Oxidized low-density lipoprotein induces calpain-dependent cell death and ubiquitination of caspase 3 in HMEC-1 endothelial cells

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Oxidized low-density lipoprotein (oxLDL) is known to induce apoptosis in endothelial cells, and this is believed to contribute to the progression of atherosclerosis. In the present study we made the novel observation that oxLDL-induced death of HMEC-1 cells is accompanied by activation of calpain. The μ-calpain inhibitor PD 151746 decreased oxLDL-induced cytotoxicity, whereas the general caspase inhibitor BAF (t-butoxycarbonyl-Asp-methoxyfluoromethylketone) had no effect. Also, oxLDL provoked calpain-dependent proteolysis of cytoskeletal α-fodrin in the HMEC-1 cells. Our observation of an autoproteolytic cleavage of the 80 kDa subunit of μ-calpain provided further evidence for an oxLDL-induced stimulation of calpain activity. The Bcl-2 protein Bid was also cleaved during oxLDL-elicited cell death, and this was prevented by calpain inhibitors, but not by inhibitors of cathepsin B and caspases. Treating the HMEC-1 cells with oxLDL did not result in detectable activation of procaspase 3 or cleavage of PARP [poly(ADP-ribose) polymerase], but it did cause polyubiquitination of caspase 3, indicating inactivation and possible degradation of this protease. Despite the lack of caspase 3 activation, oxLDL treatment led to the formation of nucleosomal DNA fragments characteristic of apoptosis. These novel results show that oxLDL initiates a calpain-mediated death-signalling pathway in endothelial cells.

Key words: apoptosis, Bid, calpain, endothelial cells, α-fodrin, low-density lipoprotein.

INTRODUCTION

Oxidized low-density lipoprotein (oxLDL) plays a central role in atherogenesis [1,2], and it is assumed that oxLDL-induced apoptosis and/or necrosis of vascular cells contributes to the progression of atherosclerosis (reviewed in [3]). OxLDL is well recognized as a pro-apoptotic agent in the endothelium, and has previously been reported to induce death of human umbilical-vein and coronary-artery cells, as well as bovine aortic endothelial cells [4–6]. Thus far, the signalling proteins implicated in oxLDL-elicited cell death include caspases, FLIP {FLICE [FADD (Fas-associated death domain)-like interleukin-1β-converting enzyme] inhibitory protein}, protein kinase C and protein tyrosine kinases [5,7,8].

Calpains are Ca^{2+}-dependent cysteine proteases that are known to be involved in the proteolysis of a number of proteins during mitosis, apoptosis and necrosis. The calpains appear to perform a key function in certain models of apoptosis, including the death program induced by tumour necrosis factor in U937 cells [9] and by glucocorticoids in thymocytes [10]. Furthermore, activation of calpain has been shown to induce apoptosis-like events in platelets [11], and also seems to be important for excitotoxic neuronal death [12,13]. The calpains constitute a large family of distinct isoenzymes that differ in structure and distribution [14], and two members of this family, namely μ-calpain and m-calpain, are ubiquitous. The specific endogenous protein inhibitor calpastatin modulates calpain activity *in vivo* [15]. Moreover, we have shown [16], as have other researchers [17], that calpastatin is cleaved during apoptosis, which may lead to increased calpain activity, and such cleavage can be achieved by calpains or caspases, depending on the cell type and the stimulus inducing apoptosis. The cytoskeletal protein α-fodrin is another death substrate that may be cleaved by calpains or caspases [9,18,19]. Additional calpain substrates known to be involved in apoptosis are Bax [20,21], Bid [22], p53 [23] and procaspases 3, 7, 8 and 9 [11,24,25].

Caspases (aspartic acid-specific cysteine proteases) have been identified as the main players in most models of apoptosis, because they are responsible for triggering and carrying out apoptotic demise induced by both death-receptor ligation and drugs (reviewed in [26]). A number of researchers have reported that caspases are involved in oxLDL-induced cell death [6,27,28], and the experiments in those studies were conducted using high (> 20 μM) concentrations of the general caspase inhibitor VAD-fmk (Val-Ala-Asp-methoxyfluoromethylketone). In that context, other investigators have observed unexpected inhibition of calpain activity by VAD-fmk [11,13,29], and it has been demonstrated that oxLDL-induced apoptosis in endothelial cells requires Ca^{2+} [4]. Furthermore, oxLDL is well known to induce oxidative stress, and oxidative stress up-regulates calpain expression [30]. In light of these findings we examined the possibility that calpains participate in oxLDL-elicited death of endothelial cells.

Our results revealed a novel type of oxLDL-initiated death signalling in endothelial cells, which involves activation of calpain, cleavage of α-fodrin and Bid, and inactivation of caspase 3 by polyubiquitination.

Abbreviations used: Ab, antibody; BAF, t-butoxycarbonyl-Asp-methoxyfluoromethylketone; $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+}; DEVD-amc, Asp-Glu-Val-Asp-aminomethylcoumarin; FBS, foetal-bovine serum; HUVEC, human umbilical-vein endothelial cells; LDH, lactate dehydrogenase; mAb, monoclonal antibody; (ox)LDL, (oxidized) low-density lipoprotein; PARP, poly(ADP-ribose) polymerase; pAb, polyclonal antibody; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; TEP, tetraethoxypropane; (z)VAD-fmk, (butyloxycarbonyl-)Val-Ala-Asp-methoxyfluoromethylketone.

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EXPERIMENTAL

Materials

BAF (t-butoxycarbonyl-Asp-methoxyfluoromethylketone) and zVAD-fmk (butyloxycarbonyl-VAD-fmk) were purchased from Enzyme Systems Products (Livermore, CA, U.S.A.). DEVD-amc (Asp-Glu-Val-Asp-aminomethylcoumarin) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and PD 151746 (binding to the Ca^{2+}-binding site of μ-calpain) was from Calbiochem (La Jolla, CA, U.S.A.). A Cell Death Detection ELISA^{PLUS} kit, a Cytotoxicity Detection Kit, Pefabloc® (a registered trademark of Pentapharm, Basel, Switzerland) and leupeptin were obtained from Roche Diagnostics (Mannheim, Germany). The cathepsin B inhibitor CA-074-Me was purchased from Peptides International (Louisville, KY, U.S.A.), Coomassie Plus Protein Assay reagent was from Pierce (Rockford, IL, U.S.A.) and PMSF was from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Fluo-3/AM (cell-permeant Fluo-3 acetoxymethyl ester) was from Molecular Probes (Eugene, OR, U.S.A.). Anti-α-fodrin monoclonal antibody (mAb) was from Affiniti Research Products Ltd. (Mamhead, Exeter, Devon, U.K.), anti-Bid polyclonal Ab (pAb) was from R&D Systems (Abingdon, Oxford, U.K.), anti-(caspase 3) mAb was from Alexis Biochemicals (Läufelfingen, Switzerland) and anti-PARP {anti-[poly(ADP-ribose) polymerase]} mAb was from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.). Anti-ubiquitin mAb and agarose-conjugated goat anti-(caspase 3) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-μ-calpain large subunit mAb was from Chemicon International (Temecula, CA, U.S.A.), Protein A–Sepharose was from Amersham Biosciences (Uppsala, Sweden), anti-α-tubulin mAb was from Oncogene Research Products (Boston, MA, U.S.A.) and horseradish peroxidase-coupled goat anti-rabbit and goat anti-mouse immunoglobulins were from Dako A/S (Glostrup, Denmark). Reagents not listed here were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated in the text.

Cell culture

The human dermal microvascular cell line HMEC-1 [31] was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, GA, U.S.A.). The cells were cultured in MCDB 131 medium supplemented with 2 mM L-glutamine, 50 i.u./ml penicillin/streptomycin (all from Gibco BRL Life Technologies, Gaithersburg, MD, U.S.A.), 10 % (v/v) heat-inactivated foetal-bovine serum (FBS), 10 ng/ml epidermal growth factor and 1 μg/ml cortisol. The cells were maintained at 37 °C in a humidified incubator under 5 % CO_2.

Isolation and oxidation of LDL

Plasma was obtained from healthy volunteers with their informed consent, and lipoproteins were isolated by sequential flotation ultracentrifugation [32]. Isolated LDL [density (ρ) = 1.019–1.063 g/ml] was desalted on an Econo-Pac 10 DG chromatography column (Bio-Rad, Hercules, CA, U.S.A.) and sterile-filtered (0.22 μm pore size; Millipore, Bedford, MA, U.S.A.); the LDL was stored under N_2 at +4 °C, but was used within 4 weeks of preparation. The protein content of newly isolated LDL was determined by the Lowry method.

To oxidize LDL, the lipoprotein (0.5 mg/ml in sterile PBS) was incubated with 5 μM $CuSO_4$ at 37 °C for 20 h. Oxidized LDL was concentrated by centrifuging in Amicon Centriplus YM-100 tubes (Millipore Corp., Bedford, MA, U.S.A.) for 2 h at 3000 ***g*** and 8 °C, and subsequently sterile-filtered. The oxidation was confirmed by measuring thiobarbituric acid-reactive substances (TBARS) using tetraethoxypropane (TEP) as a standard [33]. The TBARS assay is based on the reaction of malonaldehyde, a lipid hydroperoxide degradation product, with thiobarbituric acid. The TBARS content of oxLDL used in our study was 25–45 nmol of TEP equivalents/mg of protein. We used the oxLDL within 24 h of preparation, and endothelial cells were incubated with oxLDL dissolved in culture medium containing 1 % FBS.

To exclude unspecific effects of possible residual Cu^{2+} in the oxLDL preparation, we performed control experiments with analyses of cell toxicity and α-fodrin fragmentation, but we did not find any effects of Cu^{2+} in the relevant concentration range (results not shown).

Analysis of cytotoxicity

The level of cytotoxicity induced by oxLDL was analysed using a Roche Cytotoxicity Detection Kit, which measures release of lactate dehydrogenase (LDH) from cells with compromised membrane integrity. The analysis was performed according to the instructions of the manufacturer, and native LDL was used as control treatment. The 100 % value of cytotoxicity (LDH leakage) was obtained from samples permeabilized with 1 % Triton X-100, which resulted in the release of all LDH activity into the supernatant.

Caspase assay

Activity of caspase 3 in HMEC-1 cells was analysed with a fluorescence spectrophotometric assay, using the fluorogenic peptide DEVD-amc as a substrate. After various treatments, cells were harvested by scraping them into 300 μl of caspase lysis buffer [10 mM Tris/HCl/10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5)/130 mM NaCl/0.1 % Triton-X-100/10 mM sodium pyrophosphate] on ice. Floating cells were collected, pelleted and pooled with the lysate. The assay was performed in white Fluoronunc™ Polysorp 96-well plates (Nunc A/S, Roskilde, Denmark). Lysates (50 μl aliquots) were added to the reaction wells, together with 200 μl of Hepes buffer [20 mM Hepes (pH 7.5)/10 % (v/v) glycerol/2 mM dithiothreitol] and 4 μl of DEVD-amc (dissolved in DMSO). The reaction mixture was incubated at 37 °C for 1 h, after which amc fluorescence was analysed using a Fluostar plate reader (BMG Lab Technologies, Offenburg, Germany), employing excitation and emission wavelengths of 390 and 460 nm respectively. The protein content of lysates was assayed by using Pierce's Coomassie® Plus Protein Assay Reagent.

Immunoprecipitation

For immunoprecipitation of caspase 3, HMEC-1 cells were cultured in 100 mm-diameter culture dishes, treated in appropriate ways, and then scraped into 800 μl of immunoprecipitation buffer [150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/2 mM orthovanadate/2 mM Pefabloc®/1 % (v/v) Nonidet P40]. The resulting lysate was pre-cleared with Protein A–Sepharose, and caspase 3 was immunoprecipitated with an agarose-conjugated anti-(caspase 3) Ab (20 μg/ml). The immunoprecipitate was washed four times with the abovementioned buffer, resuspended in 2 × sample buffer [125 mM Tris/HCl (pH 6.8)/4 % (w/v) SDS/20 % (v/v) glycerol/0.05 % Bromophenol Blue/100 mM dithiothreitol], heated to 90 °C for 5 min, and then loaded on to SDS/polyacrylamide gels and subjected to Western blotting.

Preparation of cell lysates and Western-blot analysis

After various treatments in 60 mm-diameter culture dishes, floating cells were collected and pelleted, and adherent cells were harvested by scraping into 250 μl of Western lysis buffer [150 mM NaCl/20 mM Tris/HCl (pH 7.4)/5 mM EDTA/2 mM orthovanadate/4 μg/ml leupeptin/60 μg/ml PMSF/1 % Nonidet P40]. The pelleted cells were pooled with the lysate, and 60 μl of 5 × sample buffer was added to each sample, and the samples were heated to 90 °C in a heating block for 5 min. Proteins were separated under reducing conditions at 120 V in SDS/polyacrylamide gels and then Western-blotted on to PVDF filters at 100 V. Blots were blocked with Tris-buffered saline (TBS; 50 mM Tris base/150 mM NaCl/0.05 % Tween-20) containing 5 % (w/v) dry milk powder, and then incubated for 1–2 h with anti-α-fodrin mAb (1:1000), anti-(proteolysed fodrin) pAb ([34]; diluted 1:200), anti-(caspase 3) mAb (1:500), anti-Bid pAb (1:1000), anti-ubiquitin mAb (1:400), anti-α-tubulin mAb (1:500), anti-μ-calpain mAb (1:1000) or anti-PARP mAb (1:5000). The blots were subsequently washed in TBS for 30 min and then incubated with a peroxidase-conjugated secondary Ab. Bound Ab was assayed by enhanced chemiluminescence detection (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Assessment of apoptosis

To evaluate the type of death induced by oxLDL, HMEC-1 cells were cultured in 96-well plates and incubated with or without oxLDL for 12 h. Thereafter, the level of internucleosomal (apoptotic) DNA fragmentation was determined by Ab-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone–DNA complexes, using the Cell Death Detection ELISA^{PLUS} kit. The ELISA assay was performed according to the instructions of the manufacturer. Briefly, endothelial cells were washed and incubated with 200 μl of lysis buffer for 30 min. After pelleting nuclei (200 ***g***, 10 min), 20 μl of the supernatant (cytoplasmic fraction) was used in the ELISA. Finally, A_{405}, upon incubating with a peroxidase substrate for 15 min, was determined with a Fluostar microplate reader. Values from wells containing lysis buffer and substrate only were subtracted as background.

Measurement of $[Ca^{2+}]_i$ (concentration of intracellular free Ca^{2+})

$[Ca^{2+}]_i$ was measured using a fluorescence microplate assay employing the Ca^{2+} indicator Fluo-3. HMEC-1 cells grown in 96-well plates (Costar #3603) were treated as described in Figure 4, and thereafter loaded with 8 μM Fluo-3/AM in Ca^{2+} buffer (136 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/5 mM $NaHCO_3$/20 mM Hepes/1.1 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4) for 1 h at 37 °C. After incubation, cells were rinsed in Ca^{2+} buffer and analysed directly with a microplate reader employing excitation and emission wavelengths of 488 and 525 nm respectively. Thereafter the fluorescence of the Ca^{2+} signal was calibrated separately for each well. Ca^{2+} saturation was achieved by adding 5 μM ionomycin in the presence of 5 mM Ca^{2+}, and virtually zero Ca^{2+} by further addition of 10 mM EGTA. The concentration of vehicle, DMSO, never exceeded 0.2 %. $[Ca^{2+}]_i$ for experimental samples was calculated using a $K_d(Ca^{2+})$ (the Ca^{2+} dissociation constant) value of 325 nM for Fluo-3/AM. The basal level of $[Ca^{2+}]_i$ in HMEC-1 cells was found to be 83 ± 21 nM ($n = 12$).

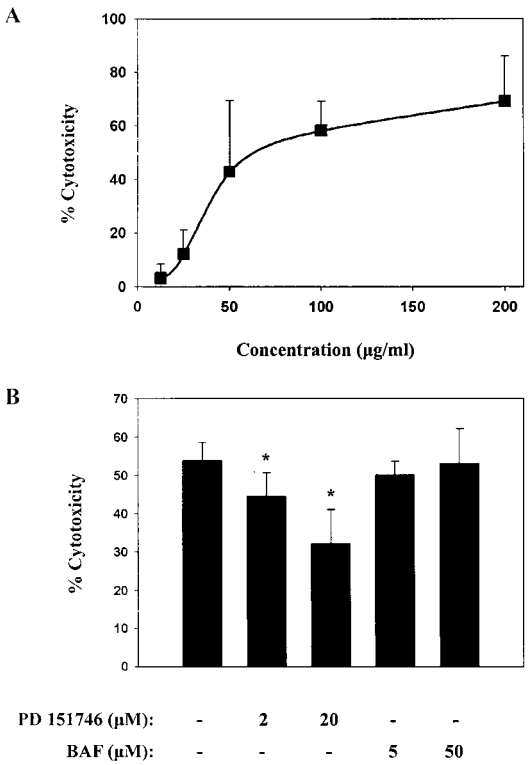

Figure 1 Cytotoxicity of oxLDL and involvement of proteases

(**A**) HMEC-1 cells were incubated in 48-well plates with the indicated concentrations of oxLDL for 24 h at 37 °C. Thereafter, plasma-membrane permeability was measured by detecting LDH release, as described in the Experimental section. Results are means + S.D. (compared with LDL-treated control, $n = 4$). (**B**) Cells were incubated as in (**A**), except protease inhibitors were added to some samples together with oxLDL (100 μg/ml), as indicated. Results are means + S.D. ($n = 4$). The statistical significance of the effects of the inhibitors was calculated using Student's *t* test; $^*P < 0.05$.

RESULTS

Cytotoxicity of oxLDL in HMEC-1 cells

The cytotoxicity and death-inducing capacity of oxLDL have been observed in several types of cells. We performed LDH assays to determine the level of toxicity in HMEC-1 cells incubated with various concentrations of oxLDL. The results show that 100 μg/ml oxLDL led to approx. 58 % cytotoxicity within 24 h (Figure 1A), and we chose to use this concentration in further experiments conducted to study the involvement of proteases in oxLDL-induced cell death.

Proteases involved in oxLDL cytotoxicity

To explore the possible involvement of caspases and calpain in oxLDL-induced cell death, we exposed cells to the general caspase inhibitor BAF and to the μ-calpain inhibitor PD 151746. BAF did not provide any protection against oxLDL toxicity (Figure 1B), and 10 μM VAD-fmk was equally ineffective (results not shown). We did not use higher concentrations of the latter inhibitor, because it has been shown that such treatment causes non-specific inhibition of calpains [11,13,29]. On the other hand, the μ-calpain inhibitor (20 μM PD 151746) did provide a considerable degree of protection against oxLDL-induced death (a decrease of approx. 40 %). Consequently, it seems that the activity of μ-calpain, but not caspases, was necessary for the execution of oxLDL-induced death of HMEC-1 cells.

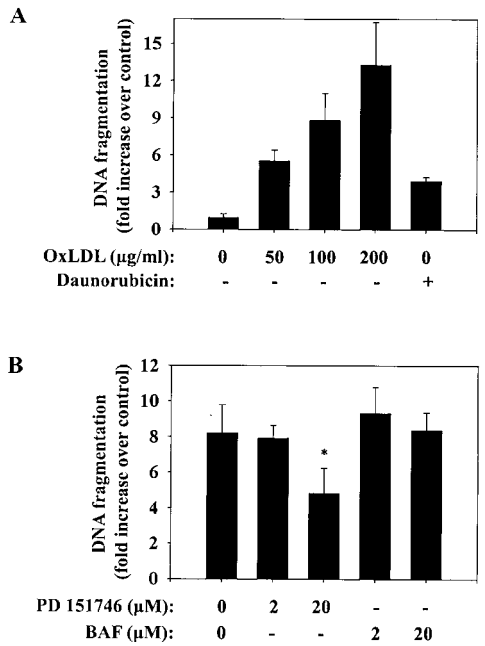

Figure 2 Formation of nucleosomal DNA fragments in oxLDL-treated HMEC-1 cells and effects of protease inhibitors

(**A**) Cells cultured in 96-well plates were exposed to indicated concentrations of oxLDL or to 1 μM daunorubicin (positive control) for 12 h. Thereafter, the cells were lysed and the amount of nucleosomal DNA fragments was measured using an ELISA assay, as described in the Experimental section. Results are presented as fold increases in absorbance over control (untreated cells) and are mean values + S.D. ($n = 3$–7). (**B**) Cells were exposed to 100 μg/ml oxLDL, together with protease inhibitors, as indicated, and thereafter processed as described for (**A**). Results are fold increases in absorbance over control and are means + S.D. ($n = 3$). The statistical significance of the effects of the inhibitors was calculated using Student's t test; $^*P < 0.05$.

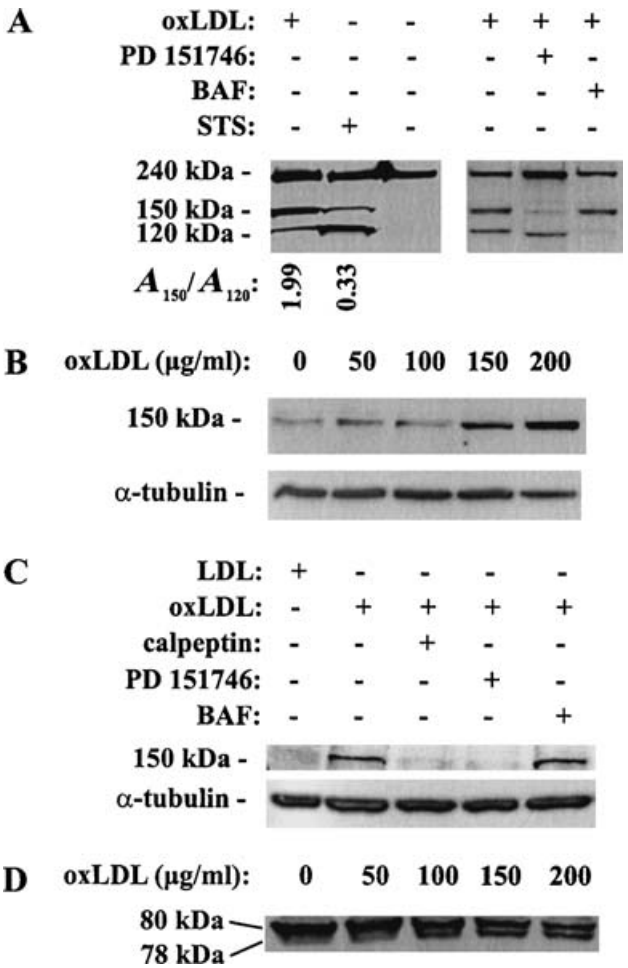

Figure 3 Proteolysis of α-fodrin and activation of calpain by oxLDL

(**A**) HMEC-1 cells cultured in 60-mm-diameter culture dishes were left untreated or were exposed to 200 μg/ml oxLDL for 20 h or to 200 nM staurosporine (STS) for 4 h. Along with oxLDL, some cells were co-treated with PD 151746 (20 μM) or BAF (50 μM). Thereafter, the cells were harvested and processed for Western blotting as described in the Experimental section. The PVDF membrane was probed with an anti-α-fodrin mAb. The absorbance (A) of the 150 and 120 kDa bands was scanned, and the ratio of the two values (A_{150}/A_{120}) is indicated below relevant samples. The illustrated blot is representative of five separate experiments. (**B**) Cells were exposed to the indicated concentrations of oxLDL for 20 h and then harvested for Western-blot analysis. The PVDF membrane was probed with an anti-(proteolysed α-fodrin) Ab specific for the 150 kDa α-fodrin fragment produced by calpain activity. The membrane was subsequently stripped and re-probed with anti-α-tubulin Ab as a loading control. The blot shown is representative of three separate experiments. (**C**) Cells were treated with 200 μg/ml LDL or oxLDL for 20 h. Along with oxLDL, some cells were co-treated with calpeptin (10 μg/ml), PD 151746 (20 μM) or BAF (50 μM). The PVDF membrane was probed with the same antibodies as in (**B**). The blot shown is representative of three separate experiments. (**D**) Cells were treated as in (**B**), lysates were subjected to SDS/7.5 % -PAGE, and the PVDF membrane was probed with anti-(μ-calpain large subunit) Ab. The blot shown is representative of four separate experiments.

Apoptotic DNA fragmentation induced by OxLDL in HMEC-1 cells

The results of LDH assays merely reflect the level of cytotoxicity and do not distinguish between the two major types of cell death: apoptosis and necrosis. Therefore we used an ELISA assay to study the possible formation of nucleosomal DNA fragments in HMEC-1 cells incubated with oxLDL for 12 h. We found that oxLDL induced a dose-dependent increase in the formation of histone-associated cytoplasmic DNA fragments (Figure 2A), showing that oxLDL indeed induced apoptosis in HMEC-1 cells. Exposure of the cells to PD 151746 in combination with oxLDL resulted in a significant decrease of DNA fragmentation, whereas BAF did not provide any protection (Figure 2B). Thus our data suggest that calpain activity, but not caspase activity, is important for oxLDL-induced apoptotic DNA fragmentation.

Cleavage of α-fodrin induced by oxLDL

Cleavage of the cytoskeletal protein α-fodrin has been observed in many types of cell death, but has not, to our knowledge, been studied in cells exposed to oxLDL. We performed Western-blot analysis, and found that treating HMEC-1 cells with oxLDL for 20 h led to a dose-dependent increase in α-fodrin proteolysis, accompanied by formation of the typical fragments of 150/145 and 120 kDa (Figure 3A). Exposing the cells to oxLDL caused a particularly pronounced increase in the 150/145 kDa fragments, whereas the dominating fragment generated by staurosporine-induced cleavage of α-fodrin was 120 kDa in size (Figure 3A). No cleavage of α-fodrin occurred in parallel incubations with native LDL (results not shown). In previous studies we have found [9], as have others [18,19], that both calpains and caspases are capable of cleaving α-fodrin, and, depending on the kind of cell being studied, either one or both of these types of enzymes may be involved in such cleavage during apoptosis. Furthermore, it has been reported that the 150/145 kDa fragments can be formed by the actions of both calpains and caspases, whereas the 120 kDa fragment is thought to be produced primarily by caspase action [19]. This was confirmed in our experiments in which we co-exposed HMEC-1 cells to oxLDL and protease inhibitors

(Figure 3A). We therefore assumed that the predominant generation of 150/145 kDa fragments of α-fodrin induced in HMEC-1 cells by treatment with oxLDL is a sign of calpain activation.

Calpain-mediated cleavage of α-fodrin during oxLDL-induced cell death

Use of Western-blot analysis and a specific Ab to detect a calpain-proteolysed α-fodrin fragment has proven to be one of the most reliable methods to demonstrate calpain activation in cell lysates [18,34]. We employed a polyclonal Ab specific for the calpain-proteolysed 150 kDa α-fodrin fragment [34] to determine whether calpain activation occurs in HMEC-1 cells treated with oxLDL. We detected increased amounts of the calpain-generated 150 kDa α-fodrin breakdown product in HMEC-1 cells exposed to 50 μg/ml oxLDL, and there was a dose-dependent increase in the intensity of this band (Figure 3B). The 150 kDa α-fodrin band became visible after 12 h of oxLDL treatment and increased in strength up to 24 h (results not shown). Furthermore, co-treatment with calpain inhibitors calpeptin or PD 151746 prevented oxLDL-induced formation of the 150 kDa fragment (Figure 3C), whereas BAF was without effect. These results confirm the calpain-catalysed cleavage of α-fodrin, and show that oxLDL induces activation of calpain in endothelial cells.

Autoproteolytic cleavage of calpain upon exposure of HMEC-1 cells to oxLDL

The autoproteolytic cleavage of the 80 kDa subunit of μ- and m-calpain is known to be associated with activation of these enzymes [14]. To further verify the activation of calpain in response to oxLDL exposure, we used a mAb against the 80 kDa subunit of μ-calpain on Western blots, and we observed a dose-dependent formation of the 78 kDa autoproteolysis product of μ-calpain upon treatment of HMEC-1 cells with oxLDL (Figure 3D). The detection of cleaved μ-calpain provided further evidence for an oxLDL-induced stimulation of calpain activity in HMEC-1 cells.

Increased level of $[Ca^{2+}]_i$ in oxLDL-treated HMEC-1 cells

OxLDL has been shown to induce elevations of $[Ca^{2+}]_i$, and the oxLDL-induced toxicity to cultured cells has been suggested to be mediated through a delayed (10–12 h) Ca^{2+} peak [35]. To confirm that oxLDL induces an increase in $[Ca^{2+}]_i$ in HMEC-1 cells, we performed measurements of $[Ca^{2+}]_i$ in oxLDL-treated cells, employing the fluorescent indicator Fluo-3. As shown in Figure 4, we found a significant elevation of the basal $[Ca^{2+}]_i$ in HMEC-1 cells treated for 10 and 12 h with 100 μg/ml oxLDL. The increase in $[Ca^{2+}]_i$ preceded the observed activation of μ-calpain and gives further support to the novel type of oxLDL-initiated death signalling in endothelial cells, which involves activation of this Ca^{2+}-dependent protease.

Proteolysis of Bid in oxLDL-treated HMEC-1 cells

To further characterize the death signal induced by oxLDL in HMEC-1 endothelial cells, we studied the pro-apoptotic BH3-domain-only protein Bid, which is reportedly a substrate for calpain [22]. Truncated Bid is thought to mediate cell death via oligomerization of Bax and/or Bak, with subsequent release of cytochrome *c* from the mitochondria [22,36]. We observed a dose-dependent decrease in the $\approx$22 kDa band recognized by the anti-Bid Ab in HMEC-1 cells incubated with oxLDL for 20 h (Figure 5A). Inasmuch as caspase 8, cathepsin B and calpain have all been shown to cleave Bid in various cell models [22,37,38], we performed co-incubations with different protease inhibitors

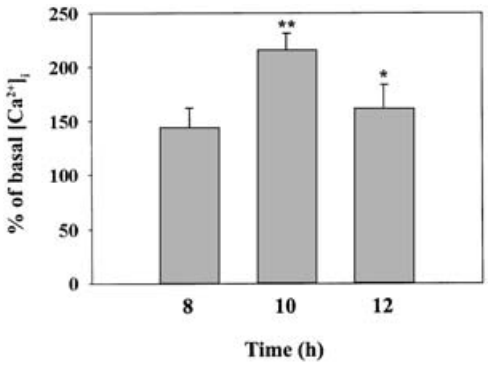

Figure 4 Increased $[Ca^{2+}]_i$ induced by oxLDL in HMEC-1 cells

HMEC-1 cells were incubated in 96-well plates with or without 100 μg/ml oxLDL at 37 °C for the indicated times. Thereafter, cytosolic free Ca^{2+} was measured using a fluorescence microplate assay with the Ca^{2+} indicator Fluo-3, as described in the Experimental section. Results are presented as percentages of the value for the untreated control. The basal level of $[Ca^{2+}]_i$ in HMEC-1 cells was found to be 83 ± 21 nM ($n = 12$). Results are means + S.E.M. ($n = 4$). The statistical significance of the increase in cytosolic free Ca^{2+} was calculated using Student's *t* test; $^*P < 0.05$, $^{**}P < 0.01$.

and oxLDL to track the protease responsible for Bid cleavage in the present system. The results show that only PD 151746 protected Bid from cleavage in HMEC-1 cells, whereas neither the cathepsin B inhibitor CA-074, nor the caspase inhibitor BAF, had any effect (Figures 5B and 5C). We used CA-074 {[l,3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-l-isoleucyl-l-proline} at a concentration of 10 μM, because we found that higher levels were toxic *per se* to the cells (results not shown). This suggests that calpain-catalysed proteolysis of Bid occurs in endothelial cells exposed to oxLDL.

Caspase 3 is not activated during oxLDL-induced death of HMEC-1 cells

Because other researchers have suggested that oxLDL-induced cell death is mediated by caspases [6,27,28], we examined the effects of oxLDL on activation of caspase 3 in HMEC-1 endothelial cells. PARP is a well-known substrate of caspase 3 that is cleaved early in most types of apoptosis. We found that cleavage of PARP in HMEC-1 cells was not induced by exposure to oxLDL for 20 h, but was very pronounced upon treatment with staurosporine (Figure 6A). Furthermore, Western-blot analysis of procaspase 3 revealed no significant proteolytic activation in oxLDL-treated HMEC-1 cells, whereas staurosporine led to production of the 17 kDa catalytic subunit of caspase 3 (Figure 6B). A weak band of 17 kDa appeared in the lysates of cells treated with the highest concentration of oxLDL. More importantly, 100 μg/ml oxLDL did not induce any formation of active p17, whereas this concentration did induce cell death (Figures 1 and 2). To further verify the absence of caspase 3 activation in our cell system, we performed a DEVD-amc-cleavage assay on lysates of HMEC-1 cells. We found no stimulation of the DEVD-amc-cleavage activity in oxLDL-treated HMEC-1 cells, as compared with untreated or LDL-treated cells, whereas exposure to staurosporine caused marked stimulation of caspase 3 activity (Table 1). Indeed, we observed that LDL and oxLDL actually induced a decrease in DEVD-amc-cleavage activity, as compared with control cells. The data presented in Table 1 represent cells subjected to the different treatments for 14 h. We also incubated cells with oxLDL for 6 and 24 h to study the effects on DEVD-amc cleavage, but in no case did we observe any stimulation (results not shown).

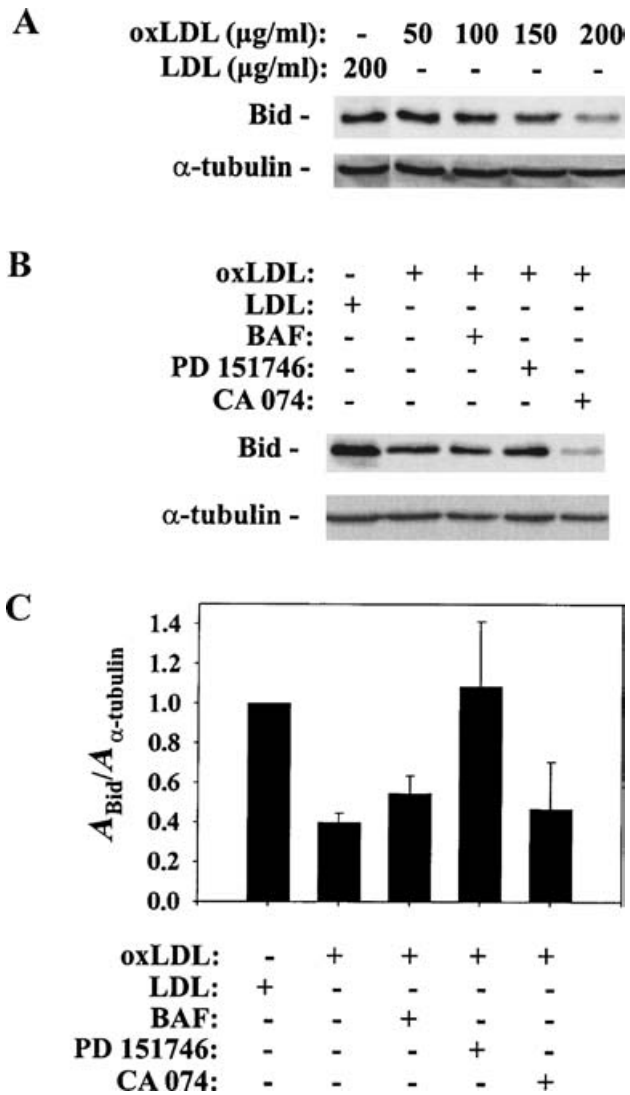

Figure 5 Cleavage of Bid induced by oxLDL in HMEC-1 cells

(**A**) Cells cultured in 60-mm-diameter dishes were exposed to the indicated concentrations of native LDL or oxLDL for 20 h and then processed for Western blotting. Cellular Bid (22 kDa) was detected with an anti-Bid Ab, and the membrane was subsequently stripped and re-probed with an anti-α-tubulin Ab as loading control. (**B**) Cells were treated for 20 h with 200 μg/ml LDL or oxLDL in combination with 50 μM BAF, 20 μM PD 151746, or 10 μM CA-074 ('CA 074') as indicated. The blots shown are representative of three similar experiments. (**C**) Cells were treated as described in (**B**). The absorbance (A) of all bands of Bid and α-tubulin was scanned, and the illustrated data represent the mean values of calculated ratios ($A_{Bid}/A_{\alpha\text{-tubulin}}$) + S.E.M. from three separate experiments.

OxLDL-induced polyubiquitination of caspase 3

Interestingly, treatment of HMEC-1 cells with oxLDL caused a dose-dependent increase in high-molecular-mass (> 100 kDa) bands recognized by the anti-(caspase 3) mAb (Figure 6B), suggesting a possible oxLDL-induced ubiquitination of caspase 3. Ubiquitination (covalent conjugation of 8.5 kDa ubiquitin molecules, in the form of multi-ubiquitin chains) of caspases has recently been reported to inactivate these enzymes [39,40]. Therefore, we immunoprecipitated caspase 3 from HMEC-1 cells and probed the Western blots of these immunoprecipitates with an Ab against ubiquitin. We found that exposing the cells to oxLDL clearly induced polyubiquitination of caspase 3, whereas treatment with native LDL did not (Figure 6C).

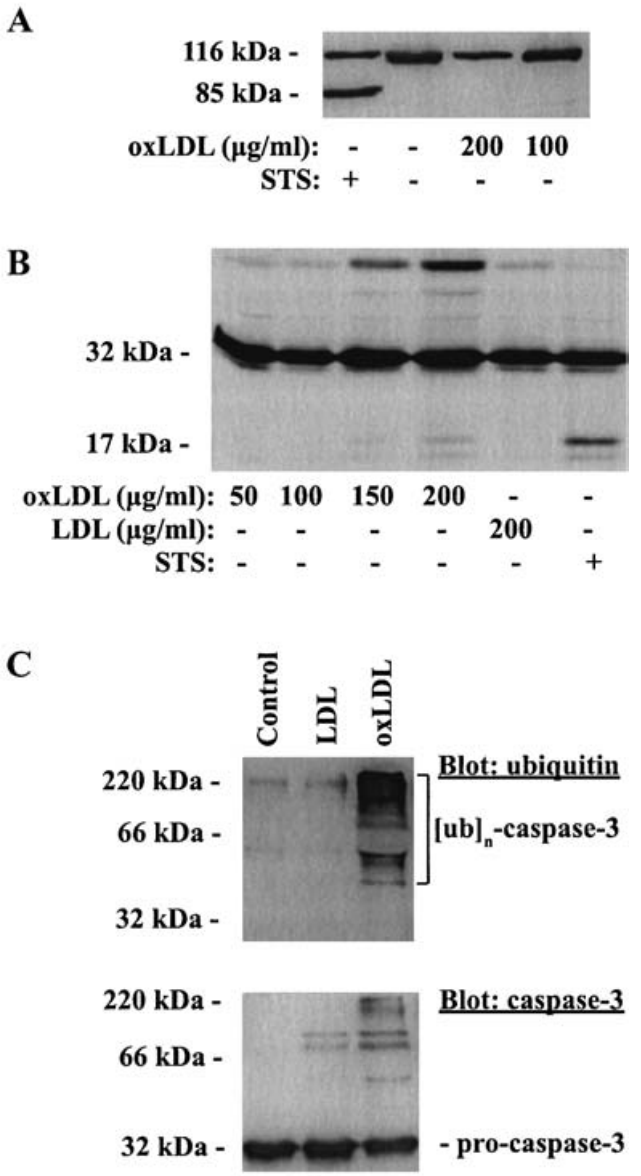

Figure 6 Caspase 3 is not activated, but is ubiquitinated, during oxLDL-induced HMEC-1 cell death

Cells were incubated with the indicated concentrations of oxLDL for 20 h and then harvested, electrophoresed, and Western-blotted. (**A**) The cellular content of PARP (116 kDa) was probed with an anti-PARP Ab as described in the Experimental section. STS denotes treatment with 500 nM staurosporine for 4 h. Lane 2 contained a control sample of untreated cells. The blot shown is representative of those from five similar experiments. (**B**) The cellular content of procaspase 3 (32 kDa) and active p17 was probed with an anti-(caspase 3) mAb. The blot shown is representative of those from three similar experiments. (**C**) Cellular caspase 3 was immunoprecipitated, and Western blots of the immunoprecipitates were probed with an anti-ubiquitin (ub) Ab and then stripped and re-probed with an anti-(caspase 3) Ab. The lipoproteins were used at a concentration of 200 μg/ml. The blots shown are representative of five similar incubations.

DISCUSSION

As mentioned in the Introduction, it is well known that oxLDL is a proapoptotic agent in endothelium, and it is assumed that vascular cell death induced by oxLDL contributes to the progression of atherosclerosis (reviewed in [3]). Calpain has previously been suggested to mediate endothelial apoptosis induced by growth-factor deprivation [41,42]. We found that calpain plays an

Table 1 Effects of LDL and oxLDL on caspase-3 activity in HMEC-1 endothelial cells

HMEC-1 cells cultured in six-well plates were left untreated (control) or were exposed to the indicated concentrations of LDL, oxLDL or staurosporine. After 14 h, cells were lysed, and caspase 3-mediated cleavage of DEVD-amc was analysed as outlined in the Experimental section. Values given represent means ± S.D. from three separate experiments.

Treatment	Caspase 3 activity (amc fluorescence/μg of cell protein)
Control	63.6 ± 7.7
LDL	
100 μg/ml	38.6 ± 15.3
200 μg/ml	50.2 ± 5.1
OxLDL	
100 μg/ml	54.3 ± 10.4
200 μg/ml	30.7 ± 7.3
Staurosporine	
500 nM	156.4 ± 10.5

important role in mediating oxLDL-induced death of endothelial cells, as illustrated by the significant protection against oxLDL-induced cytotoxicity and DNA fragmentation afforded by the μ-calpain inhibitor PD 151746. Our data demonstrate that the cytoskeletal protein α-fodrin was cleaved in a calpain-dependent fashion; that is, one of the cleavage products was recognized by an Ab specific for the 150 kDa calpain-proteolysed α-fodrin fragment, and this proteolysis was prevented by PD 151746. In addition, we observed a dose-dependent formation of the 78 kDa autoproteolysis product of μ-calpain upon treatment of HMEC-1 cells with oxLDL, supporting an oxLDL-induced stimulation of calpain activity. Results from Ca^{2+} measurements suggested a delayed peak in $[Ca^{2+}]_i$, consistent with previously published reports, and further supporting an important role for Ca^{2+}-activated protease(s) in oxLDL-induced cell death.

In further evidence of calpain involvement in oxLDL-induced cell death, we observed that this lipoprotein caused a dose-dependent decrease in cellular levels of Bid. Caspase 8, cathepsin B, and calpain have all been shown to cleave Bid in various cell models [22,37,38], but the results of our experiments suggest that a calpain was responsible for the proteolysis of Bid in HMEC-1 cells. More precisely, we found that protection against cleavage of Bid was offered by PD 151746, but not by the cathepsin B inhibitor CA-074 or the caspase inhibitor BAF.

The relevance of Bid cleavage in the present system is not yet clear, since it is presumed that the cell death caused by truncated Bid is mediated by release of cytochrome *c* and subsequent caspase activation. Inasmuch as caspase 3 seems to be inactivated in the system we studied, it is possible that other mechanisms are involved in cell death promoted by truncated Bid. OxLDL treatment also caused the appearance of a weak band corresponding to the 120 kDa α-fodrin fragment, inhibitable by BAF treatment, suggesting that a caspase was activated upon oxLDL treatment. Our observations that Bid was activated by calpain, and that α-fodrin may be cleaved partly by a caspase, are in good agreement with previous reports showing that calpain activation can lead to caspase activation [11,13,29,43]. The identity of this caspase is unclear, but more importantly, BAF did not confer any protection against oxLDL-induced cytotoxicity in HMEC-1 cells.

Caspase 3 is believed to be the most important enzyme in the apoptosis program; yet, our experiments show unequivocally that oxLDL did not stimulate caspase 3 activity in HMEC-1 endothelial cells. We detected neither cleavage of PARP, nor any proteolytic activation of procaspase 3, in oxLDL-treated HMEC-1 cells. Nevertheless, the caspase pathway seemed to be functioning normally in the cells, as evidenced by massive, staurosporine-induced activation of caspase 3. Furthermore, compared with untreated or LDL-treated cells, those exposed to oxLDL did not exhibit stimulated DEVD-amc-cleavage activity. In fact, the level of DEVD-amc cleavage was lower in cells treated with LDL and oxLDL than in control cells. It is not surprising that the basal level of caspase 3 activity was reduced by LDL, because this lipoprotein has been found to have mitogenic effects [44,45]. A plausible explanation for the oxLDL-elicited inhibition of caspase 3 activity is the ubiquitination of caspase 3 observed in our study. The discovery that caspase inhibitors in no way protected against oxLDL-mediated toxicity further supports the conclusion that cell death caused by oxLDL does not depend on the activation of caspases in HMEC-1 endothelial cells. Recent reports have indicated that caspases may be inactivated by ubiquitination [39,40], and we found that polyubiquitination of caspase 3 was induced by oxLDL in the HMEC-1 cells. To our knowledge, we are the first to demonstrate oxLDL-induced ubiquitination of caspase 3, although Vieira et al. [46] have proposed that the rate of ubiquitination of cellular proteins in general may be increased by oxLDL. Taken together, our results suggest that caspases are not important for oxLDL-induced death of endothelial cells. This is refuted by investigations suggesting that inhibition of caspases protects against oxLDL-mediated cell death [6,27,28]. Unfortunately, the identity of the death-mediating protease(s) in the cited studies remains unknown, because the high concentrations (>20 μM) of VAD-fmk used in those experiments have been shown to cause unspecific inhibition of calpains [11,13,29]. Nevertheless, studying HUVEC (human umbilical-vein endothelial cells), Dimmeler and co-workers [7] found that caspase 3 was activated by 10 μg/ml oxLDL, and this type of cell death could also be prevented by moderate concentrations of caspase inhibitors [7]. In our study we found no response to such a low concentration of oxLDL, and our observations of caspase 3 ubiquitination and calpain activation indicate novel and alternative signalling pathways initiated by oxLDL. However, the protein concentration of oxLDL is only a relative measure, and, depending on the extent of LDL oxidation, 10 μg/ml can induce various levels of oxidative stress in the cells. In the abovementioned study [7], the oxidation level of LDL was not clearly expressed, and thus it is not possible to directly compare these two studies. Importantly, despite the lack of caspase 3 activation in the present study, oxLDL treatment led to the calpain-dependent formation of nucleosomal DNA fragments characteristic of apoptosis. This observation is in good agreement with a recent report showing caspase-independent apoptotic DNA fragmentation, inhibitable by calpain inhibitors, in vitamin D_3-induced cell death of human breast-cancer cells [47].

OxLDL contains an undefined mixture of oxidized lipids (aldehydes, oxysterols and lipid peroxides), and the toxicity of oxLDL has mainly been associated with the lipid fraction [35]. Exchange of oxidized lipids with the plasma membrane and receptor-mediated uptake of oxLDL particles are the main routes thought to be involved in the initiation of cytotoxicity. HMEC-1 cells express the scavenger receptors LOX-1 and CD36, which have both been implicated in the apoptotic effect of oxLDL. HUVEC cells, however, do not express CD36, and this could perhaps explain the differences in response to oxLDL exposure. Oxidized lipids from oxLDL induce modifications of cell protein structure, elicit generation of ROS (reactive oxygen species) and cellular lipid peroxidation, and thus alter the regulation of various signalling pathways. Importantly, oxidative stress has been shown to up-regulate calpain expression [30].

OxLDL-generated intracellular ROS may inhibit plasma-membrane Ca^{2+}/ATPases pumps, thereby contributing to the rise of cytosolic Ca^{2+}, which activates Ca^{2+}-dependent endonucleases and calpains.

Our results, together with reports that calpain is involved in the death program of U937 cells, thymocytes, platelets, neurons and breast-cancer cells [9–13,47], suggest that, in certain types of cell death, calpain activation is used as an alternative to the caspase cascade. Moreover, it has been shown that calpain-proteolysed procaspases may cause inactivation of the caspase cascade [12]. Our findings further underscore the importance of Ca^{2+} in vascular biology and also illustrate a novel mechanism that may underlie the effectiveness of Ca^{2+}-channel blockers as atheroprotective agents [48,49]. This is also supported by previous results obtained in our laboratory [50], as well as by other workers [4], showing that Ca^{2+}-channel blockers inhibit apoptosis induced by 25-hydroxycholesterol or oxLDL.

We are grateful to Dr Fredrik Melander (Department of Laboratory Medicine, Lund University) for assistance with the immunoprecipitation assay. We also thank Ms Patricia Ödman, a freelance linguist, for linguistic revision of the manuscript. This work was supported by grants from Inga and John Hain's Foundation, the Crafoord Foundation, Foundations at Malmö University Hospital, Greta and Johan Kock's Foundations, Alfred Österlund's Foundation, the Royal Physiographic Society in Lund, the Swedish Medical Society, and the Medical Faculty of Lund University. The Assistant Professorship held by I. P.-A. is partly funded by a grant (no. 12871) from the Swedish Research Council.

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Received 18 December 2002/22 May 2003; accepted 30 May 2003
Published as BJ Immediate Publication 30 May 2003, DOI 10.1042/BJ20021955