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Effects of authentic and VLDL hydrolysis-derived fatty acids on vascular smooth muscle cell growth

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1 There are contradictory findings regarding the effects of free fatty acids on vascular smooth muscle cell (VSMC) growth. In the present study we investigated the effects of fatty acids released from hydrolysis of human VLDL triglycerides by lipoprotein lipase and of the fatty acids most abundant in the hydrolysed VLDL, namely oleic, linoleic, palmitic and myristic acid, all non albumin-bound, on VSMC growth.

2 The effect of fatty acids on VSMC growth was assessed by [^{3}H]-thymidine incorporation, colourimetrically, by cell counting, by determination of the cytoplasmic histone-associated DNA fragments and the caspase 3 activity. The fatty acid concentrations were determined by gas chromatography-mass spectrometry. Stimulation of ERK1/2 and p38 was determined by the chemiluminescence Western blotting method.

3 Incubation of VSMC with purified VLDL (100 μg ml^{-1}) and lipoprotein lipase (35 u ml^{-1}) led to almost complete cell death although the ERK1/2 and the p38 MAP kinases were stimulated. The EC_{50} of oleic, linoleic, myristic and palmitic acid were 4.6 ± 1.3, 2.4 ± 0.2, 116 ± 10 and 287 ± 30 μM, respectively. The estimated EC_{50} of myristic and palmitic acid when derived from hydrolysed VLDL were 10 and 8 times, respectively, lower than when used alone. Apoptosis was not involved in the fatty acid-induced VSMC growth suppression/death.

4 We conclude that (a) non albumin-bound fatty acids cause VSMC necrosis in a dose-dependent manner with a parallel ERK1/2 and p38 stimulation, (b) unsaturated fatty acids are more toxic to VSMC than saturated, and (c) saturated fatty acids are more toxic to VSMC in the hydrolysed VLDL than when used individually.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular response kinase 1/2; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TIU, trypsin inhibitor unit; VLDL, very low density lipoprotein; VSMC, vascular smooth muscle cells

Introduction

Proliferation of arterial vascular smooth muscle cells (VSMC) plays an important role in the development of atherosclerosis and hypertension and inhibition of VSMC growth may be crucial for the prevention of cardiovascular diseases (Ross, 1993; 1995). There are contradictory findings regarding the effects of free fatty acids on VSMC growth. Some investigators have observed growth stimulation (Hu *et al*., 1998; Lu *et al*., 1996; 1998; Rao *et al*., 1995) while others suppression (Huttner *et al*., 1977; Olsson *et al*., 1999). In short, it has been shown (Rao *et al*., 1995) that a weak increase in DNA synthesis and a moderate increase in VSMC number occur after a 96 h incubation with 20 μM linoleic acid. Similarly, others (Lu *et al*., 1996; 1998) have found that oleic acid at concentrations from 25–200 μM significantly increased [^{3}H]-thymidine uptake and cell number in rat VSMC after a 6 day stimulation period. Furthermore, it has been shown that linoleic acid (50 μM) induced a significant increase in VSMC DNA synthesis and cell number over a 4 day incubation period (Hu *et al*., 1998). On the other hand, others found that exposure of human arterial smooth muscle cells to 100–300 μM linoleic acid lowered their proliferation rate and altered cell morphology (Olsson *et al*., 1999). Moreover, it has been described that oleic acid (Lu *et al*., 1996; 1998) and linoleic acid (Hu *et al*., 1998; Rao *et al*., 1995) stimulate the extracellular response kinases 1 and 2 (ERK1/2, also known as $p44^{mapk}$/$p42^{mapk}$) but not the p38 mitogen-activated protein (MAP) kinase (Lu *et al*., 1998).

The fatty acids employed in the previous studies were bound to albumin. In this study we examined the effects of non albumin-bound fatty acids on VSMC growth. Furthermore, we imitated *in vivo* conditions by incubating VSMC with physiologically derived fatty acids produced by the

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addition of lipoprotein lipase, the enzyme responsible for the hydrolysis of circulating triglycerides, to human very low density lipoprotein (VLDL). By doing so, cultured VSMC were exposed to concentrations of fatty acids 'identical' to the relative fatty acid composition in the vicinity of the arterial wall *in vivo*. To compare these effects on VSMC growth to the ones of authentic fatty acids, we used commercially available water soluble oleic acid (C-18 : 1) and linoleic acid (C-18 : 2), as well as palmitic acid (C-16 : 0) and myristic acid (C-14 : 0), since these are the fatty acids with the highest concentration in hydrolysed VLDL. The fatty acid concentrations used here are within the normal range of plasma levels of normal individuals.

The MAP kinase cascade has been proposed to regulate a diverse range of biological functions, including cell growth, differentiation and death. Therefore we investigated whether MAP kinases are involved in the fatty acid signalling of VSMC growth suppression. Finally, we examined whether apoptosis contributed to the inhibitory effects of fatty acids on VSMC growth by measuring two apoptotic markers, namely the caspase 3 activity and the cytoplasmic histone-associated DNA fragments and found no evidence of its involvement.

Methods

Isolation and culture of vascular smooth muscle cells

Rat aortic VSMC were isolated from the thoracic aorta from Wistar-Kyoto rats (6–8 weeks old, Charles River Wiga, Sulzfeld, Germany) by enzymatic dispersion using a slight modification of the method of Chamley *et al*. (1979) as described previously (Sachinidis *et al*., 1995). Cells were cultured in DMEM supplemented with 10% FCS (v v^{-1}), non-essential amino acids, penicillin 100 IU ml^{-1} and streptomycin 100 μg ml^{-1} at 37°C in the Steri-cult incubator (Forma Scientific, Göttingen, Germany) in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown in 75 cm^2 flasks to confluence over 4–5 days.

Gel electrophoresis and immunostaining

VSMC were seeded in 3 cm petri dishes (4×10^5 cells per dish) and cultivated in culture medium until confluent. The medium was then replaced by serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1 : 1, v v^{-1}). Following another 24 h cultivation in serum-free medium, the cells were incubated with lipoprotein lipase, VLDL, hydrolysed VLDL (defined as VLDL incubated together with lipoprotein lipase at 37°C for 24 h in a sterile Eppendorf tube in a Steri-cult incubator), oleic acid and linoleic acid for different time periods. After removing of the medium, cells were lysed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (mM: NaCl 50, Tris-HCl 20, NaF 50, EDTA 10, $Na_4P_2O_7$ $10H_2O$ 20, 1% Triton X-100, pH 7.4) containing 1 mM Na_3VO_4, 1 mM phenylmethylsulphoxide (PMSF), 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} antipain, and 0.023 TIU ml^{-1} aprotinin. Protein determination was performed using the Bio-Rad Protein Assay. Ten μg of protein were separated in a 10% SDS polyacrylamide gel (SDS–PAGE) with a thickness of 0.75 mm using the Mini Gel Protean system (Bio-Rad, Munich, Germany).

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane overnight at 100 mA with a buffer containing 25 mM Tris-HCl, 192 mM glycin and 20% methanol, pH 8.3. Phosphorylated (activated) MAP kinases were detected using the chemiluminescence Western blotting system from NEN Life Science Products, Inc. (Boston, MA, U.S.A.) as described in the instructions, using a phospho-specific ERK1/2 rabbit polyclonal IgG primary antibody (1 : 1000), a phospho-specific p38 MAPK rabbit polyclonal IgG primary antibody (1 : 1000) and the secondary horseradish peroxidase-labelled anti-mouse IgG (1 : 5000). The primary antibodies recognize phosphorylated (Thr202/Tyr204) ERK1/2 and phosphorylated (Thr180/Tyr182) p38 MAP kinase, respectively. Phosphorylation of the MAP kinases on the appropriate amino acid residues is essential for their activation (Marshall, 1995).

Determination of DNA synthesis

The effect of VLDL, lipoprotein lipase, hydrolysed VLDL and of oleic, linoleic, palmitic and myristic acid on [^{3}H]-thymidine incorporation into cell DNA was assessed as previously described (Sachinidis *et al*., 1995). VSMC were cultured until approximately 70% confluence. Then, the medium was replaced by serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1 : 1, v v^{-1}) and after 24 h cultivation in the serum-free medium the aforementioned agents were added to the cells for 15, 30, 120, 240 min and 24 h. Twenty hours later 3 μCi ml^{-1} [^{3}H]-thymidine were added to the serum-free medium. Four hours later the experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco's phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid (w v^{-1}) and ethanol/ether (2 : 1, v v^{-1}). Acid-insoluble [^{3}H]-thymidine was extracted into 0.5 M NaOH (250 μl per well) and 100 μl of this solution were mixed with 5 ml scintillator liquid (Packard, Ultimagold, Groningen, The Netherlands) and quantified using a liquid scintillation counter (Beckman LS 3801, Düsseldorf, Germany). Fifty μl of the residual solution were used for the determination of protein using the Bio-Rad protein assay according to the method of Bradford (1976).

Determination of the cell counts

For cell counting, VSMC were seeded in 24-well culture plates (5×10^4 cells per well, well diameter 12 mm) and cultured in DMEM, supplemented with 10% FCS (v v^{-1}), non-essential amino acids, penicillin 100 iu ml^{-1} and streptomycin 100 mg ml^{-1} at 37°C for 24 h until a cell confluence of approximately 70% was reached. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1 : 1, v v^{-1}) and after 24 h the VSMC were stimulated with lipoprotein lipase, VLDL, various doses of hydrolysed VLDL, oleic, linoleic, palmitic and myristic acid for different time periods. After 24 h the cells were trypsinized and cell counting as well as determination of cell diameter was performed using the CASY-1 system based on the coulter counter principle (Schärfe, Reutlingen, Germany).

VLDL isolation and VLDL hydrolysis by lipoprotein lipase

VLDL ($d < 1.006$ g ml^{-1}) was isolated from the plasma of one normotriglyceridemic and normocholesterolemic subject (serum triglycerides <160 mg dl^{-1}, total cholesterol <200 mg dl^{-1}) by potassium bromide density-gradient ultracentrifugation according to Redgrave *et al.* (1975) as described previously (Sachinidis *et al.*, 1997). The purity of VLDL was examined as described previously (Sachinidis *et al.*, 1997). In order to hydrolyse the VLDL triglycerides, various doses of VLDL and commercially available lipoprotein lipase (35 u ml^{-1}), dissolved in sterile PBS, were either co-incubated in a sterile Eppendorf tube at 37°C for 24 h in the Steri-cult incubator or were added simultaneously in the cell culture plates for 24 h. VLDL hydrolysed by either of the above methods is referred to as 'hydrolysed VLDL'. Fatty acid determination in the mixture was performed after 24 h.

Amounts of VLDL (intact or hydrolysed) refer always to the amount of total protein in the lipoprotein and are given in μg ml^{-1}.

Free fatty acid determination

Total free fatty acid concentrations in the serum VLDL fraction, in lipoprotein lipase and in VLDL were determined using an enzymatic colorimetric assay based on the method of Shimizu *et al.* (1980) using a commercially available kit (Half-micro test, Boehringer Mannheim, Germany). Individual fatty acid concentrations were determined using a highly sensitive gas chromatography-negative chemical ionization mass spectrometry method (GC-MS). Fatty acid pentafluorobenzyl (PFB) esters were produced in a single step extraction and derivatization procedure. Briefly, the method is based on extraction of carboxylate anion-tetrabutylammonium cation pairs by dichloromethane and their reaction with PFB bromide in the organic phase. A fatty acid not occurring in mammalian species, margaric acid (C-17 : 0), was used as internal standard. The PFB ester derivatives were extracted with hexane, evaporated to dryness and redissolved in hexane prior to GC-MS analysis. Fatty acids were chromatographed on a capillary 30 m $\times$ 0.32 mm, 1.0 μm DB-5 column (J & W Scientific, Rancho Cordova, CA, U.S.A.) using nitrogen as the carrier. Negative chemical ionization was performed with methane. Selected ion monitoring on the respective fatty acid anion fragment was performed with a Trio 1000 quadrupole mass spectrometer (Fisons, Crewe, U.K.). Samples were analysed in triplicates. Mass traces were integrated and quantified using calibration curves.

Caspase 3 activity assay

Caspase 3, also called apopain, is an enzyme derived from the proenzyme CPP32 at the onset of apoptosis (Goldberg *et al.*, 1996). Apoptosis of VSMC was determined by the Fluor-Ace™ Apopain Assay Kit (Bio-Rad) based on continuous fluorometric assay of caspase 3 activity as described in the kit manual. Caspase 3 activity was monitored using the fluorogenic peptide substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (AFC). Caspase 3 enzymatically cleaves the ACF from the peptide and releases free ACF substrate that can be detected by measurement of the fluorescence at the wavelength of 530 nm by the excitation wavelength of 380 nm. Briefly, confluent VSMC in 10-cm petri dishes were preincubated in 5 ml serum-free medium for 24 h before addition of VLDL, lipoprotein lipase, hydrolysed VLDL, oleic, linoleic, palmitic and myristic acid. After 24 h the cells were scraped, centrifuged at 1500 r.p.m. for 5 min and washed three times with 5 ml PBS. Then they were suspended in 150 μl apopain lysis buffer containing (mM): HEPES 10, EDTA 2, 0.1% 3-[3-cholamidopropyl-dimethyl-ammonio]-1 propanesulphonate (CHAPS), dithiothreitol (DTT) 5, phenylmethylsulfoxide (PMSF) 1, pepstatin A 15, aprotinin 1.5 and leupeptin 43, pH 7.4, and lysed by freezing and thawing of the samples by transferring them sequentially from a methanol-dry ice bath to a 37°C water bath. The lysed cell extracts were transferred to microfuge tubes and cell debris was removed by centrifugation at 9000 r.p.m. for 30 min. Forty μl from the supernatant were used to determine caspase 3 activity in 1 ml reaction buffer containing 1 mM piperazine-N, N′-bis 2-ethane sulfonic acid (PIPES), 2 mM EDTA, 0.1% buffer containing 1 mM CHAPS, 5 mM DTT and 50 μM AFC. Fluorescence was measured at 180 min.

Apoptotic cell death detection by ELISA

This assay (Cell Death Detection Elisa^{plus} assay kit from Boehringer Mannheim) is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments whose presence is a feature of cells undergoing apoptosis (Bonfoco *et al.*, 1995). It is based on the quantitative sandwich-enzyme-immunoassay principle and uses mouse monoclonal antibodies directed against DNA and histones, thus allowing the specific determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates. Briefly, confluent VSMC in 24-well culture plates (5×10^4 cells per well, well diameter 12 mm) were preincubated in 5 ml serum-free medium for 24 h before addition of VLDL, lipoprotein lipase, hydrolysed VLDL, oleic, linoleic, palmitic and myristic acid. After 24 h the various samples (cell lysates and culture-supernatants) were incubated for 2 h with a mixture of biotin-labelled anti-histone and peroxidase (POD) conjugated anti-DNA antibodies. The anti-histone antibody binds to the histone component of the nucleosomes and the anti-DNA-POD antibody reacts with the DNA component of the nucleosomes. After removal of the unbound antibodies by a washing step, the amount of nucleosomes is quantified by the POD retained in the immunocomplex. POD is determined photometrically at 405 nm with ABTS (2,2′-azino-di[3-ethylbenzthiazolin-sulphonate]) as substrate.

Cell proliferation assay

The CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, U.S.A.) is a colorimetric method for determining the number of viable cells in proliferation assays. The CellTiter96® AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; MTS] and an electron coupling reagent (phenazine ethosulphate; PES)

which is combined with MTS in solution. The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product whose quantity as measured by the amount of absorbance at 490 nm is directly proportional to the number of living cells in culture (Riss & Moravec, 1992). Briefly, 20 μl of the CellTiter96® AQueuous One Solution Reagent are added in each well of a 96 well plate containing different concentrations of hydrolysed VLDL, oleic, linoleic, palmitic and myristic acid. The plate is then incubated for 3 h at 37°C in the Steri-cult incubator, and the absorbance at 490 nm is recorded using a 96 well plate reader.

Materials

DMEM, Ham's F-10 and PBS were obtained from Gibco BRL (Eggestein, Germany). The Cell Death Detection Elisa^{plus} and FCS were obtained from Boehringer Mannheim (Mannheim, Germany). [Methyl-^{3}H]-thymidine and the polyvinylidene difluoride membranes were obtained from Amersham (Little Chalfont, U.K.). ECL Western blotting detection system was obtained from New England BioLabs Inc. (Beverly, MA, U.S.A.). The phospho-specific ERK1/2 and p38 rabbit polyclonal antibodies were also obtained from New England BioLabs. The horseradish peroxidase-labelled anti-mouse antibody was obtained from Amersham Life Sciences (Little Chalfont, U.K.). Lipoprotein lipase (one unit releases 1 nmol of substrate per minute at 37°C at a pH of 7.2), water soluble oleic acid and linoleic acid (vehicle ß-cyclodextrin, a cone-shaped molecule with hydrophilic upper and lower surfaces and a hydrophobic centre cavity), palmitic acid and myristic acid were obtained from Sigma (Deisenhofen, Germany). FluorAce™ Apopain Assay Kit for fluorometric detection of apoptosis was obtained from Bio-Rad (Munich, Germany). The CellTiter96® AQueous One Solution Cell Proliferation Assay was obtained from the Promega Corporation, Madison, WI, U.S.A.

Statistics

Values are expressed as the arithmetic mean ± s.e.mean unless otherwise indicated. Statistical analysis of the data was performed using the Mann-Whitney *U*-test. $P<0.05$ was considered to be statistically significant. Dose response relationships were calculated by modelling individual experimental data to the Hill equation by use of a combined Simplex and Marquardt-Levenberg iteration procedure using the computer program SigFit.

Results

After incubation of VLDL (100 μg ml^{-1}) with lipoprotein lipase (35 u ml^{-1}) at 37°C for 24 h, GC-MS analysis of the fatty acid mixture was performed showing a fatty acid composition pattern as shown in Table 1. On a molar basis, the four most abundant fatty acids in the hydrolysed VLDL were: palmitic acid 152.3 ± 6 μM, oleic acid 61.4 ± 4 μM, myristic acid 43.8 ± 4 μM and linoleic acid 25.6 ± 3 μM. After incubation of VLDL (100 μg ml^{-1}) without lipoprotein lipase at 37°C for 24 h a total fatty acid concentration of 4.37 ± 2 μM was measured. No fatty acids were detected in the serum-free medium. A dose-response curve of hydrolysed VLDL showed a dose-dependent decrease in cell number with an EC_{50} of 2.0 ± 0.09 μg ml^{-1} causing complete cell death at 10 μg ml^{-1} (Figure 1a). VLDL, up to a concentration of 10 μg ml^{-1}, and lipoprotein lipase alone had neither an effect on cell number nor on DNA synthesis, while at concentrations of 100 μg ml^{-1} VLDL caused a significant increase in cell number (150 ± 40%) and in DNA synthesis (300 ± 75%) versus controls (data not shown).

Table 1 Per cent of total VLDL fatty acids after lipoprotein lipase hydrolysis (on a molar basis). Mean value ± s.d. of three GC-MS measurements of three independent experiments

Fatty acid	*Chemical formula*	*Per cent* (± s.d.) *of total*
Capric	C-10:0	< 0.1%
Lauric	C-12:0	8.2 ± 2.3%
Myristic	C-14:0	13.7 ± 1.7%
Myristoleic	C-14:1	< 0.1%
Palmitic	C-16:0	47.5 ± 2.2%
Palmitoleic	C-16:1	< 0.1%
Stearic	C-18:0	3.2 ± 0.2%
Oleic	C-18:1	19.2 ± 1.5%
Linoleic	C-18:2n-6	8.0 ± 0.08%
Linolenic	C-18:3n-6	< 0.1%
Arachinic	C-20:0	< 0.1%
Arachidonic	C-20:4n-6	0.32 ± 0.03%
Eicosapentaenoic	C-20:5n-3	< 0.1%
Docosapentaenoic	C-22:5n-3,n-6	< 0.1%
Docosahexaenoic	C-22:6n-3	< 0.1%

A dose-response experiment using the authentic fatty acids oleic, linoleic, palmitic and myristic also showed a dose-dependent decrease in cell number (Figure 1b) and in DNA synthesis (data not shown). Their calculated EC_{50} values were 4.6 ± 1.3, 2.4 ± 0.2, 287 ± 30 and 116 ± 10 μM, respectively. We then estimated, on the basis of their relative occurrence in VLDL triglycerides, the EC_{50} values (in μM) for oleic, linoleic, palmitic and myristic acid in the hydrolysed VLDL and found them to be 13 ± 0.38, 5.41 ± 0.17, 35.4 ± 1.03 and 11.5 ± 0.33, respectively. It was interesting that while the EC_{50} for oleic and linoleic acid were about twice as high in the hydrolysed VLDL than when used individually, the EC_{50} of myristic and palmitic acid were about 10 and eight times lower in the lysate than when used individually (Figure 2a–d).

The growth-suppressing effects of the EC_{50} concentrations of the fatty acids and of hydrolysed VLDL started at 6 h (except for linoleic acid whose effects started at 12 h) and increased gradually up to 24 h (Figure 3). On the other hand, when higher doses of hydrolysed VLDL were used (100 μg ml^{-1}), complete cell death started earlier, at 1 h of incubation.

Cell proliferation was also determined using a colorimetric method for the determination of viable cells. The results obtained by this method were similar to the ones obtained using the cell count method for the various authentic fatty acids as well as for the hydrolysed VLDL (data not shown). The calculated EC_{50} values for oleic, linoleic, palmitic and myristic acid were 3.1 ± 1.0, 2.8 ± 2.1, 314 ± 40 and 129 ± 36 μM, respectively, comparable to the results obtained measuring VSMC growth by cell count. The calculated EC_{50} value for VLDL was 2.02 ± 1.4 μM.

When albumin-bound oleic acid and linoleic acid were used (equimolar amounts of albumin), there was a slight but not

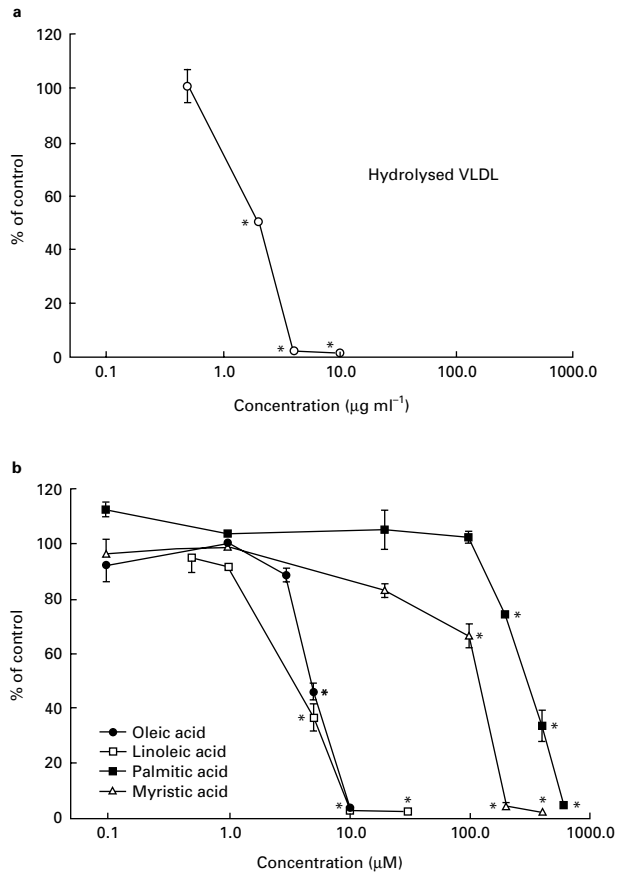

Figure 1 Effects of hydrolysed VLDL (a) and of oleic, linoleic, palmitic and myristic acid (b) on VSMC cell number. VSMC were precultured in serum-free medium for 24 h. Cells were then incubated with various concentrations of either hydrolysed VLDL or fatty acids for 24 h, trypsinized and counted as described in the Methods section. Results are obtained from three independent experiments each with triplicate determinations and are expressed as the arithmetic mean ± s.e.mean. $^*P<0.05$ for treated cells *versus* control.

significant attenuation of the oleic acid- and linoleic acid-induced VSMC growth suppression in the mid-concentration range (approximately 5 μM) (Figure 4). The calculated EC_{50} values for albumin-bound oleic acid and linoleic acid were 5.6 ± 0.7 and 5.5 ± 0.7 μM, respectively, and were statistically not significantly different from the EC_{50} values obtained with unbound oleic and linoleic acid. The time-courses of albumin-bound oleic and linoleic acid at 5 μM concentration showed decreases in VSMC growth after 6 h of incubation, with a parallel decrease in DNA synthesis (data not shown). The vehicle of the water soluble fatty acids, cyclodextrane, when used alone, did not affect cell number or DNA synthesis of VSMC.

The hydrolysed VLDL (in concentrations equal or higher than 10 μg ml^{-1}) caused pronounced changes in cell morphology, as inspected by phase contrast microscopy. The cells appeared more granular and necrotic. Representative photographs of quiescent VSMC after 24 h of incubation with VLDL alone (100 μg ml^{-1}), lipoprotein lipase alone (35 u ml^{-1}) and with hydrolysed VLDL (100 μg ml^{-1}) are shown in Figure 5.

Quantification of the band densities, performed by laser scanning densitometry obtained by three separate experiments, show that stimulation of the cells with 100 μg ml^{-1} VLDL for 5 min resulted in an approximately 3 fold increase of the phosphorylated ERK1/2 (P-ERK1/2) over the control value (Figures 6a,b). Stimulation of VSMC with hydrolysed VLDL (100 μg ml^{-1}) resulted in a much more pronounced (5.5 fold) time-dependent stimulation of ERK1/2 with a maximum at 5 min (Figures 6a,b). Lipoprotein lipase, when added alone, had no stimulatory effects on ERK1/2 phosphorylation compared to untreated cells (Figure 6a). When VSMC were stimulated with 50 μM of oleic or linoleic acid, there was a stimulation (4 fold and 5 fold, respectively) of the ERK1/2 with a maximum at 5 to 15 min for oleic acid and at 15 to 30 min for linoleic acid (Figures 6c,d).

Stimulation of VSMC with VLDL (100 μg ml^{-1}) caused a time-dependent phosphorylation of p38 MAP kinase with a maximum (6 fold increase over control) at 5 min (Figures 7a,b) returning almost back to control values at 60 min, while stimulation of the cells with 100 μg ml^{-1} hydrolysed VLDL resulted in a more pronounced (8.5 fold increase over control) stimulation of the p38 MAP kinase, starting at 5 min and continuing unchanged up to 60 min (Figures 7a,b). It is interesting to point out that at 30 and 60 min the hydrolysed VLDL caused a significantly higher p38 stimulation compared to VLDL. Lipoprotein lipase alone had no effect on p38 phosphorylation (Figure 7a). When VSMC were stimulated with 50 μM of oleic or linoleic acid there was an approximately 6 fold stimulation of the p38 MAP kinase starting at 5 min for both, oleic and linoleic acid which remained unchanged up to 60 min for oleic acid while the linoleic acid-induced p38 activation was at 60 min almost back to baseline (Figures 7c,d).

Treatment of the cells with VLDL (100 μg ml^{-1}), lipoprotein lipase (35 u ml^{-1}), hydrolysed VLDL (100 μg ml^{-1}), oleic acid (50 μM) and linoleic acid (50 μM) for 120 min did not influence the cytoplasmic histone-associated DNA fragments (Figure 8a) or the enzymatic activity of caspase 3 (Figure 8b), both being markers of apoptosis. As expected, in the Elisa assay the positive control (histone-DNA complex provided in the kit) caused a remarkable increase in absorbance at 405 nm. Also, treatment of the cells with 100 nM staurosporin, a classic apoptotic trigger (Leist *et al.*, 1997) used as a positive control in the caspase 3 activity assay, resulted in a 6 fold increase of the caspase 3 activity over its baseline value.

Furthermore, in order to examine a possible involvement of apoptosis in the VSMC growth-suppressive effect of hydrolysed VLDL and fatty acids, the aforementioned markers of apoptosis were examined before complete cell death occurs, using the EC_{50} concentrations of hydrolysed VLDL and fatty acids (24 h stimulation). While staurosporin (100 nM) caused significant apoptosis, neither the hydrolysed VLDL nor the fatty acids caused an increase in the cytoplasmic histone-associated DNA fragments (Figure 9a) or in caspase 3 activity (Figure 9b) indicating absence of apoptosis.

Discussion

There is increasing evidence that NEFA are associated with the pathogenesis of atherosclerotic lesions acting as modulators of either the extracellular matrix proteoglycans

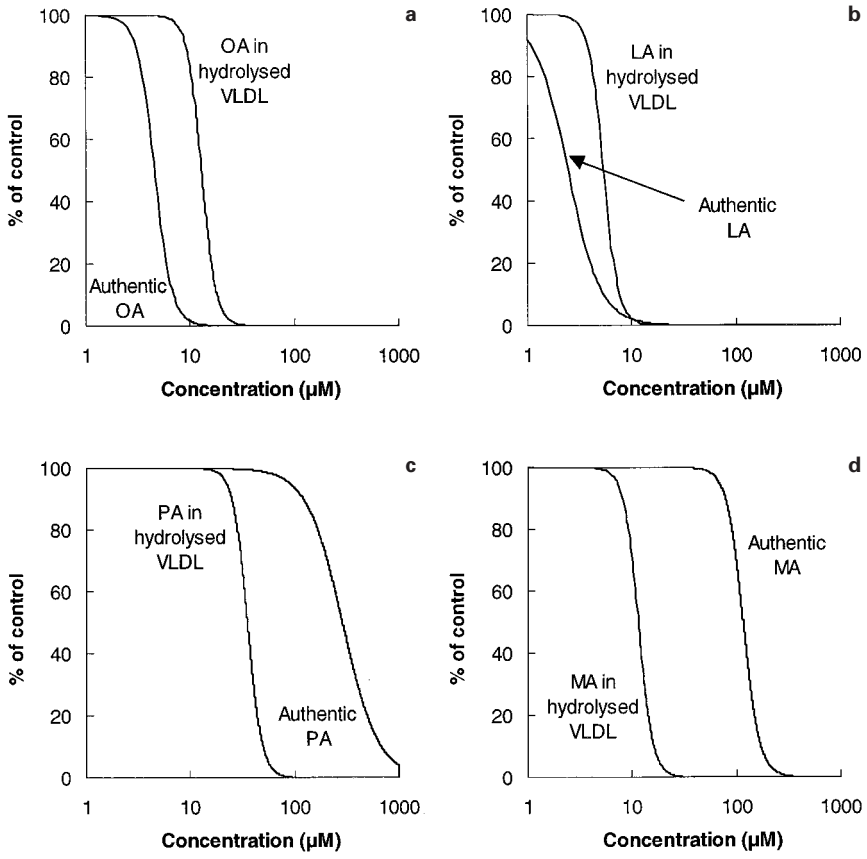

Figure 2 Comparison of the effects of authentic fatty acids *versus* VLDL hydrolysis-derived fatty acids on VSMC number (a) oleic acid (OA), (b) linoleic acid (LA), (c) palmitic acid (PA) and (d) myristic acid. Concentrations of VLDL hydrolysis-derived fatty acids were estimated based on their relative occurrence in VLDL triglycerides. The curves represent Hill plots after fitting the data of dose-response experiments ($n=3$ for each dose) performed with authentic fatty acids and hydrolysed VLDL (see Figure 1).

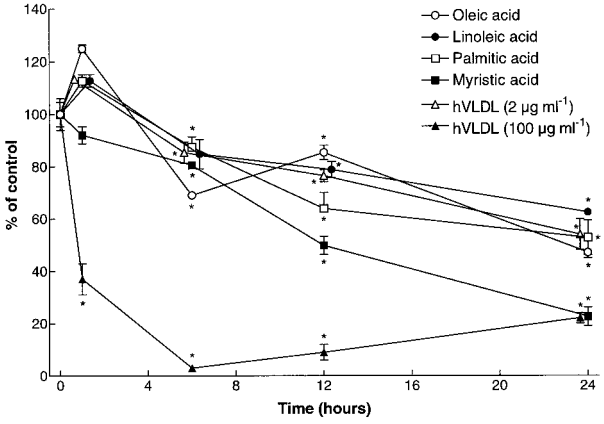

Figure 3 Time courses of the effects of the EC_{50} concentrations of oleic, linoleic, palmitic and myristic acid, or of low (2 μg ml^{-1}) and high (100 μg ml^{-1}) concentrations of hydrolysed VLDL (hVLDL) on cell number. VSMC were precultured in serum-free medium for 24 h. Cells were then incubated with fatty acids or hydrolysed VLDL, trypsinized and counted as described in the Methods section. Results are obtained from three independent experiments each with triplicate determinations and are expressed as the arithmetic mean$\pm$s.e.mean. *$P<0.05$ for treated cells *versus* control.

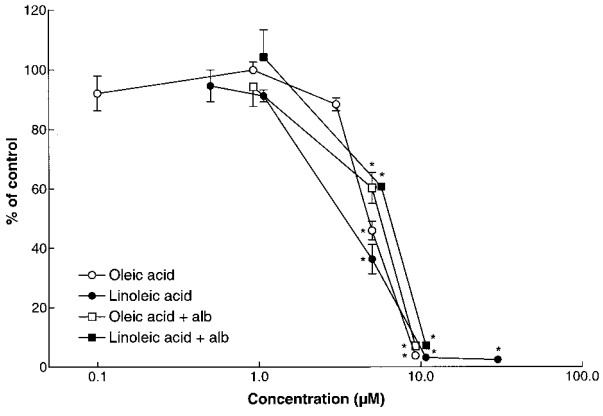

Figure 4 Effects of various concentrations of oleic and linoleic acid, either albumin (alb) bound or non albumin-bound, on VSMC cell number. VSMC were precultured in serum-free medium for 24 h. Cells were then incubated with oleic and linoleic acid either albumin-bound or non albumin-bound, trypsinized and counted as described in the Methods section. Results are obtained from three independent experiments each with triplicate determinations and are expressed as the arithmetic mean$\pm$s.e.mean. *$P<0.05$ for treated cells *versus* control.

(Camejo *et al*., 1998) or VSMC growth. The fatty acids employed in the previous studies were mainly used in their albumin-bound form since the extracellularly produced fatty acids, either from hydrolysis of triglycerides or from adipose tissue efflux, are bound rapidly by circulating albumin. It should be noted though that in the arterial intima the

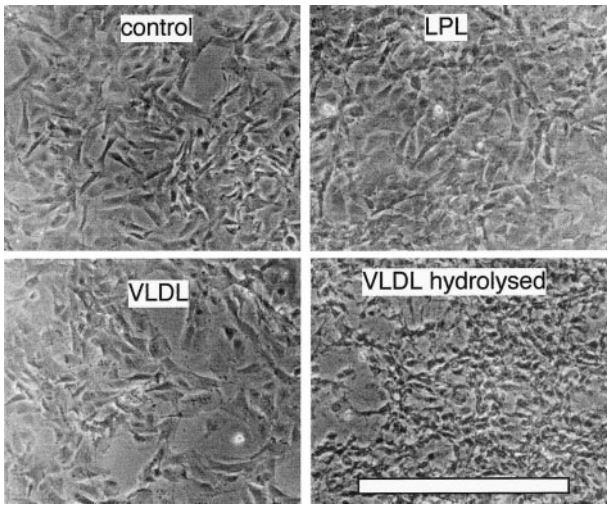

Figure 5 Effects of VLDL, lipoprotein lipase (LPL), and hydrolysed VLDL on rat VSMC morphology. VSMC were precultured in serum-free medium for 24 h. Cells were then incubated with 100 μg ml^{-1} VLDL, 100 μg ml^{-1} hydrolysed VLDL and with lipoprotein lipase (35 u ml^{-1}). After 24 h cells were photographed by phase-contrast light microscope. They appeared more granular and necrotic. The bar represents 100 μm.

amount of albumin may be three to four times lower than in plasma so that these experimental conditions do not necessarily reflect the physiological state. Furthermore, it has been suggested that the intima of large arteries may be a source of lipoprotein lipase and could thus generate fatty acids locally from apoB-containing lipoproteins, which enter the intima and are retained there by extracellular proteoglycans (Camejo *et al*., 1999). Therefore, in dyslipidemic states, the arterial wall may be exposed to an oversupply of locally generated non albumin-bound fatty acids which potentially contribute to lesion development. Moreover, the tissue concentration of non albumin-bound fatty acids would be increased in states with increased fatty acid levels such as hypertension, obesity, dyslipidemia as in type 2 diabetes, in insulin resistance states, postprandially, and in ischaemia (Rao *et al*., 1994).

The present study shows that fatty acids produced by hydrolysis of human VLDL triglycerides cause a dose- and time-dependent decrease of intact VSMC number and rapidly alter cell morphology. After only 1 h of incubation of VSMC with high concentrations of hydrolysed VLDL or with authentic fatty acids, no intact cells could be observed.

After determination of fatty acids in hydrolysed VLDL, we found four fatty acids most abundant, namely palmitic, oleic, myristic and linoleic, accounting together for almost 90% of the triglyceride-bound fatty acids. All of the aforementioned authentic fatty acids induced VSMC death with the following rank order of potency: linoleic acid > oleic acid > myristic acid > palmitic acid, with respective EC_{50} values of 2.4 ± 0.2, 4.6 ± 1.3, 116 ± 10, and 287 ± 30 (in μM). The relative toxicity of fatty acids has been confirmed by using a second cell proliferation assay (based on a formazan colorimetric method). Oxidative changes of the lipoproteins are unlikely to be considered responsible for their toxic effects since for each experiment freshly isolated VLDL was used and fatty acid samples were not used more than once.

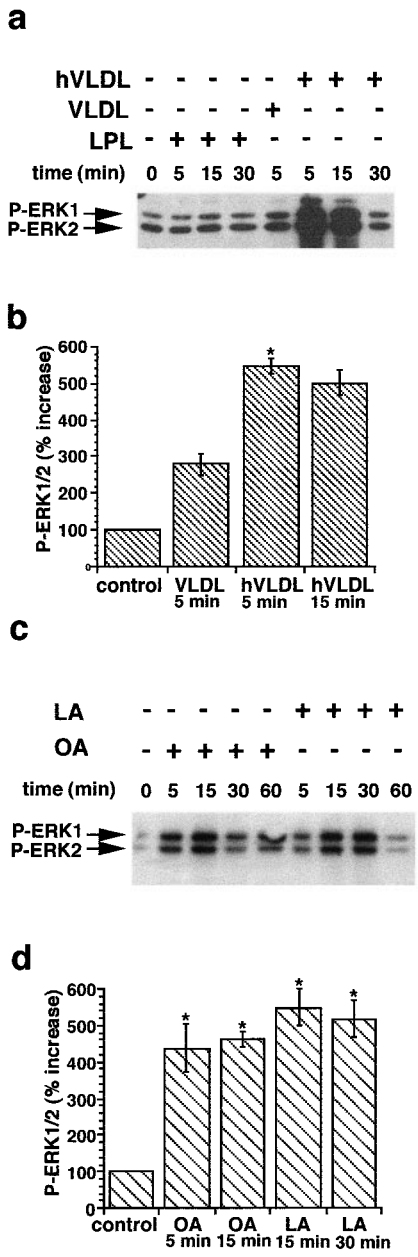

Figure 6 Effects of (a) hydrolysed VLDL (hVLDL), VLDL, lipoprotein lipase (LPL), (c) linoleic acid (LA) and oleic acid (OA) on ERK 1/2 phosphorylation. VSMC were precultured in serum-free medium for 24 h. Cells were then stimulated with VLDL (100 μg ml^{-1}), lipoprotein lipase (35 u ml^{-1}), linoleic acid (50 μM) or oleic acid (50 μM) for different time periods. Equal amounts of protein (10 μg per lane) were analysed by immunoblotting for MAP kinase activation as described in the Methods section. (a) and (c) show representative experiments from three independent experiments. (b) and (d) Densitometric analysis from data obtained from three separate experiments showing in (a) and (c). Data are expressed as per cent increase in the phosphorylated ERK1/2 (P-ERK1/2) above control (control = 100%) at 5 min of VLDL and at 5 and 15 min of hydrolysed VLDL stimulation (b) (mean $\pm$ s.e.mean, $n = 3$, $^*P < 0.05$ for hydrolysed VLDL- *versus* VLDL-treated cells at 5 min), at 5 and 15 min for oleic acid-treated cells and at 15 and 30 min for linoleic acid-treated cells (d) (mean $\pm$ s.e.mean, $n = 3$, $^*P < 0.05$ for oleic acid- and linoleic acid-treated cells *versus* control).

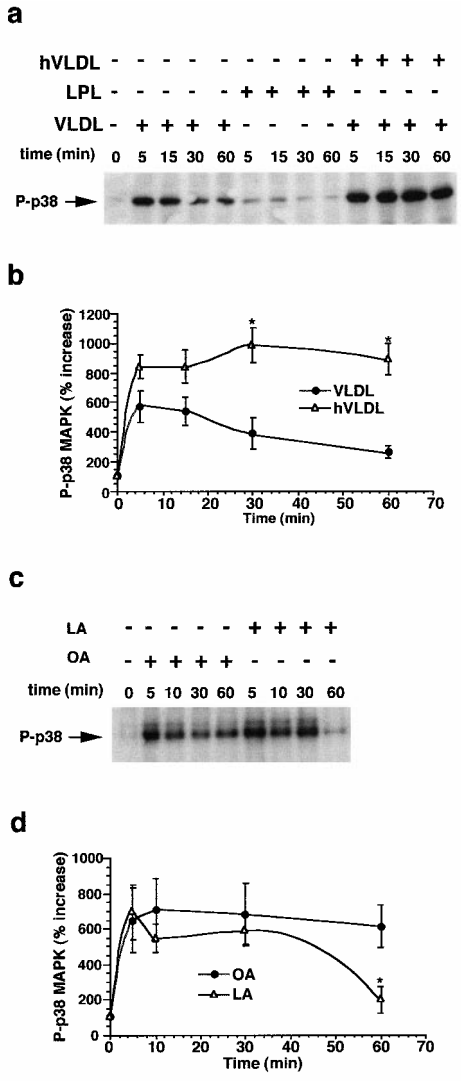

Figure 7 Effects of (a) hydrolysed VLDL (hVLDL), VLDL, lipoprotein lipase (LPL), (c) linoleic acid (LA) and oleic acid (OA) on p38 MAP kinase phosphorylation. VSMC were precultured in serum-free medium for 24 h. The cells were then stimulated with hydrolysed VLDL (100 μg ml^{-1}), lipoprotein lipase (35 u ml^{-1}), VLDL (100 μg ml^{-1}), linoleic acid (50 μM) or oleic acid (50 μM) for different time periods. Equal amounts of protein (10 μg per lane) were analysed by immunoblotting for p38 MAP kinase activation as described in the Methods section. (a) and (c) show representative experiments from three independent experiments. (b) and (d) Densitometric analysis from data obtained from three separate experiments showing in (a) and (c). Data are expressed as per cent increase in the phosphorylated p38 (P-p38) above control (control = 100%) after VLDL and hydrolysed VLDL stimulation (b) (mean $\pm$ s.e.mean, $n=3$, $^*P<0.05$ for hydrolysed VLDL- *versus* VLDL-treated cells at 30 and 60 min) and after oleic acid and linoleic acid stimulation (d) (mean $\pm$ s.e.mean, $n=3$, $^*P<0.05$ for oleic acid- *versus* linoleic acid-treated cells at 60 min).

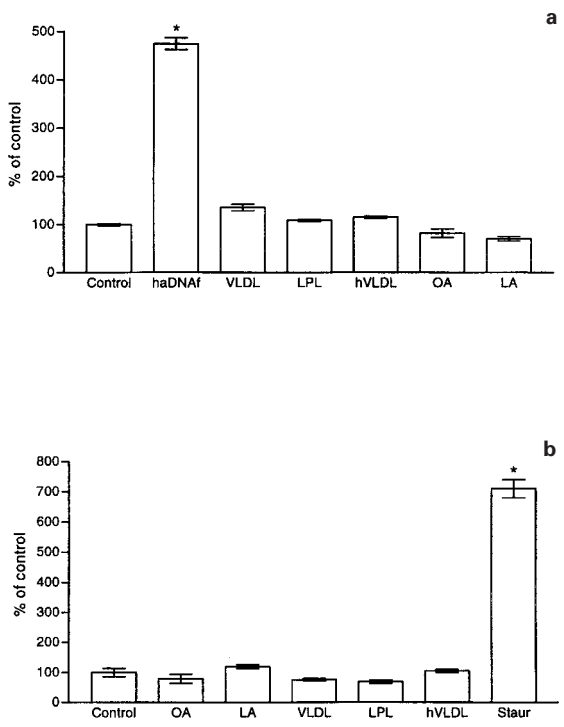

Figure 8 Effects of 100 μg ml^{-1} VLDL, 35 u ml^{-1} lipoprotein lipase (LPL), 100 μg ml^{-1} hydrolysed (hVLDL), 50 μM oleic acid (OA) and 50 μM linoleic acid (LA) on cytoplasmic histone-associated DNA fragments (haDNAf) (a) and on caspase 3 activity (b) in VSMC. (a) VSMC were precultured in serum-free medium for 24 h. They were then treated with the above agents for 24 h. Histone DNA complex from Cell Death Detection Elisa^{plus} assay kit was used as a positive control. The determination of cytoplasmic mono- and oligonucleosomes was performed as described in the Methods section. (b) VSMC were precultured in serum-free medium for 24 h. Cells were then treated with the above agents for 120 min and with 100 nM staurosporin (Staur) for 24 h. Cells were then lysed and apopain activity was measured as described in the Methods section. Results are obtained from three independent experiments for (a) and for (b) and are expressed as the arithmetic mean $\pm$ s.e.mean. $^*P<0.05$ for treated cells *versus* control.

When albumin-bound oleic and linoleic acid were used, there was a slight but not significant attenuation of the fatty acid-induced VSMC growth suppression. This indicates that albumin partially prevents but does not abolish the toxic effects of oleic and linoleic acid.

Of interest was the observation that the estimated EC_{50} values of oleic and linoleic acid in the lysate were about twice as high as those when used alone, while the ones of myristic and palmitic acid were 10 and eight times, respectively, lower in hydrolysed VLDL compared to the ones when used alone. These findings could suggest either that the effect of a specific combination of fatty acids on VSMC growth is different from that of single fatty acids, or that another unknown factor in VLDL enhances saturated fatty acid toxicity. In accordance with our findings, other investigators have also shown a diversion between the effects of single *versus* combined essential fatty acids on a human prostate cancer cell line (Motaung *et al*., 1999). However, since estimated EC_{50}s are not as valid as calculated ones, further studies are needed for verification of this observation.

Although the mechanism by which fatty acids exert their effects on VSMC has not yet been clarified, it has been shown that they are able to induce apoptosis in some cell types (Shimabukuro *et al*., 1998) and to have cytotoxic potential against macrophages (Chung *et al*., 1995). Our findings show that neither hydrolysed VLDL nor the fatty acids tested modulated caspase 3 activity or the histone-associated DNA fragments, suggesting that fatty acids promote necrosis rather

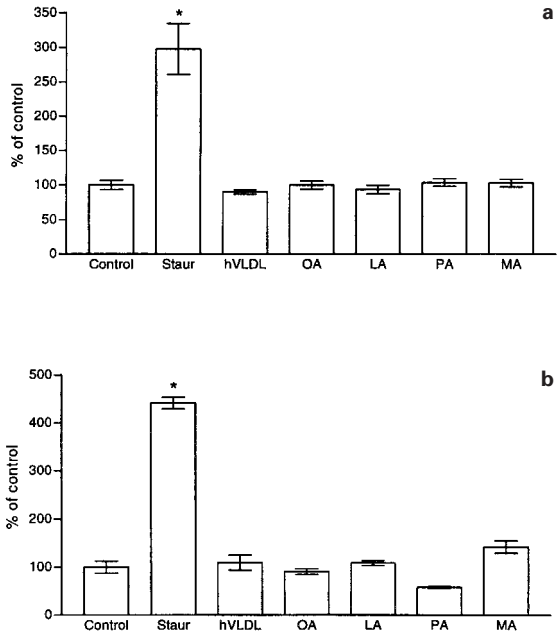

Figure 9 Effects of EC_{50} concentrations of hydrolysed VLDL (hVLDL) and of oleic acid (OA), linoleic acid (LA), palmitic acid (PA), and myristic acid (MA) on (a) cytoplasmic histone-associated DNA fragments and on (b) caspase 3 activity in VSMC. VSMC were precultured in serum-free medium for 24 h. The cells were then treated with the above agents and with staurosporin (Staur) (100 nM) for 24 h. The determination of cytoplasmic mono- and oligonucleosomes and of apopain activity was performed as described in the Methods section. Results are obtained from three independent experiments for (a) and for (b) and are expressed as the arithmetic mean ± s.e.mean. $*P<0.05$ for treated cells *versus* control.

than apoptosis of VSMC. When the same parameters of apoptosis were examined using the EC_{50} concentrations of hydrolysed VLDL and of fatty acids, also no evidence for apoptosis was found.

It is well known that p38 MAP kinase is strongly activated by stress factors causing cell death while ERK1/2 activation is usually associated with cell growth (Kyriakis & Avruch, 1996; Seger & Krebs, 1995). The present study shows that hydrolysed VLDL and fatty acids cause VSMC death with a stimulation of both p38 MAP kinase and ERK1/2. This is in accordance with recent findings of our group, describing ERK1/2 stimulation not only by growth factors but also by stress factors such as ethanol, which caused necrosis of VSMC (Sachinidis *et al*., 1999). We previously described that high concentrations of VLDL (100 μg ml^{-1}) stimulate ERK1/2 (Sachinidis *et al*., 1999). Now we were able to demonstrate that VLDL also induces a time-dependent stimulation of p38 MAP kinase.

Interestingly, compared to the effect of VLDL alone, the effect of hydrolysed VLDL on ERK1/2 and p38 MAP kinase stimulation was much more pronounced. Based on the observation that activation of the ERK1/2 pathway is a characteristic feature mainly of growth-promoting factors, it may be speculated that its stimulation by fatty acids may represent a secondary stress-induced event by which the cells attempt to counteract the toxic effects of fatty acids and escape cell death.

Recently there has been evidence that smooth muscle cell necrosis is a key mechanism involved in predisposing and/or eliciting plaque rupture (Bauriedel *et al*., 1999). Thus, it may be postulated that under pathophysiological conditions the fatty acid-induced VSMC death can cause plaque instability and rupture resulting in thrombus formation. Therefore, modulation of the fatty acid-induced VSMC death could have important implications for the prevention of acute ischaemic syndromes and of progression of atherosclerosis.

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