A metabolic role for mitochondria in palmitate-induced cardiac myocyte apoptosis

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Sparagna, Genevieve C., Diane L. Hickson-Bick, L. Maximilian Buja, and Jeanie B. McMillin. A metabolic role for mitochondria in palmitate-induced cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 279: H2124–H2132, 2000.—After cardiac ischemia, long-chain fatty acids, such as palmitate, increase in plasma and heart. Palmitate has previously been shown to cause apoptosis in cardiac myocytes. Cultured neonatal rat cardiac myocytes were studied to assess mitochondrial alterations during apoptosis. Phosphatidylserine translocation and caspase 3-like activity confirmed the apoptotic action of palmitate. Cytosolic cytochrome c was detected at 8 h and plateaued at 12 h. The mitochondrial membrane potential (ΔΨ) in tetramethylrhodamine ethyl ester-loaded cardiac myocytes decreased significantly in individual mitochondria by 8 h. This loss was heterogeneous, with a few energized mitochondria per myocyte remaining at 24 h. Total ATP levels remained high at 16 h. The ΔΨ loss was delayed by cyclosporin A, a mitochondrial permeability transition inhibitor. Mitochondrial swelling accompanied changes in ΔΨ. Carnitine palmitoyltransferase I activity fell at 16 h; this decline was accompanied by ceramide increases that paralleled decreased complex III activity. We conclude that carnitine palmitoyltransferase I inhibition, ceramide accumulation, and complex III inhibition are downstream events in cardiac apoptosis mediated by palmitate and occur independent of events leading to caspase 3-like activation.

fatty acids; mitochondrial permeability transition; cytochrome c

APOPTOSIS, or programmed cell death, has been studied in many cell types. The series of events comprising the apoptotic process may vary, but the data available in vertebrates indicate that the earliest events in apoptosis are associated with mitochondria (55). These organelles undergo the mitochondrial permeability transition (MPT) (for review see Refs. 3 and 4), the opening of a large pore in the mitochondrial inner membrane, which allows ions and small molecules (<1,500 mol wt) to pass through. Pore closure is stabilized by the immunosuppressant cyclosporin A. It has been postulated that release of cytochrome c from the mitochondrial intermembrane space is mediated through the MPT (44) due to swelling that occurs as a result of pore opening, although this is controversial (2, 48). Cytochrome c, once released, becomes part of an apoptosis complex (with apoptosis-activating factor 1 and caspase 9), which then activates caspase 3, a cysteine protease involved in later apoptotic events (34). There is evidence that accumulation of the sphingolipid ceramide may be linked to caspase 3 activation (18, 23, 30), but the results are controversial and dependent on cell type (49, 54). Ceramide accumulation leads to production of reactive oxygen species (ROS) via the mitochondrial electron transport chain (12, 15, 17, 42). During the apoptotic process, phosphatidylserine (PS), a phospholipid normally found on the inside of the plasma membrane, translocates to the outside, marking the cell for eventual digestion by macrophages (13). Later in apoptosis, cleavage of double-stranded DNA (DNA laddering) occurs.

Until recently, it was believed that heart cells do not undergo apoptosis. However, studies (6–8, 36, 50) in diseased cardiac tissue, most notably hearts that have undergone ischemia and reperfusion, have confirmed that apoptosis can occur in cardiac tissue. Healthy cardiac cells use glucose and long-chain fatty acids (LCFA) to meet their energy needs. During cardiac ischemia and reperfusion, it has been found that plasma levels as well as local tissue concentrations of LCFA increase (33, 35). During reperfusion of ischemic tissue, there is increased utilization of LCFA for energy production at the expense of glucose, which exacerbates damage to the heart (29). By increasing glucose during reperfusion, this damage may be partially prevented (27). Circulating plasma fatty acids are primarily composed of the saturated LCFA palmitate (C16:0) and the unsaturated LCFA oleate (C18:1). Recently, it was shown that addition of palmitate to cultured neonatal rat cardiac myocytes causes DNA laddering and in situ DNA strand breaks, confirming that palmitate induces apoptosis in these cells (10, 20). As part of the present study, we have extended this interesting finding by following the apoptosis-specific markers of PS translocation and caspase 3-like protein activation in
cardiac myocytes incubated with palmitate. The sequence of events leading to the palmitate-induced apoptotic response was then studied over the time course of the myocyte cell culture by a combination of microscopic and biochemical approaches.

**MATERIALS AND METHODS**

Primary cell culture. Neonatal rat cardiac myocytes were prepared according to the method reported by McMillin et al. (31) using 1- to 2-day-old Sprague-Dawley rat pups. Myocytes were plated in 60-mm dishes (2 × 10^6 cells) and maintained for 48 h in DMEM containing 0.3 g/l glutamine, 4.5 g/l glucose, and 10% calf serum. The medium was replaced with 0.5 mM fatty acid (palmitic or oleic acid) bound to 1% BSA in DMEM in the absence of serum. Fatty acid bound to BSA in the molar ratio of 2:1 was prepared using the method of Goldstein et al. (16), dialyzed against DMEM, and filter sterilized. The final concentration of fatty acid in the stock solutions was measured using a semimicroanalysis kit (Wako Chemicals).

Mitochondrial membrane potential. Cardiac myocytes were plated onto glass coverslips glued into 35-mm culture plates, and fatty acids were added (see above). In some cases, 1 μM cyclosporin A (Sigma Chemical) was also present. At the indicated time, the medium was removed, and 1 ml of 10 nM tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) (11) in PBS was added. The dye was allowed to equilibrate in the cells for 5 min at room temperature, and individual cells were imaged using a wide-field fluorescence microscope (Applied Precision) with deconvolution software (Delta Vision) by use of a ×60 PlanApo oil objective (Olympus). Image analysis was performed using ImageSpace software (Molecular Dynamics). The potential-dependent identification of mitochondria was consistent with their classic elongated shape. The ratio of fluorescence intensity inside the mitochondria to fluorescence intensity outside is directly proportional to the mitochondrial membrane potential (ΔΨ) (28). To measure these ratios in situ, we defined a region of interest of an individual mitochondrion (eliminating edge-blurring effects from the average intensity measurement) and another region of interest directly in the cytosolic space surrounding the same mitochondrion. To assess the percentage of the cell composed of energized mitochondria, we drew a region of interest in the cell that excluded the nucleus. Within that region, we selected mitochondria by thresholding intensity values. We then determined the percentage of the thresholded pixels within the total area of the region of interest to arrive at a percentage of pixels representing mitochondria.

Cytochrome oxidase immunostaining. Cardiac myocytes were plated on glass coverslips, treated with fatty acids, washed with PBS, fixed with 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100 in PBS. Cells were blocked with 10% goat serum and incubated with mouse anti-cytochrome oxidase, subunit IV (Molecular Probes), and then with goat anti-mouse Cy5 secondary antibody (Amersham). Mitochondria were visualized as described above with use of an excitation wavelength of 640 nm and an emission wavelength of 685 nm.

Separation of cytosolic and membrane fractions and cytochrome c immunoblotting. Cells were plated in 60-mm culture dishes (2 × 10^6 cells) and incubated in fatty acids, as described above. After incubation, the cell medium was removed and the cells were washed three times with serum-free, fatty acid-free DMEM. The plasma membrane was then permeabilized using digitonin (30 μM) and "permeabilizing medium," as previously described (31). The supernatant, representing the cytosolic contents (monitored by lactate dehydrogenase release), was collected. The membrane-containing pellet was suspended in buffer (0.5 ml of 1% Nonidet P-40, 0.1% SDS, 0.5% Triton X-100 in PBS). Samples (50 μg of cytosolic protein or 3 μg of membrane protein) were analyzed for the presence of cytochrome c by Western blotting and probed using an antibody to denatured cytochrome c (PharMingen). Gel band intensities were quantified using ImageSpace software (Molecular Dynamics).

Citrine synthase activity. Citrate synthase activity was measured in the cytosolic and membrane fractions used for measurement of cytochrome c. Citrate synthase activity was determined on a microplate reader (VersaMAX, Molecular Dynamics) according to the method of Srere (47) in the presence of acetyl-CoA and oxaloacetate, with correction for changes in absorbance due to acetyl-CoA hydrolysis activity.

Caspase 3-like activity. Caspase 3-like activity was measured by following the cleavage of the fluorescent substrate acetyl-Asp-Glu-Val-Asp (DEVD)-7-amino-4-methylcoumarin (Calbiochem), as described previously (20).

Ceramide assay. Myocytes were grown in 60-mm culture dishes (2 × 10^6 cells) and incubated with fatty acids, as described above. Cells were harvested, lipids were extracted, and the ceramide content was measured using a modification (40) of the sn-1,2-diaclylglycerol kinase (Calbiochem) assay (51). Ceramide levels were normalized to cellular phospholipid.

Carnitine palmitoyltransferase I activity. Carnitine palmitoyltransferase I (CPT-I) activity was measured in neonatal cardiac myocytes permeabilized in the presence of 30 μM digitonin. The assay medium contained 0.5 ml of permeabilizing medium in the absence of digitonin, 1% BSA, 30 μM palmitoyl-CoA, and 1 mM L-[14C]carnitine (specific activity 3,200 dpm/nmol). Linear rates of malonyl-CoA-sensitive (≥100 μM malonyl-CoA) palmitoylcarnitine synthesis were assayed as previously described by this laboratory after 20 min at 37°C (31).

Activity of electron transport complexes III and IV. Complex III (decylubiquinol:ferricytochrome c oxidoreductase) activity was quantified using the method of Ragan et al. (43) as modified by Gudz et al. (17). Cells were grown on 60-mm plates (2 × 10^6 cells), and fatty acids were added. After incubation, cells were washed three times with PBS, and 0.1 ml of 5% octylglucopyranoside was added to each plate. After incubation at 37°C for 10 min, the cells were scraped off. Cells were subjected to one freeze-thaw cycle, and complex III was measured as an increase in absorbance at 550 nm on 50 μl (100–150 μg) of protein per 1 ml of solution. Antimycin A-insensitive activities were subtracted from each sample. Protein was measured using the bicinchoninic acid method (Pierce). Complex IV (cytochrome oxidase) was quantified by monitoring KCN-sensitivity oxidation of reduced cytochrome c. Cytochrome c (3 mM) was reduced by sodium borohydride, and the pH was brought to 7.0 to eliminate excess reductant. Cells were grown on 60-mm plates, and fatty acids were added. After incubation, cells were washed three times with PBS and scraped off each plate with 1 ml of 0.5% octylglucopyranoside in potassium phosphate buffer, pH 7.5. Cell extract (50 μl, 10–20 μg of protein) was added to each of six wells in a microplate reader (VersaMAX), and 25 μl of reduced cytochrome c (0.08 mM final concentration) was added. The decrease in cytochrome c absorbance was monitored at 550 nm for 1 min.

PS translocation. Fluorescein isothiocyanate-labeled annexin V and propidium iodide were each added at 10 μl/ml of sample buffer provided in the apoptosis kit (R & D Systems).
After 15 min at room temperature, cells plated on precoated glass coverslips (Erie) were visualized using an Olympus IX 70 inverted fluorescence microscope with a ×20 Plano objective (Olympus).

ATP concentration. The ATP levels in fatty acid-treated cells were determined using ion-pairing reverse-phase HPLC over a C18 column (14). Cells were treated with fatty acids as described above. After incubation, cells were rinsed three times in PBS and scraped, and nucleotides were extracted overnight in 60% ice-cold methanol (46). Samples were microcentrifuged, and protein concentration was determined (bicinchoninic acid, Pierce). The supernatants were evaporated until dry, resuspended in ion-pairing mobile phase, and analyzed (14).

Statistics. The significance of the changes reported was determined using Student’s t-test for nonpaired variates measured against the control experiments with use of oleate. Values are means ± SE.

RESULTS

The pathway leading to palmitate-induced apoptosis demonstrates a time-dependent activation of caspase 3-like proteases (Fig. 1). Caspase 3-like activity increases at 12–16 h of incubation in palmitate and is further increased up to 20 h (Fig. 1). In separate experiments, the effect of the molar ratio of fatty acid to BSA on caspase 3-like activation was examined by increasing the BSA bound to palmitate (0.5 mM) from 1.6 to 3%. Molar ratios of 2:1 and 1:1 represent high and average physiological fatty acid-to-albumin ratios for circulating plasma fatty acids (37). After 16 h of incubation, the magnitude of the rise in caspase 3-like activity was decreased by 40% to 91.1 ± 3.1 fluorescence units·min⁻¹·mg⁻¹ at a molar ratio of 1:1 from 154 ± 16.4 to a molar ratio of 2:1. However, caspase 3-like activation at 1:1 molar ratios was still significantly increased by twofold over the baseline value determined in the presence of oleate (1:1 molar ratio) or with 3% BSA alone (43.8 ± 5.2 and 33.1 ± 7.5 fluorescence units·min⁻¹·mg⁻¹, respectively). Control experiments in which the specific group II caspase inhibitor Z-DEVD-fluoromethyl ketone was added, along with the fatty acid, inhibited all DEVDase activity in these cells (data not shown).

PS translocates to the outside of the plasma membrane during the early stages of apoptosis and is therefore used as a specific marker of this process (13). To assess whether cardiac myocytes undergo palmitate-induced translocation of PS, two fluorescent dyes are imaged simultaneously in cells by fluorescence microscopy: fluorescein isothiocyanate-conjugated annexin V binds to PS, and propidium iodide binds to DNA. Neither dye is able to pass through the plasma membrane in healthy cells. If the plasma membrane is compromised, a sign of necrosis, a cell will be stained with PS and annexin V. Occasional myocytes cultured in the presence of oleate-containing media may show necrotic changes (Fig. 1, insets A and B). Incubation in palmitate-containing media causes the appearance of annexin V-positive cells that stain with annexin V, indicating that these cells are apoptotic and necrotic. Bright-field microscopy of the same cells indicates a similar number of cells in both fields (data not shown).

Mitochondria play an early role in apoptosis via release of cytochrome c (22, 53). Cytochrome c release into the cytosol was determined by immunoblotting of cytosolic fractions obtained from myocytes where the sarcolemmal membrane has been permeabilized by use of digitonin (31). Little to no cytochrome c release was detected in cytosol from myocytes incubated in the oleate-containing medium (Fig. 2). Small amounts of cytochrome c are released by 4 h of incubation in palmitate, and maximal levels are released by 12–20 h (Fig. 2). Cytochrome c levels remain elevated in the cytosolic fraction to support significant changes in caspase activity beginning at 12 h in myocytes from palmitate-containing media (Fig. 1).

It is not known whether cytochrome c release precedes or is a consequence of opening of the MPT pore that leads to mitochondrial swelling. With the present technology, it is difficult to monitor the MPT directly in intact living cells without compromising cellular integrity (41). However, ΔΨ can serve as a “reporter” of pore opening, because ΔΨ approaches zero on prolonged...
opening of the permeability transition pore, and this loss is inhibited by cyclosporin A (3). The fluorescent membrane potential indicator TMRE, in combination with wide-field microscopy, is used to monitor the DC of individual mitochondria within a living cardiac myocyte (28). TMRE distributes itself across a membrane in a Nernstian manner, accumulating in the most interior negative compartment, i.e., the mitochondrial matrix. In cardiac myocytes incubated in oleate, TMRE-fluorescent mitochondria are present throughout the cytoplasm (Fig. 3A). In the presence of palmitate, a large number of these mitochondria are no longer able to accumulate TMRE, and the quantity of these fluorescent profiles is greatly decreased as a result of loss of DC (Fig. 3B).

On the basis of these images, temporal changes in the number of healthy, energized mitochondria (Fig. 3B, arrows) were determined within each myocyte and expressed as a percentage of the total cytoplasmic area, i.e., the region excluding the nucleus. The percentage of energized mitochondria in each myocyte was determined for cells cultured in oleate or in palmitate. In palmitate-incubated myocytes, the number of energized mitochondria begins to decline at 4 h and decreases significantly compared with oleate-incubated myocytes after 8 h of incubation (Fig. 4). In a separate set of experiments, the MPT inhibitor cyclosporin A was also included in the media with palmitate. The decrease in numbers of energized mitochondria was significantly delayed by the presence of cyclosporin A (Fig. 4), suggesting that the observed loss of ΔΨ is a consequence of MPT pore opening.

Mouse anti-cytochrome oxidase, along with the fluorescent dye Cy5, was used to image mitochondria in myocytes incubated with palmitate before (4 h) and after (16 h) significant release of cytochrome c into the cytosol. Anti-cytochrome oxidase fluorescence monitors energized mitochondria and mitochondria that have undergone the MPT. With palmitate incubation at 4 h, the majority of mitochondria shown in the cytosol of this myocyte (Fig. 4, left inset) demonstrate the classical elongated (or cigar-shaped) pattern. However, by 16 h in palmitate, the majority of mitochondria have swollen, spherical configurations (Fig. 4, right inset). These mitochondria are unlikely to retain TMRE and, therefore, represent mitochondria that are absent from the TMRE-stained micrograph (Fig. 3B).

To confirm that the loss of cytochrome c and associated mitochondrial swelling resulted from the MPT, the mitochondrial matrix enzyme citrate synthase (which is too large to be released via the MPT) was measured. Total citrate synthase in the cytosolic fraction was expressed as a percentage of the total membrane (mitochondrial) citrate synthase at 4, 8, 12, 16, and 20 h compared with oleate alone at 20 h. With oleate, only 0.031 ± 0.017% of the total citrate synthase was recovered in the cytosol. In the presence of palmitate, this percentage at each respective time point was also very small and, therefore, consistent.
with pore opening: 0.042 ± 0.004, 0.030 ± 0.001, 0.061 ± 0.005, 0.082 ± 0.007, and 0.165 ± 0.009% at 4, 8, 12, 16, and 20 h, respectively.

The ΔΨ of individual mitochondria can be assessed by measurement of fluorescence inside each energized mitochondrion and the fluorescence in the cytosol. The ratio of these measurements is dependent on the ability of mitochondria to accumulate TMRE and is directly proportional to ΔΨ (28). When these ratios were measured in >150 mitochondria within cytosol of a single myocyte immunostained with an antibody against cytochrome oxidase at 4 and 16 h of incubation with palmitate-BSA. Swollen, rounded mitochondria are visible at 16 h.

We previously demonstrated a 70–90% decrease in the rates of CO2 production from LCFA oxidation that is accompanied by ceramide accumulation in neonatal cardiac myocytes incubated for 20 h in palmitate (20). Because the malonyl-CoA-sensitive CPT-I is considered a regulatory step in LCFA oxidation, temporal changes in the activity of this enzyme were measured during incubation with palmitate or oleate. When expressed as a percentage of the activity of the oleate control (2.13 ± 0.15 nmol palmitoylcarnitine-min⁻¹-mg⁻¹), cardiac mitochondrial CPT-I is not changed at up to 12 h in palmitate (Fig. 6). At 16 h, there is a dramatic (60%) and significant diminution in malonyl-CoA-sensitive CPT-I activity that persisted at 20 h (Fig. 6).

Because inhibition of CPT-I would decrease the rate of removal of palmitoyl-CoA from the cell, enhanced concentrations of this metabolite could serve as a precursor for the de novo synthesis of the sphingolipid ceramide. Because ceramide is involved in the apoptotic process, we monitored the time course of accumulation of ceramide in cardiac myocytes after addition of palmitate to the culture medium. Compared with myocytes incubated in the presence of oleate, large increases in ceramide were measured in the palmitate-incubated myocytes at 16 and 20 h (Fig. 7). These changes are independent of the activation of caspase 3-like proteases, as seen by ceramide accumulation in the presence of the group II caspase inhibitor Z-DEVD-fluoromethyl ketone (Fig. 7, inset).

Ceramide has been shown to inhibit the mitochondrial respiratory chain by direct interaction at the level of complex III in isolated mitochondria (17). We investigated the possibility that ceramide synthesis in the neonatal cardiac myocytes is coincident with inhibition of complex III (decylubiquinone:ferricytochrome c oxidoreductase). Because there were no time-dependent

Fig. 4. Percentage of viable mitochondria and cytochrome oxidase staining. TMRE-stained (energized) mitochondria were assessed by thresholding fluorescent images similar to those in Fig. 3 (see MATERIALS AND METHODS). Cells were incubated in palmitate-BSA (○), oleate-BSA (●), or palmitate-BSA + 1 μM cyclosporin A (▲). Values are means ± SE; n = 3–4 cells. *P < 0.005; **P < 0.0005 compared with the average value of cells treated for 4–24 h with oleate. Insets: mitochondria within the cytosol of a single myocyte immunostained with an antibody against cytochrome oxidase at 4 and 16 h of incubation with palmitate-BSA. Swollen, rounded mitochondria are visible at 16 h.

Fig. 5. ΔΨ of individual mitochondria and ATP content. The ratio of mitochondrial to cytosolic fluorescence, proportional to ΔΨ, was determined for individual mitochondria within cells incubated with palmitate-BSA (●) or oleate-BSA (▲). Values are means ± SE; n = 7–40 mitochondria. Inset: ATP levels were determined in cells treated with palmitate by HPLC and normalized to levels obtained with oleate-treated cells. Values are means ± SE; n = 2. *P < 0.05 compared with controls treated with oleate.
alterations in complex III or complex IV (cytochrome c oxidase) over the 4- to 12-h period, the data were grouped for these time periods and compared with combined activities at 16 and 20 h (Table 1). No changes in complex III or complex IV were seen at 12 h when palmitate-incubated myocytes were compared with oleate-incubated myocytes (Table 1). Similarly, there were no differences in cyanide-sensitive complex IV activities at any time point between oleate- and palmitate-treated myocytes. However, a significant reduction (50%) in complex III was observed at 16 h ($P < 0.01$). This observation is consistent with a role for ceramide in inhibition of mitochondrial respiratory activity in the intact cardiac myocyte.

**DISCUSSION**

The ability of palmitic acid to induce apoptosis in cardiac myocytes in vitro provides a model whereby programmed cell death in the heart can be studied in detail. The importance of apoptosis in the heart is still controversial, so a need still exists for paradigms that are able to reproduce potential metabolic consequences of this pathway. A role for CPT-I, the regulatory enzyme in LCFA oxidation, has been implicated in cell death in hematopoietic cells (39). In the latter study, inhibition of mitochondrial CPT was imposed pharmacologically, leading to decreased oxidative metabolism of palmitate, ceramide synthesis, and cell death. Our data are the first to show inhibition of malonyl-CoA-sensitive CPT-I in cardiac myocytes as an integral component of the apoptotic pathway. That this pathway is a unique feature of saturated LCFA, and not a result of some nonspecific cellular interaction, is confirmed by use of oleate as a control, with both fatty acids present at physiological concentrations and bound to BSA in a physiological ratio.

In intact adult hearts, the rate of fatty acid uptake depends on the fatty acid-to-albumin molar ratio in the plasma. In the present study we employed a molar ratio of 2:1 that is representative of prior studies where fatty acids are elevated to increase the availability of fatty acids to cellular metabolism from low-affinity sites (29, 37). Only palmitate can serve as a precursor to ceramide synthesis, and its formation likely is a consequence of fatty acid extraction in the neonatal myocytes, where the oxidative capacity to metabolize LCFA may be exceeded. We (20) previously demonstrated that neonatal cardiac myocytes are able to oxidize oleate and palmitate at comparable rates after 4 h in culture. Because apoptosis is initiated by 8 h in the presence of palmitate (release of cytochrome c and loss of $\Delta\Psi$), by 20 h the palmitate-incubated myocytes exhibit significantly depressed oxidation of palmitate and oleate by the mitochondria (20). However, ATP is only slowly depleted from the myocyte, remaining high as late as 16 h in palmitate-incubated myocytes (see below). These data indicate that 1) the neonatal myocyte does not undergo contractile-dependent ATP hydrolysis to the same extent as the working adult heart and 2) the major cell death pathway is not a consequence of energy deprivation and oncosis. We observed blunted increases in caspase 3-like activity even at physiological fatty acid-to-BSA molar ratios of 1:1. This suggests that, despite decreased free fatty acid availability to the cell, continued fatty acid extraction over a
prolonged incubation period gradually may exceed the capacity of the neonatal cardiac myocytes for β-oxidation. In the adult heart, instances of cellular extraction of plasma fatty acid in vivo have also been associated with apoptosis. For example, ischemia and reperfusion in rat heart extracting fatty acids in vivo produce ceramide with associated indicators of programmed cell death (5). Chronic elevation in serum fatty acids in genetically obese (fa/fa) rats also leads to an age-dependent accumulation of ceramide, downregulation of one of the cardiac isoforms for CPT-I, and apoptosis (57). Therefore, we believe it is reasonable to regard the cardiac myocyte culture as a potential working model for understanding metabolic influences on programmed cell death under conditions that represent acute exposure to potential fatty acid overload in the adult working heart.

Programmed cell death in neonatal rat cardiac myocytes follows the hallmarks of cell death documented in other cell types. There is a progression from the initial release of cytochrome c and loss of ΔΨ to ceramide accumulation and, finally, activation of caspase 3-like proteases. However, several novel findings have been forthcoming from the present studies in cardiac myocytes. Because the heart contains an extensive network of mitochondria to supply its energy needs, the cardiac myocyte represents a unique opportunity to examine mitochondrial events during the progression to cell death. We have demonstrated that cardiac mitochondria respond to palmitate by a loss of ΔΨ from individual mitochondria within any given myocyte. The remaining mitochondria in each cell (identified by TMRE fluorescence) are energized and appear normal. We believe that this heterogeneous process allows each cell to continue to meet the ATP requirement for apoptosis. This is distinct from the process of necrosis, where loss of ATP in cardiac muscle likely reflects the intermingling of dead cells and viable myocytes (7, 8).

The mechanism by which ΔΨ is lost appears to be mediated by the MPT. This conclusion is based on the delay in ΔΨ loss when cyclosporin A is present during incubation of the cells with palmitate and the transition from normal elongated mitochondria at 4 h to swollen profiles at 16 h when TMRE staining (and ΔΨ) is greatly diminished. Further evidence that the MPT is involved in the loss of ΔΨ is the extremely low citrate synthase activity in the cytosolic component of these myocytes. Levels of this matrix protein are still very low even at very late times when the majority of the mitochondria have undergone high-amplitude swelling. Because 99.9% of citrate synthase remains associated with the membrane fraction containing mitochondria, we can conclude that the diameter of the pore opening is consistent with the MPT, where high-molecular-weight (>1,500) solutes are excluded, rather than a nonselective permeability change. A very small “creep” in citrate synthase from 8 to 20 h could reflect a small, increased sensitivity of the dying myocytes to the digitonin permeabilization procedure. Alternatively, a similar slow release of another matrix protein, malate dehydrogenase, has been demonstrated in liver mitochondria during the MPT (21). This release is sensitive to conditions that reverse the MPT and is thought to reflect a rupture-sealing cycle that requires an open, cyclosporin-sensitive pore.

Still unsolved is the mechanism by which the cardiac myocyte triggers the onset of pore opening and the role of palmitate in this process. In the palmitate model of apoptosis, ceramide accumulation occurs concomitant with CPT-I inhibition. The ceramide synthesis inhibitor fumonisin B₁ abolishes the ceramide accumulation (data not shown). This observation is consistent with the hypothesis that inhibition of CPT-I and associated elevations in cytosolic palmitoyl-CoA lead to de novo synthesis of ceramide.

Ceramide inhibits mitochondrial respiratory complex III with a half-maximal effect at 5–7 μM (17). Our data are the first to implicate ceramide in complex III inhibition in the intact myocyte. Ceramide and tumor necrosis factor-α inhibit electron transport at the level of complex III and then increase generation of ROS (15). Ceramide is able to replace tumor necrosis factor-α in inducing the MPT in L929 fibroblasts (38). ROS have been proposed to mediate pore opening (25) by a redox-sensitive pathway involving the adenine nucleotide translocase (1, 19). However, in palmitate-induced programmed myocyte death, accumulation of ceramide and inhibition of complex III (16 h) are widely separated in time from cytochrome c release and the loss of ΔΨ at 8 h. Therefore, in this model of cardiac myocyte apoptosis, the MPT is occurring upstream from ceramide inhibition of complex III and a proposed ROS-mediated pore opening.

Finally, the early pathway(s) by which palmitate induces the process of programmed cell death in the cardiac myocyte is unclear. Still unexplained are the specific effects of a saturated LCFA on the MPT and on inhibition of CPT-I activity. It is interesting to specu-

### Table 1. Activities of mitochondrial complexes III and IV after incubation in the presence of oleate-BSA or palmitate-BSA

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<thead>
<tr>
<th>Complex</th>
<th>12-h Incubation</th>
<th>16-h Incubation</th>
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<tr>
<td></td>
<td>Oleate</td>
<td>Palmitate</td>
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<tr>
<td>Complex III</td>
<td>22.42 ± 1.47</td>
<td>21.32 ± 0.95</td>
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<tr>
<td>Complex IV</td>
<td>0.348 ± 0.008</td>
<td>0.352 ± 0.012</td>
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Values (means ± SE) are expressed as nmol·min⁻¹·mg⁻¹ (complex III, n = 2–3) and –Δoptical density·min⁻¹·mg⁻¹ (complex IV, n = 8–10). Although no significant changes in either complex III or complex IV activities were seen before 16 h in palmitate compared with oleate, these data were combined for each condition to include 4, 8, and 12 h. *P < 0.01 versus oleate at 16 and 20 h.
ulate that palmitate-incubated myocytes accumulate long-chain acyl-CoA to a greater extent than do myocytes incubated with oleate. This theory is supported in part by the observation (20) that triglyceride levels are significantly higher in palmitate- than in oleate-incubated cardiac myocytes. Excessive deposition of triglyceride in nonadipose tissues (steatosis) is indicative of an enlarged pool of acyl-CoA, thereby providing substrate for ceramide synthesis (57). A combination of enhanced cellular levels of malonyl-CoA (20) and decreased intrinsic activity of CPT-I in the palmitate-incubated myocytes supports this scenario. It is also possible that the MPT observed at earlier times (8 h) can be explained by the known interaction of palmitoyl-CoA with the adenine nucleotide translocase (52). Evidence suggests that the adenine nucleotide translocase is capable of forming a pore-like structure under conditions known to induce MPT in mitochondria (45). The participation of the adenine nucleotide translocase in the pore complex has been proposed by other investigators (19). The action of palmitoyl-CoA to inhibit the adenine nucleotide translocase and open the pore complex is an attractive option but would require marked differences in the cellular concentrations of the saturated and unsaturated acyl-CoAs. Alternatively, incubation with palmitate may affect the fatty acid composition of the mitochondrial phospholipid bilayer. Changes in membrane fluidity are known to affect a variety of mitochondrial activities (32). The activity of CPT-I and its malonyl-CoA sensitivity are also markedly influenced by its phospholipid environment (24). Alternative actions of palmitate may include interaction with cell surface receptors or effector kinases, e.g., stress kinases. For example, palmitate may antagonize the membrane targeting of the antiapoptotic kinase Akt, which is necessary for constitutive activation of its survival signaling (56).

The involvement of palmitate in cardiac myocyte cell death is particularly relevant, since palmitate is deleterious to functional recovery of reperfused myocardium (26) and contributes to fatal arrhythmias in infants with inborn errors of fatty acid oxidation (9) and in patients with ischemic heart disease. In addition, cardiac dysfunction in obesity is caused by “lipoapoptosis” and can be prevented by reducing dietary fat (57). Our findings demonstrate for the first time that mitochondria in each cardiac myocyte lose their membrane potential in a heterogeneous and progressive process involving the opening of the mitochondrial pore leading to the MPT. Because cytochrome c release is separated in time from activation of caspase-3-related proteases and ceramide accumulation, our data suggest that the MPT is not induced by ceramide inhibition of mitochondrial electron transport leading to interaction of ROS with the pore.

In summary, the results suggest that palmitate or its metabolites act early in the apoptotic cascade to initiate cardiac myocyte cell death, causing release of cytochrome c and loss of ∆Ψ. Moreover, mitochondrial swelling (detected in TMRE-negative mitochondria by immunostaining with cytochrome oxidase antibodies) results from opening of the MPT pore, as manifested by cytochrome c release to the cytosol and retention of >99% of citrate synthase in the mitochondrial membrane fraction. Decreased activity of CPT-I is concurrent with ceramide accumulation and inhibition of the mitochondrial complex 3. Ceramide accumulation is independent of increased caspase 3-like activities and likely results from CPT-I inhibition and decreased oxidation of palmitoyl-CoA.

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