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TRPC1 is regulated by caveolin-1 and is involved in oxidized LDL-induced apoptosis of vascular smooth muscle cells

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Abstract

Oxidized low-density lipoprotein (oxLDL) induced-apoptosis of vascular cells may participate in plaque instability and rupture. We have previously shown that vascular smooth muscle cells (VSMC) stably expressing caveolin-1 were more susceptible to oxLDL-induced apoptosis than VSMC expressing lower level of caveolin-1, and this was correlated with enhanced Ca^{2+} entry and pro-apoptotic events. In this study, we aimed to identify the molecular events involved in oxLDL-induced Ca^{2+} influx and their regulation by the structural protein caveolin-1. In VSMC, transient receptor potential canonical-1 (TRPC1) silencing by ARN interference prevents the Ca^{2+} influx and reduces the toxicity induced by oxLDL. Moreover, caveolin-1 silencing induces concomitant decrease of TRPC1 expression and reduces oxLDL-induced apoptosis of VSMC. OxLDL enhanced the cell surface expression of TRPC1, as shown by biotinylation of cell surface proteins, and induced TRPC1 translocation into caveolar compartment, as assessed by subcellular fractionation. OxLDL-induced TRPC1 translocation was dependent on actin cytoskeleton and associated with a dramatic rise of 7-ketocholesterol (a major oxysterol in oxLDL) into caveolar membranes, whereas the caveolar content of cholesterol was unchanged. Altogether, the reported results show that TRPC1 channels play a role in Ca^{2+} influx and Ca^{2+} homeostasis deregulation that mediate apoptosis induced by oxLDL. These data also shed new light on the role of caveolin-1 and caveolar compartment as important regulators of TRPC1 trafficking to the plasma membrane and apoptotic processes that play a major role in atherosclerosis.

Keywords: oxidized low-density lipoprotein • vascular smooth muscle cell • TRPC1 • caveolin-1 • apoptosis

Introduction

Atherosclerosis is a slow degenerative process and is the underlying cause of heart attacks, strokes and peripheral artery diseases in human beings. This complex disorder is characterized by the focal accumulation of lipids and the remodelling of the arterial wall, leading to the formation of the atherosclerotic plaque. Modified lipoproteins, especially oxidized low-density lipoprotein (oxLDL), are present within atheroma plaques, and are thought to play a role in atherogenesis [1]. OxLDL exhibit a variety of atherogenic properties, by inducing foam cell formation, inflammatory response, cell proliferation at low concentration, and apoptosis at higher concentration [1, 2].

In atherosclerotic lesions, apoptosis is thought to be involved in necrotic core formation and in plaque rupture or erosion, which led finally to athero-thrombotic events [3].

The apoptotic signalling triggered by oxLDL involves both the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is mediated by death receptors and downstream, by caspase-8 and caspase-3. The intrinsic apoptotic mitochondrial pathway is activated by oxLDL through a Ca^{2+}-dependent mechanism [4, 5]. We previously showed that the oxLDL-induced rise of cytosolic Ca^{2+} requires an influx of extracellular Ca^{2+} [6]. However, the precise mechanism of the Ca^{2+} entry and the identity of Ca^{2+} channels remain to be elucidated. Furthermore, we recently reported that vascular smooth muscle cells (VSMC) overexpressing caveolin-1 were more susceptible to oxLDL-induced apoptosis, and this was correlated with enhanced Ca^{2+} entry and

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pro-apoptotic events [7]. It has been proposed that caveolae, which are cholesterol-rich microdomains of the plasma membrane, might regulate the spatial organization of Ca^{2+} signalling by contributing to the assembly of Ca^{2+} signalling complex as well as the site of Ca^{2+} entry [8]. Indeed, caveolae contain several complexes that are Ca^{2+} dependent or involved in Ca^{2+} metabolism, such as plasma membrane Ca^{2+} ATPases, endothelial nitric-oxide synthase (eNOS), phopholipase C, $G\alpha_{q/11}$ and IP_3-like receptors [8].

There is now growing evidence that members of the transient receptor potential canonical (TRPC) channels can assemble in cholesterol-rich caveolae domains to participate in Ca^{2+} influx pathways. To date, seven TRPCs (TRPC1-7) have been identified, TRPC1 is localized in caveolae where it is associated with signalling proteins including IP_3-like receptors, caveolin-1, calmodulin, phospholipase C-β/γ, protein kinase C-α, $G\alpha_{q/11}$ and RhoA [9]. The scaffolding domain of caveolin-1 is necessary for anchoring TRPC1 to caveolae and for its regulation [10, 11]. Wide-ranging biological roles of TRPC1 are proposed, including arterial contraction, endothelial permeability, salivary gland secretion, glutaminergic neurotransmission and cell proliferation [9]. In addition, it has been recently shown that TRPC1 has a proapoptotic role in intestinal cells and cardiomyocytes as a result of Ca^{2+} influx [12, 13]. The mode of activation of TRPC1-mediated Ca^{2+} entry is still under debate, but TRPC1 responds to general stimuli following depletion of intracellular Ca^{2+} stores, receptor activation or membrane stretch. At present, it is not known whether oxLDL-induced Ca^{2+} influx is directly linked to TRPC1 activation and whether caveolin-1/caveolae structures control Ca^{2+}-dependent pathways leading to apoptosis in VSMC.

The purpose of this study was to elucidate the molecular events involved in oxLDL-induced Ca^{2+} influx and apoptosis of VSMC, and their potential regulation by the structural protein caveolin-1.

Materials and methods

Reagents

Cell culture reagents were from Invitrogen (Carlsbad, CA, USA). 2-aminoethoxydiphenyl-borane (2-APB), Ethylene glycol tetraacetic acid (EGTA) and cytochalasin D were from Sigma-Aldrich (St Louis, MO, USA). The following antibodies were used: polyclonal anti-TRPC1 (Alomone Labs, Jerusalem, Israel), polyclonal anti-caveolin-1 and monoclonal anti-β-actin (Upstate Biotechnology, Billerica, MA, USA). The cell surface protein biotinylation and purification kit was from Pierce. The enhanced chemoluminescence (ECL) kit was from Amersham Pharmacia (Buckinghamshire, UK). Hiperfect transfection reagent was from Quiagen (Courtaboeuf, France). Fluo-3/AM, SYTO-13, propidium iodide were from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

Cell culture

We used different VSMC cells: human primary VSMC (hSMC) and rabbit arterial SMC stably expressing caveolin-1 because VSMC grown in primary culture are rapidly converted from a contractile to a synthetic phenotype leading to a decrease of cell surface caveolae and caveolin-1 [14]. In addition, there is a phenotype-dependent variation in the number of caveolae in VSMC both *in vivo* and *in vitro*. To avoid caveolin-1 and caveolae decrease by passages, we established a stable caveolin-1 transfected VSMC model, allowing us to obtain stable and constant level of caveolin-1 protein and caveolae.

Rabbit arterial femoral SMC (obtained from ATCC, Rockville, MD, USA) and stable caveolin-1 transfectants were established and grown as described previously [7]. To obtain stable transfectants, rabbit arterial SMC were transfected with the pCDNA3/caveolin-1 plasmid (a generous gift of Dr. P. Fielding, San Francisco, CA, USA) or by the empty plasmid pCDNA3 using LipofectAMINE Plus reagent (Invitrogen). Twenty-four hours after transfection, the cells were split into 10-cm dishes in medium containing 500 μg/ml G418, and the medium was changed every 3–4 days until G418-resistant colonies were clearly evident. Rabbit arterial VSMC expressing the empty plasmid pCDNA or the plasmid pCDNA3/caveolin-1 were, respectively, denominated SMC/ev and SMC/cav1. The levels of caveolin-1 expression and cell surface caveolae were similar in primary hSMC and SMC/cav1 (analysed by Western blotting and electron microscopy, [7]).

The hSMC were obtained from human coronary arteries at postmortem examinations. All experiments were conformed to the declaration of Helsinki in compliance with French legislation and written informed consent was obtained from patients for the use of surgery residual tissue for research. Briefly, the arteries were cut longitudinally and small pieces of the media were carefully stripped from the vessel wall and cultured. Within 1–2 weeks, hSMC migrated from the explants; they were capable of being passaged 3 weeks after the first appearance of cells. They were identified as VSMC by their characteristic hill-and-valley growth pattern and immunohistochemistry for VSMC-specific α-actin. The cultures were maintained in Doulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum at 37°C in a humidified, 5% CO_2 / 95% air atmosphere. The hSMC were used until the eighth passage. Caveolin-1 expression is checked every three passages in hSMC and SMC/cav1. For experiments, cells were transferred 24 hrs in G418-free medium.

LDL isolation and mild oxidation

LDL from human pooled sera were prepared by ultracentrifugation, dialysed against phosphate buffered saline (PBS) containing 100 μM ethylenediaminetetraacetic acid (EDTA). LDL were mildly oxidized by UV-C + copper/EDTA (5 μM) (oxLDL) as previously reported [15]. OxLDL contained 4.2 to 7.4 nmoles of TBARS (thiobarbituric acid-reactive substances)/μg apoB. Relative electrophoretic mobility and 2,4,6-trinitrobenzenesulfonic acid reactive amino groups were 1.2–1.3 times and 85–92% of native LDL, respectively.

Western blot analysis

Cells were lysed in solubilizing buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) for 30 min. on ice. Forty micrograms of protein cell extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then membranes were probed with the indicated primary antibodies

and revealed with the secondary antibodies coupled to horseradish peroxidase using the ECL chemoluminescence kit. Membranes were then stripped and reprobed with anti-β-actin antibody to control equal loading of proteins.

Detergent-free purification of caveolae/raft membrane fractions

Lipid raft-enriched domains were purified from cultured cells using a modified carbonate method [16]. Cells were lysed in 2 ml 0.5 M Na_2CO_3 (pH 11), containing protease inhibitors and homogenized sequentially with a tightly fitting Dounce homogenizer (10 strokes) and three 10-sec. bursts using a sonicator Soniprep 150 (MSE, London, UK). Equal amounts of cell lysates were then adjusted to 45% sucrose by mixing with 2 ml 90% sucrose prepared in 25 mM 2-(N-Morpholino)ethanesulfonic acid (MES) (pH 6.5) and 150 mM NaCl. This suspension was placed at the bottom of an ultracentrifuge tube and overlaid with a 5–35% discontinuous sucrose gradient. After centrifugation at 39,000 rpm for 18 hrs at 4°C in a swinging bucket rotor SW41 (Beckman, Roissy, France), a total of 12 fractions (1 ml each) were collected from the top of each gradient and analysed by Western blotting.

Evaluation of cytotoxicity, necrosis and apoptosis

Cytotoxicity was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, as previously used [4]. This method is based on the transformation and colorimetric quantification of MTT. The respiratory chain and other electron transport systems reduce MTT and other tetrazolium salts and thereby form non-water-soluble violet formazan crystals within the cell. The amount of these crystals can be determined spectrophotometrically (OD measured at 570 nm) and serves as an estimate for the number of mitochondria and hence the number of living cells in the sample. For cytotoxicity experiments cells were serum starved for 24 hrs and stimulated for the indicated times at 37°C. Cell lysis (necrosis) was evaluated by lactate dehydrogenase release. Apoptotic and necrotic cells were counted after fluorescent staining by two vital fluorescent dyes, the permeant DNA intercalating green fluorescent probe SYTO-13 (0.6 μM) and the non permeant DNA intercalating red fluorescent probe propidium iodide (15 μM) using an inverted fluorescence microscope (Fluovert FU, Leitz, Stuttgart, Deutschland). Normal nuclei exhibit a loose green coloured chromatin. Nuclei of primary necrotic cells exhibit a loose red coloured chromatin. Apoptotic nuclei exhibited condensed yellow/green-coloured chromatin associated with nucleus fragmentation, whereas post-apoptotic necrotic cells exhibited the same morphological features, but were red coloured.

Measurement of intracellular Ca^{2+}

Cytosolic Ca^{2+} was determined using fluo3/AM according to Jagnadan *et al.* [17]. Briefly, cells were incubated with a loading solution consisting of HEPES-buffered saline (HBS; 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11.6 mM HEPES, and 11.5 mM glucose, pH 7.3) supplemented with 5 μM fluo-3/AM, 0.02% pluronic F-127 and 1 mg/ml bovine serum albumin for 30 min., and then incubated in the loading solution without Fluo-3 for 30 min. to allow de-esterification of the probe. Loading solution was then replaced with HBS, and cells were placed in a fluorometer and fluorescence was recorded (495 and 525 nm excitation and emission wavelength, respectively). Results were normalized on protein levels and expressed in ratio of control.

Biotinylation of cell surface proteins

Following oxLDL treatment, cells were washed twice with ice-cold PBS and then incubated in 1 mg/ml sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 min. at 4°C. The biotinylation reaction was terminated with washing the cells three times with ice-cold PBS containing 10 mM glycine. The cells were then lysed in solubilizing buffer, isolation of labelled proteins were preceded using Cell Surface Protein Biotinylation and Purification Kit (Pierce) according to the manufacturer's instructions. The recovered proteins were analysed by Western blot as described previously, blots were probed with anti-TRPC1 antibody.

siRNA transfection

The selected siRNA specific to TRPC1 is 5′-AAG CUU UUC UUG CUG GCG UGC-3′ and to caveolin-1 are 5′-CAG GGC AAC AUC UAC AAG C-3′ and 5′-CCA GAA GGG ACA CAC AGU U-3′ (Dharmacon, Waltham, MA, USA). SiRNAs were transfected using the Hiperfect reagent (Quiagen) according to the manufacturer's recommendations.

Cholesterol and 7-ketocholesterol analysis

The cholesterol and 7-ketocholesterol content of the sucrose gradient fractions were assayed by GC-MS [18].

Determination of LDL cellular uptake

The uptake of native LDL and oxLDL was studied by using LDL labelled with the fluorescent lipid dye 3,3′-dioctadecyl-indocarbocyanine (DiI) (Molecular Probes), according to Negre-Salvayre *et al.* [19]. The DiI content was determined by fluorometry (spectrofluorometer Jobin Yvon JY3C; excitation 520 nm, emission 568 nm) using a standard of DiI similarly treated.

Statistical analysis

Data are given as means ± S.E.M. Estimates of statistical significance was performed by ANOVA analysis (Tukey test, SigmaStat software). Values of $P < 0.05$ were considered statistically significant.

Results

TRPC1 is involved in oxLDL-induced Ca^{2+} influx and apoptosis

We first investigated the molecular mechanisms involved in oxLDL-induced Ca^{2+} rise using VSMC stably expressing caveolin-1 (SMC/cav1), recently developed and characterized in our laboratory because VSMC grown in primary culture are rapidly converted from

a contractile to a synthetic phenotype leading to a decrease of cell surface caveolae and caveolin-1 (see 'Materials and methods' section for cell line details). As previously reported, oxLDL triggered a higher Ca^{2+} rise in SMC/cav1 compared to control VSMC expressing an empty vector (SMC/ev) [7]. The cytosolic Ca^{2+} rise was precluded by the extracellular Ca^{2+} chelator EGTA thus suggesting that the sustained Ca^{2+} rise requires an influx of extracellular Ca^{2+} (Fig. 1A, upper panel). Interestingly, the sustained Ca^{2+} rise was also inhibited by aminoethoxydiphenylborane (2-APB), an inhibitor of TRPC channels (Fig. 1A, upper panel). Time course analysis of cell death following oxLDL treatment showed that the number of apoptotic cells increases after 12 hrs oxLDL stimulation in accordance with the kinetic of the Ca^{2+} rise (Fig. 1A, lower panel). As expected, pre-treatment with EGTA or 2-APB before oxLDL exposure, reduces the apoptotic effect of oxLDL (Fig. 1B). Moreover, under the same conditions, the number of apoptotic cells, counted by Syto13/PI fluorescent staining, was significantly reduced (Fig. 1C and D). In addition, silencing TRPC1, the major TRPC channel expressed in VSMC, by small interfering RNA (siRNA) specific to TRPC1 (Fig. 2A) inhibited the oxLDL-induced Ca^{2+} rise (Fig. 2B) and reduced the toxic effect of oxLDL (Fig. 2C and D). To evaluate whether TRPC1 is implicated in oxLDL-induced apoptosis under more physiological experimental conditions, we performed the same experiment on hSMC. As shown in Fig. 2E, we observed that the cellular toxicity induced by oxLDL in hSMC was significantly decreased by TRPC1 silencing, thus supporting the major role of TRPC1 channel in oxLDL-induced Ca^{2+} influx and VSMC apoptosis.

Altogether, these data support the critical role of Ca^{2+} influx through TRPC1 channel in oxLDL-induced VSMC apoptosis. However, it may be noted that the Ca^{2+}-dependent apoptotic pathway induced by oxLDL is not the sole apoptotic mechanism, because the extrinsic apoptotic pathway mediated by death receptors has been shown to be activated by oxLDL [20]. But in our experimental model, the intrinsic Ca^{2+}-dependent apoptotic pathway is prevailing (≈60% of apoptotic cells), on the basis of the protective effect of Ca^{2+} chelators.

Caveolin-1 is involved in TRPC1 expression and oxLDL-induced apoptosis

We have previously reported that caveolin-1 sensitizes VSMC to oxLDL-induced apoptosis by potentiating the Ca^{2+} influx and the mitochondrial Ca^{2+}-dependent apoptotic pathway [7]. It has also been shown that caveolin-1 plays a critical role in the functional expression of TRPC1 in the plasma membrane and may serve as a scaffold to integrate TRPC1 regulation of Ca^{2+} influx pathways [10].

Interestingly, when we compared the levels of TRPC1 proteins in VSMC expressing different levels of caveolin-1 proteins, we observed that the expression of TRPC1 and caveolin-1 was correlated (Fig. 3A). In order to investigate whether TRPC1 expression was dependent on caveolin-1 expression, we induced a decrease of caveolin-1 expression by specific siRNA and examined the effect on TRPC1 expression. As shown in Fig. 3B, silencing of caveolin-1 induced a dramatic decrease in TRPC1 level, thus supporting the hypothesis that TRPC1 level may depend on caveolin-1 expression.

Because TRPC1 is involved in oxLDL-induced apoptosis, caveolin-1 expression should regulate the toxic effect of oxLDL through the regulation of TRPC1 level. As expected, caveolin-1 silencing reduced the toxicity induced by oxLDL treatment in SMC/cav1 (Fig. 4A) and in primary hSMC (Fig. 4B).

TRPC1 channels translocate to caveolar compartment upon oxLDL stimulation

The activity of TRPC1 channel is dependent on its expression and on its insertion in the plasma membrane [21]; this led us to investigate whether oxLDL may regulate either the expression of TRPC1 or its subcellular location. As oxLDL treatment did not modify the whole cellular level of TRPC1 (Fig. 5A), we investigated whether oxLDL may regulate the level of TRPC1 located at the plasma membrane. This was evaluated by using the membrane-impermeant biotinylation reagent sulfo-NHS-SS-Biotin (that reacts with cell surface proteins) followed by the recovery of biotin-labelled proteins with streptavidin beads and Western blot analysis. Under resting conditions, biotinylated TRPC1 was barely detectable at the cell surface in SMC/cav1 in spite of the high whole cellular level of TRPC1 (Fig. 5B). This suggests that, under basal conditions, the major part of TRPC1 is not located at the plasma membrane, but is rather located inside the cell (not accessible to the impermeant biotinylation reagent). In contrast, in cells treated for 8 to 14 hrs with oxLDL, the level of biotinylated TRPC1 (*i.e.* located at the plasma membrane) increased dramatically (Fig. 5B). This strongly suggests that oxLDL induce the translocation of TRPC1 to the plasma membrane. As functional TRPC1 has been shown to be located in caveolar lipid raft domains [21], we investigated whether oxLDL induced the translocation of TRPC1 into caveolar compartment. Using caveolae fractionation methods, we showed that, in untreated cells, TRPC1 was found in non-caveolar fractions (H_1 and H_2, high-density fractions) (Fig. 5C), in agreement with the intracellular location suggested above. Interestingly, oxLDL treatment of SMC/cav1 induced the translocation of TRPC1 into caveolar domains (light-density fractions L) (Fig. 5C). It may be noted that, as expected, caveolin-1 was localized in the caveolae (light-density fractions L), both in resting and in oxLDL-stimulated SMC/cav1 (Fig. 5C).

Given that translocation of TRPC1 to the plasma membrane appears to be induced by oxLDL, we next sought to determine whether TRPC1 translocation is required for oxLDL-induced apoptosis. It has been shown that local changes in the cytoskeleton or microtubules can contribute to channel trafficking and facilitate plasma membrane insertion [22]. The pre-treatment of SMC/cav1 with cytochalasin D, a widely utilized membrane-permeant inhibitor of actin polymerization, prevents TRPC1 translocation

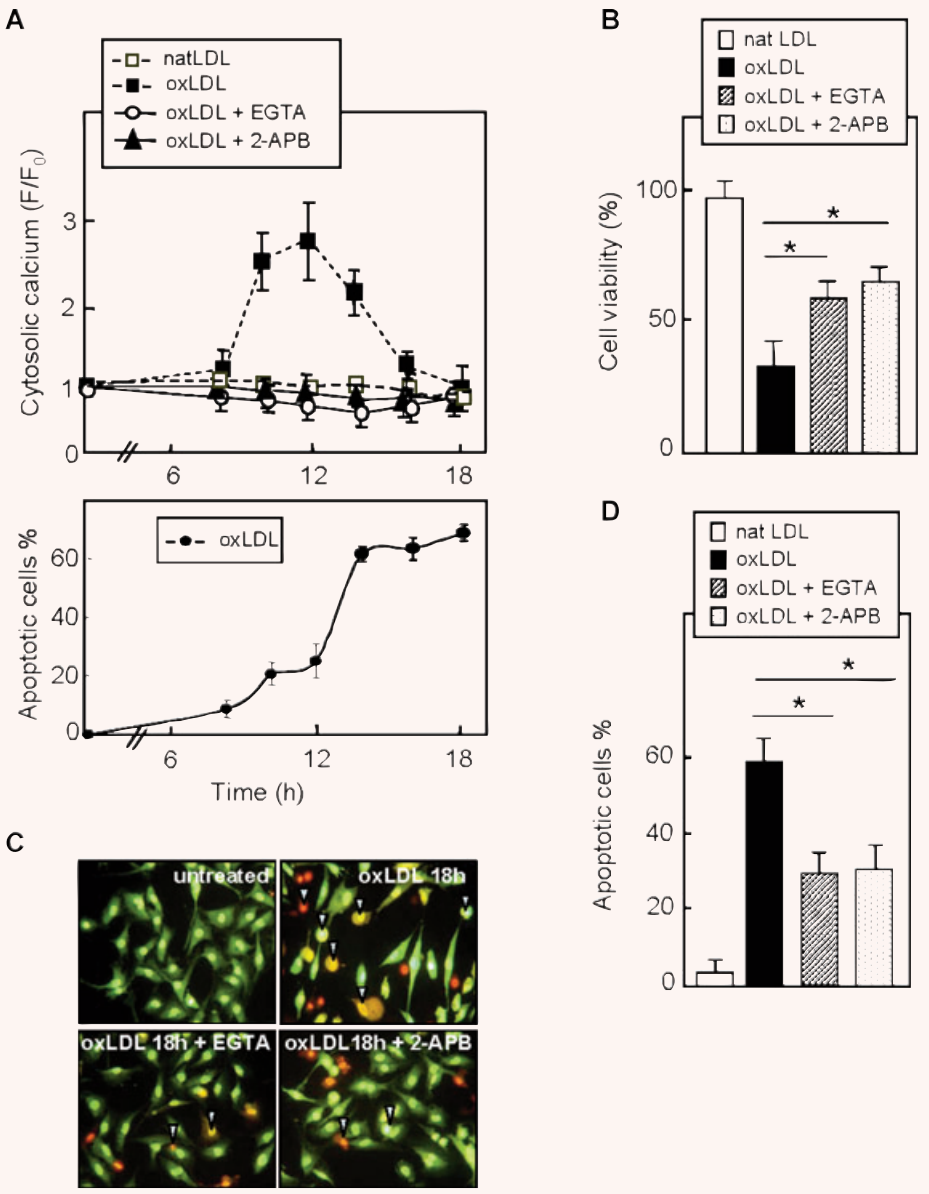

Fig. 1 EGTA and 2-APB inhibited oxLDL-induced Ca^{2+} rise and apoptosis. The Ca^{2+} rise induced by oxidized-LDL is inhibited by EGTA and 2-APB. (**A**) Upper panel, SMC/cav1 were pre-treated with 0.35 mM EGTA or 25 μM 2-APB and then incubated with oxLDL (100 μg ApoB/ml) or with native LDL (natLDL, 100 μg ApoB/ml) for 18 hrs. The variation of intracellular [Ca^{2+}] was detected by fluorescence intensity using fluo3/AM (495 and 525 nm excitation and emission wavelength, respectively) as described under 'Materials and methods'. Results were normalized on protein levels and expressed in ratio of untreated control. These results represent the mean ± S.E.M. of three independent experiments. EGTA and 2-APB shifted oxLDL-induced cell apoptosis. SMC/cav1 were pre-treated with 0.35 mM EGTA or 25 μM 2-APB and then incubated with oxLDL (100 μg ApoB/ml) or with native LDL (natLDL, 100 μg ApoB/ml). Lower panel, Time course analysis of oxLDL-induced cell death. SMC/cav1 were incubated with oxLDL (100 μg ApoB/ml) and apoptotic cells were counted after staining with SYTO13/PI at the indicated time as described under 'Materials and methods'. Results are expressed as percentage of untreated control and represent the mean ± S.E.M. of three independent experiments ($^*P < 0.05$). After 18 hrs, cell viability (**B**) was evaluated by the MTT assay and the type of cell death was evaluated by fluorescence microscopy after SYTO-13/PI staining (**C**). The arrowheads indicate nuclei exhibiting the morphological feature of apoptosis or post-apoptotic necrosis. (**D**), Apoptotic cells were counted after staining with SYTO13/PI as described under 'Materials and methods'. Results are expressed as percentage of untreated control and represent the mean ± S.E.M. of three independent experiments ($^*P < 0.05$).

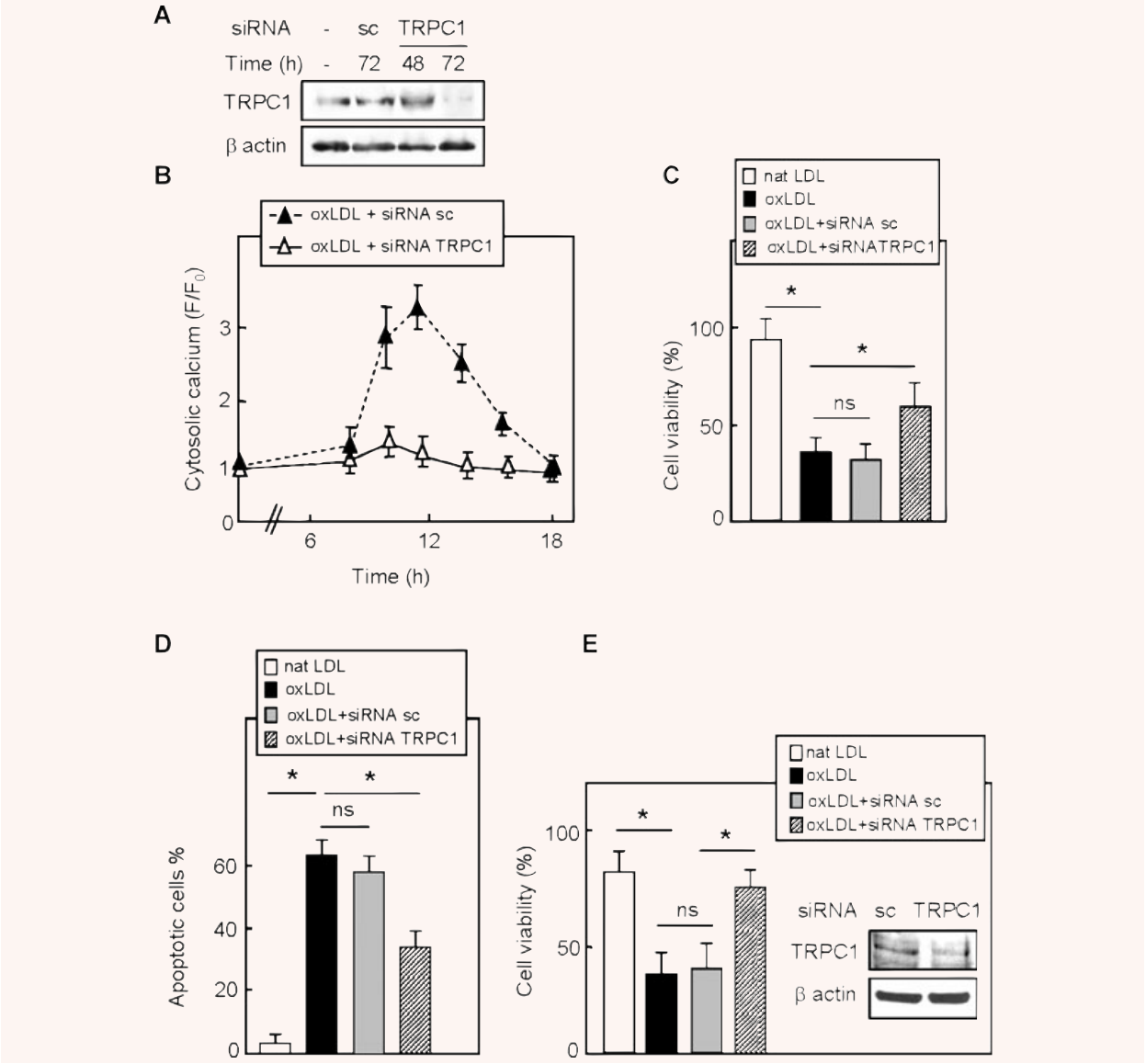

Fig. 2 TRPC1 is involved in oxidized-LDL induced Ca^{2+} rise and apoptosis. TRPC1 expression silencing by siRNA. (**A**), Expression of TRPC1 protein was analysed by immunoblotting using an anti-TRPC1 antibody, as described under 'Materials and methods', after transfection of SMC/cav1 with 100 nM scrambled siRNA (siRNA sc) or 100 nM TRPC1 siRNA (siRNA TRPC1) for 48 and 72 hrs. (**B**), The Ca^{2+} rise induced by oxidized-LDL is mediated by TRPC1. SMC/cav1 transfected with 100 nM siRNA TRPC1 or with 100 nM siRNA scrambled and then incubated with oxLDL (100 μg ApoB/ml). The variation of intracellular [Ca^{2+}] was detected by fluorescence intensity using fluo3/AM (495 and 525 nm excitation and emission wavelength, respectively) as described under 'Materials and methods'. Results were normalized on protein levels and expressed in ratio of untreated control. These results represent the mean ± S.E.M. of three independent experiments. TRPC1 silencing inhibited oxLDL-induced cell apoptosis. SMC/cav1 were transfected with 100 nM scrambled siRNA (siRNA sc) or 100 nM TRPC1 siRNA (siRNA TRPC1) then serum starved and incubated with oxLDL (100 μg ApoB/ml) or with native LDL (natLDL, 100 μg ApoB/ml) for 18 hrs. Cell viability (**C**) was evaluated by the MTT assay and apoptotic cells (**D**) were counted after staining with SYTO13/PI as described under 'Materials and methods'. Results are expressed as the mean ± S.E.M. of three independent experiments ($^*P < 0.05$). (**E**), TRPC1 is involved in oxLDL-induced cell death in human primary VSMC (hSMC). Primary hSMC (passage 5) were either transfected with 100 nM scrambled siRNA (siRNA sc) or 100 nM TRPC1 siRNA (siRNA TRPC1) then serum starved and incubated with oxLDL (100 μg ApoB/ml) or with native LDL (natLDL, 100 μg ApoB/ml) for 18 hrs. Cell viability was evaluated by the MTT assay. Results are expressed as percentage of untreated control and represent the mean ± S.E.M. of three independent experiments ($^*P < 0.05$).

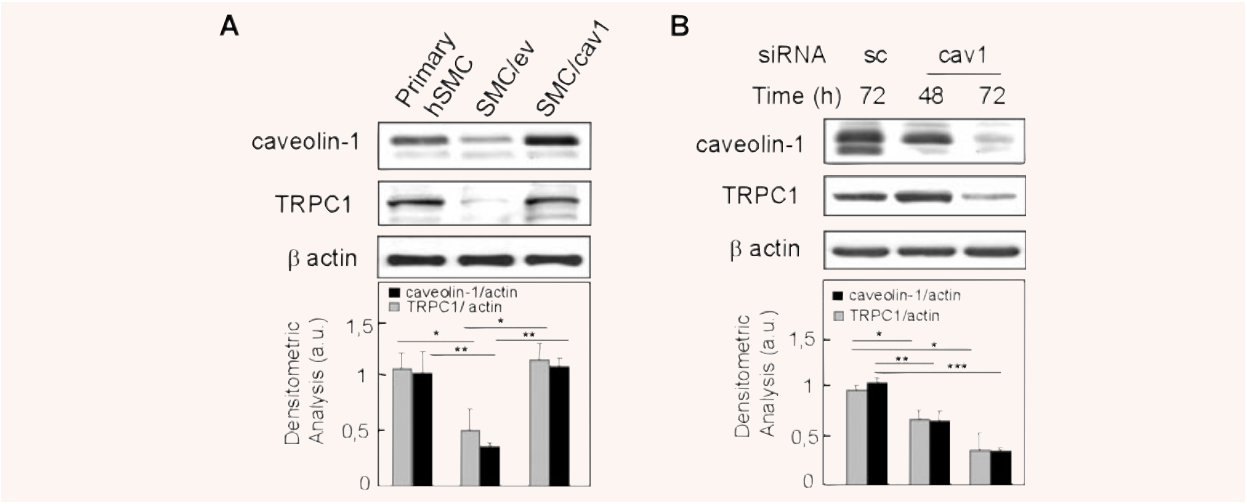

Fig. 3 TRPC1 expression is linked to caveolin-1. TRPC1 expression is correlated with caveolin-1 expression. (**A**), TRPC1 and caveolin-1 expression were analysed in human primary VSMC (hSMC, passage 5), in SMC stably expressing caveolin-1 cell line (SMC/cav1) and in control SMC expressing an empty vector (SMC/ev), by immunoblotting using anti-TRPC1 and anti-caveolin-1 antibodies as described under 'Materials and methods'. The graph represents values of caveolin-1 and TRPC1 band intensity after normalization for β-actin by densitometry (a.u., arbitrary units). Results are representative of at least three independent experiments. ($*P < 0.05$, $**P < 0.01$). (**B**), TRPC1 expression under caveolin-1 silencing. Expression of TRPC1 protein was analysed by immunoblotting using an anti-TRPC1 antibody as described under 'Materials and methods', after transfection of SMC/cav1 with 100 nM caveolin-1 siRNA (siRNA cav1) for 48 and 72 hrs or 100 nM scrambled siRNA (siRNA sc). The graph represents values of caveolin-1 and TRPC1 band intensity after normalization for β-actin by densitometry (a.u., arbitrary units). Results are representative of at least three independent experiments. ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

observed after 8 hrs oxLDL stimulation (Fig. 6A, upper panel). Accordingly to our hypothesis, we also measured a reduction in oxLDL-induced apoptosis after cytochalasin D treatment (Fig. 6B). In control experiments, we showed the integrity of cell membranes upon 8 hrs oxLDL and cytochalasin D treatment (Fig. 6A, lower panel), and we confirmed that cytochalasin D did not interfere with oxLDL uptake as measured by oxLDL labelled with the fluorescent lipid dye 3,3′-dioctadecyl-indocarbocyanine (Fig. 6C).

Because recent reports have shown that 7-ketocholesterol [18] or cholesterol [23] induce TRPC1 redistribution to raft fractions, and because oxLDL contain oxysterols (including 7-ketocholesterol) and can induce cholesterol changes [24], we investigated whether, in our conditions, mildly oxLDL may alter the level of these compounds in subcellular fractions, and more specifically in caveolar fractions. Mildly oxLDL used here induced no appreciable change in the cholesterol content of the three density gradient fractions (Fig. 7A), but increased by 300% the level of 7-ketocholesterol in the light-density L caveolar fractions (Fig. 7B).

Discussion

OxLDL-induced apoptosis of vascular cells may contribute to the erosion and instability of atherosclerotic plaques, thereby increasing the risk of subsequent thrombotic events. Various signalling pathways are involved in oxLDL-induced apoptosis [2, 25], but a sustained and intense Ca^{2+} signal plays a prominent role in triggering the mitochondrial apoptotic pathways through calpain activation [4]. We have previously shown that VSMC overexpressing caveolin-1 were more susceptible to oxLDL-induced apoptosis, and this was correlated with enhanced Ca^{2+} entry and pro-apoptotic events [7]. However, the molecular events involved in oxLDL-induced Ca^{2+} influx remain to be elucidated.

In this study, we report, for the first time, that TRPC1 is involved in oxLDL-induced Ca^{2+} influx and apoptosis of VSMC. Concerning the mechanisms of TRPC channels activation, there is evidence that they can be activated by stimulation of trafficking to the plasma membrane, or by depletion of intracellular Ca^{2+} stores, or by cell signalling involving for instance phospholipase C products (generally diacyglycerols) [26].

To date, few studies have linked TRPC channels to vascular cells apoptosis and vascular damage. But additional evidence for a role of TRPC channels in regulating cell death has been recently described in other cell types. For instance, TRPC1 expression plays a role in Ca^{2+} influx and apoptosis induced by staurosporine in intestinal cells [12]. The overexpression of TRPC3 increases apoptosis of mouse cardiomyocytes in response to ischaemia reperfusion [13]. In addition, other members of the TRP family channels, TRPL, TRPV1 and TRPM8 proteins, have been used to try to selectively kill cancer cells by enhancing the entry and

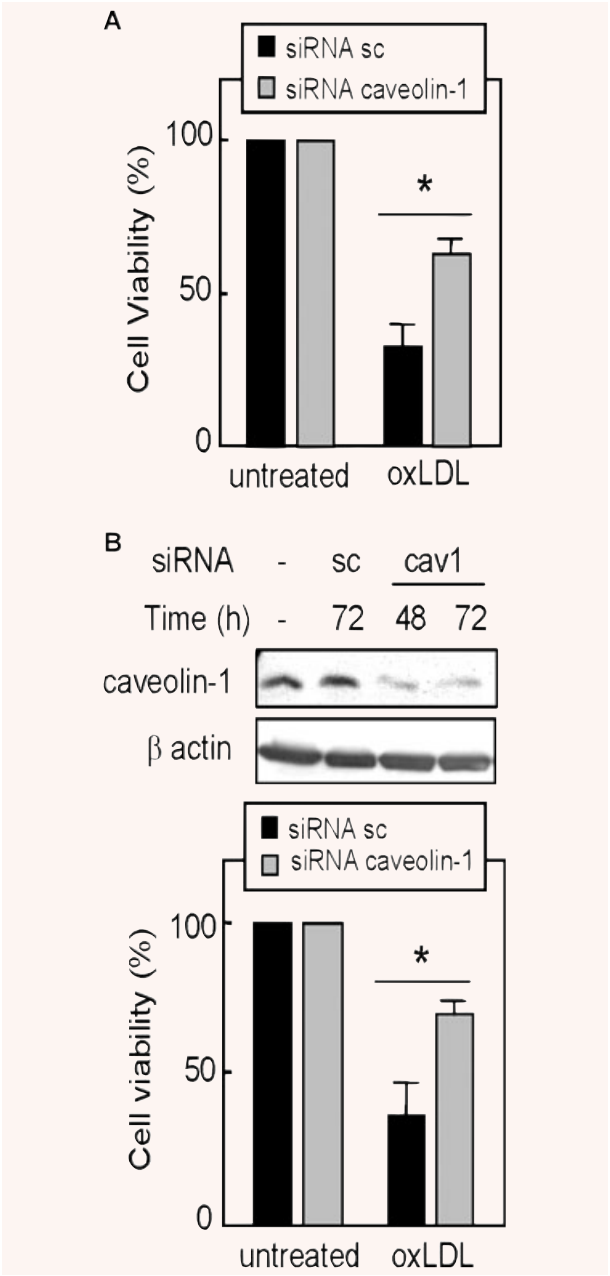

Fig. 4 Caveolin-1 silencing reduced oxLDL-induced cell death. Caveolin-1 silencing shifted oxLDL-induced cell apoptosis. SMC/cav1 (**A**) or human primary VSMC (passage 5) (**B**) were either transfected with 100 nM caveolin-1 siRNA (siRNA cav1) or with 100 nM scrambled siRNA (siRNA sc) then serum starved, and incubated with oxLDL (100 μg ApoB/ml) for 18 hrs. Expression of caveolin-1 protein was analysed by immunoblotting using an anti-caveolin-1 antibody as described under 'Materials and methods' and cell viability was evaluated by the MTT assay. Results are expressed as percentage of untreated control and represent the mean ± S.E.M. of three independent experiments ($*P < 0.05$).

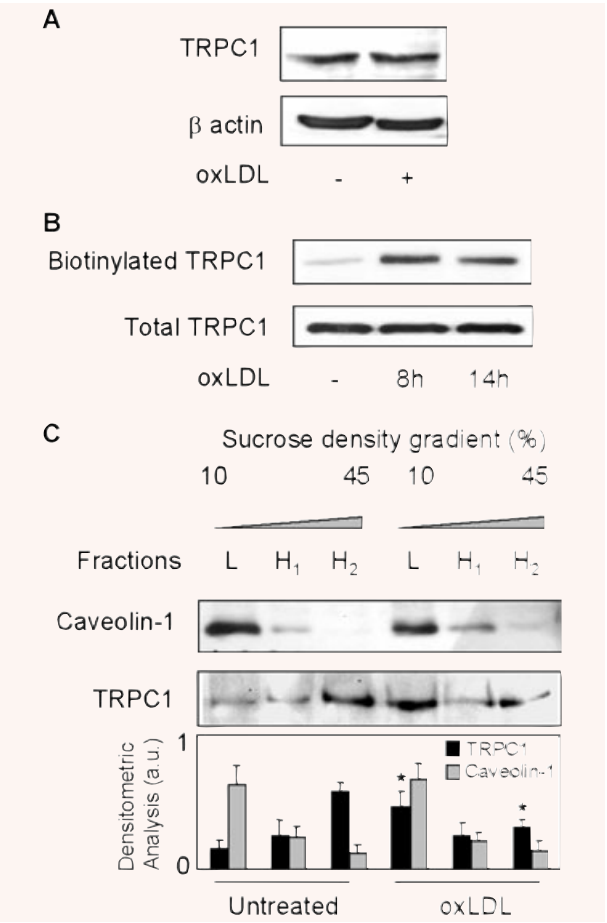

Fig. 5 OxLDL induce translocation of TRPC1 into caveolar compartment. (**A**), TRPC1 expression after oxLDL treatment. SMC/cav1 were incubated with oxLDL (100 μg ApoB/ml) for 10 hrs and expression of TRPC1 protein was analysed by immunoblotting using an anti-TRPC1 antibody as described under 'Materials and methods'. Results are representative of at least three independent experiments. (**B**), Increase in surface-expressed TRPC1 induced by oxLDL. SMC/cav1 were incubated with oxLDL (100 μg ApoB/ml) for 8 or 14 hrs, then treated with sulfo-NHS-SS-Biotin (1 mg/ml) and lysed. Biotinylated proteins were recovered as described under 'Materials and methods', and TRPC1 expression analysed by immunoblotting using an anti-TRPC1 antibody. Total lysates were analysed with TRPC1 antibody to insure for equal loading of proteins. Results are representative of at least three independent experiments. (**C**), Translocation of TRPC1 into caveolae membranes upon oxLDL stimulation. SMC/cav1 were treated with oxLDL (100 μg ApoB/ml), after 8 hrs, cells were lysed in carbonate buffer and subjected to sucrose gradient sedimentation, as described under 'Materials and methods'. A total of 50 μl of pooled fractions (4, 5 and 6 = L, light-density fractions); 7, 8 and 9 = H_1, high-density fractions; 10, 11 and 12 = H_2, high-density fractions) were used for the detection of TRPC1 and caveolin-1 by immunoblotting using anti-TRPC1 and anti-caveolin-1 antibodies. The graph represents values of caveolin-1 and TRPC1 band intensity by densitometry (a.u., arbitrary units). Results are representative of three independent experiments. ($*P < 0.05$, significantly different mean expression value from untreated conditions).

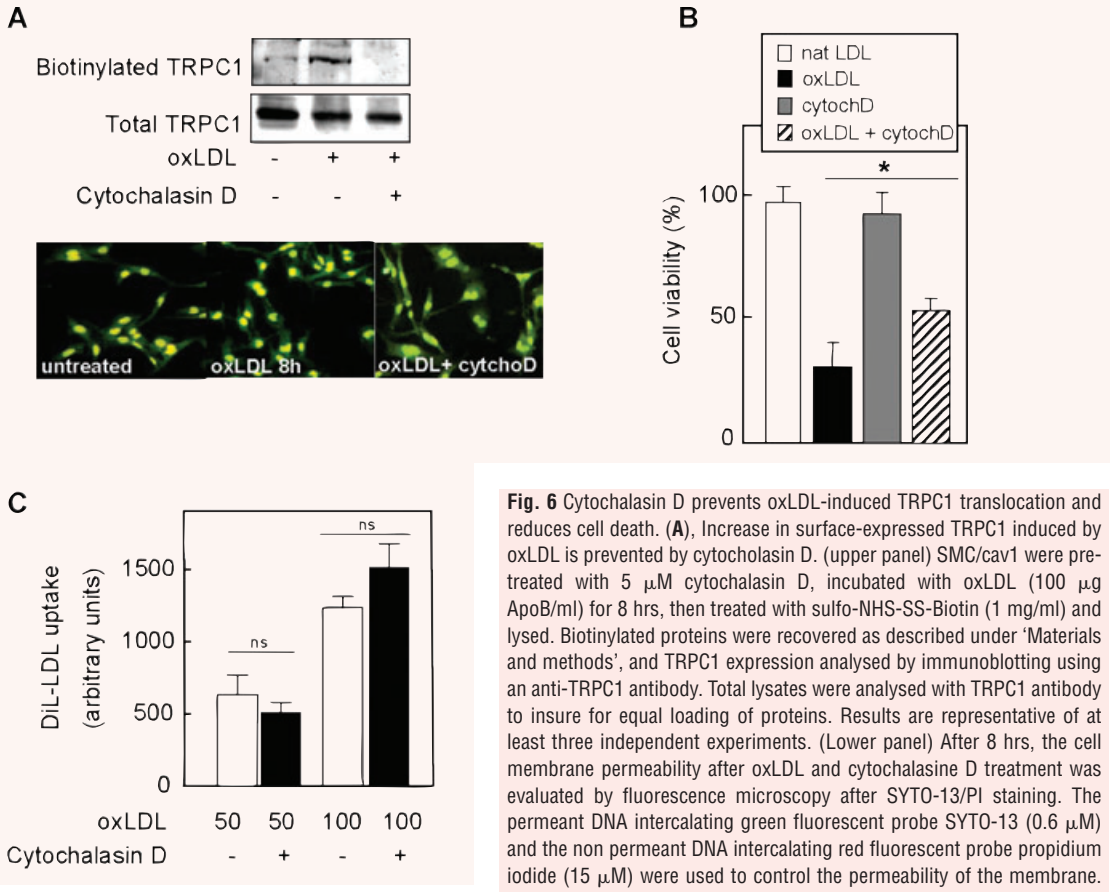

Fig. 6 Cytochalasin D prevents oxLDL-induced TRPC1 translocation and reduces cell death. (**A**), Increase in surface-expressed TRPC1 induced by oxLDL is prevented by cytocholasin D. (upper panel) SMC/cav1 were pre-treated with 5 μM cytochalasin D, incubated with oxLDL (100 μg ApoB/ml) for 8 hrs, then treated with sulfo-NHS-SS-Biotin (1 mg/ml) and lysed. Biotinylated proteins were recovered as described under 'Materials and methods', and TRPC1 expression analysed by immunoblotting using an anti-TRPC1 antibody. Total lysates were analysed with TRPC1 antibody to insure for equal loading of proteins. Results are representative of at least three independent experiments. (Lower panel) After 8 hrs, the cell membrane permeability after oxLDL and cytochalasine D treatment was evaluated by fluorescence microscopy after SYTO-13/PI staining. The permeant DNA intercalating green fluorescent probe SYTO-13 (0.6 μM) and the non permeant DNA intercalating red fluorescent probe propidium iodide (15 μM) were used to control the permeability of the membrane. Green labelled living cells confirm that upon 8 hrs oxLDL and cytochalasin D treatment cell membranes are not permeable to propidium iodide labelling. (**B**). OxLDL-induced apoptosis is reduced by cytochalasin D. Apoptotic cells were counted after staining with SYTO13/PI as described under 'Materials and methods'. Results are expressed as the mean ± S.E.M. of three independent experiments (*$P < 0.05$). (**C**), Uptake of oxLDL following cytochalasin D pre-treatment. SMC/cav1 were incubated with oxLDL labelled with 3,3′-dioctadecyl-indocarbocyanine (DiI) as indicated in 'Materials and methods' at the indicated concentrations (50 or 100 μg apoB/ml). Alternatively cells were pre-incubated for 1 hr with cytochalasin D (5 μM) then stimulated with oxLDL (50 or 100 μg apoB/ml). After 8 hrs, DiI fluorescence was measured by spectrofluorometry, the amount of oxLDL internalized are expressed in arbitrary units. Results represent mean ± S.E.M. of three independent experiments.

intracellular concentration of Ca^{2+} and Na^{+}, thereby inducing apoptosis and necrosis [27]. However, in contrast to these reports showing that TRPC contribute to increase cell death, a recent study indicate that TRPC3 and TRPC6 play a role in promoting neuronal survival in response to serum deprivation [28]. Taken together with our results, these works support the concept that TRPC channels play a critical role in regulating the cell fate.

There is growing body of evidence that caveolae are important Ca^{2+} entry sites at the plasma membrane [29]. We have previously shown that the modification of caveolin-1 expression, a major component of caveolae, modulates Ca^{2+} influx and cell death induced by oxLDL in VSMC [7]. Moreover, recent studies have demonstrated that TRPC1 is assembled in a complex with caveolin-1 and interacts with the scaffolding domain of caveolin-1. Brazer *et al.* [10], showed that expression of a truncated caveolin-1 (Cav1Δ51–169) disrupted plasma membrane localization of TRPC1 and suppressed thapsigargin- and carbachol-stimulated Ca^{2+} influx. In addition, in agreement with our results, Zhu *et al.* recently reported that overexpression of caveolin-1 increased store-operated activity, while knockdown of caveolin-1 significantly reduced store-operated activity [30]. These results support our findings that link the expression of caveolin-1 with the level of TRPC1 (present data) and with the higher oxLDL-induced Ca^{2+} influx [7].

Furthermore, we report for the first time that oxLDL induce the translocation of TRPC1 channel from an intracellular compartment to the cell surface, and, more specifically, to caveolae. The cell surface location of TRPC1 is required for both Ca^{2+} entry and apoptosis induced by oxLDL in VSMC (a schematic diagram is

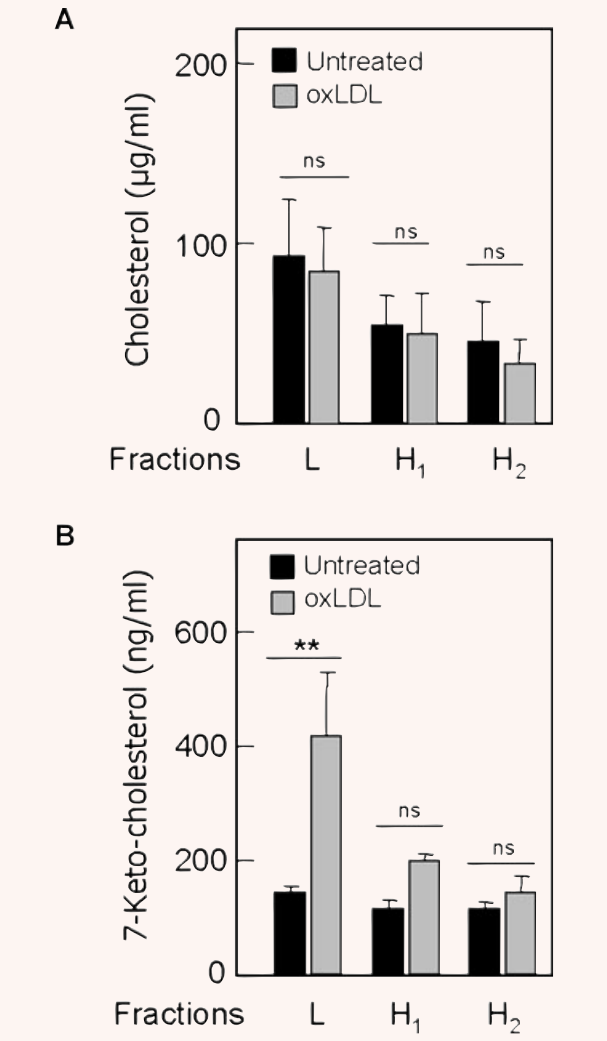

Fig. 7 The 7-ketocholesterol levels increase in caveolar fractions after oxLDL treatment. SMC/cav1 were treated with oxLDL, lysed in carbonate buffer and subjected to sucrose gradient sedimentation, as described under 'Materials and methods'. Pooled fractions (4, 5 and 6 = L, light-density fractions); 7, 8 and 9 = H_1, high-density fractions; 10, 11 and 12 = H_2, high-density fractions) were analysed by GC/MS for their cholesterol (**A**) and 7-ketocholesterol (**B**) content. Results represent mean ± S.E.M. of three independent experiments, $^{**}P < 0.01$.

proposed in Fig. 8). The oxLDL-induced translocation of TRPC1 is consistent with several studies indicating that TRPC channels translocate to the plasma membrane upon stimulation [31, 32]. In a large number of cells, TRPC appears to be present intracellularly maybe in mobile intracellular vesicles that traffic to the membrane following agonist stimulation. For example, TRPC5 translocates from intracellular vesicles to the plasma membrane upon EGF stimulation [33], an increased surface expression of TRPC1 is observed in response to thrombin stimulation of endothelial cells [34], and epoxyeicosatrienoic acids induce the intracellular translocation of TRPC6 to caveolar membranes [35]. Plasma membrane expression of TRPC1 is also determined by caveolin-1 although it is unclear whether TRPC1 trafficking to the plasma membrane is regulated by caveolin-1. Local changes in the cytoskeleton or microtubules can contribute to channel trafficking and facilitate plasma membrane insertion [22]. It has been shown that stabilization of cortical actin stimulates internalization of TRPC3 [36], and that spatial rearrangement of actin filaments promoted the association of IP3 receptor with TRPC1, the complex translocation to the plasma membrane and the Ca^{2+} entry [34]. In agreement with these data, our results showed for the first time that oxLDL-induced TRPC1 translocation to caveolar compartment involves actin cytoskeleton rearrangements. Thus, regulated trafficking of TRPC1 channels are emerging as crucial mechanisms that determine their surface expression and signals-induced activation.

Another intriguing question is to understand how oxidized LDL trigger TRPC1 translocation. As native LDL or modified (acetylated) LDL do not induce Ca^{2+} entry and apoptosis [7], it was excluded that both the apoB/E receptor and the scavenger receptors may transduce the signal leading to the Ca^{2+} rise. Moreover, the toxicity of mildly oxLDL is linked to the oxidized lipid fraction [19] that contain oxysterols. Because 7-ketocholesterol [18] and cholesterol excess [23] can induce TRPC1 redistribution to raft fractions, we investigated whether mildly oxLDL may alter the level of these compounds in subcellular fractions. The caveolar fraction of cells treated by oxLDL is notably enriched in 7-ketocholesterol, which may play the same role as exogenous 7-ketocholesterol [18]. This suggests that insertion of 7-ketocholesterol in caveolar membrane alter the intracellular trafficking of TRPC1, but the precise mechanism by which oxysterol or cholesterol activates TRPC1 translocation remains unknown. Interestingly, the cholesterol content is not altered in the caveolar fraction of cells treated with mildly oxLDL, thus suggesting that the caveolae cholesterol content is not the mediator of TRPC1 translocation by oxLDL. These data are in contrast to those of Blair *et al.* [24], who showed that extensively oxLDL causes depletion of caveolae cholesterol in endothelial cells leading to eNOS redistribution and attenuated capacity to activate the enzyme. This discrepancy may be explained by the difference in the level of LDL oxidation: mildly oxLDL contained 4–7 nmol TBARS /µg apoB, whereas the TBARS level was 15–20 nmol /µg apoB in extensively oxLDL). In addition, pre-incubation of VSMC with cyclodextrin or filipin, which extracts and disturbs the cholesterol content of plasma membrane or caveolae (as do extensively oxLDL), and potentiates the toxicity of mildly oxLDL (data not shown), in agreement with recent data on cancer cells [37]. However, further studies are required to identify the determinants of TRPC1 trafficking and targeting and how they are routed to specific subcellular domains like caveolae.

In conclusion, the current data identify for the first time TRPC1 channels as key molecules in the oxLDL-induced cytosolic Ca^{2+} rise and apoptosis of VSMC. This new emerging function of TRPC channels makes them potential targets for drugs modulating

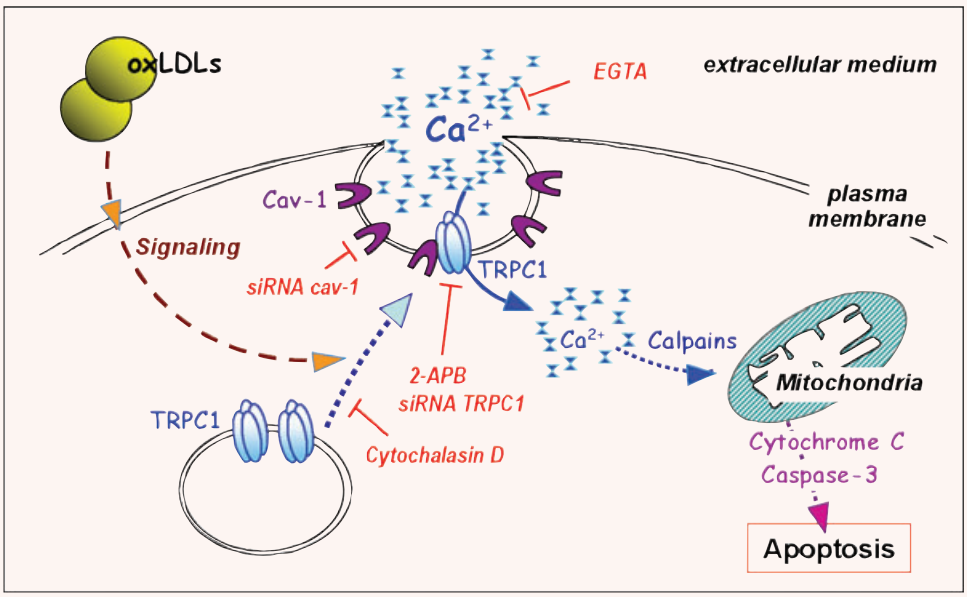

Fig. 8 Role of TRPC1 and caveolin-1 in oxLDL-induced apoptosis. TRPC1 is involved in the Ca^{2+} influx and subsequent activation of the mitochondrial apoptotic pathway. Toxic concentrations of oxLDL induce TRPC1 translocation towards caveolae/caveolin-1 compartment that trigger the activation of TRPC1 leading to a sustained Ca^{2+} influx and cytosolic Ca^{2+} rise. This elicits calpain activation, bid cleavage and the activation of the apoptotic mitochondrial pathway. The inhibitors used here and their site of action are indicated in red.

apoptosis. Furthermore, we shed new light on the role of oxLDL and some constituents (*i.e.* oxysterols) as important regulators of TRPC1 trafficking to the plasma membrane.

Our study also provides new evidence on the role of caveolae/caveolin-1 structures as organizing platform that regulate the apoptotic signalling pathway induced by oxidized lipids. Further delineation of the role of caveolin-1 and TRPC1 in the multiple signalling pathways induced by oxidized lipids will be of considerable value to the understanding of the molecular mechanisms involved in atherosclerotic plaque instability and rupture.

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