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Chemico-Biological Interactions 165 (2007) 106–116
Chemico-Biological Interactions
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A human hepatocellular *in vitro* model to investigate steatosis

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Received 25 August 2006; received in revised form 15 November 2006; accepted 16 November 2006
Available online 23 November 2006

Abstract

The present study was designed to define an experimental model of hepatocellular steatosis with a fat overaccumulation profile in which the metabolic and cytotoxic/apoptotic effects could be separated. This was accomplished by defining the experimental conditions of lipid exposure that lead to significant intracellular fat accumulation in the absence of overt cytotoxicity, therefore allowing to differentiate between cytotoxic and apoptotic effects. Palmitic (C16:0) and oleic (C18:1) acids are the most abundant fatty acids (FFAs) in liver triglycerides in both normal subjects and patients with nonalcoholic fatty liver disease (NAFLD). Therefore, human hepatocytes and HepG2 cells were incubated with a mixture of different proportions of saturated (palmitate) and unsaturated (oleate) FFAs to induce fat-overloading. Similar intracellular levels of lipid accumulation as in the human steatotic liver were achieved. Individual FFAs have a distinct inherent toxic potential. Fat accumulation, cytotoxicity and apoptosis in cells exposed to the FFA mixtures were investigated. The FFA mixture containing a low proportion of palmitic acid (oleate/palmitate, 2:1 ratio) is associated with minor toxic and apoptotic effects, thus representing a cellular model of steatosis that mimics benign chronic steatosis. On the other hand, a high proportion of palmitic acid (oleate/palmitate, 0:3 ratio) might represent a cellular model of steatosis in which saturated FFAs promote an acute harmful effect of fat overaccumulation in the liver. These hepatic cellular models are apparently suitable to experimentally investigate the impact of fat overaccumulation in the liver excluding other factors that could influence hepatocyte behaviour.

Keywords: Apoptosis; Cellular steatosis; Fatty acids; Hepatocytes; HepG2

1. Introduction

The term fatty liver identifies a liver in which lipids account for more than 5% of liver wet weight. This phenomenon is generally believed to result from an imbalance between the hepatic inflow of free fatty acids (FFAs), triglyceride synthesis and excretion [1,2]. When fat accumulates, lipids are primarily stored in the cytoplasm as triglycerides (neutral lipids), leading to micro- and macro-vesicular steatosis and balloon cell degeneration. Hepatic steatosis was long thought to be mainly a symptom of alcoholic liver disease. In recent years, however, steatosis has been found in the absence of alcohol abuse and has led to the definition of a series of disorders ranging from nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH) [2,3]. In mild or transient cases, fatty change is reversible and has no adverse effects on the organ. In severe cases

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doi:10.1016/j.cbi.2006.11.004

of steatosis however, cell dysfunction, lipotoxicity and apoptosis, as well as pathophysiological changes and increased susceptibility of steatotic liver to injury, occur [4–6]. Thus, accumulation of lipids in hepatocytes is a pathologic hallmark. Animal models with either a genetic or environmental induction of hepatic steatosis have been described to investigate mechanisms involved in both hepatic steatosis and its progression to chronic liver disease [7,8]. More recently, *in vitro* models of steatosis have been used to study the hepatocellular consequences of lipid accumulation in hepatic cells of a human origin [9,10]. These models are hepatocyte cell lines and primary hepatocytes treated in culture with monounsaturated and saturated fatty acids [9–11], which seem to reproduce the key features of NAFLD in humans. *In vitro* studies on the mechanism by which FFAs mediate lipoapoptosis [11], Fas up-regulation and increased susceptibility to liver damage [12] and the down-regulation of drug-metabolising enzymes [10] greatly correlate with *in vivo* observations [9–12]. Consequently, well-standardized and relevant *in vitro* hepatic cellular models may be very useful to help experimentally investigate the role and biochemical effects of fat accumulation in the liver due to alcohol consumption, dietary fatty acids or obesity, excluding other factors that could influence hepatocyte behaviour. Human hepatocytes in primary culture are the closest model to the human liver [13,14]. Nonetheless, the scarcity of liver samples greatly hinders their use. An alternative model is the human hepatocyte-derived cell line HepG2.

The present study was designed to define an experimental model of *hepatocellular steatosis* with a fat overaccumulation profile in which the metabolic and cytotoxic/apoptotic effects could be separated. This may be accomplished by defining the experimental conditions of exposure to exogenous FFAs that lead to significant lipid intracellular accumulation in the absence of overt cytotoxicity, allowing to differentiate cytotoxic and apoptotic effects. Ideally, similar intracellular levels of lipid accumulation, as in the human steatotic liver, should achieved for comparisons with human chronic steatosis, but with minor toxic and apoptotic effects. On the other hand, acute studies might require increased intracellular lipids leading to apoptosis and/or necrosis. FFAs appear to be important mediators of lipotoxicity and lipoapoptosis since they are potential cellular toxins and lead to lipid overaccumulation [11,15]. In addition, several reports show that individual FFAs have a distinct inherent toxic potential [6,11,15]. Palmitic (C16:0) and oleic (C18:1) acids are the most abundant FFAs in liver triglycerides in both normal subjects and patients with NAFLD [16]. Therefore, human hepatocytes and HepG2 cells were incubated with a mixture of long-chain FFAs with different proportions of saturated (palmitic acid) and unsaturated (oleic acid) FFAs to induce fat-overloading. Fat accumulation, cytotoxicity and apoptosis in cells exposed to the FFA mixtures were investigated.

2. Materials and methods

2.1. Reagents

Collagenase was obtained from Roche (Barcelona, Spain). Ac-DEVD-AMC caspase 3 and Ac-IETD-AFC caspase 8 fluorogenic substrates came from Pharmingen (San Diego, CA). Ac-LEHD-AFC caspase-9 fluorogenic substrate was purchased from Calbiochem (Nottingham, UK). The poly-caspases FLICA Apoptosis Detection Kit was from Serotec (Oxford, UK). BODIPY 493/503, propidium iodide and FITC-labelled annexin V were purchased from Invitrogen Molecular Probes (Eugene, OR). Culture media (Ham's F-12, Lebovitz L-15) were from Gibco BRL (Paisley, UK). Total lipid determination kit and triglyceride enzymatic assay GPO/POD were from Spinreact (Girona, Spain). Nile red, sodium oleate and sodium palmitate were purchased from Sigma (Madrid, Spain). All other chemicals were of an analytical grade.

2.2. Culture of HepG2 cells

HepG2 cells were cultured in Ham's F-12/Leibovitz L-15 (1:1, v/v) supplemented with 7% newborn calf serum, 50 U penicillin/mL and 50 μg streptomycin/mL. For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37 °C. Cultures were used at 75% confluency.

2.3. Isolation and culture of human hepatocytes

A total of eight non-steatotic and three steatotic (pathologist confirmation) cadaveric liver grafts obtained in conformity with the rules of the Hospital's Ethics Committee were used for cell harvesting. All liver samples were obtained from donors who were not suspected of harbouring any infectious disease and tested negative for human immunodeficiency virus and hepatitis. Hepatocytes were isolated using a two-step perfusion technique and cultured as described in detail elsewhere [14]. Cellular viability was assessed by the Trypan blue dye exclusion test. Hepatocytes were seeded on fibronectin-coated plastic dishes. The medium was changed 1 h later to remove unattached hepatocytes. By 24 h, the cells were shifted to serum-free hormone-supplemented medium (10 nM dexamethasone and insulin).

2.4. Fat-overloading induction in HepG2 cells and hepatocytes

To induce fat-overloading of cells, HepG2 cells at 75% confluency and primary cultures of human hepatocytes were exposed to a long-chain mixture of FFAs (oleate and palmitate) at different ratios. Stock solutions of 50 mM oleate acid and 50 mM palmitate prepared in culture medium containing 1% bovine serum albumin (BSA) were conveniently diluted in culture medium to obtain the desired final concentrations. The FFA mixture was added to both HepG2 cells and hepatocytes 24 and 12 h after seeding, respectively.

2.5. Fluorimetric determination of fat content in intact cells by Nile Red staining

The lipid content in cultured cells was determined fluorimetrically using Nile Red, a vital lipophilic dye used to label fat accumulation in the cytosol [17,18]. Cell monolayers were washed twice with PBS and incubated for 15 min with Nile Red solution at a final concentration of 1 mg/ml in PBS at 37 °C. Monolayers were washed thereafter with PBS and read in a microfluorimeter (excitation 488 nm and emission 550 nm) [10]. Cell protein content was determined by the Lowry method adapted to 96-well plates [19].

2.6. Nile red assay in human liver samples

Human liver tissue was obtained from cadaveric organ donors as described above for liver samples to be used for hepatocyte isolation. The extent of steatosis was graded by pathological examination, and only non-steatotic livers or those classified as >40% steatosis were included in the study. To prepare liver homogenates, small pieces of tissue were disrupted in PBS by the use of a glass homogenizer (20 mg of tissue/ml). Homogenates were diluted 1:10 in PBS and an aliquot of 25 μl was incubated with Nile Red solution (final concentration of 1 mg/ml in PBS) at 37 °C for 15 min in 96-well plates. After incubation, fluorescence was directly measured using a microfluorimeter (excitation 488 nm and emission 550 nm).

2.7. Extraction and quantification of total lipids and triglyceride

Cultured cells were scraped in PBS and disrupted by sonication. For lipidic determinations, homogenates from cells or human liver were extracted with a methanol–chloroform mixture as described [20]. After evaporation under nitrogen, the lipid residue was stored at −80 °C until used. Total lipids were measured using a commercial kit based on the vanillin-phosphoric acid reaction. This extract was also used to quantify the triglyceride content using an enzymatic assay.

2.8. Cytotoxicity assay

Cells were seeded in 96-well microtitre plates. After treatment with FFA mixtures, the cytotoxicity was assessed by measuring the neutral red uptake [21].

2.9. Caspase-3, and caspase-9 activity

After the hepatocytes were incubated for increasing periods of time with the different treatments, detached cells were collected by centrifugation at $500 \times g$ for 3 min and attached cells were scraped off. Cells were pooled and lysed at 4 °C in a buffer (10 mM Tris–ClH, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi), as described [19]. Caspases-3, and -9 activities were measured using the specific fluorogenic substrates 20 μM Ac-DEVD-AMC and Ac-LEDHD-AFC respectively, as previously described in detail [19]. Cellular protein was determined as described [19].

2.10. Flow cytometric analysis of early and late markers of apoptosis

In cultured human hepatocytes and HepG2 hepatoma cells, early stages of apoptosis were scored by detecting the exposure of phosphatidylserine (PS) on the cell membrane and the activation of caspases. Late apoptosis and secondary necrosis were assessed by the presence of fragmented cell nuclei with a sub G0 DNA content.

Annexin V-FITC was used as a sensitive probe to reveal PS exposure by flow cytometry [22]. Cells were trypsinized and resuspended in their original culture medium to avoid loss of apoptotic and necrotic cells. Annexin V-FITC binding was determined by flow cytometry following the manufacturer's instructions. Propidium iodide (PI, 5 μg/mL final concentration) was added to detect late apoptotic and necrotic cells. In another set of experiments, ongoing apoptosis was assessed by flow cytometry using the fluorescein derivative of a tripeptide-fluoromethyl ketone FAM-VAD-FMK, a fluorescent, polyspecific inhibitor of caspases (FLICA). FAM-VAD-FMK irreversibly binds to many activated caspases (caspases-1, -3, -4, -5, -6, -7, -8 and -9), and is used as a generic probe for the detection of most caspases. The Poly-Caspases FLICA Apoptosis Detection Kit was used according to the manufacturer's

instructions. Briefly, cells in monolayers were incubated for 1 h with FAM-VAD-FMK, then washed, trypsinized and resuspended in fresh medium for the flow cytometric analysis.

In order to assess late apoptosis and secondary necrosis, hepatocyte cell monolayers were kept frozen at −20 °C until the time of analysis. Then monolayers were thawed and covered with hypotonic lysis solution [23] and kept overnight at 4 °C in order to release the nuclei. Propidium iodide (50 μg/mL, final concentration) was added to the nuclei suspension for the fluorescent staining of DNA, and nuclei suspensions were incubated for 30 min at room temperature in the dark. To analyse the HepG2 cells, the culture medium was discarded and monolayers were covered with hypotonic lysis solution [23] containing propidium iodide (50 μg/mL, final concentration) and kept overnight at 4 °C. Then the nuclei were released by gentle pipetting. The degree of apoptosis was estimated from the percentage of nuclei with a DNA content lower than the diploid (2C) peak in a single-parameter histogram of PI fluorescence distribution [23].

Samples were analysed in a Cytomics FC500 flow cytometer (Beckman-Coulter, Brea, CA) using a 488 nm blue argon ion laser. Green fluorescence (FL1, 525 nm) was collected to quantify either annexin V-FITC binding or the intracellular retention of FAM-VAD-FMK. Orange fluorescence (FL3, 610 nm) was used to quantify PI uptake.

2.11. Statistical analysis

Data are expressed as mean ± S.D. The Student's *t*-test was used to determine the statistical significance of the experimental data.

3. Results

3.1. Fat overloading of hepatic cells with different ratios of FFAs

Human hepatocytes were incubated with 0.5, 1 and 2 mM of the FFA mixture (oleate/plmitate) at different ratios (3:0, 2:1, 1:1, 1:2 and 0:3) for 24 h. Human hepatocytes (Fig. 1A) and HepG2 cells (Fig. 1B) exposed to the FFA mixtures developed a clear dose-dependent increase of fat accumulation in the cytosol. Oleate/palmitate at 3:0, 2:1, 1:1 and 1:2 ratios induced similar levels of intracellular fat, and the maximal content of the FFA mixture was reached at 2 mM. However, as Fig. 1 shows, lower fat levels were found in cells treated with palmitate alone (0:3 ratio) as it is apparently toxic at such a concentration. FFAs overloaded human hepatocytes accumulated a higher fat content (8–9 fold over control cells) than HepG2 cells (5–6-fold over control cells).

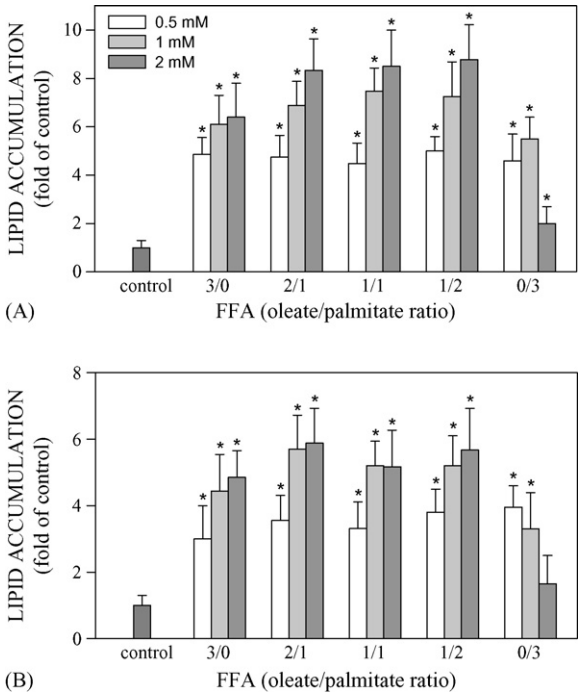

Fig. 1. Fat overloading of hepatic cells with different FFA ratios. (A) Human hepatocytes and (B) HepG2 cells were incubated with an FFA mixture (oleate/palmitate) at different ratios (3:0, 2:1,1:1, 1:2 and 0:3) for 24 h. A dose-dependent increase of intracellular fat accumulation was determined by Nile Red staining. $^{*}p < 0.05$ in relation to controls.

3.2. Cytotoxicity of FFA overloading

The cytotoxicity of the FFA mixture at different ratios (oleate/palmitate at 3:0, 2:1,1:1, 1:2 and 0:3) was also investigated after 24 h of treatment. The results show that the various ratios of the FFA mixture produced different cytotoxic effects. As Fig. 2A shows, no cytotoxic effect was observed in human hepatocytes at the oleate/palmitate 3:0, 2:1, 1:1 and 1:2 ratios up to 2 mM, while a strong cytotoxic effect was shown at 1 mM of the FFA mixture for the 0:3 ratio. However, HepG2 cells seem to be more sensitive to fat accumulation (Fig. 2B). In addition, the FFA mixture at 2 mM produced a significant toxicity at those ratios with a higher palmitic acid content (1:1, 1:2 and 0:3 ratios). Moreover, 1 mM palmitate (0:3 ratio) was shown to be highly toxic. These results suggest a cytotoxic-dependent effect of an increase in palmitate concentration in the FFA mixture (Fig. 2B).

Therefore, to achieve maximal fat overaccumulation with minimal cytotoxicity, the FFA mixture containing a low proportion of palmitic acid (oleate/plmitate 2:1

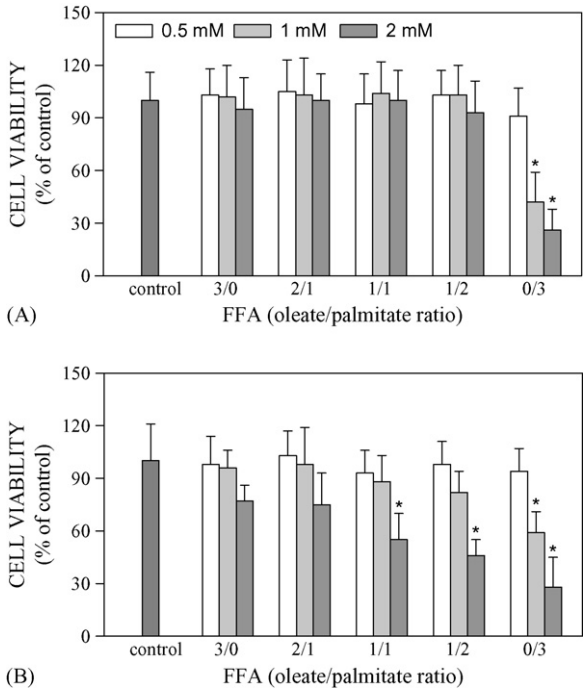

Fig. 2. Cytotoxicity of FFA overloading at different ratios. The cytotoxicity of the FFA mixture at different ratios (oleate/palmitate at 3:0, 2:1,1:1, 1:2 and 0:3 ratios) was assessed by the MTT test at 24 h after treatment. (A) Human hepatocytes and (B) HepG2 cells. $^{*}p < 0.05$ in relation to controls.

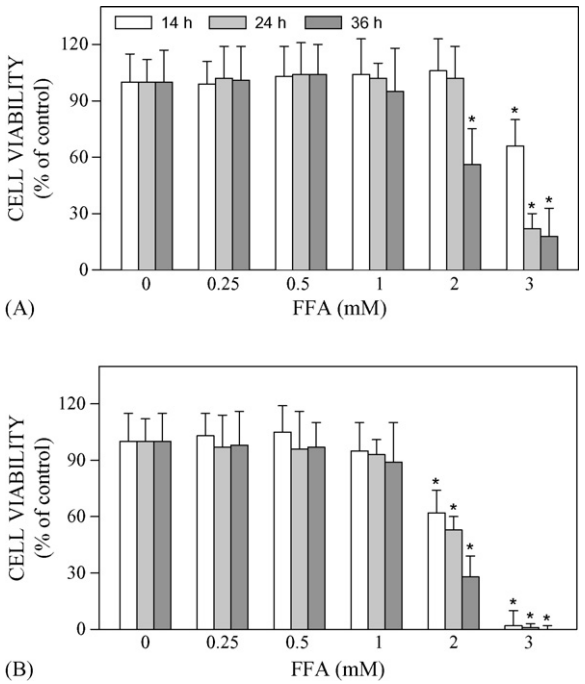

Fig. 3. Dose- and time-dependent cytotoxicity of FFA overloading (2:1 ratio). Dose- and time-dependent cytotoxicity of the oleate/palmitate 2:1 ratio at a wide range of concentrations and the exposure time were assessed by the MTT test. (A) Human hepatocytes and (B) HepG2 cells. $^{*}p < 0.05$ in relation to controls.

ratio) was selected for further experiments. Dose- and time-dependent cytotoxicities of oleate/palmitate at the 2:1 ratio were assessed in both human hepatocytes and HepG2 cells at a wide range of concentrations (Fig. 3). The results showed that 2 mM FFA was not toxic to human hepatocytes after 14 and 24 h exposure, while 2 mM showed toxicity after 36 h and 3 mM was toxic after any time of treatment (Fig. 3A). HepG2 cell were more sensitive to FFA, producing at 2 and 3 mM a high reduction of cell viability after 14 h of treatment (Fig. 3B).

3.3. Caspase activation in FFA-overloaded hepatic cells

The activation of caspases-3 and -9 was evaluated in HepG2 cells treated with various ratios of oleate/palmitate at 0.5 and 1 mM for 24 h. As shown in Fig. 4A, caspase-3 activation was only observed in cultures treated with the higher ratios of palmitic acid (oleate/palmitate, 1:1, 1:2 and 0:3 ratios) at concentrations which did not cause cell necrosis, according to the results on cell viability presented in Fig. 2A. No significant effects were found with the other FFA ratios. The caspase-9 activation was assessed to investigate the apoptotic pathway involved in the caspase activation cascade. Activation of effector caspase 9 was observed in HepG2 cell cultures which were fat overloaded with palmitic acid (oleate/palmitate, 0:3 ratio), suggesting that caspase 9, and consequently the mitochondrial pathway, are involved in fat-induced apoptosis (Fig. 4B). Based on these results, we investigated caspase activation in human hepatocytes exposed to FFAs (2:1 ratio) at subcytotoxic concentrations (0.25–1 mM). Neither caspase-9 nor caspase-3 were activated after a 24-h exposure to FFAs.

3.4. Flow cytometric markers of early and late apoptosis in FFA-overloaded hepatic cells

Early apoptosis was assessed by flow cytometry in HepG2 cells incubated up to 24 h with various ratios of the FFA mixture (oleic acid/palmitic acid 0:3, 1:2, 1:1, 2:1 and 3:0) by monitoring the binding of annexin V-FITC as a measure of the translocation of phosphatidylserine to the cell surface [22]. For the relative quantification of apoptosis and necrosis, the following criteria were followed: Live cells were scored as negative for both annexin V-FITC and PI, as compared by an unstained control. Early apoptotic

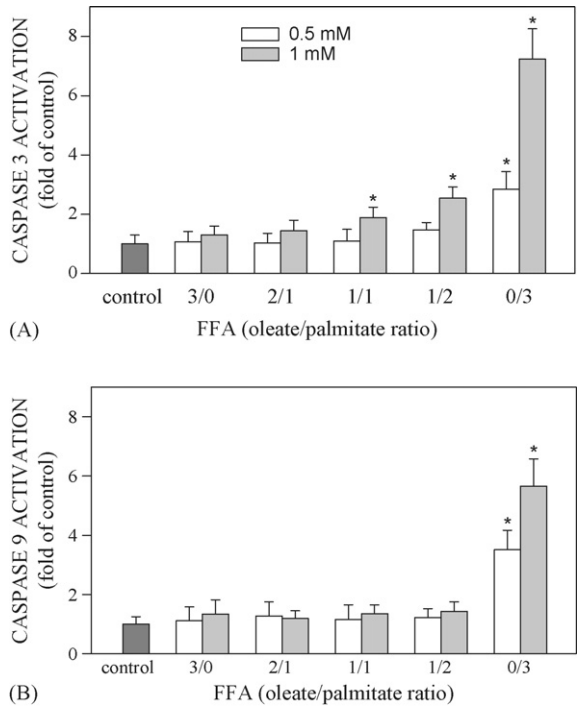

Fig. 4. Caspase activation in FFA-overloaded hepatic cells. The activation of caspases-3 and -9 was assessed in HepG2 cells treated with various ratios of oleate/palmitate at 0.5 and 1 mM for 24 h. (A) Caspase-3 activation and (B) caspase 9 activation were only observed in cultures treated with the higher ratios of palmitic acid at concentrations up to 1 mM which did not overlap cell necrosis. Concentrations: 0.25–1 mM. Neither caspase-9 nor caspase-3 were activated after a 24-h exposure to FFA. $^{*}p < 0.05$ in relation to controls.

cells were defined as being positive for annexin V-FITC and negative for PI. Late apoptotic cells were positive for both annexin V-FITC and PI, while necrotic cells and bare nuclei released from them were identified as positive for PI but negative for annexin V-FITC. The results showed a significant percentage of late apoptotic and secondary necrotic cells in cultures treated with palmitic acid alone while no significant early or late apoptosis were present with any other ratio of the FFA mixture up to 1 mM (data not shown).

No significant apoptosis was detected in human hepatocytes by flow cytometry as subG0 nuclei at 24 h with any FFA mixture containing oleate (oleate/plmitate ratios 3:0, 2:1, 1:1 and 1:2) up to 2 mM. Palmitic acid alone (oleate/palmitate, 0:3 ratio) already induced DNA fragmentation at 1mM of the FFA mixture. Analysis of DNA fragmentation by flow cytometry was also performed in HepG2 cells incubated up to 24 h with various ratios of the FFA mixture. A significant increase of the percentage of nuclei with sub-diploid DNA content (DNA fragmentation) in cultures treated with high proportions of palmitic acid (oleate/palmitate, 1:2 and 0:3 ratio) was observed, especially at 24-h incubation (Fig. 5A–F).

Consistent results were obtained when morphological parameters, which we previously showed to be sensitive flow cytometric markers of apoptosis [23], were assessed in both hepatocyte and HepG2 cultures. A significant increase of the relative nuclear size (forward scatter measurement) in diploid (2C) and tetraploid (4C) hepatocyte nuclei and in G0/G1 nuclei from HepG2 cells was observed in cultures treated with palmitate alone or at the 2:1 ratio of oleate. A similar increase was observed under the same conditions for the coefficient of variation of the DNA distribution in 2C and 4C hepatocyte nuclei and in the nuclei from G0/G1 HepG2 nuclei (data not shown). Similar changes accompany the biochemical features of apoptosis in liver cells treated with a variety of apoptosis-inducing xenobiotics, and reflect the disorganization of chromatin that characterizes apoptotic cell death [23].

In order to differentiate necrosis from secondary apoptosis in the high palmitate ratios treatments, 24-h lipid-treated HepG2 cell suspensions were examined by flow cytometry for poly-caspase activation using the FAM-VAD-FMK fluorogenic substrate. FAM-VAD-FMK probe enters each cell and covalently binds to a reactive cysteine residue of the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. Because FAM-VAD-FMK becomes covalently coupled to the enzyme, it is retained within the cell, while any unbound FAM-VAD-FMK will diffuse out of the cell. The cell-associated green fluorescence is, therefore, an evidence of active caspases being present in the cell at the time the reagent was added. In all treatments, non-vital cells, as determined by well established features of cell death (such as decreased FS and increased permeability to PI), were positive for FAM-VAD-FMK fluorescence, supporting that caspase activation, a hallmark of apoptosis, had occurred during the process of cell death (Fig. 5G–I). No significant differences in the mean fluorescence intensity of FAM-VAD-FMK (i.e. average poly-caspase activity per individual cell) were observed in the apoptotic subpopulation across treatments, indicating that the changes in caspase activity evidenced by bulk fluorimetry measurements reflect the increased percentage of apoptotic cells observed with the higher content of palmitic acid.

3.5. Kinetics of FFA overloading of hepatic cells

The kinetics of fat overloading was evaluated after exposing cells up to 3 mM oleate/palmitate at a ratio of 2:1 for 14, 24 and 36 h. A time-dependent increase in the

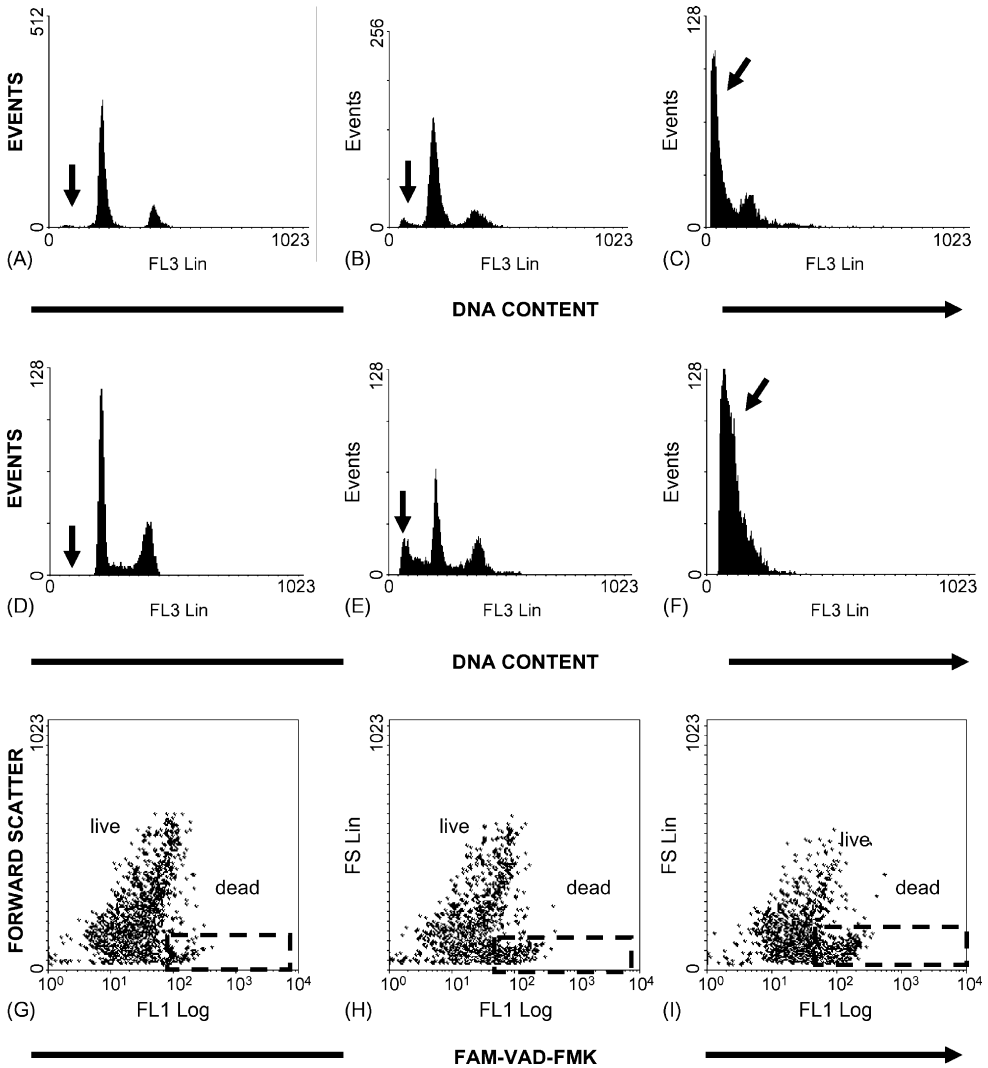

Fig. 5. Flow cytometric assessment of apoptosis in human hepatocytes (A–C) and HepG2 hepatoma (D–I). Hepatocytes were isolated from human liver with or without steatosis and maintained for 24 h under standard culture conditions. Panels A–C show the presence of subG0 nuclei (arrows) with fragmented DNA in hepatocyte cultures treated for 24 h with vehicle (A), 2 mM oleic acid (B) or 2 mM palmitic acid (C), as described in Material and Methods. Panels D–F show the presence of subG0 nuclei with fragmented DNA in HepG2 cultures treated for 24 h with vehicle (A), 2 mM oleic acid (B) or 2 mM palmitic acid (C). Panels G–I show the relative level of caspase activation in live and dead cells, as determined with the fluorescent inhibitor poly-caspase substrate FAM-VAD-FMK in HepG2 cultures treated for 24 h with vehicle (A), 2 mM oleic acid (B) or 2 mM palmitic acid (C). Dotted boxes indicate the cells with morphological signs of cell death that are positive for FAM-VAD-FMK. Graphs show a representative result of each experimental condition.

fat content of cells was observed, and the maximal lipid accumulation was reached after 24 h of incubation with FFAs in both the hepatocyte (Fig. 6A) and HepG2 cells (Fig. 6B).

3.6. Lipid content in FFA-overloaded cells versus hepatocytes from steatosic liver

The extent of lipid accumulation in the experimental model of cell steatosis was compared to that found in fatty livers. The lipid content, estimated fluorimetrically using the Nile Red assay, was 10-fold higher in homogenates of human livers with steatosis than in livers from the non-steatosis group (Fig. 7). When lipid levels were comparatively determined in hepatocytes isolated from human livers, either with or without steatosis, the lipidic charge was around 3.8-fold higher in the steatosis group (Fig. 7). As seen above (Fig. 6A), a similar increase in fat accumulation (about four-fold over control) was reached after 14 h exposure to 1 mM FFA

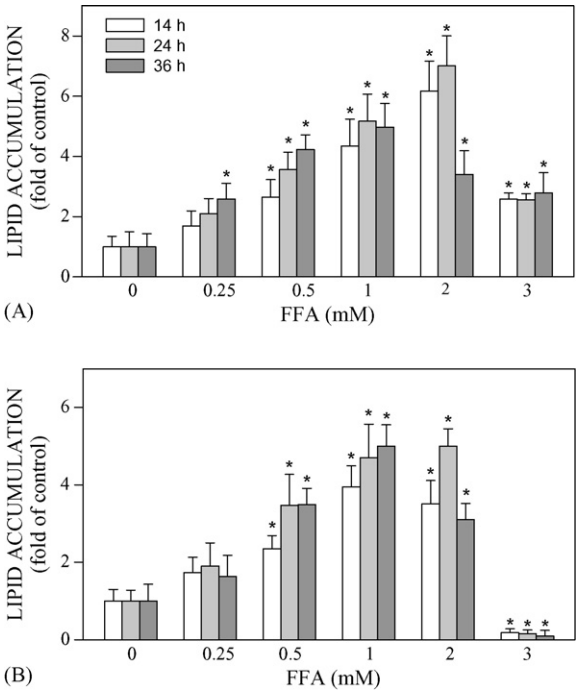

Fig. 6. Kinetics of FFA overloading (2:1 ratio). The kinetics of fat overloading after exposure of cells up to 3 mM oleate/palmitate at a ratio of 2:1 ratio 12, 24 and 36 h was assessed by Nile red staining. (A) Human hepatocytes and (B) HepG2 cells. $^{*}p < 0.05$ in relation to controls.

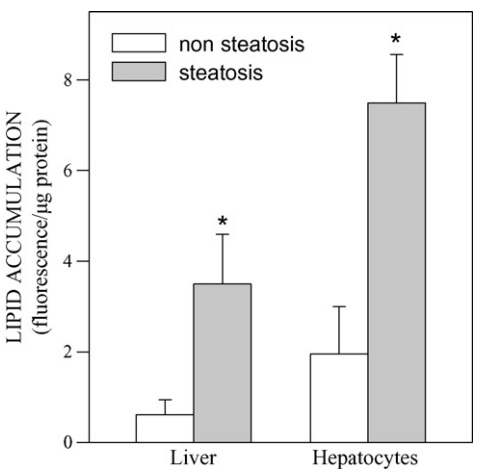

Fig. 7. Lipid content in FFA-overloaded cells versus hepatocytes from steatosic liver. The lipid content was estimated by Nile Red staining in liver homogenates from patients with steatosis or from normal livers and in 24-h cultured hepatocytes obtained from liver tissue either with or without steatosis. Results are expressed as fluorescence arbitrary units/µg of protein. $^{*}p < 0.05$ in relation to the non-steatosis group.

Table 1
Comparison of total lipids and triglyceride levels in fatty livers and in *in vitro* fat-overloaded hepatocytes

	Total lipids (µg/mg protein)	Triglycerides (µg/mg protein)
Human liver		
Non-steatosis	270 ± 95	141 ± 56
Steatosis	841 ± 297*	381 ± 110*
Hepatocytes[a]		
Control	208 ± 48	95 ± 22
FFA	459 ± 121*	232 ± 63*
HepG2 cells		
Control	212 ± 34	105 ± 19
FFA	535 ± 81*	278 ± 51*

[a] Hepatocytes obtained from normal livers (non-steatosis) and HepG2 cells were maintained under standard culture conditions (control) or treated for 20 h with 1 mM FFA (oleate/palmitate, 2:1).
* $p < 0.05$ respect to non-steatosis group or control cells.

mixture (2:1, oleate/palmitate) of hepatocytes obtained from normal livers. These results suggested that lipid accumulation in *in vitro* fat-overloaded hepatic cells was comparable to that observed in the livers of patients with steatosis. This was confirmed by the quantification of total lipids and triglycerides after extraction with a chloroform/methanol mixture (Table 1). Treatment of hepatocytes from non steatotic livers or HepG2 cells with 1 mM FFAs for 14 h produced an intracellular accumulation of total lipids and triglycerides. The amounts of total lipids and triglycerides (expressed as microgram per milligram of cell protein) reached in fat-overloaded cultured cells are lower, but comparable to that measured in fatty livers (Table 1).

4. Discussion

The hallmark of NAFLD is hepatic steatosis. This is mostly a benign noninflammatory condition that neither appears to have any adverse sequelae nor can be associated with steatohepatitis, a condition that can result in end-stage liver diseases [5,24]. In the present study, we have characterized a hepatic *in vitro* model of cellular steatosis with a fat overaccumulation profile in which the metabolic and cytotoxic/apoptotic effects could be separated. Similar intracellular levels of lipid overaccumulation, as in hepatocytes from human steatotic liver, have been achieved (Fig. 7 and Table 1) by overloading cells with various proportions of saturated (palmitic acid) and unsaturated (oleic acid) long-chain FFA mixtures which produced significantly a different toxic and apoptotic effect. Human hepatocytes and HepG2 cells, a well-characterized and widely used human hepatoma

cell line expressing a variety of liver functions [25,26], have been used.

Palmitic acid synthesized from acyl CoA, is desaturated to palmitoleic acid or elongated to stearic acid, which can be desaturated to form oleic acid. These FFAs are used to synthesize triglycerides which are stored in the cytoplasm, leading to micro- and macro-vesicular steatosis. Palmitic and oleic acids are common dietary long-chain FFAs, the most abundant in liver triglycerides in both normal subjects and patients with steatosis [16]. In addition, the liver accumulates an excess of palmitic and oleic acids in humans [16], mice [27] and cows [28] with hepatic steatosis. A mixture of oleic and palmitic acids has been used to induce fat accumulation in hepatic cells. To this end, human hepatocytes and HepG2 were overloaded with different ratios (oleate/palmitate, 3:0, 2:1, 1:1, 1:2 and 0:3) of the FFA mixture. The results show that the different FFA composition induced a dose- and time-dependent increase of fat overaccumulation; however, the combination of oleic and palmitic acids seems to be more efficient than when used individually (Figs. 1 and 6), in agreement with previous reports [29]. The results of fat overaccumulation were confirmed by flow cytometry (data not shown) using BODIPY 493/503, a fluorescent lipid probe specific for intracellular lipid droplets [30].

A major question is to what extent these experimental models reproduce lipid accumulation characteristic of fatty livers. We found that FFA-overloaded human hepatocytes and HepG2 cells reached similar levels of maximal intracellular lipid accumulation, which were very close to that determined in hepatocytes from human steatotic liver (Fig. 7 and Table 1). Moreover, culturing hepatic cells under conditions of maximal *in vitro* fat accumulation (i.e. 1 mM FFA: oleate/palmitate, 2:1) led to an increase in intracellular triglyceride content (five- to nine-fold over control cells) comparable to that found in human liver with steatosis (Table 1) [16].

When lipids overaccumulate in a non-adipose tissue, they may enter a non-oxidative deleterious pathway, leading to cell injury and death [31,32]. Important pathophysiological changes linked to steatosis and an increased susceptibility of steatotic liver to injury have been reported [5,6]. Therefore, the cytotoxicity of various proportions of oleic and palmitic acids was then investigated. Our results show a clear cytotoxic effect associated with the increase of palmitate concentration in the FFA mixture in both human hepatocytes and HepG2 cells (Figs. 2 and 3). These results are consistent with recent reports showing that increased saturated FFAs are associated with increased liver injury, using dietary models of hepatic steatosis characterized by a similar accumulation of total triglyceride, but a different composition of FFAs [6]. Similar findings have been also shown in a variety of *in vitro* systems in which saturated FFAs were substantially more lipotoxic than unsaturated FFAs, despite causing a similar magnitude of cellular fat accumulation: endothelial cells [33], pancreatic β cells [34], cardiomyocites [35], CHO cells [36], hepatocytes [37], human hepatoma HepG2 cells [11] and rat hepatoma H4IIE cells [38].

The apoptotic effects of the different ratios of oleate/palmitate were then assessed in fat-overloaded HepG2 cells (Fig. 5). Confirmation of apoptosis caused by palmitate, but not by the monounsaturated oleate, was obtained by flow cytometry by demostrating annexin V binding, DNA fragmentation, as well as by caspase activation, hallmark events of apoptosis [11]. We first observed annexin V labeling and a concentration-dependent increase of apoptotic nuclei with sub-diploid DNA content after exposure to palmitate and to the FFA mixture with a high content of palmitate, while no effect was produced in the presence of oleate. Progression of the caspase activation cascade ends with the activation of caspase-3 that occurs in the early apoptosis, long before DNA-fragmentation appears. The results showed a caspase-dependent mechanism involved in palmitate-mediated apoptosis. As shown in Fig. 4A, a concentration-dependent effect on the caspase-3 activation was found at concentrations of palmitate which did not cause necrosis, while flow cytometry analysis using the FLICA reagent FAM-VAD-FMK demonstrated that most cells with necrotic features were positive for this marker, thus supporting a previous activation of the apoptotic pathway under such experimental conditions.

Consistent with our data, it has been shown that lipoapoptosis is specifically induced by saturated fatty acids while unsaturated fatty acids had no effect. Thus, oleic acid supplementation leading to triglyceride accumulation is well tolerated, whereas excess palmitic acid causes lipoapoptosis [29,39]. Moreover, low concentrations of oleic acid completely inhibited palmitic acid-induced oxidative stress and apoptosis in rat and human β cells [39], pancreatic β cells [34] and in cardiac myocites [35].

A concentration-dependent effect was also observed when the activation of effector caspase 9 was analysed in cells overloaded with palmitate (Fig. 4B). These data indicate that cytotoxicity produced by FFAs, particularly palmitic acid, directly engage the mitochondrial cell death pathway. In addition, recent reports have excluded the contribution of the death receptor pathway in lipoapoptosis induced by fat accumulation. Malhi and co-workers [11] reported the coincidence of cas-

pase activation with mitochondrial dysfunction that was not blocked by inhibiting the death receptor pathway with a selective inhibitor of caspase 8, thus excluding the death receptor pathway in the cytotoxicity of FFAs. Consistently, no activation of caspase 8 in fat-overloaded hepatic cells was observed (data not shown). Our data are in agreement with observations in disparate cell types, showing that a cytosolic accumulation of saturated FFAs, particularly palmitic acid, despite causing a similar magnitude of cellular steatosis, induced apoptosis involving a mitochondrial basis for its pathogenesis: CHO cells [36], cardiomyocytes [35,40], testicular Leydig cells through stimulation of ceramide production and induction of Bax [41], hepatic HepG2 cells, in which JNK triggers the mitochondrial pathway [11], hepatocytes, in which levels of Bcl-2/Bax are reduced [37], and hepatic H4IIE cells [38]. These observations might suggest that toxicity and apoptosis *in vivo* are determined by the content of unsaturated fatty acids in a mixed fatty acid pool. Furthermore, it has been proposed that saturated FFA toxicity may play important roles in the transition from steatosis to steatohepatitis in humans, especially with obesity [37].

Collectively, the results show that hepatic cells are induced to fat overaccumulation by mixtures of oleic and palmitic acids, human hepatocytes and HepG2 cells, all showing a very similar behaviour. HepG2 cells are therefore depicted as a promising alternative to human hepatocytes to be used as cellular model of steatosis, as previously proposed [9]. Interestingly, despite the fact that various FFA mixtures induce a similar magnitude of fat accumulation, we have defined two different experimental models of a cellular steatosis profile. On the one hand, the FFA mixture containing the lowest proportion of palmitic acid (oleate/palmitate, 2:1 ratio) is associated with minor toxic and apoptotic effects, thus representing a cellular model of steatosis in which unsaturated FFAs serve a protective function against lipotoxicity mimicking benign chronic steatosis. On the other hand, a high proportion of palmitic acid (oleate/palmitate, 0:3 ratio) might represent a cellular model of steatosis in which saturated FFAs promote an acute harmful effect of fat overaccumulation in the liver. These hepatic cellular models are apparently suitable to experimentally investigate the impact of fat overaccumulation in the liverexcluding other factors that could influence hepatocyte behaviour.

Acknowledgements

The authors would like to express their thanks to E. Belenchón for her expert technical assistance. This work was supported by the ALIVE Foundation, and funds from the Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III of Spain, grant 03/0339, and from the European Commission, grant LSHB-CT-2004-504761 and grant LSHB-CT-2004-512051.

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