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Fatty Acid-Induced Apoptosis in Neonatal Cardiomyocytes: Redox Signaling

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ABSTRACT

Exposure of neonatal rat cardiac myocytes to palmitate and glucose produces apoptosis as seen by cytochrome *c* release, caspase 3-like activation, DNA laddering, and poly(ADP-ribose) polymerase cleavage. The purpose of this study was to understand the role of reactive oxygen species in the initiation of programmed cell death by palmitate. We found that palmitate (but not oleate) produces inhibition of carnitine palmitoyltransferase I, accumulation of ceramide, and inhibition of electron transport complex III. These events are subsequent to cytochrome *c* release and loss of the mitochondrial membrane potential. No differences in H_2O_2 production or N-terminal c-Jun kinase phosphorylation were detected between myocytes incubated in palmitate and control myocytes (non-apoptotic) incubated in oleate. These results suggest that the palmitate-induced loss of the mitochondrial membrane potential is not associated with H_2O_2 synthesis and that a membrane potential is required to generate reactive oxygen species following ceramide inhibition of complex III. Antioxid. Redox Signal. 3, 71–79.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) are important regulators of apoptosis in many systems (for review, see 19). One-electron reduction of oxygen has been shown to occur upon cytochrome *c* release from mitochondria in HL 60 cells (7), during cardiomyocyte hypoxia (11), and resulting from ceramide interaction with mitochondrial complex III (12, 15). ROS have also been proposed to be involved in the initiation of apoptosis by thiol modification of the mitochondrial adenine nucleotide translocase and stimulation of the mitochondrial permeability transition (MPT) (16). The MPT is mediated by opening of a pore in the mitochondrial inner membrane. The observation that the pore is modulated by agents that regulate the mitochondrial adenine nucleotide translocase suggests that this transporter may be part of the pore complex. In addition, amphipathic anions such as fatty acids may open the pore by altering the mitochondrial surface potential independent of depolarization (2, 5). Long-chain acyl-CoAs also competitively interact with ADP binding site(s) and stabilize the "c" conformation of the adenine nucleotide translocase (28).

Ischemia and reperfusion of ischemic heart lead to cell death, scar formation, and eventually contractile failure. Many studies have implicated ROS production in myocardial ischemia and reperfusion injury, often implying a causal relationship between ROS production

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and cell death in this pathological condition. Recent work in heart, both *in vivo* and *in vitro* (14), suggests that a portion of myocyte death can be attributed to programmed cell death. In hypoperfused zones, apoptotic cell death may contribute to ventricular remodeling, a process where regulated cell death takes place in selected cells and is less arrhythmogenic than cells with primary membrane dysfunction and large areas of necrosis (6).

As mitochondria are the source of ROS production and the initiators of apoptotic signaling, changes in substrate availability and utilization may influence the primary function of mitochondria, i.e., to provide ATP for contractile activity. Fatty acids and glucose are the major fuels for cardiac energy production under conditions of aerobic perfusion. In adults, high levels of circulating fatty acids are associated with increased fatty acid oxidation and decreases in glucose utilization. High circulating fatty acids are observed following cardiac ischemia and are preferentially oxidized at the onset of myocardial reperfusion, at which time ATP production become uncoupled from contractile function (23). Fatty acid overload and apoptosis of cardiac myocytes are also observed in obesity and are associated with reduced cardiac contractility and cardiac dilatation (35). Recent work by ourselves (18) and others (10) has demonstrated a role for saturated, but not unsaturated, long-chain fatty acids in apoptotic myocyte death. Because saturated fatty acids are precursors of ceramide synthesis as well as inhibitors of the mitochondrial adenine nucleotide translocase, palmitate may induce ROS generation and pore opening by either or both of these pathways. The present studies capitulate the role of palmitate in apoptotic myocyte death and examine the potential action of ROS in this process.

MATERIALS AND METHODS

Primary cardiac myocyte culture

Neonatal rat cardiac myocytes were prepared as previously described (27) using 1–2-day-old Sprague–Dawley rat pups. The purity of the isolated cardiac myocytes, estimated by counting striated cells stained with the filamentous actin-specific probe, Bodipy-phallicidin (Molecular Probes, Eugene, OR, U.S.A.), was ~95% (5.5 ± 2.77% fibroblast contamination). Myocytes were plated at 2×10^6 cells per 60-mm dish and maintained in Dulbecco's modified Eagle medium containing 0.3 g/L glutamine, 4.5 g/L glucose, and 10% calf serum for 72 h. The medium was then replaced with 0.5 m*M* fatty acid (palmitate or oleate) bound to 1.6% bovine serum albumin (BSA) (13) in the absence of serum. The final concentration of fatty acid in the stock solutions was measured using a semimicro analysis kit (Wako Chemicals, Neuss, Germany).

Measurements of apoptosis

DNA isolation and electrophoresis and caspase 3-like activity: These were measured as previously described (18).

Immunoblotting: For detection of cytochrome *c* release, the cytosolic contents of cardiac myocytes incubated in the presence of oleate or palmitate were separated by digitonin fractionation as previously described (27). Samples of cytosolic protein (50 μg) were analyzed by immunoblotting using an antibody to denatured cytochrome *c* (Pharmingen, San Diego, CA, U.S.A.). Mitogen-activated protein (MAP) kinase immunoblots were carried out using whole cardiac cell lysates where phospho-specific antibodies were expressed as ratios of the nonphosphorylated MAP kinase antibodies. Rabbit polyclonal antibodies to extracellular signal-regulated kinase (ERK) and phospho-ERK (New England Biolabs, Beverly, MA, U.S.A.), phospho-p38 (CalBiochem, San Diego, CA, U.S.A.), p38, and N-terminal c-Jun kinase (JNK and phospho-JNK) (Santa Cruz, Santa Cruz, CA, U.S.A.) were stained using goat anti-rabbit horseradish peroxidase (Santa Cruz). Gel band intensities were quantified using ImageSpace software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Poly(ADP-ribose) polymerase (PARP) cleavage: Whole myocytes were lysed in 1% Nonidet P40, 0.1% sodium dodecyl sulfate, 0.5% Triton X-100 in 0.5 ml of phosphate-buffered saline and analyzed for PARP cleavage using a rabbit polyclonal antibody to the carboxy terminus of PARP (Santa Cruz).

Hydrogen peroxide generation: Oxidant genera-

tion studies were carried out on glass coverslips (31). The cells were incubated for 3 or 19 h in fatty acid-containing medium; 10 μM (final concentration) of the peroxide-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DFDA) (Molecular Probes) was then added in dimethyl sulfoxide. The medium was removed after 1 h and the cells washed and placed in Dulbecco's modified Eagle medium without serum. Intracellular fluorescence was monitored using a temperature-regulated Wallach/Olympus America Concorde real-time fluorescence imaging spectrophotometer (Melville, NY, U.S.A.). Control cells were treated with 6 mM hydrogen peroxide for 5 min.

Fatty acid oxidation and carnitine palmitoyltransferase I (CPT-I) activity

Oxidation of [^{14}C]oleate and [^{14}C] palmitate was measured by a modification of the method of Awan and Saggerson (1), as previously described (18). CPT-I activity was measured in neonatal rat cardiac myocytes permeabilized in the presence of 30 μM digitonin (27). The assay medium contained 0.5 ml of permeabilization medium in the absence of digitonin, 1% BSA, 30 μM palmitoyl-CoA, and 1 mM L-[^{14}C]carnitine (specific activity 3,200 dpm/nmol). Linear rates of malonyl-CoA-sensitive palmitoylcarnitine synthesis (without and with 100 μM malonyl-CoA) were assayed as previously described by this laboratory (27).

Cellular ceramide

Cells were cultured in the presence of 0.5 mM oleate or 0.5 mM palmitate in the presence of 1.6% BSA, and ceramide was measured at the indicated times using the *sn*-1,2-diacylglycerol kinase method of Van Veldhoven *et al*. (32).

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi$) was measured on glass coverslips using the potential-sensitive fluorescent dye tetramethylrhodamine ethyl ester (TMRE; Molecular Probes). Image analysis was performed using a wide-field fluorescent microscope (Applied Precision, Issaquah, WA, U.S.A.) with DeltaVision deconvolution software and ImageSpace analysis software (Molecular Dynamics). The ratio of fluorescence intensity inside each mitochondrion to the fluorescence intensity outside is directly proportional to the mitochondrial $\Delta\Psi$ (22).

Statistical analysis

ANOVA was used when comparing the significance of incubation of cardiac myocytes in the presence of either oleate or palmitate as a function of treatment time. The ANOVA analysis reduced to Student's t test for nonpaired variates when measurements on one variable were compared either directly with control or under two different experimental conditions. Data are presented as the means ± SE.

RESULTS

Neonatal rat cardiac myocytes incubated for 20 h in the absence of serum or in the presence of oleate (plus 1.6% BSA and glucose) demonstrated no DNA fragmentation on agarose gels (10, 18). In contrast, incubation with palmitate/BSA/glucose resulted in DNA-laddering patterns consistent with internucleosomal DNA cleavage (Table 1) (10, 18). Palmitate also induced the release of cytochrome *c* from the mitochondria into the cytosol of the cardiac myocytes. This release was the earliest pro-apoptotic event measured in the palmitate-in-

TABLE 1. COMPARISON AND TIME COURSE OF APOPTOTIC EFFECTS IN OLEATE/BSA AND PALMITATE/BSA TREATED CARDIOMYOCYTES

	Oleate	*Palmitate (fold increase)*	*Incubation time (h)*
DNA laddering	—	+	20
Cytochrome *c* release	—	23.0 ± 2.8	12–20
Caspase 3-like activation*	—	2.1 ± 0.1	20

*Hickson-Bick et al., 2000.

cubated cells, with a small amount of release beginning at 4 h and plateauing to 23-fold of baseline (oleate cells) at 12 h of incubation (Table 1). This release suggests mitochondrial permeability pore opening as demonstrated by the retention of 99% of the matrix enzyme, citrate synthase, which is above the molecular weight cutoff of the pore, in the mitochondrial fraction (data not shown). The activation of caspase 3-like activity was increased twofold over background fluorescence (in the presence of oleate alone) by palmitate at 20 h (Table 1) (18). This activation is consistent with cleavage of the DNA repair enzyme PARP, which is a known substrate of caspase 3 (29). PARP cleavage was absent in the oleate-treated cells, but the major cleavage product at 85 kDa was clearly evident at both 16 and 20 h in the palmitate-incubated myocytes (Fig. 1). In summary, four independent measurements of events associated with programmed cell death can be readily demonstrated in cardiac myocytes incubated with palmitate, but not oleate.

We investigated the possibility that inhibition of CPT-I activity was a consequence of palmitate–induced apoptosis. Decreased activity of this key enzyme in the β-oxidation of long-chain fatty acids would diminish fatty acid degradation and augment accumulation of palmitate and its synthetic products, palmitoyl-CoA, triglycerides, and ceramide. Ceramide, a sphingolipid commonly associated with apoptotic signaling, can be synthesized *de novo* from fatty acyl-CoA with the initial enzymatic step demonstrating a preference for palmitoyl-CoA. After 4 h of incubation in palmitate, no significant changes in the rates of β-oxidation or malonyl-CoA-inhibitable CPT-I activity occurred. Likewise, the myocytes demonstrated no significant accumulation of ceramide over the levels measured in the presence of oleate alone (Fig. 2). In contrast, by 20 h, both CPT-I and β-oxidation were significantly depressed in the myocytes incubated with palmitate. These myocytes demonstrated low rates of oxidation when either [^{14}C]palmitate or [^{14}C]oleate was subsequently provided as energy substrates (18). Consistent with the lowered rates of fatty acid degradation, both triglyceride (18) and ceramide became prominent products of palmitate metabolism (Fig. 2).

An essential characteristic of the onset of apoptosis is initiation of the MPT, where pore opening is associated with swelling of the mitochondria, the loss of cytochrome *c*, and eventual dissipation of the mitochondrial $\Delta\Psi$. Using deconvolution microscopy of cardiac myocytes loaded with the potential-dependent dye, TMRE, incubation in palmitate for 20 h induced a dramatic loss in energized mitochondria (Fig. 3). Occasional profiles of mitochondria with a preserved $\Delta\Psi$ could be seen in the cytosol of the palmitate-incubated myocytes (see arrows), but these were greatly reduced in number compared with the oleate-incubated myocytes (Fig. 3). Therefore, the loss in $\Delta\Psi$ in each cardiac myocyte was a heterogeneous process with some energized mitochondria remaining even at late time periods.

There was no significant change in mitochondrial $\Delta\Psi$ during the 20 h of incubation of cardiac myocytes with oleic acid ($p = 0.19$, ANOVA). When the loss in $\Delta\Psi$ with myocytes incubated with palmitate alone was examined, $\Delta\Psi$ was significantly reduced at 8 h ($p = 0.026$) and at all subsequent time intervals ($p < 0.001$ at 20 h). By transforming the data to the percentage of mitochondria that are energized in the palmitate-treated myocytes versus the oleate-incubated controls as a function of time, the time-dependent loss in $\Delta\Psi$ with palmitate, but not oleate, is apparent (Fig. 4). The loss in $\Delta\Psi$ was temporally paralleled by a diminution in the activity of mitochondrial complex III in palmitate-incubated myocytes compared with oleate–incubated cells. This activity in the palmitate cells reached a nadir at 16 h (Fig. 4). As increases in ceramide were measured over the same time period as the decrease in complex III and as ceramide can inhibit complex III, the data are consistent with a direct effect of

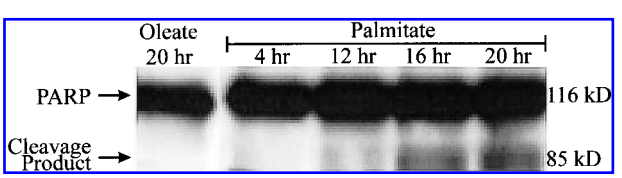

FIG. 1. PARP cleavage. Western blot of total PARP at various fatty acid incubation times in cells treated with palmitate/BSA or oleate/BSA showing full-length PARP and its 85-kDa cleavage product.

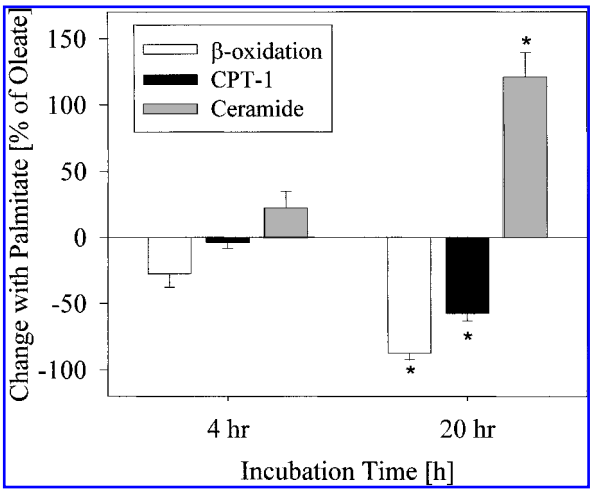

FIG. 2. Fatty acid utilization with palmitate/BSA. β-Oxidation rate, malonyl-CoA-sensitive CPT-I activity, and ceramide production were measured in cardiomyocytes incubated in palmitate/BSA and normalized to those treated with oleate/BSA expressed as a change from the oleate/BSA level. Values are expressed as means ± SE ($n = 3$–6. *$p < 0.01$ compared with controls treated with oleate/BSA).

ceramide accumulation on electron transfer through ubiquinone–cytochrome *c* reductase. Inhibition of electron transfer at this site is associated with the generation of free radicals (4), and the action of ceramide to generate ROS has been proposed to mimic the complex III inhibitor, antimycin A (12). Therefore, the effect of complex III inhibition in the palmitate-incubated myocytes on H_2O_2 production was measured and compared with H_2O_2 content in myocytes incubated with oleate. No differences in H_2O_2 content were observed at either 4 h of incubation (prior to fulminating expression of apoptotic events) or at 20 h when all indicators of apoptosis are present (Fig. 5).

Consistent with the suggestion that ROS-mediated signaling of cell death is not linked to palmitate-induced apoptosis, there were no differences in JNK phosphorylation between myocytes incubated in the presence of either oleate or palmitate at either 4 or 20 h of incubation (Fig. 6). Phosphorylation of ERK and of p38 was evident under both conditions at 4 hours and at 20 h (Fig. 6). However, at 20 h the phosphorylated ratios of ERK and p38 in the palmitate cells compared with the oleate-incubated cells increased 70–80%. This difference in phosphorylated ratios was due in part to increased phosphorylation of ERK (28%) and p38 (50%) in the palmitate-incubated myocytes at 20 h compared with 4 h. In the oleate-incubated myocytes, the phosphorylated forms of ERK and p38 decreased at 20 h compared with 4 h (Fig. 6). Both factors contribute to the differences observed in the palmitate:oleate ratios at 20 h.

DISCUSSION

Ceramide has been proposed to be a critical second messenger in the regulation of signal transduction leading to apoptosis following receptor-mediated stimulation of sphingomyelinases (33). Daunomycin elicits *de novo* ceramide generation and induces apoptosis (3). Thus, we speculated that palmitate (as a saturated fatty acid precursor of ceramide synthesis via palmitoyl-CoA) could drive the cardiac myocytes to apoptotic cell death by ceramide accumulation, whereas oleate (an unsaturated fatty acid) could not. Consistent with this expectation, we observed increases in ceramide in the neonatal cardiac myocytes incubated with palmitate, but not in myocytes incubated with oleate. This increase in ceramide production coincided with

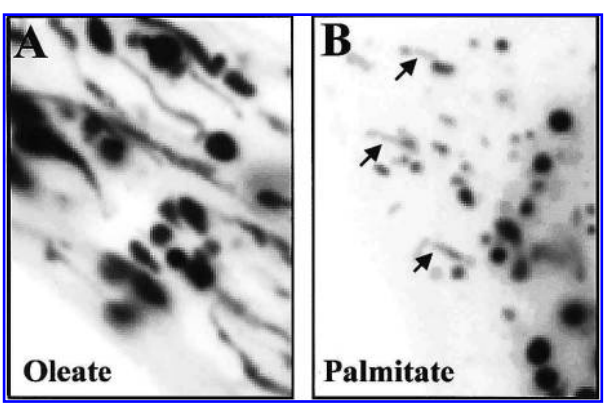

FIG. 3. Imaging of $\Delta\psi$ in individual mitochondria. Deconvolution microscopy was performed on neonatal cardiac myocytes stained with the membrane potential-sensitive indicator, TMRE. Images are portions of the cytosol of single living cardiomyocytes with intensities inverted for clarity. (**A**) The cytosol of a control cardiomyocyte incubated for 18 h in oleate/BSA. (**B**) Cytosol of a representative cardiomyocyte incubated for 18 h in palmitate/BSA. Arrows in (B) identify some of the remaining energized mitochondria. Globular features in (A) are clumps of mitochondria.

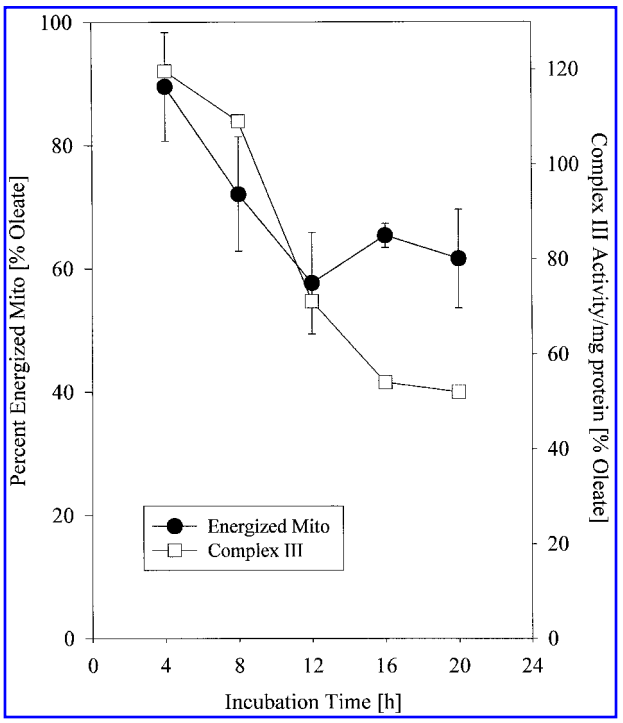

FIG. 4. Percentage of viable mitochondria and complex III activity. The percentage of cytosol containing TMRE-stained (energized) mitochondria was assessed by thresholding fluorescent images of cells treated with palmitate/BSA and normalizing to cells treated with oleate/BSA (●). Values are expressed as means ± SE (n = 3–4). The activity of mitochondrial electron transport complex III in cells incubated with palmitate/BSA was normalized to the activity with oleate/BSA (○).

a decrease in malonyl-CoA-sensitive CPT-I activity and an associated decrease in fatty acid oxidation. This event would drive the cells into fatty acid overload with acyl-CoA incorporated into triglycerides (18), as well as into ceramide.

One mechanism through which ceramide is believed to initiate apoptotic signaling is by generation of ROS by inhibition of mitochondrial complex III (15). Production of ROS is dependent on electron flow, a highly reduced matrix NAD(P)H/NAD(P), and a high $\Delta\Psi$ (12, 17). In our experiments, the production of ceramide and reduced activity of complex III in the palmitate–incubated myocytes are widely separated in time from the release of cytochrome *c* from the mitochondria and the ensuing loss of the mitochondrial $\Delta\Psi$. This suggests that although decreased activity of complex III can be measured *in vitro*, by using an exogenous electron donor and acceptor, its activity in the myocyte where the mitochondria are depolarized is limited by the absence of electron flow to site III. In this setting where $\Delta\Psi$ is absent in the majority of mitochondria from the palmitate-incubated myocytes, one-electron reduction of oxygen cannot occur at levels that can be discriminated from oleate-incubated myocytes. Also, no changes in the levels of phosphorylated JNK in the presence of oleate or palmitate were apparent at times preceding or concurrent with caspase activation. Modulation of JNK by rises in intracellular free radicals has been reported in a variety of cell types and experimental conditions (21, 26, 30). Although p38 and ERK phosphorylation was more pronounced in the palmitate-incubated myocytes at 20 h compared with oleate, their role in signal transduction is more complex. In the heart, both ERK and p38 have been reported to act as anti-apoptotic signaling molecules (8, 9, 34). In contrast, p38 induces (25), or its inhibition attenuates, cardiac apoptotic injury (24). Selective inhibition of ERK has been reported to have no effect on DNA fragmentation or caspase activation in H9c2 cells (30), and blockade of ERK 1/2 pathway has no effect on β-adrenergic receptor-mediated apoptosis (8). In the palmitate-incubated myocytes, elevations in both ERK and p38 phosphorylation, at a time when

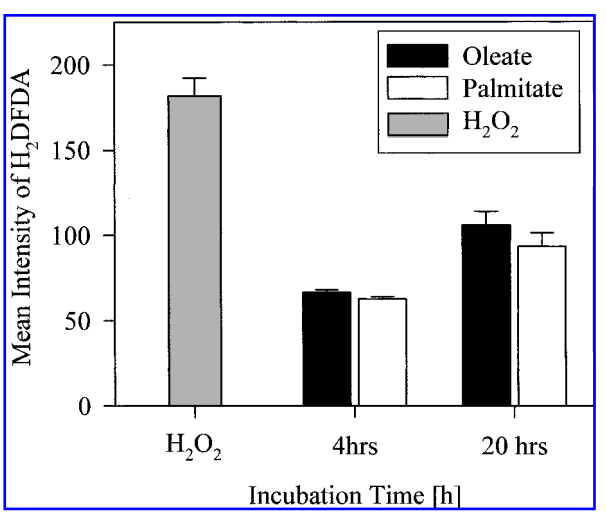

FIG. 5. H_2O_2 production in fatty acid-treated cardiomyocytes. Intensity levels of the H_2O_2-sensitive dye, H_2DFDA, were compared for cardiomyocytes incubated in palmitate/BSA and oleate/BSA or treated with 6 m*M* added H_2O_2 (5 min). Values are expressed as means ± SE (n = 4–5).

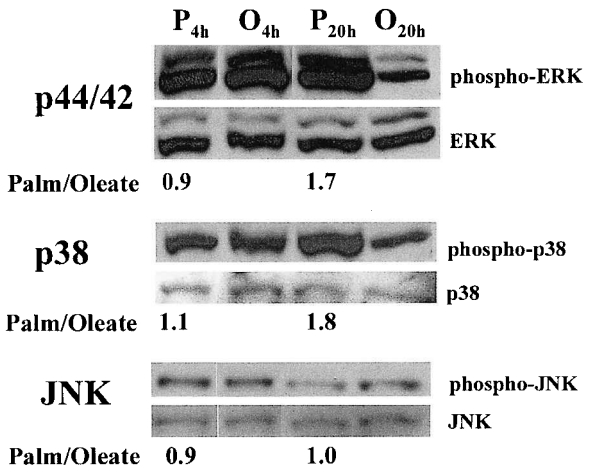

FIG. 6. Phosphorylation of MAP kinases in oleate- and palmitate-treated cardiomyocytes. Immunoblots of lysates from cardiac myocytes incubated with either oleate or palmitate for 4 and 20 h were analyzed by immunoblotting using antibodies to native and phosphorylated ERK (**top**), p38 (**center**), and JNK (**bottom**). The ratios of phosphorylated to nonphosphorylated MAP kinases were compared between the two incubation conditions after quantification of gel intensities as described in Materials and Methods. Results are representative of three different cultures.

caspase is activated, suggest either that these signaling molecules provide insufficient protection against cell death or that p38 is proapoptotic in this model.

The absence of differences in ROS content in the palmitate and oleate cells at an early time point (4 h) also suggests that the event that mediates the MPT and cytochrome *c* release is most likely not a consequence of augmented ROS production in this model. Although both palmitoyl-CoA and oleoyl-CoA are substrates for CPT-I, only myocytes incubated with palmitate lose the function of this important regulatory enzyme in β-oxidation. Modulation of CPT-I activity by membrane lipids is a well known factor influencing both catalytic activity and malonyl-CoA sensitivity of the liver isoform of CPT-I (20). Therefore, it is possible in this model that unopposed exposure of cardiac myocytes to a long-chain saturated fatty acid has significant effects on the composition of mitochondrial and cellular membrane phospholipids. Early onset of the MPT prior to ceramide accumulation and in the absence of ROS levels significantly different from background suggests that palmitate is having an action on pore opening that takes place by a pathway that precedes and is alternative to ceramide accumulation. One such pathway is the well characterized inhibition of the adenine nucleotide translocase complex by long-chain acyl-CoA or alterations in the phospholipid environment of this complex such that the conformation of this transmembrane protein becomes more rigid or "locked" into a cytosolic or "c" domain. These possibilities are being examined in ongoing studies.

In summary, incubation of cardiac myocytes with palmitic acid bound to BSA provides a model of lipoapoptosis that can be characterized both temporally and mechanistically. We have provided evidence that accumulation of ceramide in the palmitate-incubated myocytes does not occur in oleate cells. In keeping with previous studies, the cells that accumulate ceramide are well along on the pathway to programmed cell death. In contrast to suggestions that ceramide is a second messenger to apoptotic myocyte death, this model of lipoapoptosis provides evidence that the initiation of the apoptotic cascade occurs hours before the inhibition of CPT-I and the synthesis of ceramide in these cells. Also novel is our finding that ROS production is not likely to be a consequence of the ceramide-related inhibition of mitochondrial complex III. The absence of a substantial number of energized mitochondria in the palmitate-incubated myocytes argues against a role for ROS generation at site III. This observation is consistent with our findings that cellular concentrations of H_2O_2 are no different between control (oleate) and palmitate-incubated cardiac myocytes at times that are coincident with inhibition of complex III. Although this model of apoptosis uses physiologically relevant levels of long-chain fatty acids in the incubation medium, it is clear that the heart is never exposed to saturated fatty acids as the sole lipid available for energy metabolism. However, recent studies do suggest that *chronic* exposure of hearts of obese Zucker rats to fatty acid overload will eventually lead to steatosis and lipoapoptosis as a consequence of aging (35). Thus, the investigation in myocytes over a limited time frame may form the physiological basis for chronic effects of fatty acid overload and obesity on contractile dysfunction.

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ABBREVIATIONS

BSA, bovine serum albumin; CPT-I, carnitine palmitoyltransferase I; ERK (p42/44), extracellular signal-regulated kinase; H_2DFDA, 2′,7′-dichlorodihydrofluorescein diacetate; JNK, N-terminal c-Jun kinase; MAP kinase, mitogen-activated protein kinase; MPT, mitochondrial permeability transition; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; $\Delta\Psi$, membrane potential.

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