Two Distinct Calcium-Dependent Mitochondrial Pathways Are Involved in Oxidized LDL–Induced Apoptosis

Cécile Vindis, Meyer Elbaz, Isabelle Escargueil-Blanc, Nathalie Augé, Aurelia Heniquez, Jean-Claude Thiers, Anne Nègre-Salvayre, Robert Salvayre

Objective—Oxidized low-density lipoprotein (oxLDL)–induced apoptosis of vascular endothelial cells may contribute to plaque erosion and rupture. We aimed to clarify the relationship between the oxLDL-induced calcium signal and induction of apoptotic pathways.

Methods and Results—Apoptosis was evaluated by biochemical methods, including studies of enzyme activities, protein processing, release of proapoptotic factors, chromatin cleavage, and especially by morphological methods that evaluate apoptosis/necrosis by SYTO-13/propidium iodide fluorescent labeling. The oxLDL-induced sustained calcium rise activated 2 distinct calcium-dependent mitochondrial apoptotic pathways in human microvascular endothelial cells. OxLDLs induced calpain activation and subsequent Bid cleavage and cytochrome C release, which were blocked by calpeptin. Cyclosporin-A inhibited cytochrome C release, possibly by inhibiting the opening of the mitochondrial permeability transition pore (mPTP). Calcineurin, another cyclosporin-sensitive step, was not implicated, because oxLDLs inhibited calcineurin and FK-506 treatment was ineffective. Cytochrome C release in turn induced caspase-3 activation. In addition, oxLDLs triggered release and nuclear translocation of mitochondrial apoptosis-inducing factor through a mechanism dependent on calcium but independent of calpains, mPTP, and caspases.

Conclusions—OxLDL-induced apoptosis involves 2 distinct calcium-dependent pathways, the first mediated by calpain/mPTP/cytochrome C/caspase-3 and the second mediated by apoptosis-inducing factor, which is cyclosporin-insensitive and caspase-independent. (Arterioscler Thromb Vasc Biol. 2005;25:639-645.)

Key Words: calpain ■ caspase ■ mitochondria ■ apoptosis-inducing factor ■ oxidized low-density lipoprotein ■ atherosclerosis

Atherogenesis is characterized by lipid deposition, a chronic inflammatory response, and chronic wound healing processes.1-2 Apoptosis may play a role in endothelial cell lining defects, necrotic core formation, or plaque erosion and rupture.3-5 Among the variety of proapoptotic factors present in atherosclerotic plaques, oxidized low-density lipoproteins (oxLDLs) are thought to play a crucial role by concomitantly inducing lipid storage, local inflammation, and toxic events.4,5-8 OxLDLs trigger apoptosis or necrosis of cultured vascular cells7-9 and may therefore participate in vascular wall injury, plaque erosion/rupture, and subsequent atherothrombotic events.4,5

The proapoptotic effects of oxLDLs are mediated through a complex sequence of signaling events that lead to activation of several caspase-dependent or -independent apoptotic pathways.8,9 Two separate caspase-dependent apoptotic pathways have been implicated in oxLDL-induced apoptosis.7-9 The extrinsic apoptotic pathway, mediated by death receptors, Fas, and/or tumor necrosis factor receptor (TNFR) and downstream by caspase-8/caspase-3, is involved in oxLDL-induced apoptosis in endothelial cells.8,10,11 However, a recent report contests this hypothesis.12 The intrinsic mitochondrial apoptotic pathway, involving bcl-2 family members, cytochrome C, and caspase-3, is especially activated by oxLDLs.8,13 Moreover, a sustained rise of cytosolic calcium plays a central role in oxLDL-induced apoptosis/necrosis,14,15 possibly by activating the calcium-dependent calpains and, subsequently, Bid cleavage and cytochrome C release.15 However, whereas Porn-Ares et al15 found that oxLDLs induce Bid truncation without any activation of caspase-3, Chen et al12 observed that oxLDLs did not cause Bid truncation but activated caspase-3 in agreement with Dimmeler et al.16 Furthermore, oxLDLs can activate a caspase-independent apoptotic pathway mediated by the release of apoptosis-inducing factor (AIF) from mitochondria.17

Because data in the literature on mechanisms implicated in oxLDL-induced apoptosis are controversial and because calcium can trigger multiple apoptotic pathways,18 we presently investigated the potential link between oxLDL-induced cal-

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cium signaling and the activation of mitochondrial apoptotic pathways.

Methods

Extensive Materials and Methods are available online at http://atvb.ahajournals.org.

Cell Culture

Human microvascular endothelial cells (HMEC-1) were starved in serum-free medium for 24 hours before LDL treatment.

LDL Isolation and Oxidation

LDLs were prepared as previously indicated. Under standard conditions, oxLDLs contained 71 to 104 nmol lipid hydroperoxide per mg apoB and 6.4 to 9.7 nmol of thiobarbituric acid–reactive substances (TBARS) per mg apoB.

Cytotoxicity, Necrosis, and Apoptosis

Cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide test and cell lysis (necrosis) by lactate dehydrogenase (LDH) release. Apoptotic and necrotic cells were counted by fluorescence microscopy after staining by SYTO-13 and propidium iodide.

Chromatin Fragments and DNA Ladder

Chromatin fragments were determined by the procedure of McConkey et al. DNA laddering was visualized on agarose electrophoresis.

Cell Fractionation

Cytosol was separated from mitochondria by a method adapted from De Duve et al.

Enzyme Activities

Enzyme activities were determined using fluorogenic substrates.

Western Blot Analysis

Cell extracts were subjected to SDS-PAGE, as previously reported.

Immunocytochemistry

After the indicated treatment, cells were fixed, incubated with anti-AIF antibody, and revealed with fluorescein isothiocyanate–conjugated secondary antibody.

Statistical Analysis

Data are given as mean±SEM, and statistical significance was evaluated by 1-way ANOVA (Tukey test, SigmaStat software).

Results

The toxicity of oxLDLs was time- and dose-dependent and correlated with the extent of LDL oxidation. OxLDLs obtained by UV+copper/EDTA oxidation or by cell-mediated oxidation exhibited similar toxicity to cultured cells. We used preferably (UV+copper/EDTA)-oxLDLs, because cell-mediated oxLDL preparations may contain mediators secreted by cells that may interfere with oxLDL toxicity.

Time Course of the Effects of oxLDLs on the Calcium-Dependent Mitochondrial Apoptotic Pathway and Apoptosis

Figure I (available online at http://atvb.ahajournals.org) shows the time course of calpain activation and α-spectrin breakdown, Bid degradation, release of cytochrome C, and caspase-3 activation (Figure IA through ID). Cell death (Figure IE) occurred mainly by apoptosis, as shown by chromatin fragmentation, DNA laddering, and morphological changes visualized by SYTO-13/PI fluorescent staining (Figure IF through II). The level of primary necrosis was very low, as shown by low levels of LDH release (Figure IE) and by small numbers of PI-stained cells with the morphological features of primary necrosis (Figure IH and II).

Calcium/Calpain Mediates oxLDL-Induced Bid Cleavage, Cytochrome C Release, and Subsequent Caspase-3 Activation

When calcium in the culture medium was chelated by EGTA, under conditions blocking the rise in cytosolic calcium, oxLDL-induced calpain activation, cytochrome C were performed on cytosolic fractions (B). Cell lysates were used for the fluorometric measurement of DEVDase and Western blots of caspase-3 (C) and chromatin fragmentation (D). Cell viability and necrosis (E) were evaluated at 24 hours using the MTT procedure and 4,6-diamidino-2-phenylindole fluorescent staining (D), by the MTT test, and by leakage of LDH in the culture medium (the basal LDH level was very low, ~5% of the total cellular LDH; E). In A, C, D, and E, mean±SEM was of at least 4 experiments (*P<0.05). In B and C, Western blots are representative of 4 experiments.
calpain activation (Figure 2A) and prevented Bid cleavage, release of cytochrome C, caspase-3 activation (Figure 2B through 2D), and both chromatin fragmentation and cytotoxicity by oxLDLs (Figure 2E through 2F). As Bid can theoretically be cleaved by both calpains and caspases, we used the multicaspase inhibitor zVAD-fmk and the caspase-2 inhibitor zVDVAD-fmk to investigate whether caspases were involved in oxLDL-induced Bid cleavage. As shown in Figure 2B, when used at effective concentrations, zVAD-fmk and zVDVAD-fmk did not prevent Bid cleavage, thus excluding any major role for caspases-8, -3, and -2.

These data strongly support the hypothesis that calcium and calcium-dependent calpains play a pivotal role in Bid cleavage and subsequent release of cytochrome C, which are caspase-independent.

A Cyclosporin A–Sensitive but FK-506–Insensitive Mechanism Mediates oxLDL-Induced Cytochrome C Release and Subsequent Activation of the Apoptotic Pathway

Activated Bid (truncated Bid, tBid) can elicit cytochrome C release by 2 mechanisms. tBid, through its BH3 domain, triggers the translocation of Bax/Bak that oligomerizes and renders the outer mitochondrial membrane permeable (cyclosporin-A–insensitive mechanism); alternatively, tBid, through its non-BH3 domain, triggers inner mitochondrial membrane remodeling and opening of the mitochondrial permeability transition pore (mPTP; cyclosporin-A–sensitive step).27–30

In HMEC-1, cyclosporin-A inhibited the release of cytochrome C (Figure 3A) and subsequent activation of caspase-3 (Figure 3B), thereby suggesting that cytochrome C release did not result from a direct permeabilization of the mitochondrial membrane by Bax/Bak but rather involved mPTP opening which is cyclosporin-A sensitive. However, cyclosporin-A may also inhibit calcineurin, a calcium/calmodulin-dependent proapoptotic protein phosphatase that activates the Bad-Bax/Bak pathway.18 To evaluate the possible role of calcineurin, we used FK-506, a specific inhibitor of calcineurin. FK-506, used at effective concentration to inhibit calcineurin (Figure 3C), did not prevent the oxLDL-induced release of cytochrome C (Figure 3A) nor the subsequent activation of caspase-3 (Figure 3B). Moreover, oxLDLs induced marked inhibition of calcineurin (Figure 3C), indicating that the calcineurin-mediated apoptotic pathway plays no major proapoptotic role under our experimental conditions. Finally, this is consistent with the protective

**Figure 2.** The calpain inhibitor calpeptin inhibits oxLDL-induced activation of calpains, apoptotic mitochondrial pathways, caspase-3 activation, and subsequent apoptotic events. Cells were treated with or without 2.5 μmol/L calpeptin, or 25 μmol/L zVAD-fmk, or 25 μmol/L zVDVAD-fmk, and added at time 0 with 200 μg apoB per mL oxLDLs. After 20 hours or after the indicated time of incubation, cells were used to evaluate calpain activity (A), Bid cleavage (B), and cytochrome C release in the cytosol (C). Chromatin fragmentation (D), cell viability (MTT test), and LDH leakage (index of necrosis; F) were evaluated at 24 hours. In A, D, E, and F mean ± SEM was of 3 to 4 experiments (*P < 0.05). In B and D, Western blots are representative of 4 experiments.

**Figure 3.** Cyclosporin-A, but not FK-506, inhibits oxLDL-induced apoptosis. Cells were treated with or without 10 μmol/L cyclosporin-A or 100 nmol/L FK-506 with or without oxLDLs added at time 0. After 20 hours (or the indicated time) of incubation, cells were harvested and used for Western blots of cytochrome C (A), for fluorometric determination of DEVDase and Western blot of caspase-3 (B), for calcineurin determination (C), and chromatin fragmentation (D). At 24 hours, apoptosis and necrosis was evaluated after staining by SYTO-13/PI (E). In A and B (bottom), data are representative of 4 experiments. In C through E, mean ± SEM is representative of 3 to 4 separate experiments (*P < 0.05).
effect of cyclosporin-A, whereas FK-506 did not prevent oxLDL-induced apoptosis (Figure 3D and 3E).

These data demonstrate that in our model system oxLDL-induced apoptosis (i) requires the activation of the calcium/calpain/Bid pathway and the release of cytochrome C, which is mediated by a cyclosporin-sensitive mechanism, possibly mPTP; (ii) does not require the calcium/calmodulin/calcinucerin pathway; and (iii) is not mediated by a Bax/Bak oligomerization independent of mPTP.

Caspase Inhibitors Partly Prevent oxLDL-Induced Apoptosis

We next investigated whether the caspase-dependent pathway is the only apoptotic pathway activated by oxLDLs, using the caspase-3 inhibitor DEVD-CHO and the multicaspase inhibitor zVAD-fmk. These caspase inhibitors did not prevent the release of cytochrome C (Figure 4A), a finding consistent with the lack of effect of zVAD-fmk on the oxLDL-induced Bid cleavage (Figure 2B). As expected, DEVD-CHO and zVAD-fmk completely inhibited caspase-3 cleavage and activation (Figure 4B and 4C), but these inhibitors only partially blocked oxLDL-induced chromatin fragmentation and apoptosis (Figure 4D through 4F). It should be noted that cotreatment with calpeptin and zVAD-fmk exhibited the same protective effect as each inhibitor used alone (Figure 4F), thus suggesting that these inhibitors act on the same apoptotic pathway.

Because calpeptin and cyclosporin-A, like caspase inhibitors, completely blocked caspase-3 activation but only partially inhibited chromatin fragmentation and cell death (Figures 2 and 3), this led us to examine the possibility that a calcium-dependent caspase-independent apoptotic mechanism might be implicated in oxLDL-induced apoptosis.

OxLDLs Trigger the Activation of AIF Through a Calcium-Dependent Pathway

Zhang et al recently reported that oxLDLs enhance the expression of AIF, and thereby promote caspase-independent apoptosis. Therefore, we investigated whether oxLDL-induced AIF release is mediated by the same mechanism as cytochrome C release.

Western blot analysis of the cytosolic fraction of AIF showed that oxLDLs induced an increase in AIF in the cytosolic fraction (Figure 5A and 5B). Interestingly, oxLDL-induced release of AIF from mitochondria to cytosol was inhibited by EGTA but not by calpeptin, cyclosporin-A, or zVAD-fmk (Figure 5C). These data suggest that oxLDL-induced AIF release is
calcium-dependent but independent of calpain activation, mPTP opening, and caspase activation.

In control cells, immunofluorescence staining revealed the localization of AIF in mitochondria and the absence of nuclear labeling (Figure 5D). In contrast, oxLDL-treated cells exhibited a partial relocation of AIF in the cytoplasm and the nucleus. Among the inhibitors that we used, only EGTA was able to block AIF release, whereas calpeptin, cyclosporin-A, and zVAD-fmk were ineffective.

We conclude that oxLDL-induced AIF release and nuclear translocation require calcium signaling but not calpain and mPTP activation.

Discussion

OxLDLs can trigger apoptosis or necrosis of cultured vascular cells and may therefore participate in vascular wall injury, necrotic core formation, plaque erosion/rupture, and in subsequent athero-thrombotic events. Several signaling pathways activated by oxLDLs could potentially trigger apoptosis or necrosis. However, the data on signaling and apoptotic pathways triggered by oxLDLs are rather scattered. In this study, we attempted to clarify the relationship between the calcium rise and the mitochondrial apoptotic pathways triggered by oxLDLs.

The data reported here provide new evidence indicating that oxLDLs induced 2 calcium-dependent mitochondrial apoptotic pathways; one mediated by cytochrome C, the other by AIF (Figure II, available online at http://atvb.ahajournals.org).

Calcium

The crucial role of calcium in oxLDL-induced apoptotic signaling was demonstrated by the use of EGTA (at a concentration that buffered extracellular calcium but that avoided the toxic effect of higher concentrations of the chelator). In our model, the oxLDL-induced rise in cytosolic calcium level was required to trigger the activation of the mitochondrial apoptotic pathway, because the calcium chelator EGTA blocked Bid cleavage, cytochrome C release, and subsequent apoptotic events. This finding is consistent with reports pointing to the role of calcium on the toxicity of oxLDLs or oxysterols and to the protective effect of calcium channel blockers.

Because high cytosolic calcium levels can theoretically activate several proapoptotic mechanisms mediated by calcium-dependent enzymes, mPTP opening, or calcium-induced osmotic swelling/rupture of mitochondrial membranes, it was of interest to identify the calcium-dependent apoptotic pathways leading to cytochrome C in our cell system.

Calpains

The role of the calpains in apoptosis is somewhat controversial, because they have reported to be either proapoptotic or antiapoptotic. Our data suggest that calpains play a crucial role in the oxLDL-induced activation of the mitochondrial apoptotic pathway (Figure II). The data reported here reveal that calpains induce Bid cleavage and cytochrome C release and subsequent caspase-3 activation as assessed by the inhibitory effect of calpeptin. Our data also permitted us to exclude the hypothesis that calpains directly activate caspases, because the activation of executioner caspase-3 was mediated through the mitochondrial apoptotic pathway and was blocked by cyclosporin-A. Other potential candidate proteases, such as cathepsin B and proteasome, can also be excluded, because they are not calcium-dependent and because inhibitors of cathepsin B or proteasome are not effective in inhibiting the oxLDL-induced apoptosis.

Bid

Bid, a proapoptotic BH3-only member of the Bcl-2 family, can theoretically be activated by various proapoptotic pathways, including death receptors/caspase-8, caspase-2, and calcium/calpains. In our model system, oxLDL-induced Bid cleavage is mediated by calpains in agreement with Po¨rn-Ares et al but independent of caspase-8 or caspase-2, because it was inhibited by calpeptin but not by the multi-caspase inhibitor zVAD-fmk and the caspase-2 inhibitor zVDVAD-fmk.

Bid cleavage results in formation of the active truncated form tBid that can promote release of cytochrome C. Several mechanisms have been proposed for the action of tBid, including the possibility (i) that tBid promotes the oligomerization of Bak and Bax, (ii) that tBid itself homooligomerizes, and (iii) that tBid induces inner mitochondrial membrane remodeling and mPTP opening.

Cyclosporin A inhibits several proapoptotic signaling pathways that lead to mitochondrial cytochrome C release. The initial specific target of cyclosporin-A is the mPTP, which is formed by adenine nucleotide translocator, by cyclophylin D (the target of cyclosporin-A), by voltage dependent anion channel, and by the peripheral benzodiazepine receptor.

Moreover, recent data suggest that cyclosporin-A can prevent the tBid-induced destabilization of the mitochondrial membrane. Another target of cyclosporin-A is the calcium/calmodulin-dependent serine/threonine protein phosphatase calcineurin that may activate the Bad/Bax/Bak pathway. Calcineurin plays no prominent role in oxLDL-induced apoptosis because the calcineurin inhibitor FK-506 did not prevent apoptosis, and because calcineurin was inhibited by oxLDL treatment. Our data are in contrast with the activation of calcineurin reported in oxysterol-induced apoptosis. This discrepancy could result from oxidized lipids contained in oxLDLs which can inhibit calcineurin and other protein phosphatases.

Caspases

Under our experimental conditions, we observed that oxLDLs induced caspase-3 activation. Although caspases have been reported to be substrates for calpains, caspase-3 was cleaved and activated through the conventional cytochrome C pathway in our system, because it was inhibited by cyclosporin-A.
which acts downstream of calpains and blocks cytochrome C release. These data are in agreement with those of Dimmel et al. and Chen et al., but are in contrast to those of Pönn-Ares et al., who reported that oxLDLs cause ubiquitination and inactivation of caspase-3. This discrepancy may result from differences in the level of LDL oxidation because our oxLDL preparations are mildly oxidized (6 to 9 mmol TBARS per mg apoB), whereas those used by Pönn-Ares et al. are extensively oxidized (25 to 45 mmol TBARS per mg apoB). The higher level of oxidized lipids in oxLDLs may explain both ubiquitination and enzyme inactivation, because highly oxidized LDL may induce cell protein modification and enzymes inactivation.

Cell Death Receptor–Mediated Extrinsic Apoptotic Pathway

OxLDL-induced apoptosis has also been shown to be mediated by cell death receptors, including Fas and TNF-R, and subsequently by caspase-8. Interestingly, in our model system, caspase-8 played no major role because (i) apoptosis was calcium dependent, whereas Fas and TNFR signaling pathways are calcium-independent; (ii) cleavage of Bid was only poorly sensitive to zVAD-fmk, a multicaspase inhibitor able to inhibit caspase-8; and (iii) a direct activation of caspase-3 by caspase-8 was also excluded, because caspase-3 activation was mediated through a mitochondrial cyclosporin-sensitive mechanism. This minor role (if any) of caspase-8 in HMEC-1 is in agreement with the data of Chen et al. but in contrast with those of Sata and Walsh and Napoli et al., who reported a role for death receptors and caspase-8 in oxLDL-induced apoptosis. This discrepancy may result from differences in the expression of TNF or FasL in endothelial cell subtypes used by the authors.

To summarize, in our model system, the signaling apoptotic cascade triggered by oxLDLs involved a sustained calcium rise, calpain activation, Bid cleavage, and cyclosporin-sensitive release of mitochondrial cytochrome C, leading finally to the activation of apoptosome and executioner caspase-3 (Figure II).

AIF

Calpeptin and cyclosporin-A, 2 inhibitors of the oxLDL-induced cytochrome C-mediated apoptotic pathway, and the multicaspase inhibitor zVAD-fmk only partially blocked the oxLDL-induced apoptosis. It was therefore suggested that another caspase-independent apoptotic pathway was activated by oxLDLs. Zhang et al. reported recently that oxLDLs enhance the expression of the 57-kDa flavoprotein AIF, but the mechanism of release of AIF from mitochondria is indeterminate. Our data show that AIF release is calcium-dependent, but that downstream from calcium, the pathways leading to release of AIF and cytochrome C are distinct because AIF release was not inhibited by calpeptin and cyclosporin-A in contrast to cytochrome C release and caspase-3 activation. Moreover, in our system, oxLDL-induced AIF release was not inhibited by zVAD-fmk and was independent of caspase activation, in agreement with the findings of Cande et al. but in contrast with those of Arnoult et al. The mechanism by which calcium triggers AIF release is not firmly established, but poly(ADP-ribose) polymerase (PARP-1) may constitute such a link because calcium activates PARP-1 which in turn signals AIF release.

A particularly intriguing question is why the cell has evolved to use 2 different apoptotic pathways induced by toxic agents, like oxLDLs, that trigger unregulated calcium signaling. The sustained rise of cytosolic calcium constitutes a lethal hit that activates both necrosis and apoptosis pathways. Necrotic cells release proinflammatory molecules potentially dangerous to surrounding cells, whereas apoptosis may prevent or reduce the local inflammation because, in vivo, apoptotic cells are rapidly cleared by phagocytes. It may therefore be speculated that redundant apoptotic pathways selected during the evolution of multicellular organisms to effectively eliminate abnormal, infected, or damaged cells, are also activated in pathophysiological conditions to prevent the necrotic cell destruction and the subsequent local inflammatory processes.

From a pathophysiological point of view, increased apoptosis has been documented in atherosclerotic areas, particularly in lesions associated with unstable angina and ruptured plaques. This suggests that apoptosis could play a critical role in plaque erosion and rupture and, subsequently, in athero-thrombotic events. Because oxLDLs are present in atherosclerotic areas and can induce apoptosis of cultured vascular cells, it has been hypothesized that oxLDLs and other toxic compounds, including Fas/FasL and inflammatory cytokines, could converge to trigger apoptosis during atherogenesis and plaque disruption. Our data demonstrate that inhibitors of the classical mitochondrial apoptotic pathway would confer only partial protection against oxLDL-induced apoptosis, because these inhibitors are not active on the AIF apoptotic pathway. Moreover, as the oxLDL-induced sustained calcium rise is a common trigger to necrosis and apoptosis, protective drugs should act upstream from the peak of cytosolic calcium. A better understanding of upstream proapoptotic signaling activated by oxLDLs should permit the modulation of apoptosis and the prevention of plaque instability.

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Methods

**Chemicals and reagents.**

4',6-diamidino-2-phenylindole (DAPI), G-Nome kit from Bio-101, acrylamide-4X/bisacrylamide-2X solution from Bioprobe, SYTO-13 and propidium iodide from Molecular Probes, culture media and fetal calf serum from Gibco. Antibodies anti-AIF, anti-cytochrome C, anti-caspase-3, and anti-spectrin were from TEBU, anti-Bid was from RnDSystems, zVAD-fmk, zVDVAD-fmk, Ac-DEVD-CHO, Ac-DEVD-AMC were from Bachem, Taxol, EGTA, cyclosporin-A, FK-506, calpeptin, were from Sigma and other reagents from VWR or Sigma.

**Cell culture.**

HMEC-1 (human microvascular endothelial cells), obtained from CDC (Atlanta), were routinely grown in MCDB-131 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. 24h before LDL incorporation, cells were starved in serum-free medium.

**LDL isolation and oxidation.**

LDL from human pooled sera were prepared by ultracentrifugation, dialyzed against PBS containing 100 µmol/L EDTA, as previously indicated [14]. ApoB was determined by immunonephelometry. LDL were oxidized by (UV+copper/EDTA) as previously described [14]. Under standard conditions, oxLDLs contained 71-104 nmol lipid hydroperoxide/mg apoB and 6.4-9.7 nmol TBARS/mg apoB.

**Evaluation of cytotoxicity, necrosis and apoptosis**

Cytotoxicity was evaluated using the MTT (dimethylthiazolyl diphenyltetrazolium bromide) test, as previously used [17]. Cell lysis (necrosis) was evaluated by lactate dehydrogenase (LDH) release.

Apoptotic and necrotic cells were counted after fluorescent staining by 2 vital fluorescent dyes, the permeant DNA intercalating green-colored fluorescent probe SYTO-13 (0.6 µmol/L), and the non permeant DNA intercalating red fluorescent probe propidium iodide (PI, 15 µmol/l) and using an inverted fluorescence microscope (Fluovert FU, Leitz). [19]. Normal nuclei exhibited a loose green colored chromatin. Nuclei of necrotic cells exhibited a loose red colored chromatin. Apoptotic nuclei exhibited condensed yellow/green-colored chromatin associated with nucleus fragmentation, whereas post-apoptotic necrotic cells exhibited the same apoptotic features, but were red-colored [19,20]. It may be noted that necrotic cells (red-colored by propidium iodide) were also stained by trypan blue.

**Determination of chromatin fragments and visualization of DNA ladder**

Chromatin fragments were determined by the procedure of McConkey et al. [21]. Briefly, cells were lysed in 1 ml lysis buffer (5 g/l triton-X100, 20 mmol/l EDTA, 5 mmol/l Tris pH 8) and then centrifuged for 20 min at 27,000 x g to separate the chromatin pellet from cleavage products. The pellet and the supernatant were used for DNA determination by the fluorometric DAPI procedure used previously [14]. To visualize DNA ladder, cellular DNA was extracted using the G-Nome kit, resolved by agarose electrophoresis and revealed by ethidium bromide fluorescent staining.
**Cell fractionation.**

Cytosol was separated from mitochondria by a method adapted from De Duve et al. [22]. Washed cells (10⁷) were disrupted at 4°C in 20 mmol/L Hepes-KOH buffer pH 7.4, 250 mmol/L sucrose, 10 mmol/L KCl (containing 1 mg/ml bovine serum albumin, 1 mmol/L EDTA, 2 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L PMSF, 10 µg/ml leupeptin, aprotinin and pepstatin) using 25 strokes of the pestle of a tight-fitting ice-cold Dounce homogenizer. After 2 cycles clarification at 2,500 x g for 5 min, supernatant was centrifuged at 12,000 x g for 30 min. The pellet contained the mitochondrial fraction and the supernatant was ultracentrifuged (Beckman Optima) at 100,000 x g for 60 min, to obtain the cytosolic fraction.

**Determination of enzyme activities.**

Cells were lysed in ice-cold 10 mM Tris-HCl buffer pH 7.4 and enzyme activities were determined using fluorogenic substrates. Calpain activity was determined using the Suc-Leu-Tyr-AMC substrate [23]. Calcineurin activity was evaluated using methylumbelliferyl-phosphate as substrate, in the presence or absence of trifluoperazine [24]. Caspase activity was determined using the fluorogenic substrate Ac-DEVD-AMC [25].

**Western blot analysis.**

After stimulation of subconfluent cell monolayers under conditions indicated in the text, cells were washed in phosphate buffered saline (PBS) containing 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM orthovanadate and 5 mM EDTA. Then, cells were lysed with solubilizing buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 160 mM sodium fluoride, 2.5 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 2 µM pepstatin A, 10 µg/ml aprotinin, 1% Triton X-100) for 30 min, on ice. 50 µg of protein cell extracts (determined using the bichinchoninic acid method) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 100 V for 90 min), transferred onto nitrocellulose membrane (Hybond-C, Amersham). Then membranes were probed with the indicated primary antibodies and revealed with the secondary antibodies conjugated with horseradish peroxidase and developed by ECL (Amersham).

**Immunocytochemistry**

Cells, plated on glass cover slips and serum starved for 16h, were incubated with oxLDLs and chemicals (as indicated in the text). Then cells were washed with PBS and fixed in cold methanol for 10 min. After blocking with PBS containing 3% BSA-5% rabbit serum for 30 min, cells were incubated with anti-AIF polyclonal antibody in PBS for 1h and revealed with FITC-conjugated secondary antibody for 1h. The slides were washed, mounted on glass slides (in Vectashield mounting medium) and visualized using a Zeiss LSM 510 fluorescence confocal microscope.

**Results**

**Time course of activation of the oxLDL-induced calcium-dependent mitochondrial apoptotic pathway.**

As previously reported, oxLDLs induced an intense calcium rise, peaking at 12-14 h, and sustained for at least 5 h, under the used conditions [14]. The data reported in Fig.I show a rise of calpain activity at the time of the calcium peak, as assessed by the hydrolysis of the fluorogenic Suc-Leu-Tyr-AMC substrate, and cleavage of spectrin, an endogenous substrate of calpain and caspases (Fig.IA). The 150 and 120 kDa breakdown fragments resulted from
spectrin proteolysis by calpain and caspases, respectively [27]. In agreement with previous reports [12,15], we also observed the degradation of Bid, a pro-apoptotic BH3-only Bcl-2 family member (Fig.IB). Bid degradation was associated with the activation of the mitochondrial apoptotic pathway, as assessed by the release of cytochrome C (Fig.IC). As expected, caspase-3 was also activated, as shown by hydrolysis of the fluorogenic Ac-DEVD-AMC substrate and by cleavage of procaspase-3 (Fig.ID). These signaling events were followed by cell death that occurred mainly by apoptosis, as shown by chromatin fragmentation, DNA laddering and morphological changes counted after SYTO-13/PI staining (Fig.IE-I). The level of primary necrosis was very low, as shown by the low level of LDH released at 24h (Fig.IG) and by counting PI-stained cells with morphological features of primary necrosis (Fig.IH,I). It may be noted that, at 24h, the level of LDH released in the culture medium represented less than 5% of the cellular LDH content, consistently with the low level of primary necrosis and post-apoptotic necrosis. However, the level of released LDH rose after 24h, when cells underwent post-apoptotic necrosis.

Legends to the Figures

Figure I - Time course of apoptotic signaling and characterization of cell death triggered by oxLDLs.
HMEC-1 incubated with 200 µg apoB/ml oxLDLs (oxL) or native LDL (nL) or without any lipoprotein (Co) or with 1 µmol/l Taxol (Tax), used as positive control.
In A-D, time course of calpain activity and spectrin cleavage (A), Bid cleavage (B), cytochrome C release (C), DEVDase activity and caspase-3 cleavage (D) and for chromatin fragmentation evaluation (E) and DNA ladder visualization (F). Cells lysates were used in A,B,D. Cytosolic fractions were used in C.
After 24h incubation with oxLDLs, the whole toxicity was evaluated by the MTT test and LDH release (G) and the type of cell death was evaluated by fluorescence microscopy after SYTO-13/PI staining (H,I). In I, middle panel, the arrows indicate nuclei exhibiting the morphological feature of normal (no), apoptosis (a) or post-apoptotic necrosis (pan).
In A,B,C,H,I, representative of 4 experiments. In A,D,F-H, mean±SEM of 4 experiments (* p < 0.05).

Figure II - Schematic diagram of the calcium-dependent apoptotic pathways activated by oxLDLs in HMEC-1. Red arrows: existing pathways (1 and 3) (dotted red line: possible pathways 1d and 1e); grey dotted arrows: theoretical pathways, not operating in our model system (pathways 2 and 4).
**Fig. I**

A. Calpain activity (%)

B. Time (h) 0 12 16 20 24
Blot anti-Spectrin

C. DEVDase activity (%)

D. Time (h) 0 12 16 20 24

E. Chromatin fragmentation (%)

F. Blot anti-Bid

G. MTT (%) (Co oxL nL Tax)

H. Cell count (%)

I. Control oxLDL Taxol
Fig. II Vindis/ Elbaz et al. 2004

Ca\(^{2+}\) Chelator EGTA

**Ca\(^{2+}\) (10-14h)**

Calpeptin

**Calpains (12-16h)**

FK506 CSA

Calcineurine (phosphatase)

Mitochondria

**Cytochrome c (16-18h)**

Caspase-3

AIF

Nucleus