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

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Abstract

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 \( \geq 25 \mu M \), increased IL-6 production 4.1-, 14.9- and 31.4- fold over vehicle at 25, 50 and 100 \( \mu M \). Additionally, L-C16-carnitine activated JNK, ERK and p38 MAP kinases between 2.5 to 11-fold, and induced cell injury and death within 6 hours with modest activation of the apoptotic caspase-3 protein. L-C16-carnitine rapidly increased intracellular calcium, most clearly by 10 \( \mu M \), 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 ASB-16 qualitatively mimicked the L-C16-carnitine-associated cell stress outcomes, suggesting that the effects of high experimental concentrations of long-chain acylcarnitines are through membrane disruption. Herein, a model is proposed in which acylcarnitine cell membrane interactions take place along a spectrum of cellular concentrations encountered in
physiological-to-pathophysiological conditions, thus regulating function of membrane-based systems and impacting cell biology.
Introduction

Plasma acylcarnitines have long been utilized as surrogate readouts reflecting tissue acyl-CoA pools (6). Thus, these indices are used in newborn screening as diagnostic biomarkers of inherited disorders of metabolism involving enzymatic lesions in lipid and amino acid metabolism, in which there are increases or decreases in specific acyl-CoA tissue pools (7, 37). For example, long-chain fatty acid oxidation disorders (FAOD) are characterized by defects in mitochondrial oxidative enzymes, i.e., long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and carnitine palmitoyltransferase 2 (CPT2) deficiencies, and these lead to increased tissue, blood and urine long-chain fatty acylcarnitines. Under certain FAOD conditions, plasma long-chain acylcarnitines can increase several-fold: L-C16-carnitine (C16-carnitine), for instance, has been reported increase greater than 5-fold, reaching levels between ~5-44 μM in CPT2-deficient newborns (19, 23). Modest increases in tissue or blood long-chain acylcarnitines have also been noted following cardiac ischemia (17, 36, 38) and in type 2 diabetes/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 pro-inflammatory signaling pathways in RAW264.7 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, pro-inflammatory 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 that no evidence that specific Toll-like receptors or other pattern recognition receptors, are driving the pro-inflammatory 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) were increased. Furthermore, in cardiac ischemia, heart muscle acylcarnitines accumulate (17, 36, 38) and long-chain acylcarnitines have been associated with increased cardiac cellular ROS, apoptosis and 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-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. LPS was purchased from List Biologicals (Campbell, CA). Lot tested (Lot #: 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, 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). Acyl-L-carnitines of varying chain lengths and L-carnitine were purchased from Advent Bio (Downer’s Grove, IL), amidosulfobetaine-16 (ASB-16) (3-[N,N-Dimethyl(3-palmitoylaminopropyl)ammonio]-propanesulfonate) (Cat #DG062, Lot #092605) was purchased from G-Biosciences (St. Louis, MO), Toxilight and IL-6 ELISA Assays were purchased from Lonza (Basel, Switzerland) and R&D Systems (Minneapolis, MN), respectively. XTT sodium salt was purchased from Biotium (Hayward, CA) and phenazine methosulfate 98% (PMS) from Acros Organics (Geel, Belgium). Antibodies against phospho–p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cat #4370), total p44/42 MAPK (ERK) (Cat # 9102), p-JNK (Cat #4668), JNK (Cat #9252), p-p38 (Cat #4511), p38 (Cat #8690), Lamin A/C (Cat # 2032), and ER stress antibody sampler Kit (Cat #9956) were all purchased from Cell Signaling Technology (Danvers, MA). The β-tubulin (Clone Tub 2.1, Cat #ab11308) was purchased from Sigma-Aldrich (St. Louis, MO).
Cells and cell culture. C2C12 (Catalog #: CRL-1772 murine myoblast cell line) were purchased from ATCC (Manassas, VA). Cells were cultured in containing 10% FBS (Atlanta Biologicals, premium select FBS, Catalog #: S11595, Lot #: K0109, Lawrenceville, GA), 100 units/mL penicillin and 100 μg/mL streptomycin, 2 mM GlutaMAX™-I (Life Technologies, Grand Island, NY) and 100 μM L-carnitine (Advent Bio, Downers Grove, IL). Since 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 5% CO2 atmosphere until >90% confluent at which time FBS was replaced with 10% horse serum (HyClone, Logan, UT) for 4-5 days to induce myotube differentiation.

Long-term acylcarnitine treatment. Myotubes (4-5 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 in the figure legends. Myotubes were pre-treated for 1 h during starvation and overnight with sample treatment with BAPTA-AM, a calcium chelator, at the concentrations indicated in the figure legends. Conditioned media was harvested and frozen at -20°C and analyzed for IL-6. Media was further analyzed by Toxilight assay for presence of adenylate kinase, a surrogate marker of cytotoxicity.
Short-term acylcarnitine treatment. Fully differentiated myotubes were serum-starved for 2-3 h in 0.25% FBS media and then treated with corresponding L-acylcarnitines at times and doses indicated in figure legends. Plates were then placed on ice, media was removed and discarded, and cells were rinsed twice in ice 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 12000 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 (PVDF, Bio-Rad, Hercules, CA) using a Bio-Rad Trans-Blot Turbo. Membranes were blocked in a 1X PBS and 0.1% (v/v) Tween-20 (Fisher Scientific) (PBST) solution containing 2% w/v 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 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 (staurosporine, thapsigargin and tunicamycin) for 6 h in duplicate or triplicate.
At 6 h, media was harvested and cells were rinsed 2X with cold HBSS, lysed in 1X lysis buffer and replicates were pooled. Media was analyzed for AK 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, IRE-1alpha, BiP and 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 Ca\(^{2+}\)/Mg\(^{2+}\) was added to each well. Twenty-five μL of 2X Live/Dead dye (Life Technologies, Cat# R37601) was added to each well 15 min before imaging. Imaging was done on a Nikon Eclipse Ti microscope with an automated platform and Zyla Andon camera and the data acquired was analyzed using Nikon Elements HCT software. Three to four images were captured per well. The total GFP (green channel fluorescence) and RPE (red channel fluorescence) intensity 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 was removed and fresh media with treatments was added, with 0.20 mg/mL XTT and 0.001 mM PMS activation
Cells were returned to 37°C for 4 h. Absorbance measurements were read on a plate reader at 475nm with a background correction of 690 nm.

Caspase assay. C2C12 myotubes were pre-treated 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 co-treated 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 once with cold HBSS and lysed in 25 μL of reporter lysis buffer per well (Promega Cat# E3971). The samples were stored at -20°C. For assay, lysate samples were thawed quickly at 50°C and mixed on an orbital shaker for 15 min at room temperature, after which 20 μL was transferred to white round bottom 96-well plates, and 20 μL of the Caspase 3/7 Glo working reagent was added to each sample (Promega Cat # G8091). The plate was incubated (covered) at RT 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 2X in warm HBSS (phenol-red free) and 100 μL phenol-red free 0.25% DMEM was 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) was added to each well using the Synergy 2 injectors.
Fluorescence readings were taken immediately following injection and continuing every 2 seconds for a 1 min duration. Baseline to maximum values were calculated by subtracting the initial fluorescent reading value from the highest value recorded.

Data analysis. Each experiment was performed a minimum of 3 independent times, as indicated in the Results; typical experiments each included at least 3 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 to one another) post-hoc tests, and data are presented as mean ± SE. Statistical analyses were performed using PrismGraph 6.0 (GraphPad Software, San Diego, CA).
Results:

Acylcarnitine chain-length effects on IL-6 production. Since our previous studies showed that C14- and C16-acylcarnitine can elicit pro-inflammatory 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 L-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). L-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), L-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 10-25 μM were not evaluated. These effects increased in a dose-dependent manner up to 100 μM, the highest concentration tested. Adenylate kinase 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. Since 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 MAP kinase pathway. Our previous work demonstrated that C14-carnitine increases phosphorylation of the JNK and ERK mitogen-activated protein (MAP) kinases 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 10-25 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. Since 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 utilized 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. 3C,D) coupled to AK
measurement (Fig. 3E) 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 (UPR) pathways that can occur through a disruption in lipid homeostasis (4).
After 6 h of acylcarnitine treatment, none of the ER stress markers IRE1-alpha, 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 up-regulation of cleaved caspase-3 in the cells (Fig. 4B, inset showing ~10-fold increase vs. control). 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 pro-apoptotic pathways, but that the observed cell death and membrane permeability to AK was not specifically 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 in C2C12 myotubes. C16-carnitine rapidly elicited a dose-dependent increase in intracellular calcium when measured by a fluorescent calcium indicator using a microplate reader (Fig. 5A-B). While 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
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 utilized to decrease free intracellular calcium. BAPTA-AM completely blunted the stimulation of IL-6 by 25 μM C16-carnitine, and caused an approximately 78% drop at 50 μM (Fig. 5C). Notably, intracellular calcium chelation had no effect on TLR receptor-mediated IL-6 production from LPS treatment (data not shown). While 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 pro-inflammatory 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 concentrations, membrane integrity may be compromised and cell stress responses ensue. To begin to evaluate this concept, we employed the 16-carbon zwitterionic compound amidosulfobetaine-16 (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 lab 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 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. Since 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 MAP kinase 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 pro-apoptotic 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 CPTI (25). While speculative, results from the current studies 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 elicit muscle cell inflammation and stress.

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). While 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 T2DM and insulin resistance. This current study, as well as a previous study (29), raise the possibility that
under certain conditions of inefficient \( \beta \)-oxidation, an abnormal increase in long-chain acylcarnitines may contribute 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 75-100 \( \mu \text{M} \) 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, non-permeabilizing 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 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 purified caspase 3, supporting our assertion that 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), 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 apoptosis and 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, non-specific amphipathic interactions, or a combination of these,
remains to be fully elucidated.
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References:


4. Basseri S, and Austin RC. Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential. Biochemistry research international 2012:


Figure Legends:

Figure 1. L-acylcarnitine chain length dependent induction of the inflammatory cytokine IL-6 and secretion of adenylate kinase (AK) in a C2C12 murine myotube skeletal muscle cell model. Myotubes were serum-starved for 3-4 h (0.25% FBS/DMEM) and then treated for 18 h with varying concentrations (0-100 μM) and chain lengths (C2- to C18:1-) of L-acylcarnitines. Media concentrations of IL-6 (A) and AK (B) were measured by ELISA (R&D Systems Quantikine) and luminescence (Lonza Toxilight) assays, respectively. n=6 or more over 3 experiments. Two way ANOVA with Dunnett’s test (post-hoc) for dose response: *, ***, ****, p<0.05, 0.001, 0.0001, respectively vs. vehicle-treated control; mean ± SE. Data are expressed as fold of vehicle control. Mean vehicle IL-6 = 27.4 pg/mL.

Figure 2. C16-carnitine increases phosphorylation of the JNK, ERK and p38 MAP kinases in both a dose- and time-dependent manner. C2C12 myotubes were serum-starved for 3-4 h (0.25% FBS/DMEM) before treatment with varying doses of C16-carnitine (0-50μM) for the indicated times. Cell lysates were assessed via electrochemiluminescent ELISA (Meso Scale Discovery) and western blot (representative blot from 4 separate experiments). Maximum phosphorylation of p-JNK (A), p-ERK (B) and p-p38 (C) over basal was quantitated by ELISA (p-JNK, p-ERK) and densitometry (p-p38). Maximum was calculated from n=5 per treatment over 4 experiments. One way ANOVA with Dunnett’s test: **p<0.01, ***p<0.001 vs. basal; mean ± SE. Data are expressed as fold- over basal.
Figure 3. Dose-dependent cell death occurs within 6 hours of C16-carnitine treatment. C2C12 myotubes were serum-starved for 4 h (0.25% FBS/DMEM) then treated with varying concentrations of C16-carnitine (0-100µM). Concentrations greater than 40 µM resulted in increased AK release after 6 h (A). At 6 h conversion of XTT to its formazan product (B), a live/dead imaging assay (live: green, dead: red) (C,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 three experiments. One way ANOVA with Dunnett’s test: *p<0.05, ***p<0.001; mean ± SE. Data are expressed as fold- over vehicle.

Figure 4. C16-carnitine weakly activates apoptotic pathways, but not ER stress and cannot be rescued from death by caspase inhibitors in C2C12 myotubes. C16-carnitine weakly elicits the cleavage of caspase-3, but does not appear to induce markers of ER stress (IRE1-alpha, BiP, and CHOP) in myotubes (A). Inhibition of caspases does not affect release of AK (i.e. cell death) (B). Caspase inhibitors effectively inhibit staurosporine (STS) induced caspase 3/7 activity (B, inset). Three independent experiments done in duplicate (n = 6 per bar). mean ± SE. Data are expressed as fold over vehicle.
Figure 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 pre-loaded with Calcium Green-1 AM (10 μM) (A,B) or BAPTA-AM (7.5 μM) (C,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 the Methods section. Fluorescence trace is representative of 5 experiments (A). Myotubes were treated for 18 h with C16-carnitine (0, 25, 50 μM) with and without BAPTA-AM (7.5 μM) (C,D). Media IL-6 and adenylate kinase were analyzed. One way ANOVA with Tukey’s test: **p<0.01, ****p<0.0001 vs. basal; mean ± SE. Data are expressed as fold over vehicle.

Figure 6. ASB-16 elicits similar effects on IL-6 cytokine production and AK secretion in C2C12 myotubes as C16-carnitine. C2C12 cells were starved and pre-loaded as done previously, then treated with ASB-16 (0, 5, 10 or 25 μM) or ionomycin (1 μM). As seen with C16-carnitine, ASB-16 increased intracellular calcium fluorescence in a dose-dependent manner (A,B). ASB-16 also increased secretion of both IL-6 (C) and AK (D) in a dose-dependent manner. BAPTA-AM was also able to reduce IL-6 secretion but, as seen with C16-carnitine, could not prevent the myotubes from increased permeabilization to AK.
Figure 7. Proposed hypothesis and model for acylcarnitine action on cells and cell membranes. At modest concentrations, zwitterionic long-chain acylcarnitines interact with cellular membranes, which influences membrane-associated systems (i.e., certain receptors and receptor complexes) (left panel). This could be a natural physiological phenomenon that serves to link metabolism to cellular function. At least under certain conditions, the acylcarnitine-membrane associations can increase intracellular calcium, activate inflammatory pathways or decrease insulin-stimulated phosphorylation of Akt and glucose uptake. At higher concentrations of long-chain acylcarnitines that elicit cell membrane disruption, cell permeabilization ensues (right panel). Under this condition, excessive tissue long-chain acylcarnitine accumulation, singly or in concert with other stressors, may contribute to pathology outcomes (i.e. episodic myopathies in FAOD or cardiomyocyte death following cardiac ischemia).
Modestly-increased acylcarnitines impact activity of membrane-associated systems

Pathological, high acylcarnitines compromise cellular function

[Diagram showing various pathways and molecules, such as PRR, MyD88, COX-2, Akt, IR, [Ca^{2+}], cleaved caspase-3, IL-6, MAPK, JNK, ERK, p38, and pro-inflammatory genes and cytokines (macrophages).]

= Long-Chain Acylcarnitine