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Ceramide-mediated depression in cardiomyocyte contractility through PKC activation and modulation of myofilament protein phosphorylation

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Abstract

Although ceramide accumulation in the heart is considered a major factor in promoting apoptosis and cardiac disorders, including heart failure, lipotoxicity and ischemia-reperfusion injury, little is known about ceramide's role in mediating changes in contractility. In the present study, we measured the functional consequences of acute exposure of isolated field stimulated adult rat cardiomyocytes to C₆-ceramide. Exogenous ceramide treatment depressed the peak amplitude and the maximal velocity of shortening without altering intracellular calcium levels or kinetics. The inactive ceramide analog C₆-dihydroceramide had no effect on myocyte shortening or [Ca²⁺]_i transients. Experiments testing a potential role for C₆-ceramide-mediated effects on activation of protein kinase C (PKC) demonstrated evidence for signaling through the calcium-independent isoform, PKC ϵ . We employed 2 dimensional electrophoresis and anti-phospho-peptide antibodies to test whether treatment of the cardiomyocytes with C₆-ceramide altered myocyte shortening via PKC dependent phosphorylation of myofilament proteins. Compared to controls, myocytes treated with ceramide exhibited increased phosphorylation of myosin binding protein-C (cMyBP-C), specifically at Ser273 and Ser302, and troponin I (cTnI) at sites apart from Ser23/24, which could be attenuated with PKC inhibition. We conclude that the altered myofilament response to calcium resulting from multiple sites of PKC-dependent phosphorylation contributes to contractile dysfunction that is associated with cardiac diseases in which elevations in ceramides are present.

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CONFLICT OF INTEREST

None

Keywords

lipotoxicity; diabetes; troponin; myosin binding protein C

INTRODUCTION

There is emerging evidence that lipid accumulation within the myocardium is a mechanism in heart failure associated with obesity and diabetes mellitus. Ceramide, which has been deemed a cardiotoxin, is elevated in animal models of obesity [7, 40, 67] as well as in heart failure patients [8]. In addition, studies have identified ceramide as a significant contributor to the development of dilated cardiomyopathy in animal models of lipotoxicity [40], as well as to the worsening of contractile impairment in the progressively failing heart under high levels of circulating fatty acids [41]. Despite these findings, surprisingly little is known about ceramide's effects in directly altering cardiac contractility.

Our current knowledge of ceramide's ability to alter myocellular mechanics is based on studies using exogenous application of the short-chain analog C₂-ceramide to isolated rat cardiomyocytes. These studies reported a positive inotropic response to ceramide treatment, which was associated with both alterations in calcium fluxes and the sensitivity of the myofilaments to calcium [32, 46]. However, these studies were unable to determine the mechanism underlying such alterations. Moreover, treatment of cells with C₂-ceramide has been shown to produce effects that are either less potent than endogenous ceramide [19] or non-specific due to its highly hydrophilic nature [60]. C₆-ceramide, which mimics endogenous ceramide generation [14], is now recognized as a more physiologically-relevant means to assess the effects of ceramide *in vitro*. Studies in skeletal muscle using either C₆-ceramide or endogenously generated ceramide have shown reductions in maximal force production [14, 15], findings that are more consistent with the depressed contractile force typically associated with ceramide accumulation.

Our goal in the present study was to determine the role of ceramide in modulating myocyte contractility through use of C₆-ceramide. Furthermore, we sought to define the mechanism by which ceramide alters contractility. To do this we treated isolated adult rat ventricular cardiomyocytes with C₆-ceramide and assessed shortening mechanics and [Ca²⁺]_i transients, as well as post-translational modifications of the major myofilament proteins. Our findings demonstrate that acute treatment of isolated cardiomyocytes with C₆-ceramide induces a negative inotropic response through activation of PKC ϵ . Additionally, assessment of the post-translational state of the myofilaments following ceramide treatment showed significant increases in PKC-mediated phosphorylation of cardiac troponin I (cTnI) and cardiac myosin binding protein-C (cMyBP-C).

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats between 180–200 g were used for cell isolation experiments. All animal procedures were conducted according to the guidelines instituted by

the Animal Care and Use Committee at the University of Illinois at Chicago and the National Institutes of Health.

Isolation of Rat Ventricular Myocytes

Myocytes were isolated as previously described [62] Briefly, rats were heparinized (5000U/kg body weight) and then anesthetized with 100 mg/kg body weight of pentobarbital sodium, intraperitoneally. Animals were assessed for proper depth of anesthesia and then hearts were quickly excised and cannulated via the ascending aorta. Hearts were perfused at 37°C for 3 mins with a bovine serum albumin (1mg/mL) added calcium-free control solution (BSA-CS) of the following composition (in mM): NaCl 133.5, KCl 4.0, NaH₂PO₄ 1.2, HEPES 10.0, MgSO₄ 1.2, glucose 11.1; pH 7.4 with NaOH. Cells were then perfused with oxygenated BSA-CS containing 0.25 mg/mL type II collagenase (Worthington) and 0.03 mg/mL protease (Sigma-Aldrich) for 15 to 20 mins. After digestion, the ventricles were removed, and dissociated by trituration for 10 mins at 37°C in BSA-CS + 50 µM calcium. The cell suspension was filtered through a mesh collector and cells were resuspended in fresh BSA-CS containing first 100 µM calcium, then 200 µM, 500 µM, and finally 1 mM calcium. The cells were stored at room temperature (22–23°C) and used immediately, through to 4 hours post-isolation.

Experimental Solutions

In experiments measuring intact cell shortening and [Ca²⁺]_i transients, 1.25 -10 of the cell-permeable ceramide analog C₆-ceramide (*N*-hexanol-D-erythro-shingosine, Sigma-Aldrich) 5 µM C₆-dihydroceramide (Avanti Lipids), 1 µM Chelerythrine chloride (Sigma-Aldrich), 5 µM Go6976 (Tocaris), or 5 µM Go6983 was used.

Measurements of Intact Cell Shortening and Calcium Transients

Cells were loaded at room temperature with 3 µM fura 2-AM (Invitrogen) stock (in DMSO) diluted in CS-BSA with 1 mM Ca²⁺, for 10 mins in the perfusion chamber. Following loading, de-esterification of the fura 2 indicator was accomplished with 10 min perfusion using a dye-free CS + 1.5 mM Ca²⁺ solution. Cells were field stimulated at 0.5 Hz and intracellular calcium ([Ca²⁺]_i) transients and unloaded cell shortening were recorded simultaneously as previously described [53]. Recordings were stored in an acquisition software program (Felix32, Photon Technology International) for later analysis offline. [Ca²⁺]_i was measured as a function of the ratio of 340/380 wavelength emission after subtraction of the background fluorescence. [Ca²⁺]_i transient decay times (τ_{decay}, ms) were evaluated by a monoexponential fit to the declining phase of the [Ca²⁺]_i transient. After baseline (control) measurements, the perfusion solution was changed to the appropriate treatment solution and recordings were repeated at the appropriate time, as indicated in the figure legends.

Cell Culture

Isolated cells were plated for 1 hr in creatine-carnitine-aurine (CCT) medium (Medium 199, Cellgrow) on 60-mm dishes pre-coated with 10 µg/µL mouse laminin (Sigma-Aldrich). Plates were then washed twice with CCT medium to remove any dead cells and the medium

was replaced with either the selected treatment medium or normal CCT (control). Cells were incubated for 5 mins in the selected condition medium at 37°C and then washed and collected in ice-cold buffer. Cell pellets were obtained following centrifugation at 12,000 x g for 5 min.

Western Blot

Cell pellets were lysed in UTC Buffer (8 M urea, 2 M thiourea, 4% CHAPS) with sonication and stored at -80°C for further Western Blotting or 2-D DIGE (see below). Protein lysates (20µg) from cells were separated on 12% SDS gels followed by transfer of the protein to a 0.2 µm polyvinylidene difluoride (PVDF) membrane for Western blot analysis. Membranes were blocked and then re-incubated overnight at 4°C in primary antibody, [phospho-cTnI (Ser23/24) (Cell Signaling), 1:400; cTnI (C5 clone, Fitzgerald) 1:5,000; phospho-Tm (Ser283) 1:10,000; Tm (CH-1, Iowa Hybridoma) 1:1,000; [cMyBP-C (C0-C1), 1:10,000; phospho-cMyBP-C (Ser273), 1:2,000; phospho-cMyBP-C (Ser282), 1:2,000; phospho-cMyBP-C (Ser302), 1:10,000; GAPDH (Santa Cruz), 1:1,000]. Membranes were washed for 30 mins in tris-buffer saline with 0.1% Tween-20 (TBS-T) and then incubated in secondary antibody [goat anti-mouse (Sigma-Aldrich) 1:40,000 for cTnI; donkey anti-rabbit (Santa Cruz) 1:20,000 for cMyBP-C; goat anti-rabbit (GE Healthcare) 1:20,000 for all others] conjugated to horseradish peroxidase for 1 hr at room temperature. Membranes were washed for 30 mins in TBS-T then developed by enhanced chemiluminescence on a ChemiDoc XRS + imager (BioRad). Band densities were analyzed using ImageLab software (BioRad).

2 Dimensional Difference Gel Electrophoresis (2-D DIGE)

2-D DIGE was performed as previously described [26, 59]. Briefly, protein lysates (100 µg) were cleaned using a 2D Clean-up Kit (GE Healthcare) and the precipitated protein was resuspended in UTC buffer. Samples were then labeled with cyanine dyes (CyDye, GE Healthcare). 40 µg of sample was mixed with IEF buffer (8 M urea, 2 M thiourea, 4% w/v Chaps, 10 mM EDTA pH 8.0, 250 mM DTT, plus ampholytes) and samples were isoelectrically focused on nonlinear 18 cm immobilized pH gradient (IPG) 4–7 pH or 7–11 pH strips (GE Healthcare) using the standard protocols. After focusing, samples were equilibrated in 1% DTT (w/v) dissolved in IEF Equilibration buffer (IEF-EQ; 6 M Urea, 5% SDS (w/v), 30% glycerol (v/v)), then in 2.5% iodoacetamide (w/v) dissolved in IEF-EQ. The second dimension electrophoresis was run on a 12% SDS resolving gel. Gels were imaged using a Typhoon 9410 Imager (GE Healthcare) with Cy3 (532-nm laser), Cy5 (633-nm laser) and Cy2 (488-nm laser). Images were analyzed using PDQuest version 8 advanced (Biorad).

Subcellular Fractionation

All steps were carried out on ice or at 4°C. Following culturing, cells were manually scraped using 120 µL of ice cold fractionation buffer (FB; 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 0.3 mM sucrose, pH 7.4 with mammalian protease inhibitor cocktail (Sigma, 1:100) and phosphatase inhibitor cocktail (Calbiochem, 1:100)) and centrifuged for 5 mins at 20,000 x g to wash and collect cells. The supernatant was removed and cell pellets were resuspended in 30 µL of FB. Samples were then homogenized with a micro ground glass Dual homogenizer for 1 min (Figure 1, Fraction a – whole cell homogenate) and

centrifuged at 1,000 x g for 5 mins. The supernatant fraction was saved and the pellet re-homogenized 3 additional times with the final pellet (*Fraction b*) ultimately discarded. Supernatant fractions from each homogenization were pooled together (*Fraction c – enriched sample*). Cytosolic and particulate fractions were then separated by ultracentrifugation at 40,000 x g for 30 mins. The resulting supernatant fraction was kept as the cytosolic fraction (*Fraction d – cytosolic*) and the pellet was resuspended in sucrose-free FB with 1% Triton, incubated for 1 hr (*Fraction e – particulate*), then centrifuged at 80,000 x g for 15 mins. The resulting supernatant fraction was kept as the membrane fraction (*Fraction f – membrane*) and pellets were discarded (*Fraction g*). Prior to experimentation, successful fractionation of the cytosol and membrane was confirmed using Western blotting with antibodies against the cytosolic protein GAPDH and the membrane protein Na⁺/K⁺ ATPase (Millipore; 1:5,000) (Fig. 1). Protein samples (20 µg) of cytosolic and membrane fractions were separated by SDS-PAGE and protein was transferred to 0.2 µm nitrocellulose membrane. Membranes were stained with MemCode (Pierce Biotechnology) and imaged on a Chemidoc XRS+ imager to quantitate total protein. After the stain was removed, membranes were then incubated overnight at 4°C in primary antibody [PKCε (Millipore), 1:1,500; PKCδ (Santa Cruz), 1:500; PKCζ (Santa Cruz), 1:500] diluted in TBS-T with 2.5% milk. Membranes were and incubated in secondary antibody (goat anti-mouse (Sigma) 1:50,000 for Na⁺/K⁺ ATPase; goat anti-rabbit (GE Healthcare), 1:40,000 for all others) conjugated to horseradish peroxidase for 1 hr at room temperature. Membranes were and developed by enhanced chemiluminescence on the ChemiDoc XRS+ imager. Band densities were analyzed using ImageLab software and normalized to the total protein density in each respective lane. Cytosolic and membrane fractions from each sample were expressed as the percentage of total (cytosolic + membrane).

Statistical Analysis

Values are presented as means ± SEM. For measures of unloaded cell shortening and [Ca²⁺]_i transients, where 3 groups were compared statistical analyses, were performed using one-way ANOVA along with a multi-comparison Newman-Keul's post-hoc test to make comparisons among groups. All other data were analyzed using a paired or unpaired Student's *t* test, as appropriate. A level of *p* < 0.05 was considered significant throughout.

RESULTS

Acute treatment of isolated cardiomyocytes with ceramide leads to a depression in contractility without altering Ca²⁺ transients

First we assessed the functional consequence of exogenous ceramide exposure in both a concentration- and a time-dependent manner. As depicted in Figure 2A & B, treatment of isolated rat cardiomyocytes for 5 min with C₆-ceramide decreased the peak amplitude of cell shortening in a concentration-dependent manner, with a maximal decrease of 25.1 ± 3.4% at 5 µM concentration. Importantly, treatment with vehicle (0.05% DMSO) had no effect on cell shortening. The ceramide-mediated decrease in peak amplitude of shortening was also time-dependent, as shown in Figure 2C & D. Regardless of time or concentration studied, ceramide exposure had no effect on the peak Ca²⁺ transient or the rate of transient decline (τ), as assessed by the fura 2 ratio (Fig. 2A *Insert*). Based on these initial studies, further

characterization of the functional effects of ceramide on cell mechanics and intracellular $[Ca^{2+}]_i$ was conducted using 5 min exposure of myocytes to 5 μ M C₆-ceramide.

Figure 3A shows an overlay of cell shortening and $[Ca^{2+}]_i$ transient at base-line (control) and after ceramide treatment. In these experiments, the average resting cell length at base-line was $108.2 \pm 7.5 \mu$ m and did not differ with ceramide treatment. Acute exposure of isolated cardiomyocytes to 5 μ M C₆-ceramide resulted in a significant depression in the peak shortening amplitude (base-line = $8.8 \pm 0.8\%$ vs. ceramide = $7.3 \pm 0.7\%$). The peak rate of contraction was also reduced following ceramide treatment (base-line = $86.0 \pm 7.9 \mu$ m/s vs. ceramide = $72.7 \pm 6.6 \mu$ m/s), as was the peak rate of relaxation (base-line = $56.7 \pm 4.2 \mu$ m/s vs ceramide = $50.8 \pm 3.4 \mu$ m/s), although to a lesser extent (Figure 3B). These changes in cellular mechanics following ceramide treatment occurred independently of changes in $[Ca^{2+}]_i$, demonstrated by the lack of an effect of ceramide treatment on peak $[Ca^{2+}]_i$ amplitude (fura ratio; base-line = 0.34 ± 0.06 vs ceramide = 0.36 ± 0.07), the decay time constant, τ (base-line = 281.6 ± 23.9 ms vs ceramide = 290.0 ± 23.6 ms) (Figure 3C) and the resting $[Ca^{2+}]_i$ (base-line = 0.31 ± 0.03 vs ceramide = 0.31 ± 0.03). Table 1 shows the effects of ceramide, along with all treatments used, on the parameters of cell shortening and $[Ca^{2+}]_i$ expressed as a percentage of base-line measures.

To test the specificity of ceramide's functional effect, we exposed cardiomyocytes to C₆-dihydroceramide, an inactive ceramide analog [2], or 0.05% DMSO (the concentration of DMSO present in the ceramide and dihydroceramide perfusion buffers) and measured cell shortening and $[Ca^{2+}]_i$ transients. As shown in Figure 4, both DMSO and dihydroceramide failed to alter any of the parameters of cell shortening or $[Ca^{2+}]_i$ transients. These findings indicate that the negative inotropic response induced by ceramide is specific to ceramide's known bioactivity. Moreover, the negative inotropic response to ceramide treatment in the presence of unaltered $[Ca^{2+}]_i$ suggests downstream alterations at the level of the myofilament.

PKC ϵ mediates ceramide's negative inotropic response

To address whether PKC is involved in mediating ceramide's effects on cardiomyocyte function we repeated measures of intact cell shortening and $[Ca^{2+}]_i$ transients in isolated myocytes in the presence of ceramide + the general PKC inhibitor chelerythrine chloride. Cardiomyocyte contractile parameters were unchanged from base-line levels when exposed to ceramide in the presence of chelerythrine chloride (Fig. 5A), implying that ceramide signals through PKCs to depress cardiomyocyte contractility. The peak shortening amplitude was $8.1 \pm 1.8\%$, $7.8 \pm 1.8\%$ and $7.9 \pm 1.9\%$ for base-line, chelerythrine chloride treatment alone and ceramide + chelerythrine chloride treatment, respectively. Maximal shortening velocity was $82.9 \pm 15.3 \mu$ m/s, $80.3.7 \pm 15.4 \mu$ m/s, $81.2 \pm 15.4 \mu$ m/s and maximal relengthening velocity was $50.3 \pm 11.5 \mu$ m/s, $48.1 \pm 11.5 \mu$ m/s, and $49.1 \pm 11.4 \mu$ m/s for base-line, chelerythrine chloride treatment alone and ceramide + chelerythrine chloride treatment, respectively. Measurements of $[Ca^{2+}]_i$ transients were also unchanged among all groups (Table 1).

It is known that rat cardiomyocytes express 4 isoforms of PKC, α , δ , ϵ and ζ [42]. Of these, isoform α is of the conventional class of PKCs and is dependent on calcium and

diacylglycerol (DAG) for activation, while the other isoforms (novel PKCs, δ and ϵ ; atypical PKC, ζ) are calcium independent [33]. Through use of the conventional PKC inhibitor, Go6976, we were able to demonstrate that ceramide mediates its negative inotropic effects through one of the calcium-independent isoforms of PKC (Fig. 5B). Treatment of cardiomyocytes with Go6976 alone had no effect on cell shortening parameters (peak shortening = $8.3 \pm 0.5\%$ vs base-line = $8.6 \pm 0.4\%$, velocity of shortening = $90.8 \pm 10.9 \mu\text{m/s}$ vs. base-line = $90.8 \pm 9.6 \mu\text{m/s}$, velocity of relengthening = $64.0 \pm 10.8 \mu\text{m/s}$ vs base-line = $62.4 \pm 8.9 \mu\text{m/s}$) or $[\text{Ca}^{2+}]_i$ transients (peak fura amplitude = 0.44 ± 0.07 vs base-line = 0.43 ± 0.06 , decay time constant (τ) = $234.6 \pm 16.0 \text{ ms}$ vs base-line = $228.5 \pm 15.2 \text{ ms}$). Moreover, Go6976 was unable to block ceramide's functional effects when cells were exposed to Go6976 + ceramide. This is observed by ceramide's ability to depress peak shortening amplitude ($7.2 \pm 0.5\%$ vs base-line = $8.6 \pm 0.4\%$) and the maximal velocity of shortening ($74.3 \pm 10.4 \mu\text{m/s}$ vs base-line = $90.8 \pm 9.6 \mu\text{m/s}$) with a reduced, but insignificant, change in the relengthening velocity (ceramide + Go6976 = $54.2 \pm 9.0 \mu\text{m/s}$ vs base-line = $62.4 \pm 8.9 \mu\text{m/s}$). Additionally, in the presence of Go6976 neither the $[\text{Ca}^{2+}]_i$ transient (peak fura amplitude for ceramide + Go6976 = 0.41 ± 0.06 vs base-line = 0.43 ± 0.06), or decay time constant (τ) (ceramide + Go6976 = 243.6 ± 17.8 vs base-line = $228.5 \pm 15.2 \text{ ms}$) was unaltered among the treatment groups. These data were confirmed with use of G06983, another inhibitor of PKC which is selective for the α , β , δ , and γ isoforms [64] (see Table 1).

Subcellular fractionation and immunoblotting of the calcium-independent PKC isoforms were used as a reliable means to indirectly measure PKC activation through translocation. To do this, cardiomyocytes were fractionated into cytosolic and membrane compartments and the relative amount of each calcium-independent PKC isoform within that compartment was then determined using Western blotting. Cardiomyocytes were also treated with 100 nM of phorbol-12-myristate-13-acetate (PMA), a potent activator of conventional and novel PKCs, as a positive control, for PKC δ and ϵ . As expected, treatment of cardiomyocytes with 100 nM PMA resulted in translocation of virtually all of PKC ϵ and PKC δ into the membrane fraction ($98.60 \pm 0.58\%$ and $91.50 \pm 0.05\%$, respectively, $n=3$), while the subcellular distribution of PKC ζ remained unchanged by PMA treatment (membrane fraction = $33.60 \pm 2.92\%$ vs cytosolic fraction = $66.43 \pm 2.92\%$, $n=3$) (Fig. 6A). Interestingly, ceramide treatment resulted in a significant shift in the subcellular distribution of PKC ϵ , with $28.80 \pm 3.80\%$ localized within the membrane fraction compared to $16.67 \pm 1.90\%$ in untreated (control) samples. The distribution of PKC δ (membrane fraction = $55.32 \pm 3.60\%$ in ceramide treated vs $63.01 \pm 2.60\%$ in untreated controls) and PKC ζ (membrane fraction = $20.23 \pm 6.72\%$ in ceramide treated vs $37.53 \pm 8.51\%$ in untreated controls) within the myocytes remained unchanged by ceramide treatment (Fig. 6B).

Ceramide treatment results in increased phosphorylation of cTnl and cMyBP-C

Acute changes in cardiomyocyte function, as observed here, are likely the result of transient post-translational protein modifications. Moreover, it is well-known that such post-translational modifications of myofilament proteins lead to alterations in contractility [27, 54]. Therefore, we tested whether acute exposure of cardiomyocytes to ceramide, or ceramide in the presence of the PKC inhibitor chelerythrine chloride had an effect on the

post-translational state of myofilament proteins using 2D-DIGE, which allows for analysis of the post-translational state of the sarcomeric proteome on a global level. Figure 7 shows a representative scans of Cy5-labeled untreated (control) sample, Cy2-labeled ceramide treated sample and Cy3-labeled ceramide + chelerythrine chloride sample run within the same gel, along with the representative merged image for direct comparison. Spots associated with cTnI are labeled in the merged image, with a shift in the migration of phosphorylated cTnI spots toward the acidic region of the strip in an additive fashion (i.e. acidity of P4>P3>P2, etc.). Analysis of the samples showed an increase in the tris-phosphorylated (P3) spot alone in samples treated with ceramide ($22.8 \pm 1.4\%$ vs control = $14.0 \pm 2.2\%$, n=7), which was attenuated by PKC inhibition ($15.1 \pm 2.7\%$). Further 2D-DIGE analysis of the other myofilament proteins showed no significant differences in the post-translational state of Tm, RLC or cTnT (Fig. 8).

Assessment of site-specific myofilament protein phosphorylation upon C6-ceramide treatment was also assessed using phospho-peptide antibodies, where available. We used phospho-peptide antibodies (gift from Dr. Sakthivel Sadayappan) against 3 of the known phosphorylation sites for cMyBP-C. Figure 9A shows a concentration-dependent increase in the intensity of the immunoblot signal for Ser-273 and Ser-302 without changes in the Ser-282 signal in ceramide treated samples. Quantification of samples confirmed an increase in the phosphorylation state of Ser-273 (1.5-fold) and Ser-302 (1.6-fold) with no difference in Ser-282 phosphorylation (1.1-fold). Immunoblotting with anti-phospho-peptide antibodies was also carried out for cTnI at Ser23/24 and for Tm at Ser283 (gift from Dr. David Wiecek). As shown in Figure 9B & C, no difference in phosphorylation at these sites was observed in untreated vs. ceramide treated cardiomyocytes.

Previous studies have shown phosphorylation of cMyBP-C at Ser-273, Ser-282 and Ser-302 to be mediated by PKA, whereas only two of these sites, Ser-273 and Ser-302, are phosphorylated by PKC [34]. This, coupled with the observed increase in the phosphorylation state of cTnI at sites apart from Ser23/24 (putative PKA sites) strongly support the likely involvement of PKC in mediating ceramide's negative inotropic effects.

DISCUSSION

Although ceramide accumulation in the heart is known to adversely affect cardiac function, the present study demonstrates for the first time that ceramide directly alters cardiomyocyte contractility through modulation of the post-translational state of the myofilament proteins. Moreover, our data strongly support ceramide signaling to the myofilaments via PKC ϵ (Fig. 10).

Previous studies have concluded that depressions in contractility observed when ceramide levels are elevated are due primarily to cell death via ceramide-stimulated apoptosis. However, our functional observations in intact cardiomyocytes revealed that ceramide directly regulates contractile function demonstrated by a depression in shortening amplitude and velocity following ceramide exposure, which occurred despite unaltered $[Ca^{2+}]_i$ levels or kinetics. Moreover, the potential ceramide-mediated effects on apoptosis and cell death are most likely not relevant inasmuch as these were acute experiments. Although the

observed changes were modest (17–25% reduction), they would be projected to reduce force production and power output (the product of force and velocity) in the intact myocardium and contribute significantly to depressed contractile function

The mechanism by which ceramide-mediated activation of PKC might alter contractile mechanics is likely to involve both the allosteric regulation of crossbridges reacting with the thin filament and crossbridge dynamics independent of thin filament activation. PKC dependent phosphorylation of the myofilaments *in vitro* has been shown to reduce Ca^{2+} -activated actomyosin ATPase, myofilament Ca^{2+} sensitivity and cooperativity with observed depressions in the maximal tension generation [6, 25, 28, 35–39, 44, 56]. Functionally, these changes are associated with reduced force production and shortening velocity, both loaded and unloaded [31, 55], resulting in depressed power output [23]. While reductions in isometric tension generation can be attributed to an altered activation state of the myofilaments, previous studies demonstrate that shortening velocities at zero load are unaffected by myofilament activation [13, 18] and are more likely to depend on crossbridge cycling [24]. However, exactly how PKC-dependent phosphorylation of the myofilaments modifies crossbridge dynamics is unclear. Whereas earlier studies by Pyle et al [45] identified PKC-mediated effects on crossbridge detachment rates, as demonstrated by increased tension cost (an estimate of the crossbridge detachment rate [4, 11]) in transgenic mice expressing an unphosphorylatable mutant cTnI (cTnI-S43A/45A), more recent studies have shown unaltered tension costs, and therefore unaltered crossbridge detachment rates in transgenic (TG) mice in which all 3 PKC sites were pseudophosphorylated by glutamic acid substitution [26]. The authors, instead, attributed the negative inotropic response in TG mice to a decreased rate of crossbridge reaction with thin filaments and a Ca^{2+} -independent persistence of the active state. Despite the discrepancy, in general PKC-mediated phosphorylation has been shown to reduce crossbridge cycling rate [36, 37, 39] and would be expected to prolong the duty cycle, consequently reducing shortening kinetics during ejection. This is likely also to be true for the PKC-dependent effects we observed following ceramide treatment. Moreover, evidence from others indicates that the effects we observed in isolated cardiomyocytes may be more pronounced in the intact, auxotonically-loaded heart [29], translating to a marked reduction in pressure development, decreased extent of shortening during ejection and overall impairment of systolic function.

An important and novel finding of our study was that ceramide's negative inotropic response was due to PKC-mediated phosphorylation of the myofilament proteins cTnI and cMyBP-C. Our biochemical assessment revealed increases in the phosphorylation of cMyBP-C at the PKC sites upon ceramide exposure, as well as increases in the phosphorylated form (P3) of cTnI which was prevented by inhibition of PKC. Previous studies support the idea that the phosphorylated P3 spot likely contains phosphorylation of cTnI on Ser-43/45 [1, 50, 51, 66]. Specifically, it has been shown that the higher charged, more acidic cTnI species identified by isoelectric mobility in IEF gels includes phosphorylation on the PKC sites [51], while the vast majority of mono-phosphorylated (cTnI_p) and bis-phosphorylated (cTnI_{pp}) cTnI found in the basal state contain cTnI phosphorylated at Ser-23/24 [1, 50, 66].

Although it is clear that the net phosphorylation state of myofilament proteins is critical for determining the overall contractile effect in the intact heart, our data in isolated, unloaded

cardiomyocytes support a dominate role for cTnI phosphorylation in mediating ceramide's negative inotropic effects. Functionally, PKC-mediated phosphorylation of cTnI *in vitro*, specifically at Ser-43/45, has been shown to reduce isometric tension and the calcium-activated ATPase rate by promoting the blocked state of thin-filament activation [25, 39, 45], an effect which is likely to alter the economy of muscle contraction. Indeed, a recent study by Hinken et al [23] showed PKC treatment of skinned rat cardiomyocytes reduced force production and loaded shortening velocity resulting in depressed power output. Phosphorylation of cTnI was shown to play a dominate role in these responses. Consistent with that study are data presented here demonstrating reduced shortening amplitude and velocity due to alterations in the post-translational state of the myofilaments. *In vivo* phosphorylation of cTnI at Ser43/45 has been shown to play a main role in the contractile dysfunction and progression to failure associated with PKC ϵ overexpression, demonstrated by rescue of the PKC ϵ overexpressing mouse phenotype when endogenous cTnI was partially replaced with a non-phosphorylatable cTnI at Ser43/45 (S43/45A) [51]. Interestingly, a study using mice, which constitutively express pseudo-phosphorylated cTnI at Ser43/45 and Thr144 (cTnI_{PKC-P}), showed that minimal changes (7%) in cTnI phosphorylation at the PKC sites were sufficient to cause contractile dysfunction [26]. It is worth noting here that the observed increase in phosphorylation induced by ceramide treatment in the current study was about 8%. The aforementioned study suggests that in the context of the whole heart this degree of modification would be sufficient to impair contractility.

Unexpectedly, our observed increase in cMyBP-C phosphorylation was not associated with an increase in the rate of contraction or relaxation, as has been previously described [9, 49, 58]. One explanation for this lack of increase in contraction and relaxation rates is that increases in phosphorylation of the PKC sites at Ser273 and Ser302 without changes in Ser-282, as shown here, may impact contractile mechanics differently than when all three sites on cMyBP-C are phosphorylated to the same extent. In fact, studies examining the role of cMyBP-C phosphorylation on contractile mechanics have focused solely on PKA-mediated phosphorylation of cMyBP-C, in which case all three sites, Ser273, Ser282 and Ser302, are phosphorylated. However, Sadayappan et al [48] have shown that pseudo-phosphorylation of cMyBP-C at the PKC sites was not sufficient to rescue contractile dysfunction in the cMyBP-C null background, while Ser282 alone was. The authors suggest that phosphorylation of Ser282 is essential for cMyBP-C's ability to regulate contractility and may be the major determinate of cMyBP-C phosphorylation-induced alterations. Moreover, Florea et al [17] found that constitutive phosphorylation of PP1 I-1 at its PKC sites resulted in depressed systolic function that was associated, in part, with reduced phosphorylation of cMyBP-C at Ser282. Future studies examining the functional differences between PKA and PKC-mediated phosphorylation of cMyBP-C may shed light on more recent findings. A second explanation is that perhaps phosphorylation of cMyBP-C does little to change relengthening kinetics in *unloaded* cell measurements. The notion of a load-dependence of relaxation under auxotonic conditions has long been established [5]. Moreover, in the unphosphorylated state cMyBP-C has been proposed to conform to a 'viscous-load model' in which it produces an opposing drag-force through its ability to bind actin. This effect is reduced when phosphorylation of cMyBP-C blunts its binding to actin,

allowing for less force-opposition and faster contraction and relaxation kinetics [61]. In this case, the influence of cMyBP-C phosphorylation may be reduced in the context of unloaded cardiomyocytes used here, particularly in terms of relengthening and may, therefore, account for our lack of change in relengthening velocity following ceramide treatment, despite an increase in cMyBP-C phosphorylation.

Given that measurements were taken in isolated cardiomyocytes at room temperature (22–24°C) and not at physiologic temperatures of 37°C, we cannot completely rule out the possibility of altered effects of ceramide at body temperature. Reductions in temperature have been shown to reduce the relative contribution of SR Ca²⁺ release to inotropy [52], as well as accelerate relaxation by both increasing calcium removal from the cytosol [43] and by reducing myofilament calcium sensitivity [21]. The latter may partially explain why increased calcium sensitivity owing to MyBP-C phosphorylation was not observed in this study.

Beyond the acute effects shown here, ceramide-induced activation of PKC may also be of significance in cardiac remodeling and the development of dilated cardiomyopathy associated with long-term elevations in ceramide [16, 40, 65, 67]. Activation of PKCs directly by ceramide has been shown to occur in the heart, leading to both insulin resistance [8] and reduced β -adrenergic signaling [12]. Likewise, it is well known that PKC activation induces hypertrophic growth within the heart [3, 47, 57, 63] and may further play a role in modifying actin capping dynamics [22] to promote addition of new sarcomeres into the myofilament lattice. Thus we speculate that chronic elevations in ceramide may be one of the mechanistic triggers of cardiac decompensation, which leads to contractile dysfunction, ventricular dilation and the progression to failure. This hypothesis is supported by studies from Park et al [40] who found that inhibition of *de novo* ceramide biosynthesis in LpL_{GPI} overexpressing mice, which develop lipotoxic-induced dilated cardiomyopathy, resulted in reduced apoptosis, enhanced myocardial energetics and improved overall systolic function and survival. Interestingly, in a rat model of metabolic syndrome, elevations in TAG and long-chain acyl CoAs (ceramide content was not measured) caused membrane translocation and activation of PKC ϵ leading to contractile dysfunction [10]. Nevertheless, future studies examining ceramide accumulation in human diseases of cardiac lipotoxicity will be critical for establishing a role for ceramide as a lipotoxic mediator in human pathology.

Although novel, these findings are limited by the use of exogenous treatment of a non-biological ceramide analog. The shorter chain length differs from endogenously produced ceramide, which is composed of a sphingoid base with an acyl chain length ranging from 14–28 carbons, the most abundant of which C20:0 and C24:0 in the heart [30]. Nevertheless, it has been demonstrated that 1–20 μ M of short-chain ceramide analog administered exogenously to cells results in a cellular accumulation of ceramide that is within the range of endogenous levels and which elicits specific biological responses [20]. Moreover, *in vitro* use of C6-ceramide has been shown to promote negative inotropic responses similar to endogenously generated ceramide [15]. In the failing human heart, 1.5 fold elevations in total ceramide content have been shown [8]. This increase was associated with higher insulin resistance in heart failure patients, mediated by activation of PKCs. Interestingly, implantation of a left ventricular assisted device was able to return ceramide levels to

normal, attenuated PKC activation and improved insulin resistance. This study highlights the clinical relevance of moderate elevations in ceramide content and PKC activation.

Conclusion

In summary, this study provides a novel mechanism by which ceramide may depress cardiac contractility through activation of PKC and subsequent phosphorylation of myofilament proteins and further suggests that accumulation of ceramide in pathological conditions should be considered as a significant factor contributing to the depression in cardiac function.

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Fig. 1. Western blot verifying efficient subcellular fractionation

Representative Western blot of GAPDH and Na⁺/K⁺ ATPase performed on samples taken during each step of the subcellular fractionation protocol (see *Materials and Methods*). The presence of GAPDH predominately within the cytosolic fraction and Na⁺/K⁺ ATPase only within the corresponding membrane fraction confirms proper subcellular fractionation.

Fig. 2. Concentration and time-dependent effects of exogenous C₆-ceramide treatment on contractile mechanics and [Ca²⁺]_i in ventricular cardiomyocytes

(A) Overlay of unloaded cell shortening measurements from a single cardiomyocyte under field stimulation at 0.5 Hz at baseline and during treatment with sequentially increasing concentrations of C₆-ceramide. *Insert*, overlay of simultaneous Ca²⁺-transient measures corresponding to each cell shortening measure. (B) The quantified change in the percentage of cell shortening relative to control following treatment with vehicle (DMSO) and each concentration of C₆-ceramide. * *p* < 0.01 vs DMSO; *n* = 6. (C) Representative cell shortening measurements taken under steady-state pacing at baseline conditions and following 30 sec, 2 min and 5 min of exogenous 5 μM C₆-ceramide treatment. ((D) The quantified change in the percentage of cell shortening relative to control during the time course for 5 μM C₆-ceramide treatment. **p* > 0.05 vs. Baseline, #*p* < 0.01 vs. 0.5 min, *n* = 8.

Fig. 3. Effects of acute C_6 -ceramide treatment on contractile mechanics and $[\text{Ca}^{2+}]_i$ in ventricular cardiomyocytes

(A) Overlay of representative unloaded cell shortening measurements with simultaneous Ca^{2+} transient measurements in a single cardiomyocyte under field stimulation (0.5 Hz) before (black) and after (red) exogenous treatment with 5 μM C_6 -ceramide. (B) Quantification of the contractile parameters (percent of shortening and maximal rates of contraction and relaxation) and (C), Ca^{2+} transient parameters (peak amplitude of the Fura-2 ratio and the time constant, τ , for the declining phase of the transient). * $p < 0.05$ vs Control, ** $p < 0.001$ vs Control, $n = 8$.

Fig. 4. Assessment of functional specificity using the non-bioactive sphingolipid C₆-Dihydroceramide

(A) Quantification of contractile parameters (percent of shortening and maximal rates of contraction and relaxation) before treatment, after vehicle treatment (0.05% DMSO) and following treatment with 5 μM C₆-dihydroceramide. (B) Quantification of Ca²⁺ transient parameters (peak amplitude of the Fura ratio (340 nm/380 nm) and the time constant, τ, for the declining phase of the transient) before treatment, after vehicle treatment and following treatment with 5 μM C₆-dihydroceramide. *n*=5.

A.

B.

Fig. 5. Attenuation of the functional effects of acute C₆-ceramide treatment by inhibition of PKCs

(A) Quantification of contractile parameters (percent of shortening and maximal rates of contraction and relaxation) following treatment with 1 μM chelerythrine chloride and 1 μM chelerythrine chloride + 5 μM C₆-ceramide. $n=6$ (B) Quantification of contractile parameters (percent of shortening and maximal rates of contraction and relaxation) following treatment with 5 μM Go6976 and 5 μM Go6976 + 5 μM C₆-ceramide. * $p<0.01$ vs Control, $n=6$.

Fig. 6. Subcellular fractionation of ventricular cardiomyocytes treated with C₆-ceramide to assess translocation of the calcium-independent PKC isoforms

(A) Representative Western blots of the calcium-independent PKC isoforms in the cytosolic and membrane subcellular fractions of isolated cardiomyocytes in control cells, cells treated with 100 nM PMA, and cells treated with 10 μM C₆-ceramide. (B) Quantification of the cytosolic and membrane subcellular fractions from control and C₆-ceramide treated samples. White bars represent the amount of PKC in the cytosolic fraction and grey bars represent the amount of PKC within the membrane fraction. Each band density was normalized to the total protein within that lane. * $p < 0.05$ vs Control, $n = 3$.

Fig. 7. The role of PKC in C₆-ceramide-mediated effects on cTnI phosphorylation
cTnI, cardiac troponin I; P, phosphorylated cardiac troponin I. *Top*, images of the cTnI region of interest from Cy5 labeled control samples (channel 1), Cy2 labeled samples treated with 10 μ M C₆-ceramide (channel 2) and Cy3 labeled samples treated with 10 μ M C₆-ceramide in the presence of 1 μ M Chelerytherine chloride (Channel 3) and the merged image (channel 1 + 2 + 3). Samples were pooled together and focused in the first dimension on a 18 cm nonlinear IPG 7–11 pH strip followed by standard 12% SDS-PAGE for the second dimension. *Bottom*, quantitative results for cTnI phosphorylation spots 1–4 in which each spot density is shown relative to the total density for all cTnI spots. * $p < 0.05$ as indicated; $n = 7$.

Fig. 8. 2D-DIGE of myofilament protein phosphorylation for Tm, RLC, cTnT and cMyBP-C
Tm, tropomyosin; RLC, regulatory light chain; cTnT, cardiac troponin T, MyBP-C, myosin binding protein-C; P, phosphorylation, M, modification. A, *Top*, representative gel images for the region of interest including Tm, RLC and cTnT are shown in a merged image of control samples labeled with Cy3 (pseudo-colored green) and C₆-ceramide treated samples labeled with Cy5 (pseudo-colored red). *Bottom*, Results from the quantification of phosphorylation sites from Tm, RLC and cTnT expressed as a percentage of all spot density for the protein of interest. B, *Top*, representative gel image for cMyBP-C shown as a merged image of control samples labeled with Cy3 and C₆-ceramide treated samples labeled with Cy5. *Bottom*, Results from the quantification of modification sites on cMyBP-C expressed as a percentage of all spot density. * $p < 0.05$ vs Control; $n = 7$.

Fig. 9. Site-specific immunoblotting for cMyBP-C, Tm and cTnI phosphorylation

(A) *Left*, Representative images from Western blots using cMyBP-C phospho-specific antibodies against Ser-273, Ser-282 and Ser-302 in untreated samples and in samples treated with increasing doses of C₆-ceramide. *Right*, Quantification of cMyBP-C phosphorylation levels in isolated cells treated with and without 10 μM C₆-ceramide at Ser-273, Ser-282 and Ser-302. GAPDH was used as a loading control. *n*=5 (B) Western blot quantification of isolated cells treated with and without 10 μM C₆-ceramide using a phospho-Tm (Ser283) antibody. *n*=5 (C) Western blot quantification of isolated cells treated with and without 10 μM C₆-ceramide using a phospho-cTnI (Ser23/24) antibody. *n*=6; **p*<0.05 vs Control.

Fig. 10. Schematic diagram of the mechanism by which ceramide leads to negative inotropy Accumulation of ceramide within the cardiomyocyte results in activation of PKCε, leading to phosphorylation of the myofilament proteins cTnI and cMyBP-C. These post-translational modifications within the sarcomere alter the basal function and cause a reduction in the ability of the contractile machinery to generate force.

Table 1
Parameters of Cellular Mechanics and Intracellular Ca^{2+} Transients (% Control)

	DMSO (n = 5)	5 μM C_6 -ceramide (n = 8)	1 μM Chel Cl (n = 6)	5 μM C_6 -ceramide + Chel Cl (n = 6)	5 μM Go6976 (n = 6)	5 μM C_6 -ceramide + 5 μM Go6976 (n = 6)	5 μM Go6983 (n = 7)	5 μM C_6 -ceramide + 5 μM Go6983 (n = 7)
Peak Shortening	99.8 $\pm$ 1.3	83.3 $\pm$ 1.9*	93.4 $\pm$ 2.6	93.4 $\pm$ 3.1	96.3 $\pm$ 1.5	80.4 $\pm$ 3.4*	97.1 $\pm$ 1.9	84.2 $\pm$ 2.6*
- dL/dt	101.2 $\pm$ 1.8	84.9 $\pm$ 2.0*	96.1 $\pm$ 2.8	97.8 $\pm$ 2.0	98.7 $\pm$ 2.1	81.9 $\pm$ 4.1*	99.4 $\pm$ 1.2	87.4 $\pm$ 3.9*
+ dL/dt	105.2 $\pm$ 3.5	90.4 $\pm$ 3.2*	96.0 $\pm$ 4.5	98.6 $\pm$ 4.7	98.2 $\pm$ 3.6	89.6 $\pm$ 5.2	102.4 $\pm$ 2.0	91.0 $\pm$ 5.2
Peak $[\text{Ca}^{2+}]$ Amplitude	99.9 $\pm$ 2.9	102.0 $\pm$ 3.9	102.5 $\pm$ 2.6	108.3 $\pm$ 4.1	102.1 $\pm$ 4.7	100.3 $\pm$ 7.8	97.4 $\pm$ 2.2	101.6 $\pm$ 3.2
Tau (τ)	101.2 $\pm$ 3.3	99.3 $\pm$ 3.4	119.3 $\pm$ 13.3	107.5 $\pm$ 7.3	103.7 $\pm$ 5.7	106.9 $\pm$ 4.3	102.8 $\pm$ 3.0	100.8 $\pm$ 3.2

Results are means $\pm$ S.E.M.

* $p < 0.05$ where paired observations are found to be statistically different based on a one-way ANOVA with a Newmann-Keuhl's post-hoc analysis. Chel Cl: Chelerythrine Chloride.