Palmitate-induced apoptosis in neonatal cardiomyocytes is not dependent on the generation of ROS

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RECENT STUDIES HAVE SHOWN that the saturated long-chain fatty acid palmitate induces apoptosis in neonatal rat cardiomyocytes. This apoptosis is associated with early mitochondrial release of cytochrome c and a subsequent loss of mitochondrial membrane potential. Recent reports implicate a role for reactive oxygen species (ROS) in palmitate-induced apoptosis. We studied the role of ROS in palmitate-induced apoptosis in the neonatal rat cardiomyocyte and report no evidence of ROS involvement. ROS production, nitric oxide production, and nuclear factor-κB activation were not increased above those observed using the nonapoptotic fatty acid oleate. Indeed, the production of ROS was significantly higher in cells treated with oleate. Furthermore, the presence of antioxidants and ROS scavengers did not attenuate the induction of apoptosis by palmitate. Variations in the fatty acid-to-albumin ratio from 2:1 to 7:1 had no effect on the extent of apoptosis. No evidence was found for an increase in oxidative protein modification in palmitate-treated cells. Our results lead us to conclude that oxidative stress does not play a role in palmitate-induced apoptosis.

antioxidants; nitric oxide; mitochondria; nuclear factor-κB

Hickson-Bick, Diane L. M., Genevieve C. Sparagna, L. Maximilian Buja, and Jeanie B. McMillin. Palmitate-induced apoptosis in neonatal cardiomyocytes is not dependent on the generation of ROS. Am J Physiol Heart Circ Physiol 282: H656–H664, 2002. First published October 4, 2001; 10.1152/ajpheart.00726.2001.—The saturated fatty acid palmitate induces apoptosis in neonatal rat cardiomyocytes. This apoptosis is associated with early mitochondrial release of cytochrome c and a subsequent loss of mitochondrial membrane potential. Recent reports implicate a role for reactive oxygen species (ROS) in palmitate-induced apoptosis. We studied the role of ROS in palmitate-induced apoptosis in the neonatal rat cardiomyocyte and report no evidence of ROS involvement. ROS production, nitric oxide production, and nuclear factor-κB activation were not increased above those observed using the nonapoptotic fatty acid oleate. Indeed, the production of ROS was significantly higher in cells treated with oleate. Furthermore, the presence of antioxidants and ROS scavengers did not attenuate the induction of apoptosis by palmitate. Variations in the fatty acid-to-albumin ratio from 2:1 to 7:1 had no effect on the absence of ROS production or on the extent of apoptosis. No evidence was found for an increase in oxidative protein modification in palmitate-treated cells. Our results lead us to conclude that oxidative stress does not play a role in palmitate-induced apoptosis.

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methods

Primary cell culture. Neonatal rat cardiac myocytes were prepared according to McMillin et al. (16) using 1- to 2-day-old Sprague-Dawley rat pups. Myocytes were plated at 2 × 10⁶ cells/60-mm dish or on glass coverslips for microscope studies and maintained for 60 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 BSA in DMEM in the absence of serum. Fatty acid concentration was measured using a semimicrodetermination of fatty acid concentrations. Unbound fatty acids were measured using the acetylated intestinal fatty acid-binding (ADIFAB) protein (Molecular Probes; Eugene, OR) method developed by Richieri (20). The concentration of unbound fatty acids was calculated using the ratio of fluorescence intensities of bound to unbound ADIFAB indicator at 505 and 432 nm, respectively, using calculations and constants outlined on the product data sheet.

Caspase-3-like activity. Caspase-3-like activity was measured by following the cleavage of the fluorescent substrate, Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC; Calbiochem; San Diego, CA), as described previously (8).

Microscopy for ROS generation. Oxidant generation studies were carried out on glass coverslips. The cells were incubated for 3 or 19 h in fatty acid-containing medium; 5 μM (final concentration) of either the peroxide-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), or the superoxide-sensitive dye dihydroethidiom (DHE) (both from Molecular Probes) was then added in DMSO. The medium was removed after 1 h at 37°C, and the cells were washed and placed in HEPES-DMEM without serum. Intracellular fluorescence was monitored using a temperature-regulated (37°C) Wallach/Olympus America Concorde real-time fluorescence imaging spectrophotometer and an Olympus IX70 inverted fluorescence microscope with a ×40 objective. Image acquisition was with a fast-scan 12-bit charge-coupled device camera. Signal-based averaging was used to quantitate the fluorescence signal from five to six fields of cells. Control cells were treated with 6 mM hydrogen peroxide for 1 h before the addition of the fluorescent dye.

Protein oxidation. The changes in oxidatively modified protein were measured using the Oxyblot protein oxidation detection kit (Intergen) and SDS-PAGE. The hydrogen peroxide control was obtained by incubation of cells with 6 mM H₂O₂ for 1 h.

NO measurement. Total nitrate plus nitrite was measured using a colorimetric assay kit (Cayman; Ann Arbor, MI) based on the Griess reaction. Where noted, 0.5 mM Nω-monomethyl-l-arginine (L-NMMA) was added along with the fatty acid to inhibit NO formation.

Electrophoretic mobility shift assays. Nuclear extracts from primary rat neonatal cardiac myocytes were prepared as previously described (17). Double-stranded DNA probes containing the sequences for the NF-κB-binding region (CTAGCAGTTGGGGACTTTCCCAGGCG) or sequence-specific mutated strands (CTACAGTTGGGGTCTGTCCAGGGC) were synthesized by Operon Technologies (Alameda, CA). Electrophoretic mobility shift assay reaction mixtures included 6 μg nuclear extract, 25 mM HEPES, 100 mM KCl, 0.1% Nonidet P-40 (vol/vol), 1 mM dithiothreitol, 5% glycerol, and 50 ng PolydIdC as a nonspecific competitor in a 20-μl reaction volume. After incubation for 10 min at room temperature, 0.3 ng of radiolabeled probe was added, and the reaction was incubated for 20 min. When included, an antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology; Santa Cruz, CA) or 100-fold molar excess of cold probe was added during the first incubation. Protein-DNA complexes were separated on a 4% nondenaturing polyacrylamide gel at 25°C.

Statistics. The significance of the changes reported was determined using Student’s t-test for nonpaired variates measured against the control experiments using olate. Data are presented as means ± SE.

results

ROS production by myocytes. The membrane-permeable dye DCFH-DA enters cardiomyocytes and produces a fluorescent signal after intracellular oxidation by ROS such as hydrogen peroxide and the hydroxyl radical (29). Neonatal cardiomyocytes were incubated in palmitate or the control fatty acid olate for 4 or 20 h. We have previously shown that exposure of these cells to palmitate for 4 h is sufficient to induce mitochondrial cytochrome c release, whereas after 20 h, palmitate causes caspase-3-like activation, increased ceramide production, and DNA laddering (8, 26). With the use of video microscopy of live cells and examination of multiple fields of cells, we were unable to detect any increase in fluorescence in palmitate-treated cells over control olate-treated cells at either time. Addition of hydrogen peroxide to these cells elicited an intense signal (Fig. 1).

DHE also freely enters myocytes and is oxidized by ROS, particularly superoxide, to yield fluorescent ethidium (1, 29). Ethidium can pass into the nucleus, and subsequent binding of ethidium to DNA results in an amplified fluorescent signal. Incubation of cardiomyocytes with palmitate did not lead to an increased oxidation of DHE. After both 4 and 20 h in the nonapoptotic fatty acid olate, the DHE fluorescence was significantly higher than in the palmitate-treated cells (Fig. 2). Because under physiological conditions an estimated 2–5% of O₂ utilized by mitochondria is reduced by single electron transfer by electrons that escape from the electron transport chain (27, 28), this increased production of ROS by olate-treated cells may be attributed to the increased level of substrate β-oxidation over palmitate-treated cells (8). Inhibition of mitochondrial electron transport at complex I with rotenone or complex III with antimycin A resulted in increased superoxide formation (Fig. 2). The fluorescent signals for cells treated with antimycin A and rotenone were essentially saturated under the conditions employed, preventing a quantitative comparison between these cells and the cells incubated in fatty acids.

Because we were unable to detect any increase in ROS production associated with palmitate-induced apoptosis in neonatal myocytes by fluorescent microscopy, we studied the effect of various antiangiogenic...
dants and ROS scavengers on palmitate-induced apoptosis, as assessed by the activation of caspase-3-like proteins (Table 1). Incubation of cells in the presence of palmitate and 4,5-dihydroxy-1,3-benzene disulphonic acid (DBDA, Tiron), a cell-permeable nonenzymatic superoxide scavenger, or 5-aminosalicylic acid (5-ASA), a powerful scavenger of hydroxyl radicals, did not prevent activation of caspase-3-like proteins. Pyrrolidine dithiocarbamate (PDTC), a free radical scavenger and metal chelator, also did not reduce caspase-3-like activation. Trolox (water-soluble vitamin E), a scavenger of lipid peroxyl radicals, was not effective in reducing caspase-3-like activation by palmitate. Glutathione plays a central role in cellular antioxidant defense, and its loss may render cells more prone to oxidative stress. Cardiomyocytes were therefore treated with N-acetyl cysteine (NAC), a precursor compound for glutathione formation and previously shown to increase the glutathione content of cardiomyocytes (9). NAC was also unable to attenuate palmitate-induced apoptosis in these cells (Table 1).

Effect of levels of unbound fatty acids and cell type on ROS production. Other investigators have reported that in Chinese hamster ovary (CHO) cells, palmitate induces the formation of ROS (14). Because these results do not agree with our observations in cardiomyocytes, we hypothesized that these differences were a result of different fatty acid-to-BSA ratios employed or, alternatively, cell type-specific differences. These authors used a fatty acid-to-BSA ratio of 8:1, as opposed to the physiological ratio of 2:1 that we employed. We predicted that their preparations would contain a
higher concentration of unbound fatty acid, which could be deleterious to the cell. We measured spectrofluorimetrically, using the ADIFAB protein, the unbound fatty acid in fatty acid bound to BSA at a ratio of 7:1 and 2:1 (Fig. 3). Increasing the fatty acid-to-BSA ratio from 2:1 to 7:1 resulted in a nearly sixfold increase in unbound fatty acid (66.7 ± 26.4 vs. 381.0 ± 63.6 nM). However, varying the fatty acid-to-BSA ratio did not increase the DCFH-DA fluorescence in cardiomyocytes (Fig. 4), indicating that an increased level of media free fatty acids does not lead to increased ROS production. Similarly, we were unable to observe any increases in fluorescence in rat fibroblasts (Fig. 4) or CHO cells (data not shown) after incubation with either 2:1 or 7:1 palmitate-to-BSA ratios. This suggests that the lack of a ROS response is not cell type specific or restricted to cells with an increased dependency of fatty acid metabolism over glucose metabolism. It has been documented that fibroblasts are subject to apoptosis induction during serum deprivation (12). An increased level of ROS production in fibroblasts maintained in serum-free media without fatty acid supplementation (Fig. 4B) indicates that apoptosis induced by serum deprivation may involve ROS synthesis.

**NO production and palmitate-induced apoptosis.** NO production by inducible NO synthase (iNOS) has been implicated in ischemia-reperfusion injury and apoptosis of cardiomyocytes (31). Superoxide generated by mitochondria can interact with NO to form peroxynitrite (ONOO⁻), which can induce mitochondrial dysfunction and apoptosis and exacerbate the apoptotic effect of superoxide alone. We examined the role of NO production in palmitate-induced apoptosis of neonatal cardiomyocytes by measuring the total production of nitrite plus nitrate over the course of a 20-h incubation in fatty acid-BSA (2:1) or serum-free medium alone (Fig. 5). NO production by palmitate-treated cells was signifi-

**Table 1. Caspase-3-like DEVDase activities with FA plus various antioxidants**

<table>
<thead>
<tr>
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<th>Oleate</th>
<th>Palmitate</th>
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<tr>
<td>FA alone</td>
<td>51.8 ± 7.4</td>
<td>334.2 ± 91.2</td>
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<tr>
<td>FA + DBDA</td>
<td>60.0 ± 6.0</td>
<td>384.8 ± 30.8</td>
</tr>
<tr>
<td>FA + 5-ASA</td>
<td>42.6 ± 11.2</td>
<td>359.2 ± 29.0</td>
</tr>
<tr>
<td>FA + NAC</td>
<td>51.2 ± 8.2</td>
<td>380.8 ± 60.9</td>
</tr>
<tr>
<td>FA + Trolox</td>
<td>51.4 ± 4.8</td>
<td>379.8 ± 52.4</td>
</tr>
<tr>
<td>FA + PDTC</td>
<td>61.0 ± 9.4</td>
<td>396.8 ± 59.4</td>
</tr>
<tr>
<td>No FA treatment</td>
<td>48.13 ± 5.9</td>
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</table>

Values are means of DEVDase fluorescence per minute per milligram of protein ± SE; n = 6 cardiomyocytes. Cardiomyocytes were treated with fatty acids (FA) ± antioxidants for 20 h. All antioxidants were added at a final concentration of 100 μM. DBDA, 4,5-dihydroxy-1,3-benzenes disulfonic acid; 5-ASA, 5-aminosalicylic acid; NAC, N-acetyl cysteine; PDTC, pyrrolidine dithiocarbamate.
cantly lower than that of oleate-treated cells for periods up to 12 h, at which time no difference was seen. Addition of the cell-permeable iNOS inhibitor L-NMMA reduced the production of nitrate plus nitrite in both oleate- and palmitate-treated cells during a 16-h incubation (Fig. 6A) but failed to reduce the induction of caspase-3-like activity at this time (Fig. 6B).

**NF-κB binding activity and effect of superoxide dismutase inhibition on palmitate-induced apoptosis.** Binding activity of NF-κB, a redox-regulated transcription factor, was found to be very low in the nucleus of cardiomyocytes maintained in serum alone. Addition of 0.5 mM fatty acid-1.6% BSA increased the translocation of NF-κB from the cytosol to the nucleus (Fig. 7A). No significant difference was observed between NF-κB translocation in oleate- or palmitate-incubated cells. To confirm the specificity of the NF-κB binding activity, we performed supershift assays with a polyclonal antibody to the NF-κB p65 subunit (Fig. 7B). The binding of NF-κB was also competed with 100-fold excess of unlabeled probe but not with mutant probe (Fig. 7B). Quantification and averaging of the palmitate-to-oleate signal ratio for several gels confirmed that there was no significant time-dependent difference in NF-κB binding between cells maintained in these two fatty acids (Fig. 7C). Thus while incubation of cardiomyocytes with fatty acids increases NF-κB binding, this signaling mechanism is not involved in palmitate induction of apoptosis.

**Inhibition of superoxide dismutase.** Our data demonstrate that cardiomyocytes do not increase production

<table>
<thead>
<tr>
<th>Cardiomyocytes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fibroblasts&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oleate</strong></td>
<td><strong>Palmitate</strong></td>
</tr>
<tr>
<td>2:1 FA:BSA</td>
<td>36.7±2.7</td>
</tr>
<tr>
<td>7:1 FA:BSA</td>
<td>50.1±2.8</td>
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<sup>a</sup>Cardiomyocytes were treated with fatty acids for 4 h.

<sup>b</sup>Fibroblasts were treated with fatty acids for 16 h.

Values are average intensity units ±SE, N= 8-12
ROS when incubated with palmitate. We examined whether increasing the level of ROS production in these cells increased the apoptotic response of these cells as assessed by the activation of caspase-3-like activity. Addition of diethyldithiocarbamic acid (DDC), an inhibitor of cytosolic (Cu, Zn) superoxide dismutase, to cells incubated in palmitate for 16 h causes an increase in the nuclear translocation of NF-κB (Fig. 8).

DDC inhibition of superoxide dismutase has been shown to increase superoxide generation in neonatal rat cardiac myocytes (24), and we demonstrated that the caspase-3-like activity of both oleate- and palmitate-incubated cells increased in the presence of increasing concentrations of DDC (Table 2). However, the caspase-3-like activity of oleate-treated cells in the presence of DDC, even at a concentration (100 μM) previously shown to induce apoptosis (24), never attained the levels achieved by palmitate incubation alone (without DDC).

Protein oxidation and palmitate-induced apoptosis.

Oxidative stress-induced injury can involve the selective modification of intracellular proteins. We measured the levels of oxidatively modified proteins using Oxyblot analysis, which detects protein carbonyl formation. We were unable to detect changes in protein carbonylation in cardiomyocytes incubated with either oleate or palmitate for 4 or 20 h (Fig. 9) compared with cells incubated in serum-free medium alone. A change in the protein carbonylation pattern was observed when cells were incubated in hydrogen peroxide for 1 h.

DISCUSSION

Palmitate, but not oleate, induces apoptosis in neonatal cardiac myocytes. We have previously shown that this apoptosis is characterized by an early mitochondrial release of cytochrome c and a loss of mitochondrial membrane potential (26). Later events include an increase in ceramide synthesis, inhibition of mitochondrial electron transport complex III, acti-

Table 2. Caspase-3-like activity of cardiomyocytes incubated for 20 h in the presence of FA and varying concentrations of DDC

<table>
<thead>
<tr>
<th>DDC, μM</th>
<th>0</th>
<th>50</th>
<th>100</th>
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<tbody>
<tr>
<td>Palmitate</td>
<td>182 ± 8.0</td>
<td>316 ± 61.4</td>
<td>581.2 ± 14.9</td>
</tr>
<tr>
<td>Oleate</td>
<td>42. ± 2.0</td>
<td>66.1 ± 6.0</td>
<td>68.1 ± 5.9</td>
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</table>

Values are means of caspase-3-like activity (in fluorescence units·min⁻¹·mg protein⁻¹) ± SE. DDC, diethyldithiocarbamic acid.

Fig. 6. NO inhibition does not affect caspase-3-like activity. Cardiomyocytes were treated with either BSA (no FA), oleate, or palmitate (Palm) for 16 h in the presence (open bars) or absence (solid bars) of N-monomethyl-l-arginine (L-NMMA). Total nitrate plus nitrite (A) and caspase-3-like activity (B) were quantified from the same samples. Error bars represent means ± SE; n = 3.
tion of caspase-3-like enzymes, and DNA laddering (8, 26). Increased production of ROS have been implicated in stress induction of apoptosis in cardiac myocytes and reported to be involved in palmitate-induced apoptosis of CHO cells (14).

Mitochondrial oxidative phosphorylation represents a process by which the oxidation and reduction of a
metals, H2O2 can give rise to the more reactive hydroxyl radical (OH·) by Fenton chemistry. Superoxide may also be converted to the very reactive peroxynitrite in the presence of NO.

We employed a variety of techniques to measure the production of ROS species. We could not detect an increased production of ROS by cells incubated in palmitate at 4 h, a time when mitochondrial cytochrome c release can be measured, or 20 h, when caspase-3-like activity and ceramide accumulation is maximal. Similarly, the presence of ROS scavengers (DBDA, 5-ASA, and PDTC) during palmitate incubation was unable to prevent apoptosis as measured by caspase-3-like activity. No increase in NO synthesis in palmitate-over oleate-incubated myocytes was observed. Increasing myocyte ROS by the inhibition of superoxide dismutase with DCC increases the level of caspase-3-like activation and apoptosis, indicating that ROS can induce apoptosis in these cells but that this is distinct from, and additive to, the effect of palmitate.

Putative downstream targets of ROS signaling are alterations in the intracellular redox state and the oxidative modification of proteins. The intracellular redox state is controlled primarily by the buffering capacity of the intracellular thiols glutathione and thioredoxin. These thiols reduce oxidative stress by reducing H2O2 levels. Increasing the thiol buffering capacity of myocytes with the glutathione precursor NAC (9) did not prevent palmitate-induced apoptosis. We were also unable to detect any oxidative modification of proteins.

ROS have been implicated in signal transduction pathways leading to a modulation of the DNA-binding activities of the transcription factor NF-κB (15), implying a role for alterations in gene transcription as a response to oxidative stress. We report a significant increase in the DNA-binding activity of NF-κB in cardiomyocytes incubated in the presence of fatty acids. However, no significant differences were observed between the NF-κB activities of cells incubated in palmitate or oleate, implying no role for this signaling pathway in palmitate-induced apoptosis.

We observed that incubation of neonatal cardiac myocytes with fatty acids may lead to measurable ROS production. This ROS production is not, however, associated with palmitate-induced apoptosis because higher levels of production are observed in oleate-incubated cells. Oleate-incubated cells have elevated rates of β-oxidation compared with palmitate-incubated cells (8), providing increased electron movement through the electron transport chain and oxidative phosphorylation. Some small percentage of electrons escape from the electron transport chain, potentially at complex I and complex III. These electrons are capable of forming ROS, explaining the higher measurable levels of ROS in oleate-incubated nonapoptotic cells.

Free fatty acids act as uncouplers of mitochondrial electron transport by means that are not fully defined but are probably related to uncoupling proteins (UCPs) present within the cell (for a review, see Ref. 25). The heart has an abundant level of these proteins, particularly UCP2 (5). It has been postulated that the mild uncoupling ability of fatty acids is sufficient to maintain the mitochondrial membrane potential below a threshold level and prevent ROS formation by the respiratory chain (11). Differences in the uncoupling ability of oleate and palmitate have also been reported in model mitochondrial vesicles (22). Uncoupling by fatty acids decreases production of ROS and oxidative stress. Generation of ROS from the electron transport chain requires a high mitochondrial membrane potential (7). We previously reported that palmitate-induced apoptosis in the neonatal cardiomyocyte is associated with a decrease in the mitochondrial membrane potential, also decreasing the ability of the mitochondria to produce ROS.

Our results are in direct opposition to those reported for palmitate-induced apoptosis in CHO cells measured using similar fluorescent dyes. These differences cannot be explained by the different ratios of fatty acid to BSA employed. The pathologically high 8:1 fatty acid-to-BSA level employed by Listenberger et al. (14) is associated with an increased level of unbound fatty acids. However, even under similar conditions (7:1 fatty acid-to-BSA ratio), neither oleate nor palmitate demonstrated augmentation of the ROS production observed using a 2:1 fatty acid-to-BSA ratio. Our measurements were made on beating myocytes using realtime video fluorescence microscopy. Because this requires visualization of fluorescence, it could lead to an overestimation, rather than an underestimation, of ROS production. We (26) previously reported that palmitate-induced apoptosis is associated with a mitochondrial loss of cytochrome c. Cytochrome c is a potent catalyst of dichlorofluorescein oxidation, leading to an increase in DCFH-DA oxidation despite a lowered rate of ROS production (3). Nonetheless, no evidence of DCFH-DA oxidation could be observed.

In conclusion, and contrary to other reports, we are unable to find any evidence of ROS involvement in palmitate-induced apoptosis. In considering the bioenergetics of ROS production by mitochondria and the known biophysical properties of fatty acids, this result is not unexpected. Recent studies from our laboratory have shown that the mitochondrial release of cytochrome c is directly related to a decrease in mitochondrial cardiolipin and associated alterations in phospholipid metabolism (18). Although ROS production plays an important role in ischemia-reperfusion injury and apoptosis induced by other means, palmitate-induced apoptosis involves other pathways that lead to perturbations in cellular metabolism.
REFERENCES


