal deoxynucleotidyl transferase and biotinylated UTP. Detection of labeled cell nuclei was carried out using horseradish peroxidase and diaminobenzidine. Cultures were counterstained briefly in hematoxylin, rinsed, and mounted in aqueous mountant (DakoCytomation, Carpinteria, CA) for viewing.

**Measurement of ceramide and diacylglycerol content.** Muscle lipids were extracted according to the method of Bligh and Dyer (5), and diacylglycerol and ceramide were extracted and quantified according to the methods of Preiss et al. (36). Briefly, diacylglycerol kinase and [32P]ATP (15 mCi/mmol cold ATP) were added to cell lysates preincubated with cardioliipin/octylglucoside, and the reaction was stopped after 2 h by the addition of chloroform-methanol (2:1). Samples were spotted onto thin-layer chromatography plates and developed. [32P]-labeled phosphatidic acid (corresponding to diacylglycerol) and ceramide 1-phosphate (corresponding to ceramide) were identified, dried, scraped from the TLC plate, and counted in a liquid scintillation analyser (Tri-Carb 2500TR; Packard, Canberra, Australia).

**Short interfering RNA.** Stearoyl-CoA desaturase-1 (SCD-1) is an endoplasmic reticulum-bound lipogenic enzyme that catalyzes the conversion of saturated fats (palmitoyl and stearoyl-CoA) to mono-unsaturated fats (palmitoleoyl- and oleoyl-CoA). Because palmitate is preferentially channeled into ceramides, we reasoned that reducing SCD-1 content would increase ceramide content. Two siRNA sequences of rat SCD-1 were tested for their capacity to decrease SCD-1 protein levels in L6 myotubes. Gene silencing was achieved with equal efficacy with the following pairs of 21 bp oligonucleotide sequences: accession no. NM139192, siRNA ID 58064, and siRNA ID 57973 (Ambion, Austin, TX). A negative control siRNA consisting of a scrambled sequence was purchased (catalog no. 4613) to account for off-target effects. For transfection, the cell culture medium was changed to DMEM, and 50 pmol of the respective siRNA were added with Lipofectamine 2000 (Invitrogen) to give a final concentration of 30 nM. The transfection medium was removed after 16–20 h, and cells were washed with PBS and differentiated for 3 days prior to experiments.

**2-deoxy-o-[14C]glucose uptake experiments.** For determination of 2-deoxy-o-[14C]glucose (2-DG) uptake, cells were grown as described and incubated in 750 μM palmitate conjugated to BSA or BSA alone for 6 h. Caspase inhibitors were purchased from Calbiochem. Ac-DEVAD-aldehyde (DEVAD-CHO) is a cell permeable inhibitor of caspase-3, -6, -7, -8, and -10, and Z-DQMD-FMK is a cell-permeable caspase-3 inhibitor. The efficacy of the inhibitors was tested in pilot experiments (data not shown). The inhibitors were reconstituted in DMSO and were added to cells at this time. An equal volume of DMSO was added to control wells and did not exceed 0.07% of the total medium volume. Cells were washed with warm PBS and incubated in αMEM without (basal) or with 100 nM insulin for 30 min. The medium was removed, and 2-DG uptake (0.5 μCi/ml, 10 μM cold 2-DG) was added. The assay was stopped after 20 min by washing with ice-cold PBS three times. Cells were lysed in 30 mM NaOH, and an aliquot was used for protein determination (Pierce Kit; Progen Industries, Darra, Australia). Radioactivity was determined by liquid scintillation counting.

**Western blot analysis.** Cell lysates normalized for protein concentration (bicinchoninic acid method; Pierce Kit) were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 8–14% polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% BSA overnight, and probed with the appropriate polyclonal primary antibody for 1 h. After incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence and quantified by densitometry (Scion Image, Frederick, MD). Primary antibodies for anti-insulin receptor substrate (IRS)-1 were obtained from Upstate (Lake Placid, NY), anti-p85 and anti-Akt from Cell Signaling Technology (Beverly, MA), anti-myo-erydin (FSD) and anti-actin HRP from Santa Cruz Biotechnology (Santa Cruz, CA), anti-actin that recognizes the carboxy-terminal 11 amino acids of skeletal muscle α-actin from Sigma-Aldrich (St. Louis, MO), and anti-cytokine c from BioVision (Mountain View, CA).

**Statistics.** Data are expressed as means ± SE. Statistical analyses were conducted using Sigma Stat 2.03. Statistical analyses were conducted using one-way or two-way analysis of variance where appropriate. The Student-Newman-Keuls test was used for post hoc analysis. Statistical significance was set a priori at P < 0.05.

**RESULTS**

**Palmitate induces caspase-3 activity in a dose- and time-dependent manner.** Caspases are a key component of the proapoptotic machinery of cells, and caspase-3 is considered a key effector that signifies full commitment to cellular disassembly. To assess the effective dose at which palmitate induced apoptosis, we incubated L6 myotubes in increasing concentrations of palmitate prior to caspase-3 determination. Caspase-3 activity was not increased above vehicle treatment at 125 and 250 μM, increased in a dose-dependent manner at 500 and 750 μM, and plateaued at 1,000 μM (data not shown). Since the most robust response was observed at 750 μM, without significant cell death as determined by the enzymatic determination of lactate dehydrogenase in medium, this concentration was used for all subsequent experiments. Caspase-3 activity was elevated after 2 h of palmitate exposure and increased progressively until 6 h. Caspase-3 activity declined to basal levels after 24 h (Fig. 1B).

To determine whether apoptosis is FA specific, we examined caspase-3 activation after 6 h of incubation with FAs of varying chain length and degree of saturation. The long-chain
FA palmitate (16:0) and stearate (18:0) increased caspase-3 activity 2.5-fold above control (Fig. 1B), whereas the short-chain saturated FAs, laurate (12:0) and myristate (14:0), and the mono- and polyunsaturated FAs, oleate (18:1) and linoleate (18:2), did not induce caspase-3 activation. The effects of coinubcation with saturated and unsaturated FAs were also investigated because unsaturated FAs can prevent saturated FA-induced apoptosis in other cell types (25, 28). Incubating L6 myotubes with linoleate before, and in conjunction with, palmitate reduced caspase-3 activity to basal levels (Fig. 1C). Interestingly, linoleate could not rescue myotubes from apoptosis if cells had been preincubated with palmitate, indicating that the order in which cells are exposed to various FAs determines the eventual apoptotic response.

**Palmitate induces apoptosis in L6 myotubes.** Phosphatidylserine (PS) is located on the cytoplasmic surface of viable cells and is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis. PS exposure was examined by viewing annexin V-FITC binding to PS by fluorescence microscopy. Thus an increase in FITC staining indicates an increase in apoptosis. Annexin V binding was abundant in multinucleated L6 myotubes after 24 h palmitate treatment, whereas negligible binding was detected in vehicle-treated cells (Fig. 2A). These results were confirmed by fluorescence-activated cell sorter analysis demonstrating increased PS translocation after 16 and 24 h palmitate treatment compared with vehicle treated cells (Fig. 2B). This can be observed in representative plots in Fig. 2C that show a dramatic rightward shift in the cell population, which represents an increase in annexin V-FITC staining. DNA fragmentation is considered a hallmark of apoptosis. DNA laddering was assessed in L6 myotubes after supplementation with palmitate for various time periods and was confirmed after 8 h and, to a greater degree, after 16 h (Fig. 2D). Although there was evidence of DNA laddering at 24 h, there was also marked smearing, indicating late-stage apoptosis/secondary necrosis. This is consistent with the red PI staining observed by immunofluorescence microscopy (Fig. 2A, 2 and 3). Exposure of L6 myotubes to 750 μM palmitate resulted in an increased number of cells with TUNEL-positive nuclei (Fig. 2E). Collectively, these data from numerous analytical approaches demonstrate that palmitate induces apoptosis in L6 skeletal muscle myotubes.

**Ceramide accumulates with palmitate treatment and causes apoptosis.** L6 myotubes cultured with 750 μM palmitate displayed an increase in ceramide content in a time-dependent manner. Ceramide content was increased by 60% after 4 h of palmitate exposure and was further increased with duration of exposure (Fig. 3A). We also examined diacylglycerol content because diacylglycerols accumulate with palmitate treatment (11, 35) and are implicated in apoptosis in fission yeast (49). Diacylglycerol content was elevated at 8 h of palmitate treatment and remained elevated over the 24-h treatment (Fig. 3B). SCD-1 catalyzes the desaturation of the saturated FAs palmitoyl-CoA and stearoyl-CoA. Because saturated FAs are preferentially shuttled into ceramides, we reasoned that preventing SCD-1 function would result in ceramide accumulation and apoptosis. SCD-1 protein content was reduced by 40% with the introduction of SCD-1 siRNA (data not shown). Analysis of the lipid extracts revealed marked ceramide accumulation in these cells after 5 h of exposure to both FA-free and FA medium (Fig. 4A). The increased ceramide content in SCD-1 siRNA myotubes was associated with elevated caspase-3 activity (Fig. 4B) and annexin V binding (Fig. 4C). It is noteworthy that the level of apoptosis observed in SCD-1 siRNA myotubes exposed to FA-free medium was similar to control myotubes exposed to a scrambled siRNA and palmitate medium. These results indicate that ceramide accumulation may play a role in skeletal muscle lipoapoptosis. It is noteworthy that there was an apparent uncoupling between ceramide content, caspase-3 activity, and annexin V binding in L6 myotubes treated with SCD-1 siRNA and palmitate. Although we are unable to experimentally explain this disconnect, it is possible that some SCD-1 siRNA cells were highly sensitive to palmitate and had detached prior to annexin V-FITC determination. This would be consistent with the observation of “palmitate-resistant” cells in an apparently homogenous population of MIN-6 β-cells (8).

To directly determine the involvement of ceramides in lipoapoptosis, we assessed the effects of a short-chain ceramide analog (C2-ceramide) or a structurally related, metabolically inactive control (C2-dihydroceramide) on caspase-3 activity. L6 myotubes cultured with C2-ceramide for 16 h displayed an increase in caspase-3 activity that was identical to control cells treated with palmitate (Fig. 5A). This was associated with increased ceramide but not diacylglycerol content (Fig. 5, B and C), indicating a role for ceramide but not diacylglycerols in lipoapoptosis. Addition of the ceramide synthase inhibitor fumonisins B1 (50 μM) to myotubes cultured in palmitate abrogated the increase in caspase-3 activity (Fig. 5A) concomitant with reduced ceramide content (Fig. 5B). These data indicate that elevated ceramide production was attributable to increased de novo ceramide synthesis, and blocking this effect diminished the proapoptotic effect of palmitate.

**Inhibiting caspase-3 activity improves insulin-stimulated glucose disposal.** Studies in cardiac and pancreatic β-cells have demonstrated that lipoapoptosis leads to loss of tissue function (11, 35). We investigated the possibility that inhibiting caspase-3 activity may abrogate the well-documented negative effects of palmitate on insulin-stimulated glucose disposal. We initially tested the efficacy of the structurally unre-
medium was unaffected by the addition of the caspase inhibitors (Fig. 6, B and C). Insulin increased 2-DG uptake and palmitate preincubation completely blunted insulin’s ability to stimulate 2-DG uptake. Whereas both caspase inhibitors did not affect basal 2-DG uptake, the caspase inhibitors almost completely restored insulin-stimulated 2-DG uptake in palmitate-treated myotubes (72 and 91% restoration for DEVD and FMK, respectively). These effects persisted despite the finding that both ceramide and diacylglycerol, known inhibitors of insulin action, were elevated in all cells pretreated with palmitate (data not shown).

**DISCUSSION**

The results of the present study provide several lines of evidence demonstrating lipoapoptosis in skeletal muscle myotubes. This process is at least partially mediated by increased de novo ceramide synthesis; however, other signaling mechanisms are also likely to activate effector caspases and induce apoptosis in response to palmitate. Lipoapoptosis was associated with reduced insulin-stimulated glucose uptake, which was partially ameliorated by inhibiting downstream apoptotic signaling. These data indicate that elevated levels of saturated FAs induce skeletal muscle apoptosis that may contribute to the development of skeletal muscle insulin resistance in vitro.

Elevated levels of FAs are implicated in the pathogenesis of pancreatic β-cell and liver dysfunction and the loss of func-

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**Fig. 3.** Palmitate exposure leads to a progressive increase in cellular lipids. L6 myotubes were incubated with 750 μM palmitate and lysed at the indicated times. Ceramide (A) and diacylglycerol (B) were determined. *Different from 0 h, P < 0.05. Values are means ± SE for 4 independent experiments. The total number of observations for each data point is n = 10.

**Fig. 4.** Reducing steroyl-CoA desaturase 1 (SCD-1) content increases ceramide content and apoptosis in L6 myotubes. L6 myoblasts were treated with scrambled (Con) or SCD-1 siRNA and differentiated to myotubes. The cells were incubated for 5 h without (vehicle) or with (palmitate) 750 μM palmitate and harvested for determination of ceramide (A), caspase-3 activity (B), and AV binding (C). Values are means ± SE for 2 independent experiments. The total number of observations for each data point is n = 6–8. *Different from vehicle within the same group; †different from Con siRNA for the same treatment, P < 0.05.
tional tissue mass (37, 42, 46), a process mediated by apoptosis. Although the FA-induced signaling pathways mediating these effects are not well described, they nevertheless support the concept that prolonged exposure to FAs, as is observed in obese, insulin-resistant phenotypes, contributes to the development of type 2 diabetes. Insulin resistance and type 2 diabetes are also characterized by elevated plasma FAs and intramyocellular FA metabolite accretion (48). The purposes of the present study, therefore, were to determine whether FAs induce skeletal muscle apoptosis and investigate proapoptotic signals in skeletal muscle cells. We have demonstrated that lipoapoptosis occurs in skeletal muscle myotubes. Apoptosis occurred with exposure to long-chain saturated FAs, whereas unsaturated FAs were without effect and actually prevented palmitate-induced apoptosis, as demonstrated in other cell lines (16, 20, 25, 28). Skeletal muscle apoptosis was confirmed by several specific measures, including caspase-3 activation, extracellular exposure of phosphatidylserine, and DNA degradation. Thus skeletal muscle, like other nonadipose tissues involved with glucose homeostasis, is susceptible to lipoapoptosis.

Accumulation of ceramide in nonadipose tissues is linked to the development of several deleterious clinical manifestations, including type 2 diabetes and the metabolic syndrome (45). Ceramides have been demonstrated to induce apoptosis in a

Fig. 5. Ceramides induce apoptosis in L6 myotubes. Cells grown in fatty acid-free medium (basal) were treated with C2-ceramide (C2) or the metabolically inactive C2-dihydroceramide (C2-di). Other cells were exposed to 750 μM palmitate for 16 h in the presence or absence of fumonisin B1 (FB1), a ceramide synthase inhibitor. Cells were harvested and assessed for caspase-3 activity (A) and ceramide (B) and diacylglycerol (C) contents. Values are means ± SE for 2 independent experiments conducted in triplicate. The total number of observations for each data point is n = 6. *Different from basal; †different from palmitate, P < 0.05.

Fig. 6. Caspase-3 inhibition partially ameliorates fatty acid-induced insulin resistance in L6 myotubes. L6 myotubes were treated without or with palmitate for 6 h and in the presence or absence of a pan caspase inhibitor [AcDEVD-aldehyde (DEVD-CHO)] or a specific caspase-3 inhibitor (Z-DQMD-FMK). A: agarose gel electrophoresis showing DNA fragmentation in palmitate-treated L6 myotubes and attenuation of this response with the caspase inhibitors. 2-Deoxy-D-[14C]glucose (2-DG) uptake was assessed with or without insulin. Values are means ± SE of 12 samples for each data point from 3 independent experiments. *Different from vehicle (i.e., no palmitate, no insulin, and no inhibitor treatment), P < 0.05. ◦, vehicle; ●, palmitate.
myotubes in FA-free medium reproduced the apoptotic effects in myotubes exposed to palmitate. This was not evident with exposure to the metabolically inactive C2-dihydroceramide. Finally, the increased ceramide content was inhibited by fumonisin B1, a ceramide synthase inhibitor, indicating that the newly synthesized ceramide was derived from de novo synthesis as opposed to sphingomyelin degradation or caspase-dependent ceramide production in response to inflammatory cytokines. The prevention of ceramide synthesis abrogated the proapoptotic effects of palmitate in myotubes. Collectively, these studies indicate that the ceramide pathway is central to the lipoapoptotic cascade in skeletal muscle myotubes. Although the precise mechanisms for ceramide effects remain unresolved, these may include induction of mitochondrial membrane permeability transition and activation of the intrinsic apoptotic cascade (Supplemental Fig. S1; Supplemental Material for this article is available at the AJP-Endocrinology and Metabolism web site),1 generation of reactive oxygen species (30), or activation of c-Jun-NH2-terminal kinase (38).

The suggested mechanisms by which palmitate induces apoptosis in nonmuscle cells includes nitric oxide synthesis (29, 41), generation of reactive oxygen species (26), endoplasmic reticulum stress (24), diminished cardiolipin synthesis (32), and ceramide (28, 40) and diacylglycerol (49) synthesis. Although the present data clearly ascribe a role for ceramides in lipoapoptosis, it is likely that some of the aforementioned processes and other unidentified mechanisms are involved. Indeed, we detected cytochrome c release and caspase-9 activation, indicating an involvement of intrinsic mitochondrial-mediated apoptosis. Decreases in cardiolipin content can occur rapidly with palmitate exposure in other cell types (19, 32), which activates mitochondrial-mediated apoptosis. However, this was not assessed in the present study. FA metabolites would also appear to be a logical trigger, and diacylglycerol is suggested to induce apoptosis (49), possibly via activation of PKC-δ, as demonstrated in insulinoma cells (16). Our data demonstrating a temporal dissociation between diacylglycerol accumulation and the induction of apoptotic signaling, and the induction of apoptosis with C2-ceramide in the absence of diacylglycerol accumulation, argues against a proapoptotic role for diacylglycerol in skeletal muscle. Aside from changes in FA metabolites/phospholipid content, we have detected endoplasmic reticulum stress in L6 myotubes with palmitate exposure (Lancaster GI, Febbraio MA, and Watt MJ unpublished observations). Further studies will assist in assessing the contribution of these and other signaling mechanisms in mediating skeletal muscle apoptosis.

FAs exert powerful apoptotic effects in β-cells, leading to loss of function and mass (37). FAs cause skeletal muscle dysfunction, which presents as a resistance to insulin and is mediated, at least partially, by the accumulation of fatty acyl metabolites and activation of inflammatory pathways (48). We examined whether reducing apoptotic signaling in a lipoapoptotic setting would improve skeletal muscle function with regards to insulin-stimulated glucose disposal. To achieve this, cell permeable peptide-based caspase inhibitors were used, partly because they proved to be effective in other disease

1 The supplemental material (Supplemental Fig. S1) for this article is available online at http://ajpendo.physiology.org/cgi/content/full/00095.2006/DC1.
models (34) and partly because caspase-3 is central to the lipoprotective cascade in skeletal muscle. The addition of caspase inhibitors largely reversed palmitate-induced insulin resistance in myotubes, independent of changes in ceramide content. This result was surprising because ceramides independently inhibit Akt/PKB activation and reduce insulin-stimulated glucose transport (11, 39, 45). Others (14) have shown that caspase-3 activation induces proteolysis in L6 myotubes, suggesting that degradation of insulin-signaling proteins may underpin the reduction in glucose uptake. Although we observed α-actin degradation, we observed no reductions in IRS-1, p85, and Akt protein content (all key components of the insulin-signaling pathway), indicating that proteolysis was not involved in the reduced glucose disposal with palmitate. The targeting of α-actin for proteolytic degradation and not insulin-signaling proteins is consistent with other models, such as cancer cachexia, where selective targeting of skeletal muscle gene products is exhibited (1). Although it is also apparent that the cellular remodeling associated with apoptosis affects glucose uptake, the precise mechanisms remain unresolved. Thus the evidence presented indicates that palmitate exposure activates proapoptotic signaling and induces nuclear decay in postmitotic multinucleated myotubes, ultimately leading to cellular decay and “blebbing” (cell death). Although these in vitro studies imply that apoptosis results in actual fiber loss, studies in a physiological system (i.e., skeletal muscle in vivo) are required to further characterize these events.

In summary, we have demonstrated that lipoproteins occurs in skeletal muscle myotubes via de novo ceramide accumulation and that inhibiting downstream apoptotic signaling improves cellular function. These data raise the possibility that elevated circulating saturated FAs, and/or defective FA handling, contributes to skeletal muscle insulin resistance in a manner analogous to the deleterious FA effects on pancreatic β-cell function. Future studies using animal and/or human models of lipid oversupply will determine whether these processes are relevant in vivo.

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LIPOTOXICITY IN SKELETAL MUSCLE


