Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes

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Abstract The energy need of cardiac muscle cells in vivo is largely covered by the oxidation of saturated and mono-unsaturated fatty acids (FA). However, in vitro studies have shown that the saturated FA C16:0 at physiological concentrations exerts detrimental effects on primary cultures of neonatal rat ventricular myocytes by, as yet, unknown mechanisms. To evaluate the noxious effects of FA in more detail, neonatal cardiomyocytes were exposed to saturated (C16:0; C18:0) or mono-unsaturated (C16:1; *cis*-C18:1; *trans*-C18:1) FA, or combinations thereof for up to 48 h. FA (0.5 mM) complexed to bovine serum albumin (BSA) (0.15 mM) were added to a glucose-containing defined medium. Irrespective of the length and degree of unsaturation of the aliphatic chain, FA supplied to the cells were readily incorporated in the phospholipid pool. In the presence of mono-unsaturated FA, cardiomyocytes remained healthy and accumulated substantial amounts of triacylglycerol. In contrast, within 24 h after application of the saturated FA C16:0 or C18:0, cells had become irreversibly damaged, as evidenced by the presence of pyknotic nuclei and massive release of the cytosolic markers lactate dehydrogenase (LDH) and fatty acid-binding protein (FABP). Moreover, the occurrence of DNA-laddering indicated that apoptosis was involved. Induction of apoptotic cell death by C16:0 was counteracted by the co-administration of equimolar amounts of *cis*-C18:1, whereas *trans*-C18:1 delayed, but did not prevent, loss of cardiomyocyte viability. The present findings suggest that the incorporation of saturated, but not mono-unsaturated, fatty acids induces alterations in the phospholipid membrane, which initiate apoptotic cell death in neonatal cardiomyocytes.—**de Vries, J. E., M. M. Vork, T. H. M. Roemen, Y. F. de Jong, J. P. M. Cleutjens, G. J. van der Vusse, and M. van Bilsen.** Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J. Lipid Res.* 1997. **38:** 1384–1394.

Supplementary key words cardiomyocytes • fatty acids • apoptosis • lactate dehydrogenase • fatty acid-binding protein

Long-chain fatty acids play an important role in a variety of biological processes in cardiac muscle cells. Not only do they serve as constituents of membrane phospholipids, but they are also involved in cellular signal transduction (1–3). Furthermore, the oxidation of fatty acids provides an important source of energy for the heart (4, 5). This notion is supported by studies in which the substrate preference of isolated cardiomyocytes was investigated by short-term incubations with radiolabeled glucose and palmitic acid (6, 7). On the other hand, circumstantial evidence indicates that fatty acids may exert adverse effects on cardiac function as well. For instance, increasing the exogenous supply of fatty acids through intravenous lipid infusion in the intact animal was associated with myocardial neutral lipid accumulation and depressed myocardial function (8). Moreover, various in vitro studies have shown that fatty acids impair mitochondrial function and modify ion channel activity of cardiac cells (reviewed in ref. 5). These noxious effects may also contribute to the fatty acid-induced cell death observed in other cell types, such as endothelial cells, fibroblasts, and lymphocytes (9–14). Information on the effect of prolonged exposure of cardiomyocytes to saturated fatty acids is scarce. The recent observation that long-term incubations with physiological levels of saturated fatty acids compromised the viability of neonatal rat ventricular cardiomyocytes by, as yet, unknown mechanisms (15), prompted us to investigate in more detail the potentially harmful effect of long-term exposure of cardiomyocytes to fatty acids.

Thereto, neonatal rat ventricular myocytes were exposed to various fatty acid species for up to 48 h. The cells were incubated with palmitic (C16:0) or oleic acid (*cis*-C18:1), complexed to bovine serum albumin. These fatty acid species were chosen as they constitute

Abbreviations: FA, fatty acids; LDH, lactate dehydrogenase; FABP, fatty acid-binding protein; TUNEL, terminal transferase-mediated dUTP-biotin nick end labeling; P/S, penicillin/streptomycin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; m.p., melting point; T_M, membrane transition temperature.

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the quantitatively most important fatty acids in blood plasma (16). To elucidate whether possible effects could be ascribed to the degree of saturation, cells were also exposed to palmitoleic (C16:1), stearic (C18:0), or elaidic acid (*trans*-C18:1). Being mono-unsaturated, elaidic acid is of special interest because the *trans*-double bond provides a structural conformation to the hydrocarbon chain reminiscent of that of a saturated fatty acid. Cellular viability was assessed morphologically and by the release of cytosolic proteins. Putative mechanisms that explain cell death induced by fatty acids involve alterations in the composition and content of the intracellular lipid pools (13). Therefore, the metabolic fate of fatty acids taken up by the cells was visualized by staining neutral lipids with Oil Red O, and by analyzing the fatty acid composition of the endogenous lipid pools with gas chromatography. Furthermore, it was investigated whether cell death was due to necrosis or apoptosis. To this end the appearance of internucleosomal DNA-strand breaks, a hallmark of apoptosis, was determined both with the TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling) assay (17) and by gel electrophoresis of genomic DNA to detect DNA-laddering (18).

The present study explored the susceptibility of cultured rat neonatal cardiomyocytes for different saturated and mono-unsaturated fatty acid species, with respect to cell survival, and the mechanism of cell death involved. Current findings indicate that saturated fatty acids induce apoptosis and that this effect is counteracted by co-addition of equimolar amounts of mono-unsaturated fatty acids.

METHODS

Cell culture

Neonatal ventricular myocytes were isolated as described by Iwaki et al. (19) with slight modifications. Briefly, hearts were removed from decapitated 1–3-day-old Wistar/Kyoto rats (local strain Maastricht). Atria were trimmed off and ventricular tissue was cut into pieces, transferred to a spinner flask, and digested to single cells with collagenase type I (Gibco, Gaithersburg, MD) and pancreatin (Gibco) in Ads-buffer (pH 7.35) consisting of (in mM):NaCl (116), HEPES (20), NaH_2PO_4 (0.9), glucose (5), KCl (5.4), $MgSO_4$ (0.8). The cell suspension was loaded on a discontinuous gradient of Percoll (Sigma Chemical Co., St. Louis, MO) with two different densities (1.059 and 1.082 g/ml) to separate the cardiomyocytes from non-myocytes, mainly fibroblasts and endothelial cells. Cardiomyocytes were plated at low density (40,000 cells/cm^2) and allowed to adhere in tissue culture dishes coated with 1% gelatin type B (Sigma G-9382) in a 4:1 mixture of DMEM (Gibco 42430) and M199 (Gibco 31153) supplemented with 10% horse serum (Gibco 16050), 5% newborn calf serum (Sera-Lab, Sussex, England), and antibiotics (penicillin 100 IU/ml (P), streptomycin 0.1 mg/ml (S), Gibco). Overnight incubation in this serum-rich medium was followed by 24 h incubation in serum-free medium of a 4:1 mixture of DMEM/M199 and P/S. Following this procedure cell cultures with >90% cardiomyocytes were obtained as determined by immunocytofluorescence with the monoclonal antibody 9D10 specific for titin (20), kindly provided by Dr. G. Schaart (Department of Molecular and Cellular Biology, Maastricht University, the Netherlands).

Preparation of BSA/fatty acid complexes

Palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (*cis*-C18:1), or elaidic acid (*trans*-C18:1) (Sigma) was dissolved in 4 ml ethanol to yield a final concentration of 18.75 mM. An equal volume of 10 mM Na_2CO_3 was added. Ethanol was evaporated at 50–60°C under continuous N_2-flow and the fatty acid-containing mixture was added dropwise to 10 ml of 10% bovine serum albumin (BSA) (Sigma, A-7906) in phosphate-buffered saline (PBS) at 40°C. The BSA/fatty acid complexes were subsequently dialyzed four times at 4°C during 4–6 h against 250 ml 0.1 M $[NH_4]HCO_3$, and lyophilized.

BSA and BSA/fatty acid complexes were dissolved in glucose-free medium composed of a 4:1 mixture of DMEM (Gibco, 11963) and glucose-free M199 (Gibco, 31153) with P/S to yield a final BSA concentration of 1% (0.15 mM). After filter sterilization (0.2 μm pore width) the media were stored at 4°C until use. The actual concentration of fatty acids in the media, measured by gas chromatography (see below), averaged 0.5 mM. Accordingly, the fatty acid/BSA ratio in the medium amounted to 3.3 to 1.

For control experiments BSA was prepared similar to the procedure as described above, but in the absence of added fatty acids. Under these conditions the concentration of fatty acids in the medium averaged 0.01 mM, which corresponds to a FA/BSA ratio of 0.07 to 1.

Experimental culture protocol

After 24 h of incubation in serum-free medium the myocytes were rinsed once with a glucose-free 4:1 mixture of DMEM (Gibco, 11963) and glucose-free M199 (Gibco, 31153) containing P/S, and subsequently cultured for up to 48 h in the same medium supplemented with 10 mM glucose (Gibco), 0.25 mM L-carnitine (Sigma), 0.25 mU/ml insulin (Sigma I-6634), and either with 0.15 mM BSA (control cardiomyocytes) or with fatty acids complexed to BSA. During the 48-h culture

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period the concentration of both glucose and fatty acids in the culture medium declined by less than 10%, indicating that substrate availability did not became a limiting factor.

Cell morphology and lipid staining

Cellular morphology was investigated by staining the cells with hematoxylin (Sigma, GHS-2-16). Neutral lipids (triacylglycerol) were visualized with Oil Red O (Aldrich Chemical Company, Milwaukee, WI) according to Clark (21). Cardiomyocytes were washed twice with PBS and fixed in 70% ethanol for 2 min at room temperature. Subsequently, the cells were stained with Oil Red O (0.5% in isopropanol) for 10 min, rinsed briefly with 70% ethanol and once with doubly distilled water, followed by a second fixation with formalin/PBS for 2 min and washed with doubly distilled water. Cells were counterstained with hematoxylin, immediately embedded in Immu-mount (Shandon, Pittsburgh, PA), and sealed with rubber cement.

Determination of LDH and FABP

Cell culture medium was sampled at the indicated time points and centrifuged (10 min, 4°C, 11,000 *g*). The supernatants were stored at −80°C for analysis of lactate dehydrogenase (LDH) activity and heart-type fatty acid-binding protein (FABP) content in order to assess the extent of irreversible cellular damage. Cells were washed twice with ice-cold PBS and lysed on ice in 0.1 M K_2HPO_4/KH_2PO_4 (pH 7.9), 0.5% Triton X-100, and 1 mM EDTA. The cell lysate was transferred to plastic cups and stored at −80°C for subsequent determination of LDH activity and FABP content. LDH activity was assayed spectrophotometrically by measuring the conversion of NADH into NAD^+ (22). The amount of FABP was measured with a sandwich ELISA using polyclonal antibodies raised against rat heart FABP (23).

Nuclear DNA fragmentation

Genomic DNA extraction was performed according to Coligan et al. (24). Cardiomyocytes (10^6 cells/plate) were washed twice with PBS and lysed in 0.5 ml of 10 mM Tris/HCl (pH 7.8), 1 mM EDTA, and 0.2% Triton X-100. The lysate was transferred to a 1.5 ml Eppendorf tube, vigorously vortexed, and centrifuged at 11,000 *g* for 10 min. The supernatant was transferred to a fresh tube to which 0.1 ml 5 M NaCl and 0.7 ml isopropanol were added in order to precipitate genomic DNA at −20°C overnight. After centrifugation the pellet was rinsed with 70% ice-cold ethanol, air-dried at room temperature, and redissolved in 25 μl 10 mM Tris/HCl (pH 8.0) and 1 mM EDTA. Electrophoresis was performed in a 2% agarose gel to assess the appearance of a DNA laddering pattern.

TUNEL assay: Cardiomyocytes were washed twice with PBS and fixed in 2% PBS-buffered formaldehyde at room temperature for 30 min. Subsequently, the cells were rinsed with 0.1 M citrate buffer (pH 7.0), 0.1% Triton X-100, followed by the TUNEL assay (17), which was performed with the Apoptag kit (Oncor) according to manufacturer's instructions. This in situ assay is based on the specific labeling of 3′-OH strands of DNA fragments by terminal deoxynucleotidyl transferase. The percentage of TUNEL-positive cells was scored by light microscopy by two independent observers.

Lipid analysis

Cardiomyocytes were washed twice with ice-cold PBS and scraped from the tissue culture dish in 1.5 ml ice-cold methanol, which contained 0.01% butylated hydroxytoluene to prevent auto-oxidation of unsaturated fatty acids. Samples were stored at −80°C for subsequent analysis as described by Van der Vusse and Roemen (25). Briefly, 3 ml chloroform was added to obtain a 2:1 (vol/vol) extraction mixture of chloroform and methanol. The lipids extracted were separated by one-dimensional thin-layer chromatography (TLC) on TLC plates coated with silica gel 60 (Merck, Darmstadt, Germany). The spots corresponding to fatty acids, triacylglycerols, and total phospholipids were visualized with rhodamine 6G and a spray of fluorescein dissolved in methanol. The lipid spots were scraped from the TLC plate and transferred to test tubes. Fatty acids were methylated with 7% BF_3 in methanol at 20°C for 15 min. Triacylglycerols and phospholipids were methylated in 3.5% BF_3 in methanol–toluol 4:1 (v/v) and in 14% BF_3 in methanol, respectively, at 100°C for 30 min. Methylation was stopped through addition of an equal volume of doubly distilled water. The fatty acid–methylesters were extracted into pentane, which was subsequently evaporated, followed by dissolution in 2,2,4-trimethylpentane to quantify the methylesters by gas capillary chromatography (25). Under the current gas chromatographic settings, the peaks corresponding to the methylesters of *trans*- and *cis*-C18:1 were fully separated (retention times 11.63 and 11.81 min, respectively). The cellular content of triacylglycerol and phospholipid was expressed as the amount of fatty acyl chains that are incorporated in these lipid fractions.

Statistics

Results are presented as the mean ± standard deviation (SD). Comparisons between groups were performed with one-way analysis of variance (ANOVA). In case the F-ratio obtained indicated that significant differences between groups were present, a two-tailed student's *t*-test for unpaired data was carried out, applying Bonferroni's adjustments for multiple comparisons (26). Differences were considered significant when $P < 0.05$.

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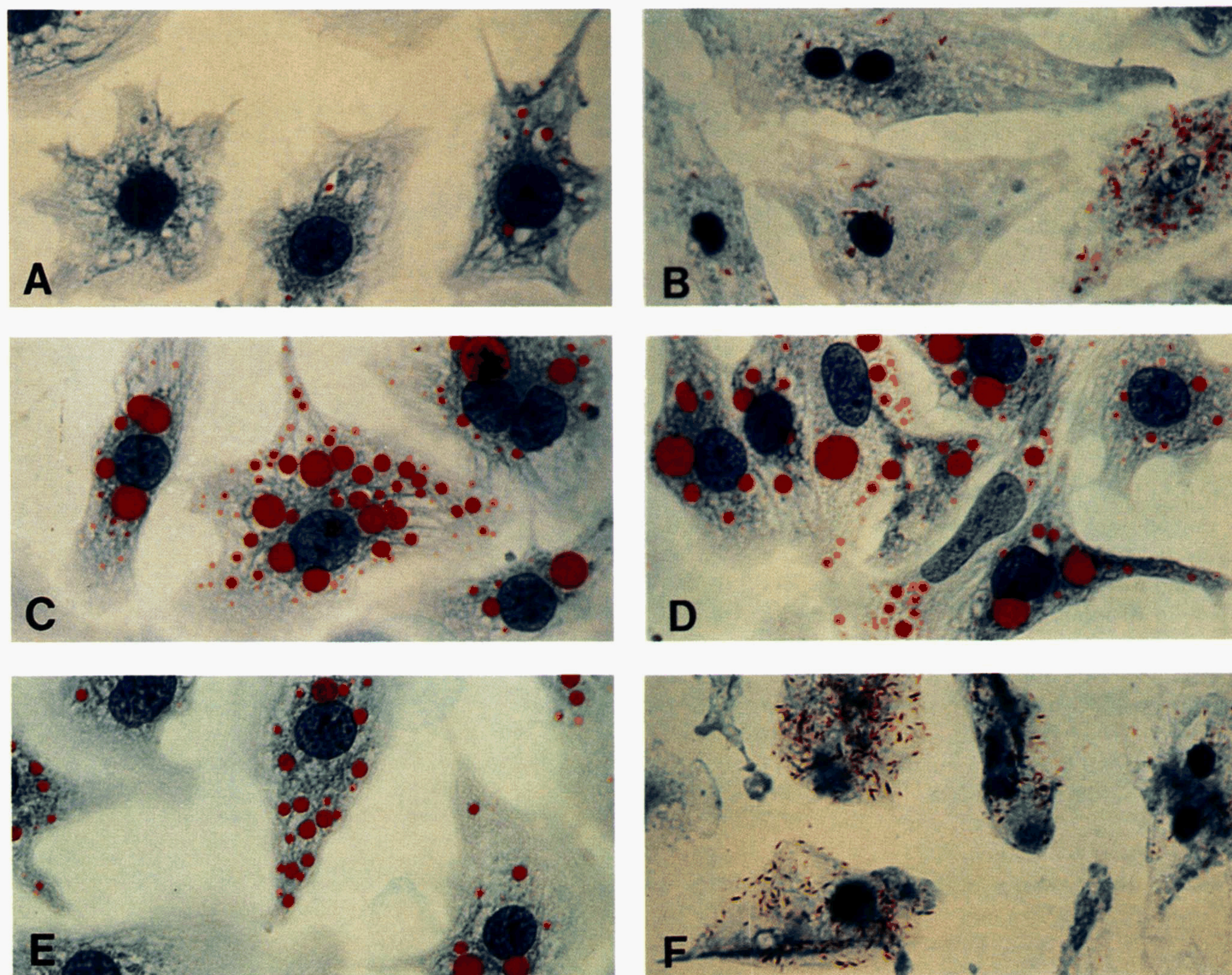

Fig. 1. Neonatal rat ventricular myocytes stained with hematoxylin and Oil Red O after 2 days of culture in medium containing 0.15 mM BSA in the absence of fatty acids (None) or with different fatty acids (0.5 mM) or combinations of fatty acids (0.25 mM each) complexed to BSA. The following conditions are shown: None, i.e., in the presence of nominally fatty acid-free BSA (A); C16:0 (B); *cis*-C18:1 (C); *trans*-C18:1 (D); C16:0/*cis*-C18:1 (E); and C16:0/*trans*-C18:1 (F). Note the presence of the Oil red O-stained lipid droplets in viable cardiomyocytes and the small red specks in non-viable cardiomyocytes (magnification 850×). The fatty acids are denoted by their chemical notation.

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RESULTS

Fatty acids affect neonatal rat ventricular myocyte morphology

Absence of fatty acids (control). After 48 h of culture in the presence of 1% BSA, but without exogenous fatty acids, the morphological appearance of the cardiomyocytes was similar to the morphology displayed at the onset of incubation. The cells were mononucleated with a centrally located, round, and dense nucleus, sharply defined by an intact nuclear membrane. Cell shape was irregular with a coarse, granular cytoplasm. Neutral lipids were occasionally observed and appeared as small red vesicles in the cytoplasm as assessed by staining with Oil Red O (**Fig. 1A**).

Saturated fatty acids. Microscopic analysis of the cardiomyocytes incubated with 0.5 mM C16:0 for 48 h indicated that the cells had not survived this treatment. Nuclei had become small and hyperchromatic, reminiscent of pyknotic nuclei, which in general is taken as a marker of cell death (27). The cells were unable to exclude trypan blue, which is a well-accepted criterion for loss of cellular integrity (28). Diffuse small red specks were occasionally observed in the cytoplasmic area. Some cells were found to be almost devoid of these structures, whereas others contained a substantial number, covering almost the entire cellular compartment (Fig. 1B). The nature of these structures was not elucidated, but the observation that the specks appeared after the formation of pyknotic nuclei suggested that they reflect post-mortem changes. Reducing the concentration of C16:0 to 0.25 mM or 0.1 mM (keeping the

BSA concentration constant) did not prevent cell death (data not shown). Furthermore, cardiomyocytes incubated with 0.5 mM C18:0 displayed detrimental morphological changes similar to those seen in cells exposed to C16:0.

Mono-unsaturated fatty acids. The presence of 0.5 mM *cis*-C18:1 for up to 48 h did not affect the normal nuclear and cytoplasmic morphological appearance, but a marked increase in number and size of vesicles filled with neutral lipids was observed (Fig. 1C). A comparable increase of lipid droplets also occurred in cardiomyocytes cultured with either 0.5 mM C16:1 (not shown) or with 0.5 mM *trans*-C18:1 (Fig. 1D). Under these conditions the cytoplasmic and the nuclear appearance did not differ from control cardiomyocytes.

Combinations of fatty acids. The presence of both C16:0 and *cis*-C18:1 (0.25 mM each) was accompanied by morphological features identical to cardiomyocytes cultured with 0.5 mM *cis*-C18:1 alone. After 48 h the cells had a healthy appearance and contained numerous lipid droplets (Fig. 1E). The fact that cardiomyocytes remained viable could theoretically be due to either the lowering of the C16:0 concentration (0.25 mM instead of 0.5 mM), or the simultaneous presence of *cis*-C18:1. However, as discussed above, exposure to 0.25 mM C16:0 was also toxic to the cells. This implies that the co-addition of *cis*-C18:1 rather than the reduction of the C16:0 concentration from 0.5 mM to 0.25 mM prevented cell death.

In addition, cardiomyocytes were incubated with a mixture of C16:0 and *trans*-C18:1 (0.25 mM each) for up to 48 h. The co-administration of *trans*-C18:1 instead of *cis*-C18:1 markedly affected cellular survival. During the first 24 h no morphological signs of a loss of cellular viability could be observed. However, after 2 days of culturing, the majority of the cells contained pyknotic nuclei embedded in cytoplasmic remnants (Fig. 1F). Small red specks with a striped appearance were present, the number of which varied considerably from cell to cell.

Release of lactate dehydrogenase (LDH) and fatty acid-binding protein (FABP)

The release of two cytosolic proteins, i.e., LDH (140 kDa) and FABP (15 kDa), into the incubation medium was used as a marker of cell death. As the release curves of LDH and FABP were superimposable, only the FABP data are presented in **Fig. 2**. The levels of LDH and FABP in the incubation medium of control cardiomyocytes showed a small but steady increase over 48 h (Fig. 2A). After 48 h LDH activity and FABP amount in the incubation medium corresponded to 20% and 18% of the total cellular LDH activity and FABP content, respectively (**Fig. 3**).

As compared to control cardiomyocytes, the release

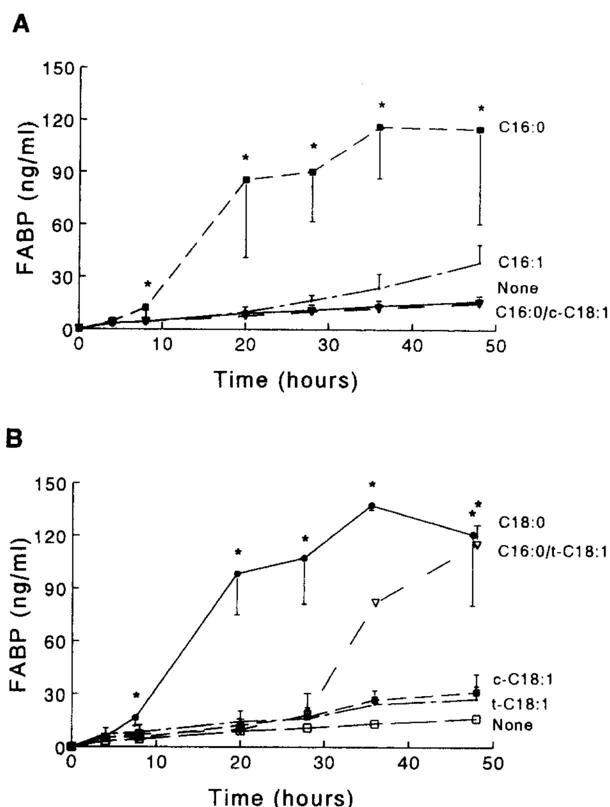

Fig. 2. Release of FABP into the culture medium of cells incubated in the absence or presence of different fatty acids (0.5 mM) or fatty acid combinations (0.25 mM each) as a function of time. Data are presented as means ± SD (n = 4). Asterisk indicates significant difference from control cardiomyocytes (i.e., cells cultured in the absence of fatty acids, depicted as None) at the corresponding time point.

of LDH and FABP from cells cultured in the presence of 0.5 mM C16:0 started to increase rapidly after 8 h (Fig. 2A). The percentage release of LDH and FABP exceeded 95% of the total cellular content after 48 h (Fig. 3), indicating massive loss of cellular integrity over this time interval. The release of LDH from cardiomyocytes exposed to 0.25 mM or 0.1 mM C16:0 after 48 h equalled the rate of release observed with 0.5 mM C16:0 (not shown), which strengthens the notion that low levels of saturated fatty acids also induced cell death. The LDH and FABP release curves in the presence of C18:0 (Fig. 2B) were found to be comparable to the release curve of C16:0, with almost complete loss of cell viability, as indicated by the near total release of LDH and FABP after 48 h (Fig. 3). Prolonged exposure of the cells to either C16:1, *cis*-C18:1, or *trans*-C18:1 did not significantly affect the release of LDH and FABP, although after 48 h it tended to be somewhat higher for cells incubated with C16:1 or *cis*-C18:1 as compared to control cardiomyocytes (Figs. 2 and 3).

When cells received a combination of C16:0 and *cis*-C18:1 (0.25 mM each) the release of LDH and FABP

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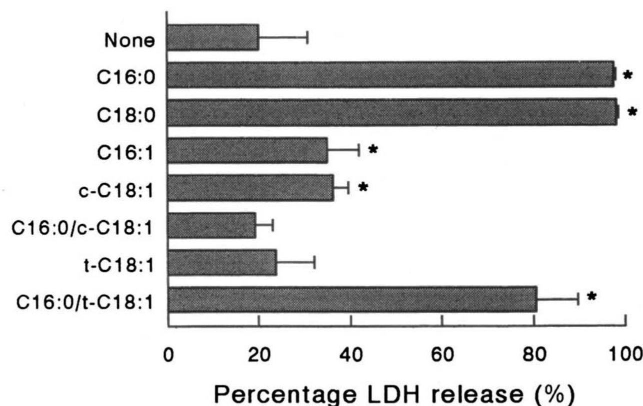

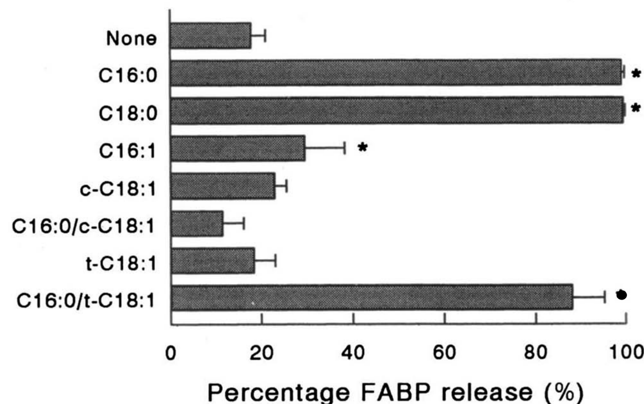

Fig. 3. Percentage release of LDH (A) and FABP (B) after 48 h from cells cultured in the absence or presence of different fatty acids or fatty acid combinations complexed to BSA. Data are presented as means ± SD (n = 4–5). Asterisk indicates significant difference from control cardiomyocytes (i.e., cells cultured in the absence of fatty acids, depicted as None).

remained low and was similar to that of control cardiomyocytes (Figs. 2A and 3). In the presence of a mixture of C16:0 and *trans*-C18:1 (0.25 mM each) the release of LDH and FABP remained at base line levels during the first 24 h, but increased rapidly thereafter (Fig. 2B), resulting eventually in a 81% and 87% release of LDH and FABP, respectively, after 48 h (Fig. 3).

Exogenous fatty acids are incorporated into endogenous lipid pools

To assess whether metabolic processing of saturated fatty acids differed from mono-unsaturated fatty acids, the cellular content and composition of the phospholipid and triacylglycerol pools were analyzed.

Phospholipids. The phospholipid content of neonatal cardiomyocytes cultured without fatty acids averaged 92 nmol of fatty acyl moieties/10^6 cells after 48 h. C16:0, C18:0, *cis*-C18:1, C18:2, and C20:4 were the main fatty acid constituents of the phospholipid pool (**Table 1**). When cells were cultured in the presence of C16:0 for 48 h, the cellular phospholipid content was significantly lower (68% of control group). The mol % of C16:0 in the phospholipid pool amounted to 14 ± 2% at t = 0 and increased to 38 ± 6% and 48 ± 3% after 8 h and 16 h of exposure to C16:0, respectively (n = 5 each). After 48 h the mol % of C16:0 averaged 56%, thereby increasing the mol % of all saturated fatty acids in the phospholipid fraction to almost 70% (Table 1). In cardiomyocytes cultured with *cis*-C18:1 the total amount of phospholipids did not change, but the mol % of *cis*-C18:1 increased from 20% to 67% after 48 h. Hence, the mol % of all mono-unsaturated fatty acids in the phospholipid pool was raised nearly 3-fold. After 48 h of exposure of the cells to *trans*-C18:1, the mol % of this fatty acid species in the phospholipid pool amounted to 52%, implying that over 50% of the original fatty acid moieties in the phospholipid pool had been replaced by *trans*-C18:1.

The combined presence of C16:0 and *cis*-C18:1 in the incubation medium did not alter the total cellular phospholipid content. Nonetheless, the phospholipid pool was highly enriched with both C16:0 and *cis*-C18:1. A substantial decline of the cellular phospholipid content was observed in cardiomyocytes incubated with the combination of C16:0 and *trans*-C18:1 for 48 h. The fatty acyl composition of the phospholipids was roughly similar to the phospholipid composition of C16:0/*cis*-C18:1 cultured cardiomyocytes, with *trans*-C18:1 substituting *cis*-C18:1 (Table 1).

Triacylglycerols. The total amount of triacylglycerols in control cardiomyocytes, cultured with nominally fatty acid-free BSA, averaged 5 nmol fatty acyl moieties/10^6 cells after 48 h. The triacylglycerol pool was mainly composed of C16:0, C18:0, and C18:1 (**Table 2**). In cells exposed to C16:0 there was a small, but significant, initial rise in triacylglycerol content to 8 ± 1 (n = 5) and 7 ± 1 (n = 5) nmol/10^6 cells after 8 h and 16 h, respectively. Thereafter the cellular triacylglycerol content did not change any further.

In cardiomyocytes cultured with *cis*-C18:1 or *trans*-C18:1 the triacylglycerol content increased to an average level of 66 and 43 nmol/10^6 cells, respectively, after 48 h. Likewise, the simultaneous supplementation of C16:0 and *cis*-C18:1 in the incubation medium induced a time-dependent increase of the triacylglycerol content to 32 and 80 nmol/10^6 cells after 16 and 48 h, respectively. After 48 h C16:0 and *cis*-C18:1 comprised 38% and 58% of the fatty acyl moieties of the TG pool, respectively (Table 2). In contrast, the triacylglycerol content of cells exposed to the combination of C16:0

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TABLE 1. Phospholipid content and fatty acyl composition of phospholipids in cardiomyocytes

	Experimental Conditions					
	None	C16:0	*c*-C18:1	C16:0/*c*-C18:1	*t*-C18:1	C16:0/*t*-C18:1
			nmol/10⁶ cells			
Total amount	92 (13)	63 (10)[a]	87 (18)	113 (16)	73 (5)	59 (2)[a]
			%			
14:0	0.4 (0.3)	0.5 (0.5)	0.1 (0.2)	0.4 (0)	ND	ND
16:0	12.3 (0.8)	56.6 (2.6)[a]	4.9 (0.9)[a]	20.4 (1.2)[a]	3.0 (0.3)[a]	23.3 (0.9)[a]
16:1	1.2 (0.2)	0.2 (0.3)[a]	0.1 (0.2)[a]	0.5 (0.1)[a]	0.2 (0.3)[a]	0.2 (0.3)[a]
18:0	20.2 (1.5)	11.9 (0.7)[a]	7.6 (0.5)[a]	10.9 (0.6)[a]	6.3 (0.3)[a]	8.7 (0.3)[a]
c-18:1	20.3 (2.5)	9.9 (1.5)[a]	67.6 (1.5)[a]	44.6 (2.6)[a]	9.5 (1.1)[a]	7.4 (0.2)[a]
t-18:1	ND	ND	ND	ND	52.7 (2.3)[a]	38.1 (0.9)[a]
18:2	19.1 (2.8)	5.7 (0.7)[a]	5.3 (0.3)[a]	6.9 (0.1)[a]	11.2 (0.4)[a]	6.2 (0.2)[a]
18:3	0.2 (0.2)	0.2 (0.2)	0.1 (0.1)	0.2 (0.2)	ND	ND
20:0	0.2 (0.2)	ND	0.1 (0.1)	0.2 (0.1)	ND	ND
20:4	21.2 (2.3)	11.3 (0.5)[a]	11.0 (0.6)[a]	12.7 (1.3)[a]	13.4 (0.7)[a]	12.1 (0.3)[a]
22:4	1.5 (0.2)	1.1 (0.2)	1.1 (0.1)[a]	0.9 (0)[a]	1.1 (0.1)[a]	1.1 (0.0)[a]
22:6	3.4 (0.7)	2.5 (0.2)	1.5 (0.2)[a]	2.3 (0.3)[a]	1.8 (0.1)[a]	1.8 (0.1)[a]
24:0	0.1 (0.1)	ND	ND	0.1 (0)	ND	ND

Neonatal rat ventricular myocytes were cultured for 48 h. The final concentration of fatty acids (denoted by their chemical notation) in the culture medium was 0.5 mM in case of C16:0, *c*-C18:1, *t*-C18:1, and 0.25 mM/0.25 mM when combinations of fatty acids (C16:0/*c*-C18:1 and C16:0/*t*-C18:1) were added. Data are presented as means (± SD) of 4 experiments.

[a]Indicates significant differences from control cardiomyocytes (None); ND, not detectable.

and *trans*-C18:1 did not differ from control cardiomyocytes after 48 h. Nevertheless, the fatty acyl composition had changed markedly and consisted of about equal amounts of C16:0 and *trans*-C18:1 residues (Table 2).

Apoptotic traits characterize fatty acid-induced cell death

A hallmark of apoptosis is the development of numerous cleavages in genomic DNA, specifically in between nucleosomes (18), leading to a typical DNA-fragmentation pattern of multiples of approximately 200 base pairs. DNA-laddering was not detected in cardiomyocytes cultured with C16:0/*cis*-C18:1 (0.25 mM each) throughout the time-course studied. However, DNA-laddering was observed in cardiomyocytes exposed to either 0.5 mM C16:0 or 0.25 mM C16:0 (**Fig. 4**). The laddering pattern was most prominent in cells exposed to C16:0 for 16 h. A faint DNA-laddering pattern

TABLE 2. Triacylglycerol content and fatty acyl composition of triacylglycerols in cardiomyocytes

	Experimental Conditions					
	None	C16:0	*c*-C18:1	C16:0/*c*-C18:1	*t*-C18:1	C16:0/*t*-C18:1
			nmol/10⁶ cells			
Total amount	5 (2)	5 (1)	66 (27)[a]	80 (17)[a]	43 (4)[a]	8 (1)
			%			
14:0	ND	ND	ND	0.1 (0.2)	ND	ND
16:0	34.6 (7.9)	76.6 (16.1)[a]	2.6 (0.5)[a]	38.4 (1.8)	3.4 (1.1)[a]	51.2 (4.4)[a]
16:1	ND	ND	0.2 (0.3)	0.4 (0.2)	ND	ND
18:0	32.9 (11.2)	3.6 (3.8)[a]	1.1 (0.1)[a]	2.0 (0.2)[a]	1.4 (0.1)[a]	2.2 (2.4)[a]
c-18:1	22.4 (13.1)	18.7 (18.5)	95.8 (1.3)[a]	58.1 (1.6)[a]	5.0 (2.6)[a]	ND
t-18:1	ND	ND	ND	ND	88.5 (4.1)[a]	46.6 (4.4)[a]
18:2	9.0 (5.2)	ND	0.1 (0.2)[a]	0.4 (0.2)[a]	ND	ND
18:3	ND	ND	ND	ND	ND	ND
20:0	ND	ND	ND	ND	ND	ND
20:4	1.1 (1.9)	ND	0.2 (0.3)	0.7 (0.1)	1.0 (0.6)	ND
22:4	ND	ND	ND	ND	ND	ND
22:6	ND	ND	ND	ND	ND	ND
24:0	ND	1.2 (2.0)	ND	ND	ND	ND

Neonatal rat ventricular myocytes were cultured for 48 h. The final concentration of fatty acids (denoted by their chemical notation) in the culture medium was 0.5 mM in case of C16:0, *c*-C18:1, *t*-C18:1, and 0.25 mM/0.25 mM when combinations of fatty acids (C16:0/*c*-C18:1 and C16:0/*t*-C18:1) were added. Data are presented as means (± SD) of 4 experiments.

[a]Indicates significant differences from control cardiomyocytes (None); ND, not detectable.

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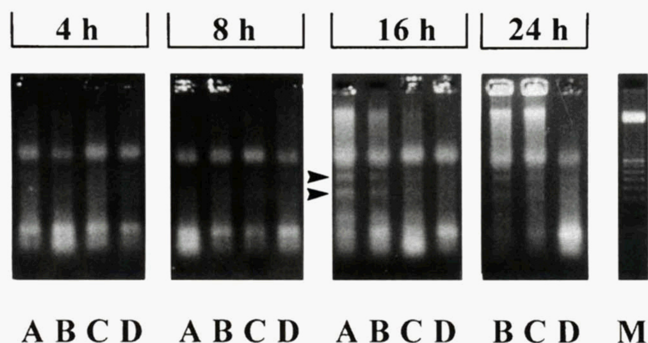

Fig. 4. Gel electrophoresis of genomic DNA isolated from cardiomyocytes exposed to different fatty acid species or combinations thereof; (A) 0.5 mM C16:0, (B) 0.25 mM C16:0, (C) 0.25 mM each of C16:0/*trans*-C18:1, and (D) 0.25 mM each of C16:0/*cis*-C18:1. Cells were harvested at the indicated time points. Position of characteristic n × 200 bp fragments are indicated by arrows. M refers to molecular weight marker (100 base pair ladder, Gibco BRL). Note the clear ladder pattern in cells exposed to 0.5 mM and 0.25 mM C16:0 for 16 h or longer, and its absence in cells exposed to C16:0/*cis*-18:1.

became visible in cells cultured in the presence of C16:0/*trans*-C18:1 (0.25 mM each) for 24 h. In support of the DNA-laddering assay was the in situ detection of DNA strand breaks by means of the TUNEL assay (**Fig. 5**). The percentage TUNEL positive cells was 5- to 10-fold higher in those conditions where DNA-laddering was apparent (data not shown).

DISCUSSION

In the present study the effect of long-term exposure of neonatal rat ventricular cardiomyocytes to fatty acids was investigated. It was demonstrated that cardiomyocyte viability was dependent on the degree of saturation of the fatty acid supplied. Incubation in the presence of the saturated fatty acids C16:0 or C18:0 resulted in massive cell death within 24 h, as evidenced morphologically by the presence of pyknotic nuclei, by the occurrence of internucleosomal DNA strand breaks, indicative of apoptosis, and the release of the cytosolic proteins LDH and FABP in the surrounding medium. In contrast, neither C16:1, *cis*-C18:1 nor *trans*-C18:1, being mono-unsaturated fatty acids, were found to be harmful to the cardiomyocytes. The simultaneous administration of equimolar amounts of *cis*-mono-unsaturated and saturated fatty acids counteracted the toxic effects of saturated fatty acids completely, whereas co-administration of *trans*-C18:1 delayed, but did not prevent apoptotic cell death.

The fact that saturated fatty acids are harmful to various cell types has been reported before (9–14). To this list neonatal ventricular myocytes can now be added. However, the nature of the process of cell death, i.e., necrosis or apoptosis, has not been subject of study. To the best of our knowledge, this study is the first to demonstrate that saturated fatty acids induce apoptosis, as exemplified by the relatively early appearance of DNA laddering and the marked increase in percentage of TUNEL-positive cells in C16:0-exposed cardiomyocytes. Nonetheless, the stimulus for apoptosis has not been defined yet. It has been shown that the mere absence of serum factors is sufficient to initiate apoptosis in cultured cells (29). Serum-deprived neonatal myocytes are likely to undergo a similar process (30, and K. R. Chien, personal communication). Indeed, in the present study we also observed a slow release of intracellular proteins from serum-deprived myocytes cultured in the absence of fatty acids, or in the presence of *cis*-mono-unsaturated fatty acids. According to this line of reasoning, exposure of the cells to saturated fatty acids somehow appreciably accelerates apoptotic cell death in cardiomyocytes cultured under serum-free conditions. Exposure to the different fatty acid species was associated with major alterations in the endogenous lipid pools, both in content and composition. Hence, it is feasible that these alterations are responsible for cell death. The potential link between fatty acid handling by neonatal cardiomyocytes and apoptotic cell death is discussed below.

Under normal circumstances both saturated and *cis*-

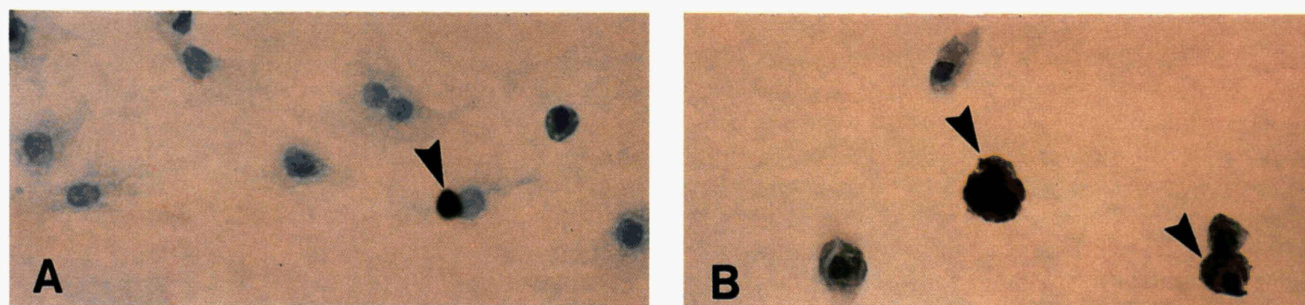

Fig. 5. In situ analysis of DNA strand breaks in neonatal cardiomyocytes by TUNEL assay. Cells were exposed (A) to 0.25 mM each of C16:0/*cis*-C18:1 and (B) to 0.5 mM C16:0 for 16 h. TUNEL-positive cells are indicated by an arrow.

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monounsaturated fatty acids are abundantly present in the circulating blood. Together these fatty acid species represent approximately three quarters of the fatty acids in rat blood plasma [16]. Normally, *trans*-unsaturated fatty acids, such as elaidic acid (*trans*-C18:1), are present in minute amounts. Nonetheless, the myocardium is able to cope with *trans*-unsaturated fatty acids. When supplied in the diet *trans*-unsaturated fatty acids and their metabolic derivatives are found to be sequestered in the cardiac lipid pools (31, 32). In addition, the myocardium is able to oxidize *trans*-C18:1 and *cis*-C18:1 at a comparable rate (33). From a physico-chemical point of view the mono-unsaturated elaidic acid resembles a saturated fatty acid. This is illustrated by the fact that, similar to saturated fatty acids, *trans*-mono-unsaturated fatty acids are preferentially esterified to the *sn*-1 position of phospholipids (34).

Neonatal cardiomyocytes already have the capacity to utilize fatty acids at a significant rate (7, 35). This was substantiated in our own laboratory, in which it was shown that the capacity to oxidize [^{14}C]palmitate by freshly isolated neonatal and by adult myocytes was comparable (J. J. F. P. Luiken, unpublished observations). Furthermore, in the present study it was found that the fatty acids, irrespective of the species supplied, were rapidly incorporated in the phospholipid pool. Moreover, in the presence of mono-unsaturated fatty acids the cells do form substantial amounts of triacylglycerols. Altogether, these findings indicate that neonatal rat ventricular myocytes are already able to extract and to metabolize fatty acids from the extracellular environment.

It is clear from the present study that the saturated fatty acids C16:0 and C18:0 induce apoptosis in neonatal cardiomyocytes. To date the mechanism underlying this phenomenon is incompletely understood. First, a detergent effect of the fatty acids supplied to the cells via the incubation medium could be envisaged. However, such an effect is most likely to manifest itself rapidly after application (36). For instance, Hoffmann et al. (37) showed that the application of 30 μM of polyunsaturated fatty acids (in the absence of BSA) to neonatal cardiomyocytes resulted in rapid alterations in cellular calcium handling, resulting in calcium overload and cell death within 30 min. In the present study fatty acids were complexed to BSA in a 3.3 to 1 ratio. Based on this fatty acid/BSA ratio and the dissociation constants estimated by Richieri, Anel, and Kleinfeld (38), the unbound fatty acid concentration is less than 50 nM, i.e., far below the critical micellar concentration (38–40). These considerations argue against the involvement of a detergent effect in the present experimental set-up (41). The second putative explanation is based on the fact that triacylglycerol composed of saturated fatty acyl chains that have a relatively high melting point (m.p) are insoluble at 37°C. It has been argued that immediately after formation the saturated triacylglycerol molecules precipitate at the site of synthesis, i.e., the sarcoplasmic reticulum. These precipitates are thought to hamper sarcoplasmic reticulum function and, hence, to compromise cellular viability (42). This mechanism was proposed on the basis of electron microscopic observations of human neutrophils incubated in the presence of saturated fatty acids (14). In line with this hypothesis is the observation that C16:0 was hardly used for the synthesis of triacylglycerols, but was avidly incorporated in the phospholipid pool of the cardiomyocytes. Although it is evident that the de novo synthesis of triacylglycerols is impaired under these conditions, it still remains to be established whether this process is linked to apoptosis.

Recent studies indicate that signal transduction through the sphingomyelin–ceramide pathway activates apoptosis in various cell types, including neonatal myocytes (43). In this respect it is tempting to speculate that the loss of phospholipids, as observed in myocytes cultured in the presence of C16:0, also involves hydrolysis of sphingomyelin. Alternatively, an increase in the cellular levels of C16:0 and C18:0, but not of C16:1, has been shown to stimulate the formation of ceramide intermediates that will initiate programmed cell death (44). However, the latter observation does not provide an explanation for our findings that co-addition of equimolar amounts of C18:1 prevents C16:0-induced apoptosis.

Finally, the cytotoxic effects of saturated fatty acids may relate to their relative enrichment in the phospholipid fraction. A relative increase of fatty acids with a high melting point in the phospholipid pool lowers the membrane fluidity (36) and, consequently, elevates the transition temperature (T_M) of the membrane (45). At temperatures below the T_M phospholipid bilayers are in the gel-like crystalline phase, which severely hampers membrane function (36, 45–47). The increase in the mol % of saturated fatty acyl chains in the membrane phospholipid pool to nearly 70% in neonatal myocytes will diminish membrane fluidity and, hence, compromise cell function. Indeed, loss of cellular viability was demonstrated in cardiac cells exposed to either C16:0 or C18:0 (m.p. 63°C and 70°C, respectively), but not when exposed to *cis*-C16:1 or *cis*-C18:1 (m.p. 0.5°C or 13°C, respectively). Apparently, an intermediate position is taken by the *trans*-mono-unsaturated fatty acid *trans*-C18:1 (m.p. 46°C). When supplied to cardiomyocytes as sole fatty acid, the cells survive. However, in combination with C16:0 apoptotic cell death is retarded, but not prevented. The combination of C16:0/*cis*-C18:1 appears to be fully compatible with cardiomyocyte function. The observation that the composition of the endogenous lipid pools of the cardiomy-

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ocytes in vitro is determined by the fatty acids present in the incubation medium, resembles the in vivo situation where the fatty acyl profile of intracellular lipids in cardiac tissue reflects the fatty acids present in the diet (5). This study indicates that the common observation that the heart in situ tolerates exogenous fatty acids very well lies in the fact that these substances are supplied as a mixture of saturated and unsaturated fatty acids.

Singh and coworkers (48) provided evidence that an increase in the mol % of saturated fatty acids in the phospholipid pool, and hence in membrane fluidity, forms an essential part of the apoptotic process. Furthermore, alterations in the composition of phospholipid membranes may act as a trigger for apoptosis (49, 50). For instance, a genetic defect in the synthesis of phosphatidylcholine was found to trigger apoptosis in CHO cells (50). Also, the pharmacological inhibition of arachidonate redistribution among the various phospholipid classes was demonstrated to induce apoptosis in a promyelocytic cell line (49). Based on these observations, we hypothesize that the over-representation of saturated fatty acids in the phospholipid pool, and the consequent effects on membrane fluidity, drive the cardiomyocytes into apoptosis.

In conclusion, the present findings indicate that saturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes, which can be counteracted by co-addition of equimolar amounts of *cis*-mono-unsaturated fatty acids. A corollary of the present observations is that in studies aiming at delineating the role of fatty acids in energy metabolism or signal transduction, the use of saturated fatty acids as sole agent in long-term experiments should be discouraged, because noxious effects of these fatty acids on overall cardiomyocyte function during prolonged exposure must be anticipated.

The research of MVB has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. The assistance of Mr. J. Willems for the LDH measurements was greatly appreciated. The authors wish to thank Drs. J. F. C. Glatz, W. Th. Hermens, and M. J. M. de Groot for stimulating discussions.

Manuscript received 23 December 1996 and in revised form 7 April 1997.

REFERENCES

1. Bishop, W. R., and R. M. Bell. 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. *Annu. Rev. Cell. Biol.* **4:** 579–610.
2. Liscovitch, M., and L. C. Cantley. 1994. Lipid second messengers. *Cell.* **77:** 329–334.
3. Van Bilsen, M., and G. J. Van der Vusse. 1995. Phospholipase-A2-dependent signalling in the heart. *Cardiovasc. Res.* **30:** 518–529.
4. Neely, J. R., and H. E. Morgan. 1974. Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annu. Rev. Physiol.* **36:** 413–459.
5. Van der Vusse, G. J., J. F. C. Glatz, H. C. Stam, and R. S. Reneman. 1992. Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol. Rev.* **72:** 881–940.
6. Rosenthal, M. D., and J. B. Warshaw. 1973. Interaction of fatty acid and glucose oxidation by cultured heart cells. *J. Cell. Biol.* **58:** 332–339.
7. Rosenthal, M. D., and J. B. Warshaw. 1977. Fatty acid and glucose oxidation by cultured rat heart cells. *J. Cell. Physiol.* **93:** 31–40.
8. Hexeberg, S., I. Hessevik, and E. Hexeberg. 1995. Intravenous lipid infusion results in myocardial lipid droplet accumulation combined with reduced myocardial performance in heparinized rabbits. *Acta Physiol. Scand.* **153:** 159–168.
9. Moskowitz, M. S. 1967. Fatty acid-induced steatosis in monolayer cell cultures. *In* Lipid Metabolism in Tissue Culture Cells. G. H. Rothblat, and D. Kritchevsky, editors. The Wistar Institute Press, Philadelphia. Vol. 6. 49–59.
10. Rosenthal, M. D. 1981. Accumulation of neutral lipids by human skin fibroblasts: differential effects of saturated and unsaturated fatty acids. *Lipids.* **16:** 173–182.
11. Buttke, T. M. 1984. Inhibition of lymphocyte proliferation by free fatty acids. I. Differential effects on mouse B and T lymphocytes. *Immunology.* **53:** 235–242.
12. Zhang, C. L., V. Lyngmo, and A. Nordoy. 1992. The effects of saturated fatty acids on endothelial cells. *Thromb. Res.* **65:** 65–75.
13. Doi, O., F. Doi, F. Schroeder, A. W. Alberts, and P. R. Vagelos. 1978. Manipulation of fatty acid composition of membrane phospholipid and its effects on cell growth in mouse LM cells. *Biochim. Biophys. Acta.* **509:** 239–250.
14. Gordon, G. B. 1977. Saturated free fatty acid toxicity. II. Lipid accumulation, ultrastructural alterations, and toxicity in mammalian cells in culture. *Exp. Mol. Pathol.* **27:** 262–276.
15. De Vries, J. E., T. H. M. Roemen, G. J. van der Vusse, and M. van Bilsen. 1995. Saturated, but not mono-unsaturated, fatty acids induce cell death in neonatal rat ventricular cardiomyocytes. *Eur. J. Physiol.* **S430:** R67.
16. Van der Vusse, G. J., T. H. M. Roemen, W. Flameng, and R. S. Reneman. 1983. Serum-myocardium gradients of non-esterified fatty acids in dogs, rat, and man. *Biochim. Biophys. Acta.* **752:** 361–370.
17. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119:** 493–501.
18. Majno, G., and I. Joris. 1995. Apoptosis, oncosis, and necrosis: an overview of cell death. *Am. J. Physiol.* **146:** 3–15.
19. Iwaki, K., V. P. Sukhatme, H. E. Shubeita, and K. R. Chien. 1990. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. *J. Biol. Chem.* **265:** 13809–13817.
20. Van der Loop, F. T. L., G. J. J. M. Van Eys, G. Schaart, and F. C. S. Ramaekers. 1996. Titin expression as an early indication of heart and skeletal muscle differentiation in vitro. Developmental re-organization in relation to cytoskeletal constituents. *J. Muscle Res. Cell Motil.* **17:** 1–14.

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21. Clark, G., editor. 1981. Staining Procedures. 4th ed. Williams and Wilkins, Baltimore. 228–229.
22. Bergmeyer, H. U., and E. Bernt. 1974. UV assay for LDH with pyruvate and NADH. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. 2nd ed. Vol. 2. Academic Press, Inc., New York. 574–579.
23. Vork, M. M., J. F. C. Glatz, D. A. M. Surtel, H. J. M. Knubben, and G. J. Van der Vusse. 1991. A sandwich enzyme-linked immuno-sorbent assay for the determination of rat heart fatty acid-binding protein using the streptavidin-biotin system. Application to tissue and effluent samples from normoxic rat heart perfusion. *Biochim. Biophys. Acta.* **1075:** 199–205.
24. Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevarh, and W. Strober, editors. 1991. Current Protocols for Immunology. Greene Publishing Associates and Wiley-Interscience, New York. 3.17.7–3.17.8.
25. Van der Vusse, G. J., and T. H. Roemen. 1995. Gradient of fatty acids from blood plasma to skeletal muscle in dogs. *J. Appl. Physiol.* **78:** 1839–1843.
26. Wallenstein, S., C. L. Zucker, and J. L. Fleiss. 1980. Some statistical methods useful in circulation research. *Circ. Res.* **47:** 1–9.
27. Kerr, J. F. R., G. C. Gobe, C. M. Winterford, and B. V. Harmon. 1995. Anatomical methods in cell death. *In* Methods in Cell Biology. L. M. Schwartz, and B. A. Osborne, editors. Vol. 46. Academic Press, San Diego. 1–27.
28. Van der Laarse, A., J. C. Altona, and C. H. F. Bloys van Treslong. 1985. Anoxia in neonatal rat heart cell cultures. *Basic Res. Cardiol.* **80S1:** 137–142.
29. Galli, G., and M. Pratelli. 1993. Activation of apoptosis by serum deprivation in a teratocarcinoma cell line: inhibition by L-acetylcarnitine. *Exp. Cell. Res.* **204:** 54–60.
30. Sheng, Z., D. Pennica, W. I. Wood, and K. R. Chien. 1996. Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. *Development.* **122:** 419–428.
31. Ohlrogge, J. B., E. A. Emken, and R. M. Gulley. 1981. Human tissue lipids: occurrence of fatty acid isomers from dietary hydrogenated oils. *J. Lipid Res.* **22:** 955–960.
32. Ratnayake, W. M., Z. Y. Chen, G. Pelletier, and D. Weber. 1994. Occurrence of 5c,8c,11c,15t-eicosatetraenoic acid and other unusual polyunsaturated fatty acids in rats fed partially hydrogenated canola oil. *Lipids.* **29:** 707–714.
33. Lanser, A. C., E. A. Emken, and J. B. Ohlrogge. 1986. Oxidation of oleic and elaidic acids in rat and human heart homogenates. *Biochim. Biophys. Acta.* **875:** 510–515.
34. Kinsella, J. E., G. Bruckner, J. Mai, and J. Shimp. 1981. Metabolism of *trans* fatty acids with emphasis on the effects of *trans, trans*-octadecadienoate on lipid composition, essential fatty acid, and prostaglandins: an overview. *Am. J. Clin. Nutr.* **34:** 2307–2318.
35. Ross, P. D., and R. L. McCarl. 1984. Oxidation of carbohydrates and palmitate by intact cultured neonatal rat heart cells. *Am. J. Physiol.* **246:** H389–H397.
36. Stubbs, C. D., and A. D. Smith. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta.* **779:** 89–137.
37. Hoffmann, P., D. Richards, I. Heinroth-Hoffmann, P. Matthias, H. Wey, and M. Toraason. 1995. Arachidonic acid disrupts calcium dynamics in neonatal rat cardiac myocytes. *Cardiovasc. Res.* **30:** 889–898.
38. Richieri, G. V., A. Anel, and A. M. Kleinfeld. 1993. Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry.* **32:** 7574–7580.
39. Richieri, G. V., R. T. Ogata, and A. M. Kleinfeld. 1992. A fluorescently labeled intestinal fatty acid binding protein. *J. Biol. Chem.* **267:** 23495–23501.
40. Rose, H., M. Conventz, Y. Fischer, E. Jungling, T. Hennecke, and H. Kammermeier. 1994. Long-chain fatty acid-binding to albumin: re-evaluation with directly measured concentrations. *Biochim. Biophys. Acta.* **1215:** 321–326.
41. Cistola, D. P., J. A. Hamilton, D. Jackson, and D. M. Small. 1988. Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule. *Biochemistry.* **27:** 1881–1888.
42. Hawley, H. P., and G. B. Gordon. 1976. The effects of long chain free fatty acids on human neutrophil function and structure. *Lab. Invest.* **34:** 216–222.
43. Krown, K. A., T. M. Page, C. Nguyen, D. Zechner, V. Gutierrez, K. L. Comstock, C. C. Glembotski, P. J. E. Quintana, and S. A. Sabbadini. 1996. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. *J. Clin. Invest.* **98:** 2854–2865.
44. Paumen, M. B., Y. Ishida, M. Muramatsu, M. Yamamoto, and T. Honjo. 1997. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **271:** 3324–3329.
45. Lenaz, G., and G. P. Castelli. 1985. Membrane fluidity. Molecular basis and physiological significance. *In* Structure and Properties of Cell Membranes. G. Benga, editor. Vol. 1. CRC Press Inc., Boca Raton, FL. 93–136.
46. Innis, S. M., and M. T. Clandinin. 1981. Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid. *Biochem. J.* **198:** 167–175.
47. Gould, R. J., B. H. Ginsberg, and A. A. Spector. 1982. Lipid effects on the binding properties of a reconstituted insulin receptor. *J. Biol. Chem.* **257:** 477–484.
48. Singh, J. K., A. Dasgupta, T. Adayev, S. A. Shahmehdi, D. Hammond, and P. Banerjee. 1996. Apoptosis is associated with an increase in saturated fatty acid-containing phospholipids in the neuronal cell line, HN2-5. *Biochim. Biophys. Acta.* **1304:** 171–178.
49. Surette, M. E., J. D. Winkler, A. N. Fonteh, and F. H. Chilton. 1996. Relationship between arachidonate-phospholipid remodeling and apoptosis. *Biochemistry.* **35:** 9187–9196.
50. Cui, Z., M. Houweling, M. H. Chen, M. Record, H. Chap, D. E. Vance, and F. Tercé. 1996. A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in Chinese Hamster Ovary cells. *J. Biol. Chem.* **271:** 14668–14671.

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