Long-chain acylcarnitines activate cell stress and myokine release in C2C12 myotubes: calcium-dependent and -independent effects

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1Molecular, Cellular and Integrative Physiology Graduate Group, University of California, Davis, California; 2Obesity & Metabolism Research Unit, United States Department of Agriculture-Agricultural Research Service Western Human Nutrition Research Center, Davis, CA; 3Department of Nutrition, University of California, Davis, Davis, California; and 4Arkansas Children’s Nutrition Center and Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

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McCoin CS, Knotts TA, Ono-Moore KD, Oort PJ, Adams SH. Long-chain acylcarnitines activate cell stress and myokine release in C2C12 myotubes: calcium-dependent and -independent effects. Am J Physiol Endocrinol Metab 308: E990–E1000, 2015. First published April 7, 2015; doi:10.1152/ajpendo.00602.2014.—Acylcarnitines, important lipid biomarkers reflective of acyl-CoA status, are metabolites that possess bioactive and inflammatory properties. This study examined the potential for long-chain acylcarnitines to activate cellular inflammatory, stress, and death pathways in a skeletal muscle model. Differentiated C2C12 myotubes treated with L-C14, C16, C18, and C18:1 carnitine displayed dose-dependent increases in IL-6 production with a concomitant rise in markers of cell permeability and death, which was not observed for shorter chain lengths. L-C16 carnitine, used as a representative long-chain acylcarnitine at initial extracellular concentrations ≥25 μM, increased IL-6 production 4.1-, 14.9-, and 31.4-fold over vehicle at 25, 50, and 100 μM. Additionally, L-C16 carnitine activated c-Jun NH2-terminal kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase between 2.5- and 11-fold and induced cell injury and death within 6 h with modest activation of the apoptotic caspase-3 protein. L-C16 carnitine rapidly increased intracellular calcium, most clearly by 10 μM, implicating calcium as a potential mechanism for some activities of long-chain acylcarnitines. The intracellular calcium chelator BAPTA-AM blunted L-C16 carnitine-mediated IL-6 production by >65%. However, BAPTA-AM did not attenuate cell permeability and death responses, indicating that these outcomes are calcium independent. The 16-carbon zwitterionic compound amidosulfobetaine-16 qualitatively mimicked the L-C16 carnitine activity, which was not observed for shorter chain lengths. L-C16 carnitine, for instance, has been reported to increase insulin resistance (1, 18, 20, 24, 26).

In addition to their use as biomarkers, there is recent evidence supporting the hypothesis that acylcarnitines have bioactivities. Our previous work has shown that long-chain fatty acylcarnitines activate proinflammatory signaling pathways in RAW 264.7 murine macrophages (1, 29) and in HCT-116 cells (29), and blunt the insulin signaling pathway in both murine C2C12 and human primary skeletal muscle cells (2). These activities were found to occur at initial extracellular concentrations of C14 carnitine and C16 carnitine as low as 5 μM. Thus, whether or not these metabolites contribute to, or exacerbate, disease phenotypes or normal physiological processes remains an open question. The mechanisms by which long-chain fatty acylcarnitines impinge upon inflammatory systems and insulin-associated cell signaling pathways in muscle have yet to be fully elucidated. Evidence to date suggests that, at least in macrophages, proinflammatory actions of acylcarnitines can occur in a MyD88-dependent manner (29), and MyD88 is considered an important adaptor protein that can serve as part of the membrane proximal signaling complexes, i.e., for many pattern recognition receptors (14). However, we found no evidence that specific Toll-like receptors or other pattern recognition receptors are driving the proinflammatory effects of C14- and C16-acylcarnitines (29).

In the course of studies examining the effect of long-chain fatty acylcarnitines on insulin resistance and inflammation, we noted changes in cell viability and function under conditions of high acylcarnitines (2, 29). In our previous work, it was observed that, at a higher concentration of C16 carnitine, release of interleukin-6 (IL-6) in myocytes (2) and adenylate kinase (AK, a cell permeability/death marker) in macrophages (29) was increased. Furthermore, in cardiac ischemia, heart muscle acylcarnitines accumulate (17, 36, 38) and long-chain acylcarnitines have been associated with increased cardiac cellular reactive oxygen species (ROS), apoptosis, and endoplasmic reticulum (ER) stress (32) and increases in intracellular calcium (39, 42). Considering that muscle is a site of robust acylcarnitine generation, we have begun to consider if long-acylcarnitine generation, we have begun to consider if long-
chain acylcarnitines impact myocyte cell function. In the current cell culture experiments, it was hypothesized that the naturally occurring zwitterionic metabolites, long-chain acylcarnitines, can elicit cell stress and cell death responses in a model of skeletal muscle myotubes and that calcium-associated pathways play a role.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS) was purchased from List Biologicals (Campbell, CA). Lot-tested (lot no. K0109) premium-select fetal bovine serum (FBS) and horse serum were purchased from Atlanta Biologicals (Lawrenceville, GA) and Hyclone (Logan, UT), respectively. Dulbecco’s Modified Eagle’s Medium (DMEM), phenol red-free Hank’s Balanced Salt Solution (HBSS), penicillin/streptomycin, sodium pyruvate, GlutaMax, and calcium green-1 AM, were all purchased from Atlanta Biologicals (Lawrenceville, GA) and Hyclone (Logan, UT), respectively. Mammary epithelial growth factor (EpdG), fetal bovine serum (FBS) and horse serum were purchased from Atlanta Biologicals (Lawrenceville, GA), 100 U/ml penicillin, 100 µg/ml streptomycin, sodium pyruvate, GlutaMax, and calcium green-1 AM, were all purchased from Life Technologies (Grand Island, NY). Ionomycin, BAPTA-AM, caspase-3 inhibitor II (Z-DEVD-FMK), and caspase inhibitor I (pan inhibitor, Z-VAD-FMK) were purchased from EMD Millipore (Billerica, MA), and cyclosporine A was purchased from Cell Signaling Technologies (Danvers, MA). Aetyl-l-carnitines of varying chain lengths and t-carnitine were purchased from Advent Bio (Downers Grove, IL), amidosulfobetaine-16 (ASB-16) [3-N-(3-amidino-3-palmitoylaminopropyl)ammonium]-propanesulfonate (catalog no. DG062, lot no. 990265) was purchased from G-Biosciences (St. Louis, MO), and Toxilight and IL-6 ELISA Assays were purchased from Lonza (Basel, Switzerland) and R&D Systems (Minneapolis, MN), respectively. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt (XTT) was purchased from Biotium (Hayward, CA) and phenazine methosulfate 98% (PMSE) from Acros Organics (Geel, Belgium). Antibodies against phospho-p4/42 mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase (ERK1/2)] (Thr202/Tyr204) (catalog no. 4370), total p44/42 MAPK (ERK) (catalog no. 9102), p-c-Jun NH2-terminal kinase (JNK) (catalog no. 4668), JNK (catalog no. 9252), p-p38 (catalog no. 4511), p38 (catalog no. 8690), lamin A/C (catalog no. 2032), and ER stress antibody sampler kit (catalog no. 9956) were all purchased from Cell Signaling Technology (Danvers, MA). The β-tubulin (Clone Tub 2.1, catalog no. ab11308) was purchased from Sigma-Aldrich (St. Louis, MO), and cells were cultured in DMEM containing 10% FBS (premium select FBS, catalog no. S11595, lot no. K0109; Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX-I (Life Technologies, Grand Island, NY), and 100 µM t-carnitine (Advent Bio). Because penicillin and streptomycin were included in all treatment conditions, any potential effects on mitochondrial function or metabolism would not influence treatment-associated differences. C2C12 myoblasts were maintained at 37°C in a 90% confluent at which time FBS was removed, and fresh media with treatments were added, with 0.20%/H11002 20°C for 15 min at room temperature, after which 20 µl of reporter lysis buffer per well (catalog no. E3971; Promega) were transferred to white bottom-96-well plates, and 25 µl of the Caspase 3/7 Glo working reagent were added to each sample (catalog no. G8091; Promega). The plate was incubated (covered) at room temperature for 30 min on an orbital shaker. Luminescence was measured on a Synergy 2 plate reader.

Long-term acylcarnitine treatment. Myotubes (4- to 5-day differentiated) were grown in 0.25% FBS starvation media for 2–4 h and then treated at various doses with different-chain-length acylcarnitines for 18 h, as indicated. Myotubes were cotreated with the respective caspase inhibitor and various compounds as indicated in the text for 6 or 20 h. Supernatants were collected and stored at −20°C. C2C12 myotubes were cotreated with the respective caspase inhibitor and various compounds as indicated in the text for 6 or 20 h. Supernatants were collected and stored at −20°C. C2C12 were washed one time with cold HBSS and lysed in 1X Cell Signaling Lysis Buffer + Pierce HALT phosphatase inhibitors (Rockford, IL). Sample supernatants were sonicated and subject to centrifugation at 12,000 g for 10 min at 4°C.

Immunoblotting. Lysates were resolved via 4–12% Bis-Tris SDS-PAGE (Life Technologies) and transferred to polyvinyl difluoride membranes (Bio-Rad, Hercules, CA) using a Bio-Rad Trans-Blot Turbo. Membranes were blocked in a 1X PBS and 0.1% (vol/vol) Tween 20 (Fisher Scientific) (PBST) solution containing 2% wt/vol dry milk. Membranes were probed for 1 h at room temperature or overnight at 4°C with primary antibody in 1X PBST followed by incubation with horseradish peroxidase-conjugated secondary antibody (Southern Biotech, Birmingham, AL) at a 1:10,000 dilution in 1X PBST + 2% milk for 1 h at room temperature. Bands were visualized using Bio-Rad Clarity Western ECL reagent and imaged on a Bio-Rad ChemiDoc XRS system.

ER stress analysis. Myotubes were grown in 96-well tissue culture plates and differentiated for 4 days. Cells were serum starved (0.25% FBS/DMEM) for 4 h and treated with C16 carnitine at 0, 5, 10, 25, 30, 40, 50, 75, and 100 µM and positive controls (stauroporine, thapsigargin, and tunicamycin) for 6 h in duplicate or triplicate. At 6 h, media were harvested, cells were rinsed two times with cold HBSS and lysed in 1X lysis buffer, and replicates were pooled. The media were analyzed for AK, and concentrations and lysate protein concentrations were measured and subjected to SDS-PAGE and Western blotting to determine levels of ER stress markers [cleaved caspase 3, inositol-requiring protein-1α (IRE-1α), binding immunoglobulin protein (BiP), and CCAAT/enhancer-binding protein homologous protein (CHOP)].

Live/dead assay. C2C12 myoblasts were seeded into 96-well clear-bottom, black wall plates (BD Falcon) and differentiated as described above. The cells were serum starved in 0.25% FBS phenol red-free DMEM for 3–4 h before treatment for 6 h with various compounds in the same medium. Supernatants were removed, and 25 µl of HBSS with Ca2+/Mg2+ were added to each well. Twenty-five microliters of 2X live/dead dye (catalog no. R37601; Life Technologies) was added to each well 15 min before imaging. Imaging was performed on a Nikon Eclipse Ti microscope with an automated stage and Zyla camera and the data acquired were analyzed using Nikon Elements HCT software. Three to four images were captured for each well. The total GFP intensities and channel fluorescence and RPE (red channel fluorescence) intensities were calculated by the Nikon Element HCT software. The ratio of GFP to RPE intensity was determined. A lower ratio indicates increased cell death.

XTT viability assay. Myotubes were grown, starved, and treated as done above for live/dead assay. After 6 h treatment, media were removed, and fresh media with treatments were added, with 0.20 mg/ml XTT and 0.001 mM PMS reagent and PBS addition. Cells were returned to 37°C for 4 h. Absorbance measurements were read on a plate reader at 475 nm with a background correction of 690 nm.

Caspase assay. C2C12 myotubes were pretreated with caspase inhibitors, caspase-3 inhibitor II (Z-DEVD-FMK) or caspase inhibitor I (Pan inhibitor, Z-VAD-FMK) (EMD Millipore), for 1 h after a serum starvation in 0.25% FBS for 2–3 h. C2C12 myotubes were cotreated with the respective caspase inhibitor and various compounds as indicated in the text for 6 or 20 h. Supernatants were collected and stored at −20°C. C2C12 were washed one time with cold HBSS and lysed in 25 µl of reporter lysis buffer per well (catalog no. E3971; Promega). The samples were stored at −20°C for assay. Lysates were thawed quickly at 50°C and mixed on an orbital shaker for 15 min at room temperature, after which 20 µl were transferred to white round-bottom 96-well plates, and 25 µl of the Caspase 3/7 Glo working reagent were added to each sample (catalog no. G8091; Promega). The plate was incubated (covered) at room temperature for 30 min on an orbital shaker. Luminescence was measured on a Synergy 2 plate reader.

Intracellular calcium readouts. C2C12 myotubes in a black 96-well tissue culture plate were serum starved for 2–3 h in 0.25% FBS plus
10 μM calcium green-1 AM. Cells were rinsed two times in warm HBSS (phenol red free), and 100 μl phenol red-free 0.25% DMEM were added back. The plate was incubated at room temperature on a Synergy 2 plate reader, and 100 μl of a 2X treatment solution (containing acylcarnitines or other factors indicated in the text) were added to each well using the Synergy 2 injectors. Fluorescence readings were taken immediately following injection and continuing every 2 s for a 1-min duration. Baseline to maximum values was calculated by subtracting the initial fluorescent reading value from the highest value recorded.

Data analysis. Each experiment was performed a minimum of three independent times, as indicated in RESULTS; typical experiments each included at least three replicates per treatment. Depending on the nature of the experiment, results were analyzed by one- or two-way ANOVA with Dunnett’s (comparing against control) or Tukey’s (comparing all treatments with one another) post hoc tests, and data are presented as means ± SE. Statistical analyses were performed using PrismGraph 6.0 (GraphPad Software, San Diego, CA).

RESULTS

Acylcarnitine chain-length effects on IL-6 production. Because our previous studies showed that C14- and C16-acylcarnitine can elicit proinflammatory gene and cytokine expression in RAW 264.7 murine monocyte/macrophages and (at higher concentrations) in C2C12 murine myotube models (2, 29), we sought to fully characterize a panel of short-, medium-, and long-chain t-acylcarnitine treatments on media IL-6 cytokine and AK concentrations in the C2C12 myotube model. These markers were chosen as factors reflecting activation of inflammatory cascades (IL-6) or cytotoxicity typically tracking cell death (AK). t-Acylcarnitines of acyl chain lengths from C2- through C12- did not alter IL-6 cytokine production or AK release into the medium after 18 h of treatment (Fig. 1). Beginning with C14 chain-length acylcarnitine and continuing with the higher chain lengths tested (C16, C18, and C18:1 carnitines), t-acylcarnitine induced both IL-6 production and AK release in parallel. The minimum concentration tested that elicited an increase in IL-6 cytokine production was 25 μM C16- and C18- carnitine, with the caveat that other concentrations between 10 and 25 μM were not evaluated. These effects increased in a dose-dependent manner up to 100 μM, the highest concentration tested. AK tracked IL-6 production under all conditions except for C18:1 carnitine, where AK release increased at 50 μM without a corresponding increase in media IL-6.

Because C16 carnitine had the most robust effects on the C2C12 myotubes, all further experiments were performed using C16 carnitine as a representative long-chain acylcarnitine. Previously, the lots of FBS and C16 carnitine were subjected to endotoxin testing and found to be negative (29). These results indicate that the inflammatory IL-6 and AK responses are a direct result of C16 carnitine and not endotoxin contamination. Furthermore, in experiments not shown, LPS treatment of cells failed to elicit an AK response despite the expected robust IL-6 response.

Acylcarnitine activation of MAPK pathway. Our previous work demonstrated that C14 carnitine increases phosphorylation of the JNK and ERK MAPKs in RAW 264.7 murine macrophages. Furthermore, IL-6 cytokine production is known to be mediated in part by the MAPK cell stress signaling pathway in the C2C12 model (13). As shown in Fig. 2, C16 carnitine elicited an increase in phosphorylation of p38, JNK, and ERK in C2C12 myotubes. The pathway activation occurred in both time- and concentration-dependent manners that were consistent for all MAPK pathways; the effects appeared to be triggered by concentrations between 10 and 25 μM. This effect suggests that C16 carnitine can elicit global activation of MAPK pathways in the C2C12 model in a dose-dependent manner.

C16 carnitine at higher concentrations elicits cell death within 6 h. Because it was observed that C16 carnitine at concentrations greater than 25 μM cause significant increases in AK after 18 h (Fig. 1B), a time course of treatment was designed to determine when and at what concentration this effect occurs. By 6 h, media AK had increased at concentrations of 40 μM and higher (Fig. 3A), indicating that cells had permeabilized and begun to release intracellular contents into the media. As a secondary readout for cell damage, lactate dehydrogenase (LDH) was also assessed in the media. While variable, LDH increased in a dose-dependent manner with C16 carnitine treatment, similar to AK (data not shown).

When cells are stressed and begin to die, mitochondrial function is compromised (21, 22). To further characterize the C2C12 response to C16 carnitine on cell stress and viability, XTT was used to assess mitochondrial redox potential at 6 h of treatment (Fig. 3B). The ability of mitochondria to convert XTT to formazan was significantly reduced starting at 40 μM...
C16 carnitine after a 6-h treatment, suggesting mitochondrial dysfunction. The results for AK, LDH, and XTT support significant cell damage and are suggestive of cell death. A live/dead imaging assay (Fig. 3, C and D) coupled to AK measurement (Fig. 3 E) confirmed that higher concentrations of C16 carnitine cause C2C12 myotube cell death.

Long-chain acylcarnitines do not activate ER stress pathways but weakly activate caspases. After observing that C16 carnitine can elicit myotube cell death, the mechanism by which death occurs was examined. Cellular death can occur through a number of well-described pathways, including activation of ER stress/unfolded protein response pathways that can occur through a disruption in lipid homeostasis (4). After 6 h of acylcarnitine treatment, none of the ER stress markers, IRE-1α, BiP, nor CHOP, were increased, in contrast to positive control treatments (Fig. 4A). Interestingly, C16 carnitine modestly increased the apoptotic marker cleaved caspase-3, beginning at 10–25 μM.

Staurosporine is a well-known activator of caspase-associated cell death and as expected triggered a large upregulation of cleaved caspase-3 in the cells (showing ~10-fold increase vs. control; Fig. 4B, inset). Addition of either a caspase-3/7 or pan-caspase inhibitor was capable of preventing the cell from eliciting a staurosporine-mediated increase in caspase-3/7 (Fig. 4B, inset). However, neither of the caspase inhibitors was capable of protecting the cells from AK release following acylcarnitine treatment (Fig. 4B). Together, these results indicate that acylcarnitines are capable of activating cell stress
Fig. 3. Dose-dependent cell death occurs within 6 h of C16 carnitine treatment. C2C12 myotubes were serum starved for 4 h (0.25% FBS/DMEM) and then treated with varying concentrations of C16 carnitine (0–100 µM). Concentrations >40 µM resulted in increased AK release after 6 h (A). At 6 h, conversion of 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt (XTT) to its formazan product (B), a live/dead imaging assay (live: green, dead: red) (C and D), and release of AK (E) confirmed concentrations of C16 carnitine 40 µM and greater result in dose-dependent injury/death of C2C12 myotubes. C: representative images of at least 3 experiments. One-way ANOVA with Dunnett’s test: *P < 0.05 and ***P < 0.001; mean ± SE. Data are expressed as fold over vehicle.
pathways but that the observed cell death and membrane permeability to AK was not likely mediated through classical caspase-dependent apoptosis.

Acylcarnitine treatment rapidly increases intracellular calcium and promotes IL-6 production. Increased intracellular calcium is required for caspase-3 activation (34) and has been associated with increases in acylcarnitines during cardiac ischemia (39). Additionally, increases in cytosolic calcium are well established to induce IL-6 transcription (3), which can be blunted by a calcineurin inhibitor (12). Thus, we assessed whether addition of exogenous C16 carnitine could elicit an increase in intracellular calcium when measured by a fluorescent calcium indicator using a microplate reader (Fig. 5, A and B). Whereas the rate of the calcium increase was slower than that of the positive control ionophore, ionomycin (Fig. 5A), the average maximum fluorescence from baseline was comparable between 50 μM C16 carnitine and 1 μM ionomycin (Fig. 5B). Increased calcium above vehicle control was detectable by 10 μM C16 carnitine.

We next investigated whether C16 carnitine-mediated increases in intracellular calcium were responsible for the increases in media IL-6. The intracellular calcium chelator BAPTA-AM was used to decrease free intracellular calcium. BAPTA-AM completely blunted the stimulation of IL-6 by 25 μM C16 carnitine and caused an ~78% drop at 50 μM (Fig. 5C). Notably, intracellular calcium chelation had no effect on Toll-like receptor-mediated IL-6 production from LPS treatment (data not shown). Whereas BAPTA-AM strongly blunted the production of IL-6 with acylcarnitine treatment, it did not reduce the AK marker of cell death (Fig. 5D). The data indicate that changes in intracellular calcium are important for some, but not all, of the effects of C16 carnitine in myotubes.

**Mechanism of acylcarnitine-mediated cell death.** Observations from the current experiment and our previous studies of inflammation (29) and insulin sensitivity (2) in response to acylcarnitines have illustrated that types and magnitude of bioactivities differ dramatically when considering lower (i.e., 5–10 μM) and higher (i.e., >25 μM) concentrations of these metabolites. The latter consistently has led to cell permeability in both RAW cells and C2C12 myotubes, whereas this effect is not observed at lower long-chain acylcarnitine concentrations, despite proinflammatory or insulin-resistance phenotypes at the lower levels of the metabolites. It is possible that acylcarnitines elicit some or all of their effects through interaction with cellular membranes by virtue of their zwitterionic character, perhaps even at the lower concentrations. At very high con-
centrations, membrane integrity may be compromised, and cell stress responses ensue. To begin to evaluate this concept, we employed the 16-carbon zwitterionic compound ASB-16 that displays similar chemical characteristics with C16 carnitine (Fig. 6). In dose-response studies under identical conditions to those used for C16 carnitine, ASB-16 elicited comparable patterns for increasing intracellular calcium (Fig. 6A). Additionally, IL-6 and AK release were evident at similar concentrations to those seen for acylcarnitine (Fig. 6B). Although the studies were not designed to statistically compare ASB-16 and L-C16 carnitine, overall the molecules displayed qualitatively similar activities on the parameters tested. These results support the idea that at least some of the effects of acylcarnitine involve cell components sensitive to zwitterion interaction with cellular membranes.

DISCUSSION

A growing body of research related to inefficient or impaired long-chain fatty acid oxidation has implicated some lipid intermediates as being associated with insulin resistance, inflammation, and cell stress responses (1, 20). Recent studies by our laboratory have focused on the bioactive properties of acylcarnitines in eliciting an inflammatory response in murine macrophages (29) and in blunting insulin signaling in murine and human myotubes (2). These effects were qualitatively equivalent with either C14- or C16-acylcarnitines, suggesting a similar mechanism. One particularly striking finding was that higher concentrations of long-chain acylcarnitines (i.e., C14- or C16-acylcarnitines) elicited apparent cell death in macrophages; however, the pathophysiological relevance of these observations, if any, remains to be determined. Because skeletal muscle is a major source of whole body acylcarnitine production, we sought to identify the inflammatory and cell stress effects of long-chain acylcarnitines on murine C2C12 myotubes and explored the mechanism by which these effects might occur.

Herein, we provide evidence that C16 carnitine, a representative long-chain acylcarnitine elevated under certain disease conditions such as FAOD (23), cardiac ischemia (17, 36, 38), or more modestly in insulin resistance/type 2 diabetes (1, 18, 20, 24, 26), elicits the activation of cell death and stress pathways in a concentration-dependent manner in murine myotubes. C16 carnitine rapidly activated the JNK/ERK/p38 MAPK stress pathways (in concert with AK release), increased intracellular calcium, and elicited markers of cell death within 6 h. C16 carnitine also modestly activated the proapoptotic caspase-3 catalytic protein but did not increase markers of ER stress. These results are in line with a study by Mutomba and colleagues who reported activation of recombinant caspase-3 enzyme activity by palmitoylcarnitine (25). In the latter study, the idea that long-chain acylcarnitines can activate caspases was also supported by the observation that staurosporine-mediated apoptosis was blunted in cells lacking CPT1 (25). While speculative, results from the current study and the literature are consistent with the hypothesis that, under certain disease conditions or events, such as FAOD or severe lipotoxicity, increases in long-chain acylcarnitines could elicite muscle cell inflammation and stress.

**Fig. 5.** Intracellular calcium is rapidly increased with C16 carnitine treatment, and its chelation blunts C16 carnitine-mediated IL-6 production but does not protect from cell death. Differentiated C2C12 myotubes were serum starved in 0.25% FBS/DMEM for 4 h and preloaded with calcium green-1 AM (10 μM) (A and B) or BAPTA-AM (7.5 μM) (C and D) for the last hour of starvation. Cells were treated with C16 carnitine (0, 5, 10, or 25 μM) or positive control ionomycin (1 μM) for 1 min, and maximum fluorescence over basal levels (B) was determined by plate reader as described in MATERIALS AND METHODS. Fluorescence trace is representative of 5 experiments (A). Myotubes were treated for 18 h with C16 carnitine (0, 25, and 50 μM) with and without BAPTA-AM (7.5 μM) (C and D). Media IL-6 and AK were analyzed. One-way ANOVA with Tukey’s test: *P < 0.05, **P < 0.01 and ****P < 0.0001 vs. basal; mean ± SE. Data are expressed as fold over vehicle.
Lipotoxicity is a well-described phenomenon that occurs when lipids and lipid intermediates accumulate abnormally throughout the body, as seen in insulin resistance and more severely in poorly controlled type 2 diabetes (30, 35). There are a host of cellular consequences to lipotoxic conditions, including increases in cellular ROS, ER stress, inflammation, and, in its most severe manifestation, cell death (5, 30). Increases in cardiac tissue acylcarnitines have been noted during ischemia-reperfusion and associated with a range of cellular complications, including derangements in ionic flux that control cardiac electrophysiology (9, 32, 40–42) and stress and death pathway activation (32). Although the skeletal muscle metabolic effects of lipotoxicity associated with type 2 diabetes or insulin resistance are well documented, the specific metabolites that trigger cell stress are less conclusive. There have been reports of increased caspase-3/apoptosis (33), ROS, and mitochondrial damage (31) in type 2 diabetes mellitus and insulin resistance. This current study, as well as a previous study (29), raise the possibility that, under certain conditions of inefficient oxidation, an abnormal increase in long-chain acylcarnitines contributes to lipid-associated cell stress.

The mechanisms-of-action of long-chain acylcarnitines on cell inflammation (29), insulin sensitivity (2), and cell stress responses has been elusive. Based on the aggregate of results, it is speculated that these effects involve associations with cell membranes (28) and fall along a spectrum from modest (i.e., impacting insulin signaling or inflammation) to severe (i.e., triggering cell death and loss of membrane integrity). At higher concentrations of long-chain acylcarnitines, disruption of membrane integrity may be associated with a host of negative consequences, including arrhythmias, myopathies, and necrosis, since loss of proper membrane function overrides the cells’ ability to maintain homeostasis (10, 11). Long-chain acylcarnitines at high concentrations were also shown to trigger red blood cell lysis (8). Consistent with our perspective, it was observed that the zwitterionic compound ASB-16, often used to solubilize membranes and membrane proteins (16), displayed effects qualitatively similar to high-concentration long-chain acylcarnitines with respect to IL-6 release, cell death, and permeabilization in C2C12 myotubes. C16 carnitine and ASB-16 are structurally similar compounds. Furthermore, the C16 carnitine effects on cell death were seen at concentrations within the same order of magnitude as its 75- to 100-µM estimated critical micelle concentration (CMC) (27). As depicted in a working model (Fig. 7), it is proposed that the long-chain acylcarnitine interaction with cell membranes also manifests at lower, nonpermeabilizing concentrations that impact cell signaling outcomes. In other words, long-chain acylcarnitines at concentrations that are normally seen in vivo may hypothetically interact with cell membranes to impact cellular function and receptor signaling. In contrast, at pathophysiologically high concentrations, the membrane effects could also lead to cellular damage.

While this concept provides a provocative perspective on acylcarnitine biology, a major limitation is that it remains to be determined if effects seen using the initial extracellular concentrations in cell culture studies mimic acylcarnitine actions at concentrations circa or within cells in vivo. It is notable that, in studies by Mutomba et al. (25), the lowest concentration of palmitoylcarnitine tested, 1 µM, activated recombinant puri-
fied caspase-3, supporting our assertion that long-chain acylcarnitine accumulation could trigger some cell stress mechanisms well below the CMC. We have also observed inflammatory phenotypes in immune cells treated with as low as 5 μM long-chain acylcarnitines (29). The working model of membrane associations would apply most clearly to long-chain acylcarnitines and would be less likely to explain bioactivities, if any, of shorter-chain acylcarnitines. Indeed, we observed a sharp increase of cell stress outcomes with increasing acyl chain length, consistent with the relative affinities for acylcarnitines for membranes, as indicated by a decrease in CMCs. For instance, Haeyaert et al. (15) reported CMCs of C12 carnitine (700 μM), C14 carnitine (100 μM), and C16 carnitine (40 μM). The absolute CMCs are highly dependent on the system being tested and the pH, yet, despite variability of these values in the literature, the chain-length associations would still hold. As a final consideration, it is likely that the free acylcarnitine concentrations in situ, and hence their cellular activities, are modified through binding to other lipids and proteins. Thus, future studies to examine the validity of the model components could include, for instance, determination, albeit challenging, of the in situ cellular concentrations of acylcarnitines (i.e., immune and myocyte cells), monitoring cellular effects of manipulation of intracellular generation of acylcarnitines (i.e., genetic modifications such as CPT2 knockdown, specific enzyme inhibitors), protein binding, and experiments that determine if acylcarnitine association with membrane preparations corresponds with receptor- or membrane protein-based outcomes.

In summary, we have shown that long-chain acylcarnitines have the potential to rapidly increase intracellular calcium and to activate skeletal muscle cell stress pathways, which under certain conditions elicit cell death. Our studies employed acylcarnitine administration externally to cells, which were in line with the high concentrations of long-chain acylcarnitines within plasma and muscle under conditions of FAOD (23, 43).
However, it is acknowledged that the amounts used herein may differ from the concentrations found at the sarcolemma or within muscle cells. Thus, it remains speculative as to the potential role of long-chain acylcarnitine accumulation in the episodic myopathy typical of certain FAOD conditions. Whether acylcarnitines trigger cellular responses through specific receptors, membrane-associated proteins, nonspecific amphi-pathic interactions, or a combination of these remains to be fully elucidated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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