Different mechanisms of saturated versus polyunsaturated FFA-induced apoptosis in human endothelial cells

Michaela Artwohl,[1,*] Andrea Lindenmair,[*] Veronika Sexl,[†] Christina Maier,[*] Georg Rainer,[§] Angelika Freudenthaler,[*] Nicole Huttary,[**] Michael Wolzt,[*,††] Peter Nowotny,[*] Anton Luger,[*] and Sabina M. Baumgartner-Parzer[*]

Department of Internal Medicine III, Division of Endocrinology and Metabolism,* Institute of Pharmacology,† Department of Ophthalmology,§ Clinical Institute of Pathology,** Department of Clinical Pharmacology,†† Medical University of Vienna, Vienna A-1090, Austria

Abstract **Apoptosis and underlying mechanisms were evaluated in human umbilical vein endothelial cells (HUVECs), in target tissues of late diabetic vascular complications [human aortic endothelial cells (HAECs) and human retinal endothelial cells (HRECs)], and in endothelial progenitor cells (EPCs) exposed to FFAs, which are elevated in obesity and diabetes. Saturated stearic acid concentration dependently induced apoptosis that could be mediated via reduced membrane fluidity, because both apoptosis and membrane rigidity are counteracted by eicosapentaenoic acid. PUFAs triggered apoptosis at a concentration of 300 μmol/l in HUVECs, HAECs, and EPCs, but not HRECs, and, in contrast to stearic acid, involved caspase-8 activation. PUFA-induced apoptosis, but not stearic acid-induced apoptosis, strictly correlated ($P < 0.01$) with protein expression of *E2F-1* ($r = 0.878$) and *c-myc* ($r = 0.966$). Lack of *c-myc* expression and activity owing to quiescence or transfection with dominant negative In373-Myc, respectively, renders HUVECs resistant to PUFA-induced apoptosis. Because *c-myc* is abundant in growing cells only, apoptosis triggered by PUFAs, but not by saturated stearic acid, obviously depends on the growth/proliferation status of the cells. Finally, this study shows that FFA-induced apoptosis depends on the vascular origin and growth/proliferation status of endothelial cells, and that saturated stearic acid-induced apoptosis and PUFA-induced apoptosis are mediated via different mechanisms.**—Artwohl, M., A. Lindenmair, V. Sexl, C. Maier, G. Rainer, A. Freudenthaler, N. Huttary, M. Wolzt, P. Nowotny, A. Luger, and S. M. Baumgartner-Parzer. **Different mechanisms of saturated versus polyunsaturated FFA-induced apoptosis in human endothelial cells.** ***J. Lipid Res.*** **2008.** 49: **2627–2640.**

Supplementary key words aortic endothelial cell • retinal endothelial cell • endothelial progenitor cell • membrane rigidity • caspases • c-myc • E2F-1 • XRCC1 • mad

FFAs, elevated in visceral obesity and diabetes, play a vital role in atherogenesis and acute coronary syndromes (1, 2). In vitro, high FFA concentrations contribute to accelerated apoptosis of the endothelium (3–7), which ranks among the most endangered target tissues in diabetes. In diabetic retinopathy, endothelial apoptosis occurs before other histopathology is detectable (8), and procoagulatory apoptotic endothelial cells, topographically associated with microthromboses (9), could contribute to vascular occlusion. In atherosclerosis, in addition to its contribution to initial lesion formation owing to detachment of endothelial cells from the underlying intimal layer, endothelial apoptosis leads to increased vascular permeability, plaque erosion, and plaque rupture (10, 11).

At least two observations render obsolete the former assumption that loss of endothelial cells owing to apoptosis is solely accomplished by an increased mitotic response/turnover of endothelial cells located nearby: *i*) re-endothelialization appears to be more likely attributable to cells migrating over long distances than to local endothelial cells (12); and *ii*) bone marrow-derived endothelial progenitor cells (EPCs) are presumably responsible for postnatal vasculogenesis in physiological and pathophysiological neovascularization (13). Increasing evidence suggests that loss of endothelial integrity, owing to damage/apoptosis induced by atherosclerotic risk factors, might be repaired by circulating EPCs (14, 15), which on recruitment are capable of differentiating into endothelial cells, displaying classical morphology and characteristics. Thus, evaluation of the effects of risk factors such as FFAs should not be

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Abbreviations: DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; EPA, eicosapentaenoic acid; EPC, endothelial progenitor cell; FCS, fluorescence correlation spectroscopy; HAEC, human aortic endothelial cell; HREC, human retinal endothelial cell; HUVEC, human umbilical vein endothelial cell; XRCC1, X-ray repair cross-complementing 1.

[1] To whom correspondence should be addressed.
e-mail: michaela.artwohl@meduniwien.ac.at

restricted to damaged target cells (e.g., aortic or retinal endothelial cells), but should also include subsequent "repairing" cells, the EPCs.

To date, the pro-apoptotic activity of different FFAs has been examined primarily in immortalized endothelial cell lines (EA.hy926, ECV304) and human umbilical vein endothelial cells (HUVECs) (3–6). Studies involving target tissues directly affected by the metabolic syndrome are rare (7, 16, 17) and have not been performed in EPCs. Hitherto, FFAs' chain length, degree of saturation, and/or position of double bonds (3, 7), p38 mitogen-activated protein kinase (p38 MAPK) (5, 16), NF-κB (7, 16), bcl-2 family members (3, 4), and executioner caspases, i.e., caspase-3 and/or -7 (5, 17, 18) have been reported to be involved in the pro-apoptotic action of selected FFAs. Characterization of underlying mechanisms for a broad spectrum of both selected nutritional FFAs and different human endothelial cell types is missing as yet.

The present study therefore evaluated the effects of a broad spectrum of nutritional FFAs (saturated/monounsaturated/polyunsaturated; ω3/ω6/ω9) on endothelial apoptosis in target tissues of late diabetic vascular complications, i.e., human aortic endothelial cells (HAECs) and human retinal endothelial cells (HRECs) as well as in human EPCs, which presumably have a role in vascular repair. Our study shows that FFA-induced apoptosis depends on the vascular origin of endothelial cells, the growth/proliferation status of the cells, and the FFA structure. Whereas the pro-apoptotic activity of saturated stearic acid is apparently related to the membrane rigidity of the cells, PUFA-induced apoptosis is mediated via *c-myc/E2F-1/XRCC1/caspase-8*.

MATERIALS AND METHODS

Endothelial cell culture

HUVECs, HAECs, and HRECs were isolated, cultured, and identified as described (3, 18–20) and used in the 1st, 5th, and 2nd subcultures, respectively.

EPC culture

Circulating EPCs from peripheral blood of healthy human volunteers were obtained with the approval of the local ethics board from the Department of Clinical Pharmacology and were isolated by density-gradient centrifugation (400 *g* for 30 min) using Histopaque-1077 (Sigma; St. Louis, MO) overlaid with an equal volume of fresh anti-coagulated whole blood at room temperature. The fraction containing the peripheral blood mononuclear cells was then transferred into a fresh vial, washed with PBS (Hyclone; Logan, UT), and cultured on fibronectin- (Sigma) coated culture dishes in Medium 199 (M199; Sigma) supplemented with 20% fetal calf serum (Hyclone), 5 U/ml heparin (Biochrom; Berlin, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 ng/ml fungizone (Hyclone). EPCs grew out from colonies and differentiated into endothelial cells of >90% purity, as characterized by triple staining showing the following expression pattern: $CD31^+/CD144^+/CD14^-$, representing positive staining for the endothelial marker proteins CD31

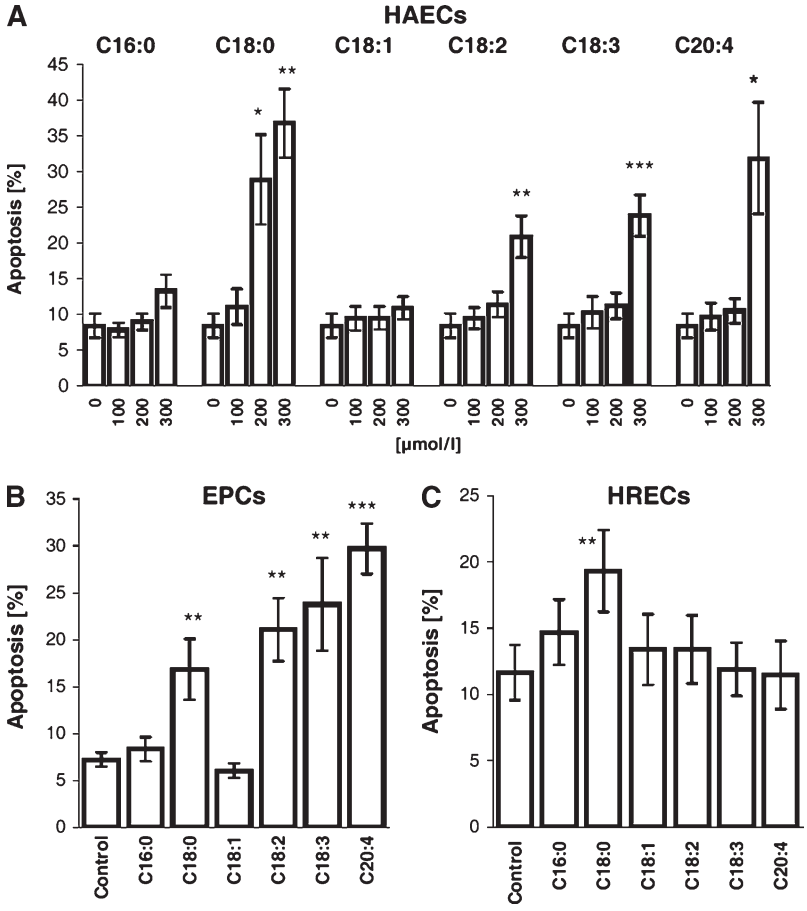

Fig. 1. FFA-mediated apoptosis in endothelial cells. Apoptosis rates in growing (A) human aortic endothelial cells (HAECs) (n = 7), (B) endothelial progenitor cells (EPCs) (n = 6), and (C) human retinal endothelial cells (HRECs) (n = 9) after exposure to palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) compared with intra-individual control cells treated with ethanol (solvent, 0 μmol/l FFA). HAECs were exposed to 100–300 mol/l FFAs for 24 h. EPCs and HRECs were exposed to 300 μmol/l for 24 h and 48 h, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ FFA-induced apoptosis versus ethanol-control. Data are mean ± SEM.

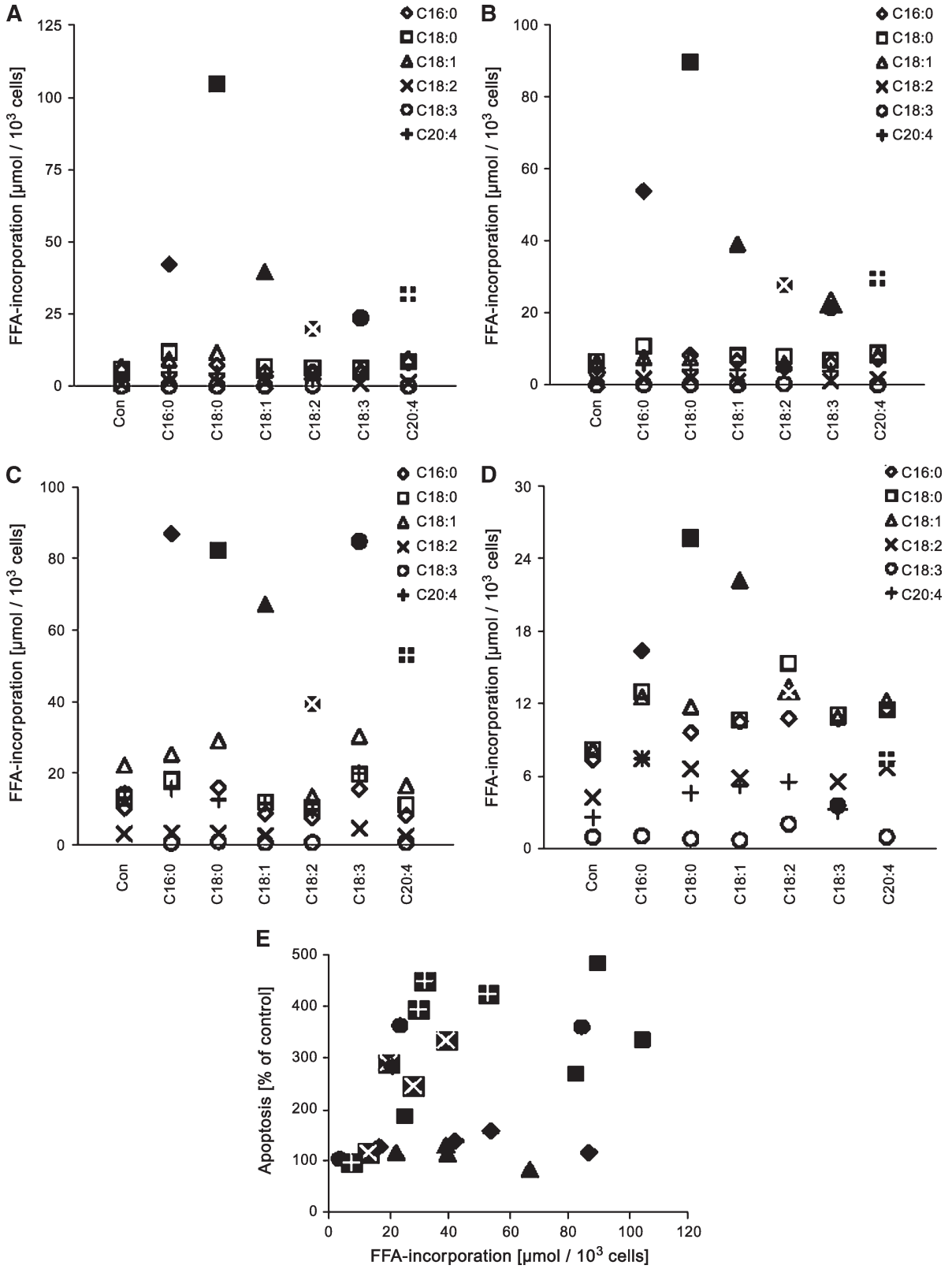

Fig. 2. FFA incorporation into cell membranes. Exposure (24 h) of one pool of (A) human retinal endothelial cells (HUVECs) (n = 4), (B) HAECs (n = 5), (C) EPCs (n = 3), and (D) HRECs (n = 10) to 300 µmol/l of a defined FFA (C16:0, diamonds; C18:0, squares; C18:1, triangles; C18:2, crosses; C18:3, circles; C20:4, plus signs) primarily resulted in incorporation of the respective FFAs (full symbols) into the cell membranes. However, FFA incorporation (*x* axis) of the respective FFAs did not correlate with FFA-induced endothelial apoptosis (*y* axis) in HUVECs, HAECs, EPCs, and HRECs (E).

(platelet-endothelial cell adhesion molecule-1; BD Pharmingen, San José, CA) and CD144 (vascular endothelial-cadherin; Bender MedSystems, Vienna, Austria), but negative staining for the monocyte marker CD14 (BD Pharmingen). Additionally, the isolated EPCs expressed the endothelial marker proteins CD146 (S-endo; BD Pharmingen) and endocan (endothelial specific molecule-1; R and D Systems, Minneapolis, MN).

Incubation of endothelial cells with FFAs

Growing HUVECs, HAECs, HRECs, and EPCs were exposed either to FFAs (100–300 μmol/l; palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), arachidonic acid (C20:4ω6) (Sigma), or to the respective ethanol concentration (1–3 ppm; used as solvent control) in M199 supplemented with 20% delipidated FBS (Sigma; final concentration of albumin, 0.4%) and antibiotics. Solely for analysis of *c-myc* dependence of apoptosis, confluent HUVECs were, as well, exposed to 300 μmol/l of palmitic acid, stearic acid, oleic acid, linoleic acid, and α-linolenic acid.

Apoptosis assays

[^{3}H]thymidine- (1 μCi/ml) labeled endothelial cells, trypsinized and seeded into 24-well culture plates, were exposed to FFAs for 24 h (HUVECs, HAECs, EPCs, HRECs) and for 48 h (HRECs), and experimental as well as intra-individual control cultures (exposed to ethanol) were tested for apoptosis (3, 18, 20). In brief, the cells were lysed (20 mmol/l Tris-HCl, pH 7.5, 0.4% Triton X-100; 10 min on ice), and fragmented (apoptotic) DNA was separated by centrifugation (3, 18, 20). Radioactivity of fragmented versus total DNA (digested with 180 μg/ml DNase I; Boehringer Mannheim, Germany) was quantified using a Tri-Carb 3100 TR liquid scintillation analyzer (Packard Instruments; Meriden, CT). Experiments were performed in triplicate. In examination of FFA-induced endothelial apoptosis, we have already shown that these assays lead to results comparable to those of different commercially available kits for apoptosis detection (i.e., TdT-FragEL™, Oncogene, Boston, MA; MitoCapture™, BioVision, Palo Alto, CA) (3, 18).

In some experiments, apoptosis was determined in HUVECs, HAECs, EPCs, and HRECs coincubated with the different FFAs

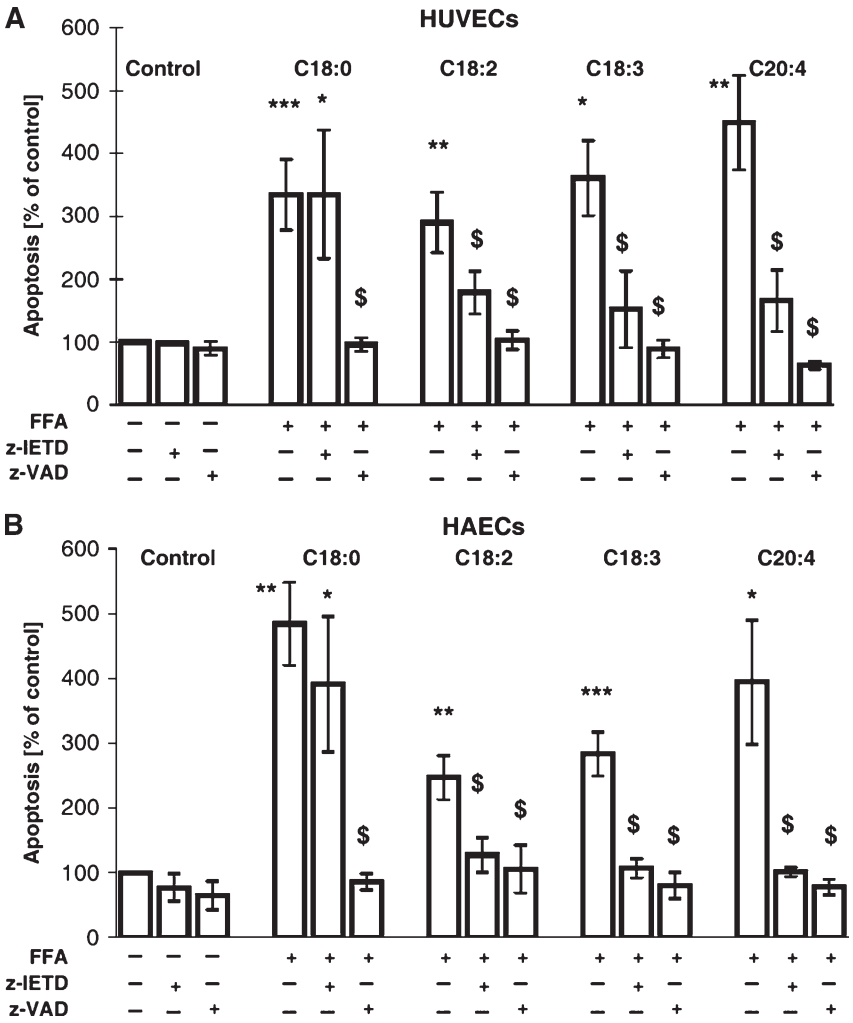

Fig. 3. Caspase activation. Modulation by *caspase-8* inhibitor z-IETD.fmk (20 μmol/l) and pan-caspase inhibitor z-VAD.fmk (30 μmol/l) of stearic acid (C18:0; 300 μmol/l) and PUFA (C18:2, C18:3, C20:4; 300 μmol/l) induced apoptosis in (A) HUVECs (n = 4), (B) HAECs (n = 3), (C) EPCs (n = 4), and (D) HRECs (n = 4); for HRECs, data are presented solely for the only pro-apoptotic FFA, stearic acid. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ FFA-induced apoptosis versus ethanol-control; $^{\$}P < 0.05$, inhibition by caspase inhibitors of apoptosis induced by 300 μmol/l of the respective FFA. Data are mean ± SEM.

along with caspase inhibitors (30 μmol/l z-VAD.fmk or 20 μmol/l z-IETD.fmk; Calbiochem, Palo Alto, CA) or eicosapentaenoic acid (EPA, 5–20 μmol/l; Sigma).

FFA incorporation into cell membranes

HUVECs, HAECs, EPCs, and HRECs were exposed (24 h) to FFAs (300 μmol/l) followed by cell membrane preparation. In brief, the cells were lysed on ice in hypotonic buffer (final concentrations: 5 mmol/l $MgCl_2$, 10 mmol/l HEPES, pH 7.4, 40 mmol/l KCl) followed by shearing the lysate 10 times through a 30 G needle. After centrifugation (10 min, 200 *g*, 4°C), the supernatant was subjected to an ultracentrifugation step (30 min., 28,000 rpm, 4°C, rotor: TLA 55), and the pelleted cell membranes were resuspended in PBS, followed by lyophilization. Lyophilized cell membrane preparations were reconstituted in distilled water, and an internal standard (C17:0, heptadecanoic acid; Riedel de Haën, Seelze, Germany) was added to each sample and to an FFA standard mixture (Altech, Deerfield, IL). The samples were then extracted and transesterified to methyl esters by a one-step reaction as follows. After addition of the reaction mixture [2-propanol-*n*-heptane-H_2SO_4 (0.25 mol/l) 40:10:1; all Merck, Darmstadt, Germany], samples were mixed vigorously before extraction with *n*-heptane and distilled water. After centrifugation (10 min, 1,000 *g*, 4°C), the resulting upper layer was transferred into reaction vials (Pyrex; Bibby Sterilin Ltd, Staffordshire, UK) and evaporated under nitrogen. Methanolysis was performed by addition of methanol-benzene (4:1) and acetyl chloride (Fluka; Buchs, Switzerland) at 100°C for 75 min under continuous stirring. The reaction was stopped by addition of 6% K_2CO_3, and the organic phase was collected, followed by extraction with benzene. Fatty acid methyl esters were analyzed using a Hewlett-Packard (Boise, ID) GC-MSD 5973 system equipped with a 30 m, 0.12 μm DB23 fused silica column, inner diameter 0.25 mm·(J and W Scientific, Folsom, CA). Gas chromatography was operated at 50°C for 2 min, rising to 180°C at 10°C/min, followed by a 5 min hold, rising to 240°C at 5°C/min, followed by a 2 min hold and rising at least to 250°C at 3°C/min under constant flow (1.1 ml/min) of helium as a carrier gas.

The above-mentioned standards (internal standard and FFA standard mixture) were detected by electronic impact ionization mass spectrometry using total ion current and their extracted ionized total mass peak (M+) values to calculate quantification factors in relation to the intensity of the internal standard. FFAs incorporated in the cellular membranes were identified by their retention times and were quantified in relation to the intensity of the internal standard.

Membrane rigidity

HUVECs were cultured on a poly-D-lysine-coated LabTek chambered coverglass and then exposed to stearic acid ± EPA or to arachidonic acid (used as PUFA control) versus ethanol (solvent). Cells were then incubated (20 min) with 10 μmol/l

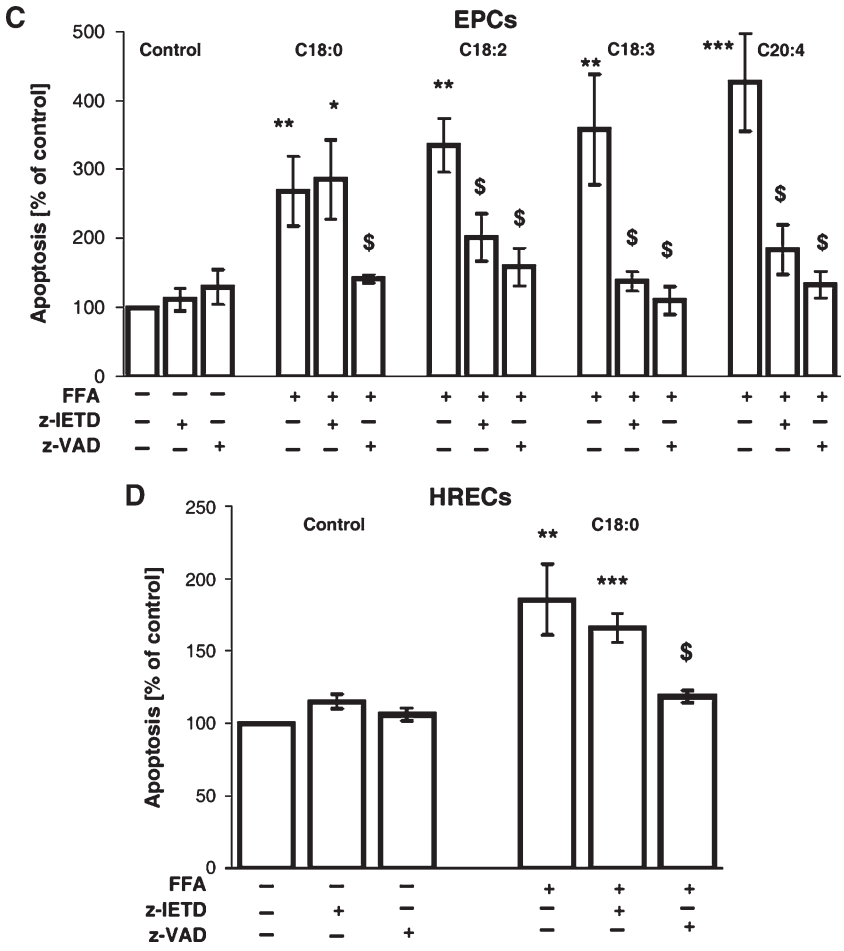

Fig. 3.—*Continued.*

of the lipophilic membrane marker 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes, Eugene, OR) in a nonfluorescent binding buffer (21). Fluorescence correlation spectroscopy (FCS) was used to evaluate the diffusion behavior of the fluorescent marker DiO in the cell membrane. Details of the method have been published elsewhere (21). Briefly, FCS measurements were carried out on a Confocor spectrofluorimeter (Carl Zeiss-Evotec, Jena, Germany). The pinhole diameter was set to 45 μm, resulting in a confocal volume element of 0.17 μm in the radial dimension and 2.4 μm in the axial dimension. The confocal volume was positioned in the cells using an x-y stage with 1 μm resolution, whereas the correct focus positioning on the cell membrane was ascertained by a scanning procedure in 1 μm steps. Autocorrelation curves were best-fitted to the two-component model, with component τ_1 corresponding to the diffusion time of DiO in solution and component τ_2 representing DiO diffusion on the cell membrane derived from the fitting procedure. Autocorrelation curves (n = 20–34) taken at the membrane position from six individual cells were evaluated for each experimental subgroup.

Protein expression

Associated protein expression was determined by Western blot analyses as described (3, 19) in growing HUVECs with/without FFAs after 24 h exposure. In brief, endothelial cells were lysed in cold Weinberg buffer (3, 19) and total protein was measured using a BCA protein assay (Pierce; Rockford, IL) according the manufacturer's instructions. An aliquot containing exactly 10 μg total protein was loaded in each lane and subsequently subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes (Schleicher and Schüll; Kassel, Germany). After controlling for homogenous sample loading by staining the blots with Ponceau S (Sigma), blocking of unspecific binding sites with nonfat dry milk (5% in TBS) containing 0.05% Tween-20 (Bio Rad; Hercules, CA) was followed by incubation with primary antibodies (*mad*, 1:2,000, PharMingen, San Diego, CA; *c-myc*, 1:200, BioSource, Camarillo, CA; *XRCC1*, 1:500, Kamiya Biomedical Co., Seattle, WA; *E2F-1*, 1:200, NeoMarkers, Fremont, CA) and detection with HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Pharmacia; Buckinghamshire, UK) using Super Sig-

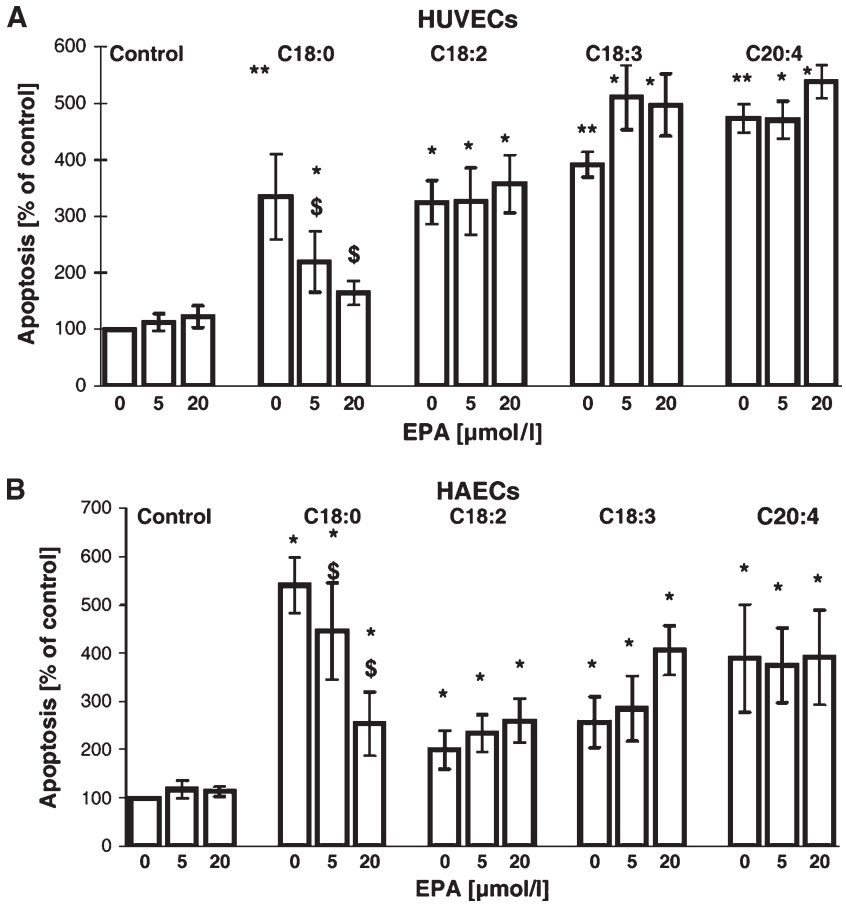

Fig. 4. Modulation by eicosapentaenoic acid (EPA) of apoptosis and membrane rigidity. (A–D). Modulation by EPA (C20:5ω3; 5–20 μmol/l) of apoptosis induced by 300 μmol/l stearic acid (C18:0), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) in (A) HUVECs (n = 5) and in (B) HAECs (n = 3) or by 300 μmol/l stearic acid (C18:0) in (C) EPCs (n = 4) and (D) HRECs (n = 6) compared with intra-individual control cells treated with ethanol (solvent, control). E: Diffusion time as a measure of membrane rigidity after exposure (24 h) of HUVECs (n = 4 cultures; 6 single cells per culture were measured) to 300 μmol/l stearic acid (C18:0) ± EPA, 20 μmol/l. $^{*}P < 0.05$, $^{**}P < 0.01$ FFA-induced apoptosis versus ethanol-control; $^{\$}P < 0.05$, inhibition by EPA of apoptosis induced by 300 μmol/l stearic acid (C18:0); $^{***}P < 0.01$, stearic acid-induced membrane rigidity versus ethanol-control in HUVECs; $^{\$\$\$}P < 0.001$, inhibition by EPA of stearic acid-induced prolongation of diffusion time in HUVECs versus cells exposed to 300 μmol/l stearic acid. Data are mean ± SEM.

nal Substrate (Pierce). Results are expressed in relation to intra-individual control cells (set to 100%).

Transfections

Confluent monolayers of primary HUVECs were transiently transfected (4 h) with 2 μg/ml In373-Myc (generously provided by V. Sexl, Vienna) or with the empty vector (pSRαMSV) using polyethyleneimine 25,000 (pH 7.0, Aldrich; Milwaukee, WI) (22) in serum-free M199. After overnight equilibration in full growth medium, HUVECs were trypsinized and seeded into 35 mm culture plates (2×10^7 cells/plate). After adherence (6 h) and exposure (24 h) to the different FFAs versus intra-individual control cells (incubated with ethanol as solvent), the cells were fixed (75% ice-cold ethanol) and DNA fragmentation was determined after staining with propidium iodide (1 μg/ml) by fluorescence-activated cell sorting analyses using an FACS Calibur (Becton Dickinson; Heidelberg, Germany) as described previously (3, 19). Transfection efficiency was controlled by immunocytochemical determination of cotransfected CD8.

Statistics

Data are expressed as means ± SEM. Statistical analysis was performed using paired or independent samples *t*-test (SPSS for Windows 7.5.1), as appropriate, with Bonferroni correction for multiple comparisons.

RESULTS

Apoptosis in growing endothelial cells

Macrovascular endothelial cells. In growing HAECs, saturated stearic acid concentration dependently induced apoptosis (**Fig. 1A**), whereas PUFAs linoleic acid, α-linolenic acid, and arachidonic acid triggered programmed cell death only at the highest concentration used (Fig. 1A). These findings mimic our recent observations in growing HUVECs (3), another macrovascular endothelial cell type. Palmitic acid and oleic acid, however, increased apoptosis neither in HUVECs (palmitic acid, 138 ± 25%; oleic acid, 112 ± 19%; n = 6; not significant) nor in HAECs (Fig. 1A).

EPCs. Owing to the severely limited number of EPCs, these cells could be analyzed only after exposure to a concentration of 300 μmol/l of the different FFAs. At this concentration, neither palmitic acid nor oleic acid induced cell death in EPCs (Fig. 1B), whereas stearic acid and PUFAs triggered EPC apoptosis (Fig. 1B). Therefore, EPCs resemble HUVECs (3) and HAECs (Fig. 1A) in regard to their pro-apoptotic response to FFAs.

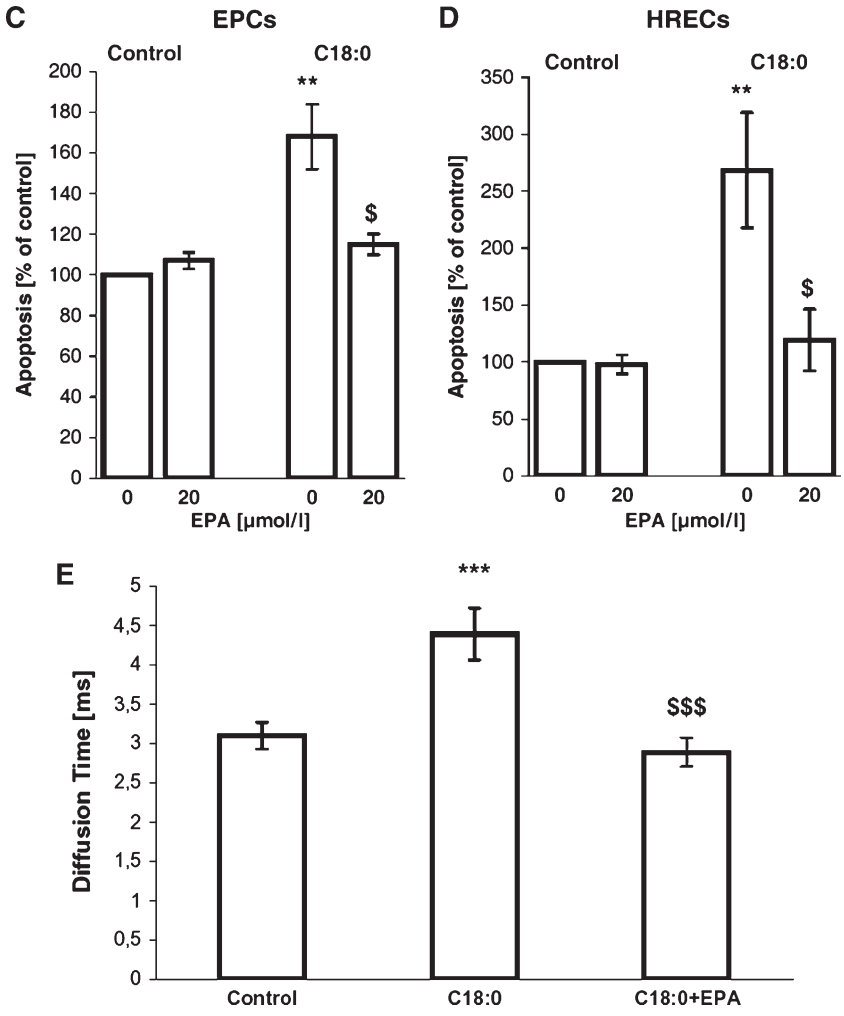

Fig. 4.—*Continued.*

Microvascular endothelial cells. Because of their limited availability, HRECs, as well, could be analyzed only after exposure to 300 μmol/l of the different FFAs. In HRECs, only stearic acid induced apoptosis, whereas palmitic acid, oleic acid, and PUFAs did not. Even enhancing the incubation time with PUFAs up to 48 h did not result in increased pro-apoptotic activity in HRECs (Fig. 1C).

FFA-induced endothelial apoptosis is independent of membrane incorporation of FFAs

Upon exposure of endothelial cells to FFAs, the cellular membranes represent the first cell components interacting with the FFAs. Therefore, FFA incorporation into the cell membranes was investigated. Although exposure of endothelial cells to a defined FFA primarily increased the incorporation of that particular FFA into the cell membranes (**Fig. 2A–D**, filled symbols), no correlation could be found between FFA incorporation into the membranes and endothelial apoptosis (Fig. 2E).

FFA-induced endothelial apoptosis requires caspase activation

Recently, we described stearic acid activation of caspase-3/7 in HUVECs (3). In the present study, we found that the pan-caspase inhibitory peptide z-VAD.fmk completely inhibited FFA-triggered endothelial apoptosis (HUVECs, **Fig. 3A**; HAECs, Fig. 3B; EPCs, Fig. 3C; HRECs, Fig. 3D), thereby unequivocally demonstrating that FFA-induced apoptosis in endothelial cells is mediated by caspases. Using z-IETD.fmk, a specific caspase-8 inhibitor, allowed for further delineation of the underlying mechanisms, because it inhibited PUFA-induced apoptosis but had no impact on the pro-apoptotic activity of stearic acid, irrespective of endothelial origin (HUVECs, Fig. 3A; HAECs, Fig. 3B; EPCs, Fig. 3C; HRECs, Fig. 3D).

Reduction by C20:5ω3 of stearic acid-induced apoptosis and membrane rigidity

Stearic acid-induced apoptosis could be reduced by addition of EPA, an effect of general importance that was observed in HUVECs (**Fig. 4A**), HAECs (Fig. 4B), EPCs (Fig. 4C), and HRECs (Fig. 4D). PUFA-induced apoptosis remained, however, unaffected by the addition of EPA or even tended to increase, as shown in HUVECs (Fig. 4A) and HAECs (Fig. 4B).

To test membrane fluidity of FFA-exposed endothelial cells, HUVECs were labeled with DiO. Incorporation of this lipophilic tracer resulted in characteristic diffusion behavior at the membrane position. As compared with control cells, exposure of HUVECs to 300 μmol/l palmitic

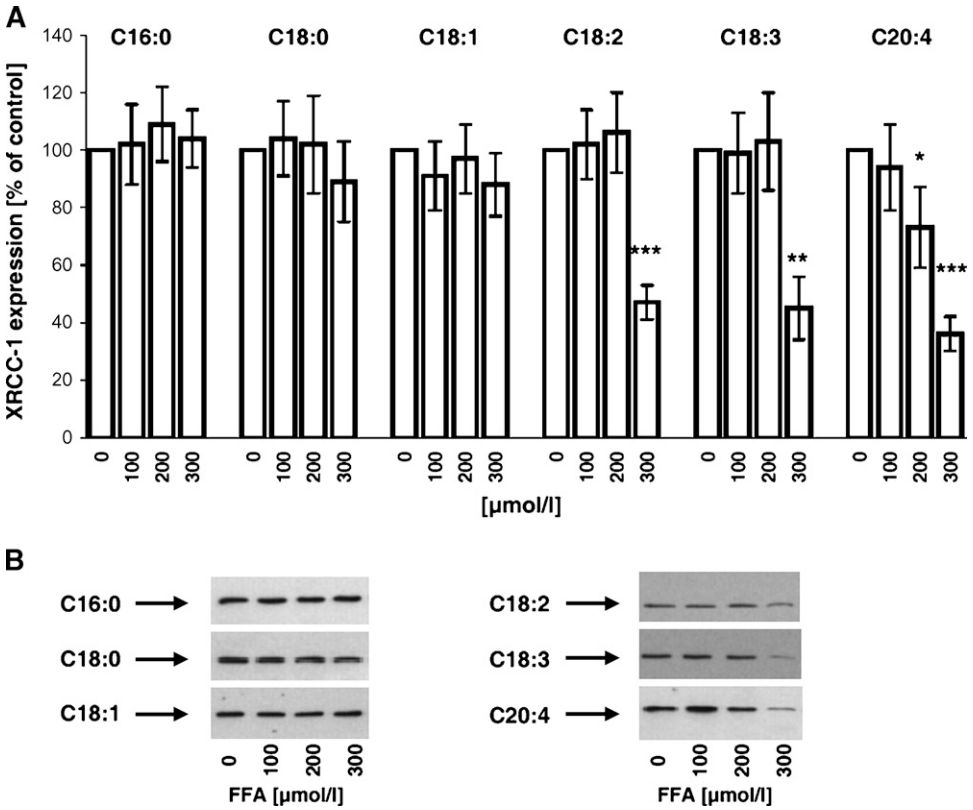

Fig. 5. *XRCC1* expression. A: *XRCC1* expression in HUVECs (n = 5) after exposure (24 h) to 100–300 μmol/l palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) compared with intra-individual control cells treated with ethanol (solvent = 0 μmol/l FFA). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ FFA-induced *XRCC1* expression versus ethanol-control. Data are mean ± SEM. B: Autoradiographs of one representative experiment for every tested FFA.

acid had no effect on membrane rigidity (+10.5 ± 11.1% of control, set to 100%; not significant), and PUFAs tended to contribute to membrane fluidity, as shown for arachidonic acid (−21 ± 7% of control, set to 100%; $P = 0.052$). However, 300 μmol/l stearic acid significantly prolonged the diffusion time of DiO in the membrane, indicating increased membrane rigidity (Fig. 4E). Like apoptosis, the prolonged diffusion time of stearic acid-exposed cells was completely reversed by the addition of 20 μmol/l EPA (Fig. 4E).

Stearic acid and PUFAs modulate apoptosis-associated protein expression differently

Protein expression of the base excision repair protein XRCC1 (**Fig. 5**) in HUVECs (n = 5) exposed (24 h) to FFAs negatively correlated ($r = -0.90$, $P < 0.01$) with apoptosis, with the exception of stearic acid (C18:0), which induced endothelial apoptosis, but had no effect on XRCC1 expression.

The pro-apoptotic activity (**Figs. 6A**, **7A**, filled circles) in growing endothelial cells exposed to FFAs positively correlated with protein expression of *E2F-1* ($r = 0.878$, $P < 0.01$; Fig. 6A) and *c-myc* ($r = 0.966$, $P < 0.01$; Fig. 7A), with the exception of stearic acid, which induced neither *E2F-1* (Fig. 6A) nor *c-myc* (Fig. 7A) in HUVECs. As an aside, *c-myc* was not detectable in confluent HUVECs (n = 5). The antagonist of *c-myc*, *mad*, remained unaffected during exposure to all FFAs tested (data not shown).

PUFA-induced but not stearic acid-induced apoptosis is mediated by *c-myc*

To determine whether FFA-induced apoptosis is mediated by *c-myc*, we then exposed confluent HUVECs, which are devoid of *c-myc* expression, to 300 μmol/l of the respective FFAs. Comparably to growing cells, palmitic acid (1.05 ± 0.12-fold, not significant) and oleic acid (1.01 ± 0.16%, not significant) were ineffective. But of interest, PUFAs lost their pro-apoptotic activity in confluent HUVECs (linoleic acid, 1.07 ± 0.11-fold; α−linolenic acid, 1.05 ± 0.08-fold; n = 3; not significant), whereas stearic acid-induced apoptosis was sustained, although less markedly (1.61 ± 0.06-fold; n = 3, $P < 0.05$) compared with growing HUVECs (~3-fold) (3).

To unequivocally link *c-myc* to PUFA-induced apoptosis, HUVECs were transfected with a dominant negative version of *c-myc* (In373-myc), which competitively inhibits intracellularly synthesized *c-myc*. Compared with either control cells transfected with the empty vector (Fig. 7C, black bars) or with nontransfected HUVECs (Fig. 7C, white bars), apoptosis induced by PUFAs was counteracted by overexpression of dominant negative *c-myc* (In373-myc; Fig. 7C, hatched bars). As expected, stearic acid-induced

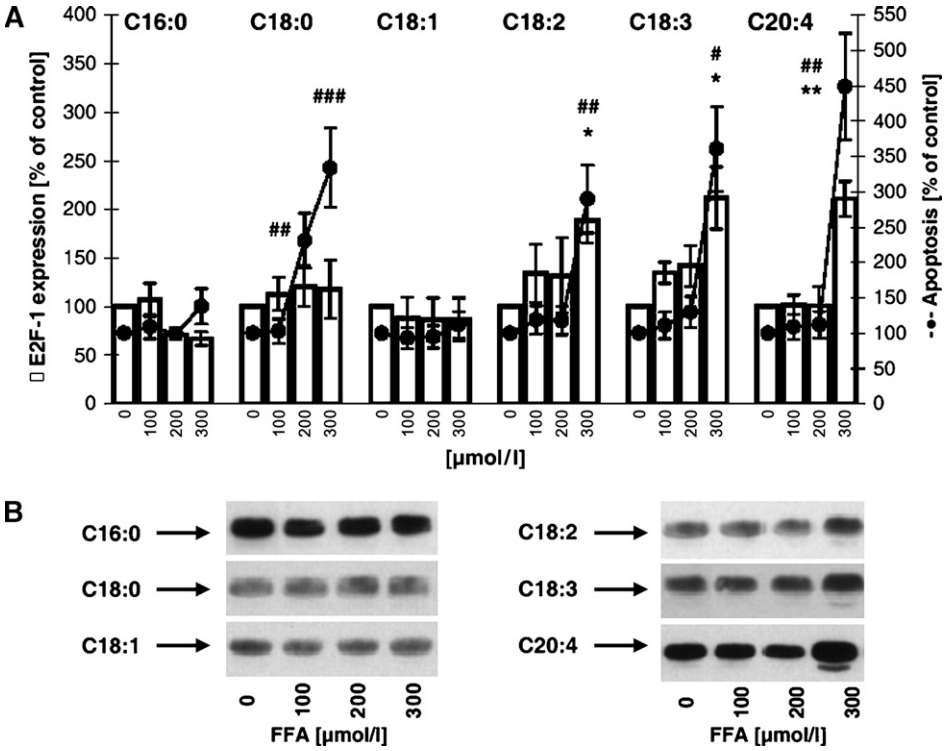

Fig. 6. *E2F-1* expression. A: *E2F-1* expression (n = 5; white bars) in relation to apoptosis [n = 6; filled circles; as published (3)] in HUVECs after exposure (24 h) to 100–300 μmol/l palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) compared with intra-individual control cells treated with ethanol (solvent = 0 μmol/l FFA). $^{*}P < 0.05$, $^{**}P < 0.01$ FFA-induced *E2F-1* expression (white bars) versus ethanol-control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ FFA-induced apoptosis (filled circles) versus ethanol-control, as published (3). Data are mean ± SEM. B: Autoradiographs of one representative experiment for every tested FFA.

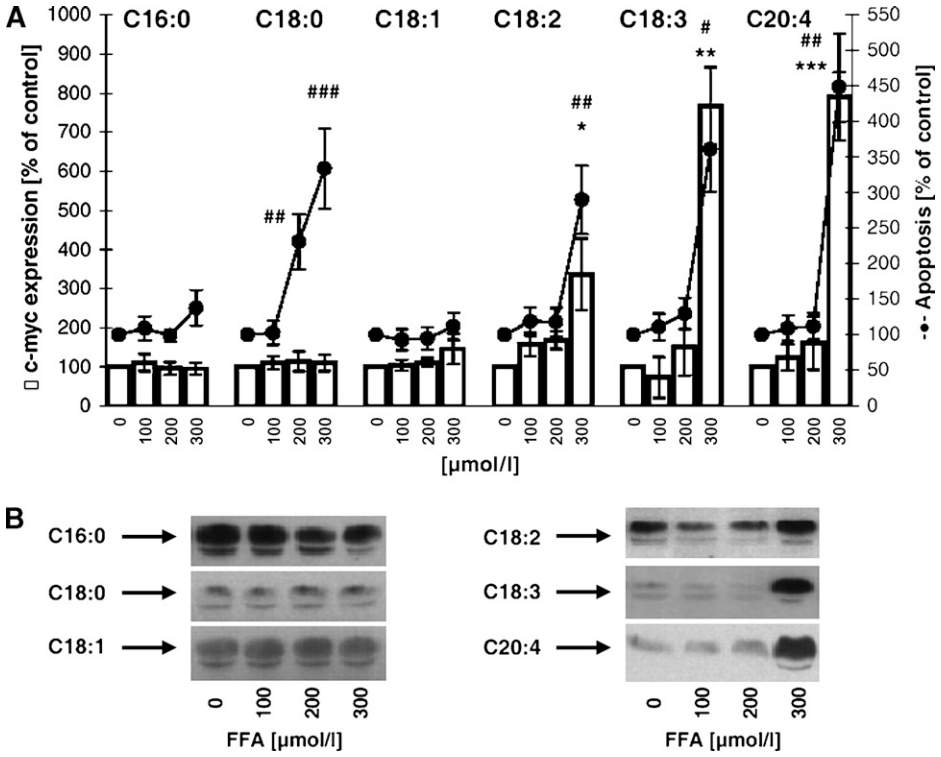

Fig. 7. *c-Myc* expression and dependence of FFA-induced apoptosis on *c-myc* abundance. A: *c-Myc* expression (n = 5; white bars) in relation to apoptosis (n = 6; filled circles), as published (3), of HUVECs after exposure (24 h) to 100–300 μmol/l palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) compared with intra-individual control cells treated with ethanol (solvent = 0 μmol/l FFA). B: Autoradiographs of one representative experiment for every tested FFA. C: Apoptosis in nontransfected (non; white bars), vector-transfected (vec; black bars), and In373-Myc-transfected (myc; hatched bars) HUVECs (n = 5) after exposure (24 h) to 300 μmol/l stearic acid (C18:0), linoleic acid (C18:2ω6), α-linolenic acid (C18:3ω3), and arachidonic acid (C20:4ω6) compared with intra-individual control cells treated with ethanol (solvent = control). D: Representative photos of vector- (vec) and In 373-Myc- (myc) transfected HUVECs exposed (24 h) to 300 μmol/l FFAs showing that stearic acid-induced apoptosis is sustained in In373-Myc-transfected cells, whereas these cells are resistant to PUFA [linoleic acid (C18:2ω6)]-, α-linolenic acid (C18:3ω3)-, and arachidonic acid (C20:4ω6)-induced apoptosis. Intra-individual control cells were treated with ethanol (solvent = Con). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ FFA-induced *c-myc* expression (white bars) versus ethanol-control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ FFA-induced apoptosis (filled circles) versus ethanol-control, as published (3). $^{+}P < 0.05$, $^{++}P < 0.01$ FFA-induced DNA fragmentation versus ethanol-control. $^{\$}P < 0.05$ inhibition by transfected In373-Myc of DNA fragmentation induced by 300 μmol/l of the respective FFAs. Data are mean ± SEM.

apoptosis persisted in HUVECs transfected with myc-In373 (Fig. 7C), thereby confirming our results of persisting stearic acid-triggered apoptosis obtained in confluent HUVECs, which lack *c-myc* expression.

DISCUSSION

Extending our previous report of FFA-triggered cell death in HUVECs (3), we studied the effects of FFA on endothelial apoptosis in human EPCs as well as in target tissues of late diabetic vascular complications, i.e., HAECs and HRECs. From the present study we learned that FFA-induced endothelial apoptosis depends on the vascular origin of endothelial cells, the respective FFAs, and the growth/proliferation status of the endothelial cells. In addition, our study shows that FFAs not only interfere with endothelial cells of target tissues known to be affected in diabetes, but also compromise their potential "repairing" cells, the EPCs.

The selected FFAs were chosen because of their importance in human nutrition, inasmuch as they are abundant in milk products (C16:0), meat (C16:0, C18:0), plant fats (C18:0) and oils (C18:1ω9, C18:2ω6, C18:3ω3), lard, egg yolk, and tuna (C20:4ω9). The different nutritional concentrations of FFAs employed in this study are, with respect to palmitic acid, stearic acid, oleic acid, and linoleic acid, within the range of physiologic concentrations (23). Under conditions relating to the metabolic syndrome (24, 25), systemic plasma FFA concentrations can rise to the millimolar range, and endothelial cells in particular might be exposed to excessively high FFA concentrations owing to local lipolysis of lipoprotein triglycerides by endothelium-bound lipoprotein lipase.

Saturated stearic acid caused apoptosis in all human endothelial cell types studied, i.e., macrovascular [HUVECs

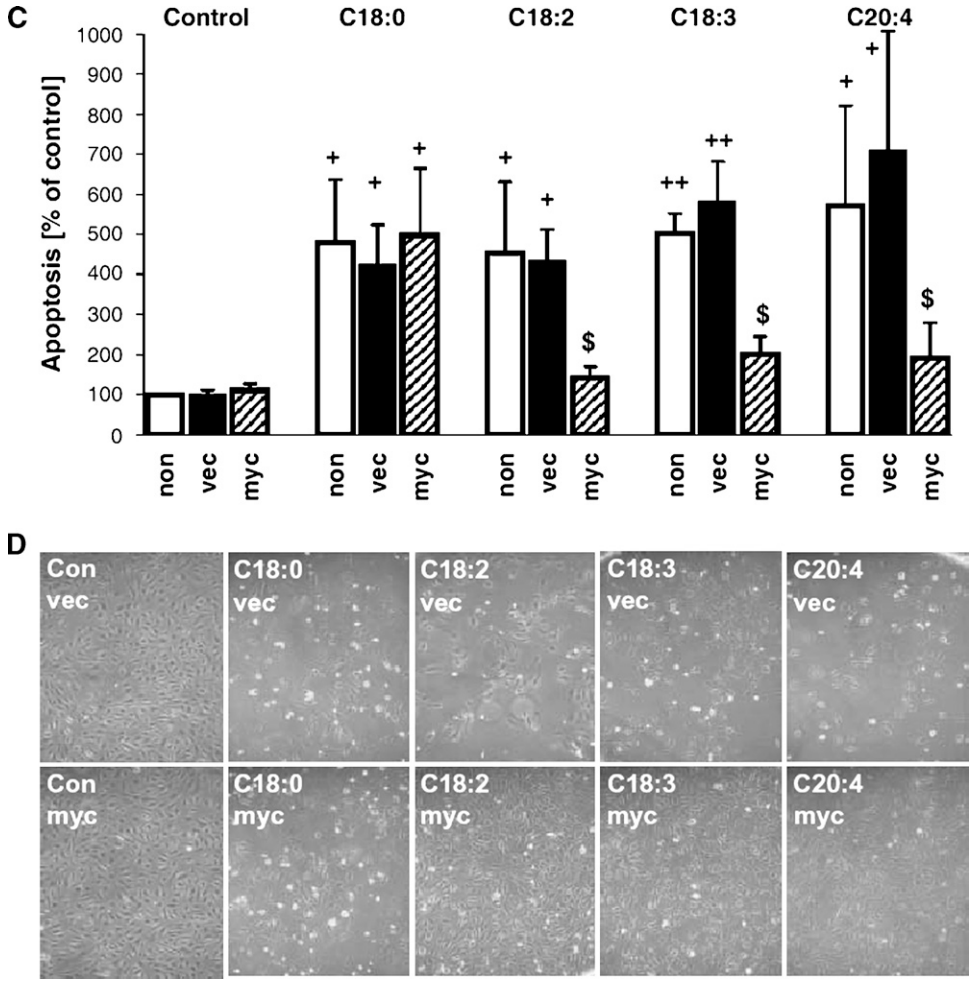

Fig. 7.—*Continued.*

(3) and HAECs] and microvascular endothelial cells (HRECs), and in EPCs, whereas palmitic acid and oleic acid did not provoke cell death in any of these cell types.

PUFAs were pro-apoptotic in HUVECs (3), HAECs, and EPCs, but not in HRECs. To ascertain that the onset of apoptosis in HRECs is not only delayed, apoptosis was additionally determined after 48 h, but even then, stearic acid remained the only FFA inducing cell death in HRECs. These data support the assumption that endothelial cells isolated from different vascular regions might respond varyingly to different FFAs, as has been suggested for human skin microvascular cells (26) and coronary artery endothelial cells (7).

It must be taken into consideration that the "resistance" of HRECs to PUFA-induced apoptosis might be a consequence of their lower FFA uptake, as could be deduced from reduced incorporation of most FFAs into the cell membranes of HRECs. However, this does not appear very likely, given that membrane incorporation of FFAs does not correlate with endothelial apoptosis and that HUVECs and HRECs comparably incorporate linoleic acid (19 nmol/10^3 cells vs. 13 nmol/10^3 cells) while completely differing in their apoptotic response. Particularly, insusceptibility of HRECs to PUFA-induced apoptosis might result from an adaptive mechanism of retinal endothelial cells to their highly unsaturated environment in vivo (27, 28): the retina possesses specific retinal fatty acid binding proteins that act as anti-oxidants by preferably binding PUFAs (29, 30). Moreover, the phenomenon of HREC "resistance" to and HAEC"susceptibility" to PUFA-induced cell death could provide one explanation for the association between high plasma FFAs and (diabetic) retinopathy, which is less obvious than the relationship between chronic elevation of FFAs in diabetes and/or visceral obesity and atherosclerosis (1, 31, 32).

We found that low concentrations of EPA, which have already been shown to inhibit detachment-induced apoptosis (anoikis) of endothelial cells (33), inhibited stearic acid-, but not PUFA-induced apoptosis in all endothelial cell types examined.

EPA, primarily found in fish oils, is a highly unsaturated ω3 fatty acid that is known to increase membrane fluidity in several cell types, including endothelial cells (34). Yet EPA not only inhibited stearic acid-triggered apoptosis in HUVECs, HAECs, HRECs, and EPCs, but also reduced stearic acid-induced membrane rigidity as shown in HUVECs. In this context, it is of note that saturated palmitic acid (C16:0) increased neither apoptosis nor mem-

brane rigidity, thereby strengthening the hypothesis that endothelial apoptosis could be mediated by biophysical effects. Although similar associations between reduced membrane fluidity and apoptosis have already been described (35, 36), particularly for T-cells exposed to stearic acid (36), a causal relationship between stearic acid-induced membrane rigidity and apoptosis in human vascular endothelial cells remains to be proven.

Stearic acid- and PUFA-triggered apoptosis in HAECs, HUVECs, and EPCs, as well as stearic acid-induced cell death in HRECs, was completely abolished by the addition of the pan-caspase inhibitor z-VAD.fmk, inhibiting caspase-1/3/4/7. Activation of caspases is a characteristic feature of apoptosis, and activation of caspase-3/7 has already been shown to take place in different models of FFA-triggered apoptosis (5, 17, 18, 37).

By contrast, z-IETD.fmk, a selective inhibitor of caspase-8, reduced PUFA-induced apoptosis but not stearic acid-induced apoptosis. Of note, activation of *caspase-8*, originally regarded as "initiator" caspase, has recently been shown to occur downstream of "effector/executioner" caspases in the *c-myc*-induced mitochondrial apoptotic pathway (38, 39). In this case, *caspase-8* is activated by interchain cleavage through effector/executioner caspases and switches on an intracellular feedback amplification loop driving apoptosis in an autonomous manner to ensure completion of the death process (38, 39). The involvement of caspase-8 in the PUFA pro-apoptotic activity is of particular interest, because, to date, neither has a specific role for caspase-8 been described in FFA-induced endothelial apoptosis, nor has there been any knowledge of differences in the downstream events of stearic acid- versus PUFA-induced endothelial apoptosis.

The hypothesis that different mechanisms are responsible for stearic acid- versus PUFA-induced apoptosis is corroborated, in that only the latter was associated with reduced protein expression of X-ray repair cross-complementing 1 (*XRCC1*). Lack of this base excision repair molecule results in reduced cell survival in different in vitro models (40, 41), including endothelial cells (41), and downregulation of *XCRR1* is also seen in apoptosis-prone coronary atherosclerotic plaques of patients with acute coronary syndromes (42). Our data thus support the assumption that reduced expression of anti-apoptotic *XRCC1* enables DNA fragmentation, as evidence of PUFA-induced but not of stearic acid-induced apoptosis.

The same holds true for *E2F-1* and *c-myc*, which were dramatically upregulated only by PUFAs, thereby positively correlating with PUFA-induced but not with stearic acid-induced apoptosis in growing HUVECs. *E2F-1* and/or *c-myc* have already been shown to prime mitochondria for apoptosis by downregulating anti-apoptotic *bcl-2* (43) and upregulating pro-apoptotic *bak* (44), in agreement with our recent observation that FFA-induced endothelial apoptosis strongly correlates with an increased ratio of *bak/bcl-2* (3).

Because *c-myc* and *E2F-1* interconnect cell cycle and cell death (38), we assume that in addition to the specific FFA and the endothelial cell type, it is the current growth/proliferation status that determines the apoptotic response of FFA-exposed endothelial cells. Indeed, we found that basal apoptosis is considerably lower in confluent than in growing HUVECs and that PUFAs, in contrast to stearic acid, no longer trigger cell death in confluent HUVECs. Given that *c-myc* is not expressed in quiescent cells (45, 46), including, as shown in the present study, confluent HUVECs, and that both *c-myc* and *E2F-1* are upregulated by PUFAs but not by stearic acid in proliferating HUVECs, we assume that *c-myc* has an important role in FFA-induced endothelial apoptosis.

To evaluate this hypothesis, HUVECs were transfected with dominant negative *c-myc* (In373-myc), which cannot bind to DNA and which competes with native, intracellularly synthesized *c-myc* (43, 47). Whereas PUFAs show no pro-apoptotic activity in In373-myc-transfected HUVECs, stearic acid-induced apoptosis persisted in those cells. Thus, our findings in confluent HUVECs, which lack *c-myc* expression, as well as in In373-myc-transfected HUVECs, which are devoid of *c-myc* activity, demonstrate that *c-myc* is critical in PUFA-induced but not stearic acid-induced endothelial apoptosis. Different patterns of FFA-induced apoptosis in the vascular endothelium (3, 7) could thus not only be tissue-specific, but could also depend on the proliferation status of the cells.

Of note, *mad*, the antagonist of *c-myc*, is not affected by any of the tested FFAs, which emphasizes previous findings that particularly in apoptosis, *mad* does not always directly antagonize the transcriptional effects of *c-myc* (48).

By inducing HREC and HAEC apoptosis, FFAs might contribute to induction and development of micro- and macrovascular disease, including diabetic retinopathy and premature atherosclerosis. Thus, our finding that oleic acid does not trigger endothelial apoptosis is in line with observations that this monounsaturated fatty acid, predominant in olive oil and in the Mediterranean diet, attenuates the development of atherosclerosis (23), whereas a high intake of saturated stearic acid and PUFAs apparently contributes to endothelial dysfunction and premature atherosclerosis (49, 50).

Stearic acid and PUFAs, however, induce endothelial apoptosis via different mechanisms. Of note, saturated stearic acid-triggered cell death affects confluent and proliferating endothelial cells, and therefore stearic acid should be considered the most hazardous FFA tested in the present study.

The enhanced susceptibility of the proliferating endothelium to PUFA-induced apoptosis could, however, particularly harm those vascular regions that are characterized by accelerated endothelial turnover rates. This applies to sites of disturbed blood flow, which are primarily prone to formation of atherosclerotic plaques (10). Because of their role in new blood vessel formation and vascular repair, FFA-induced apoptosis of human bone marrow-derived EPCs could result in reduced regenerative capacity of the endothelium and could thus further contribute to and accelerate the progression of vascular dysfunction.

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