Role of protein kinase C δ in ER stress and apoptosis induced by oxidized LDL in human vascular smooth muscle cells

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During atherogenesis, excess amounts of low-density lipoproteins (LDL) accumulate in the subendothelial space where they undergo oxidative modifications. Oxidized LDL (oxLDL) alter the fragile balance between survival and death of vascular smooth muscle cells (VSMC) thereby leading to plaque instability and finally to atherothrombotic events. As protein kinase C δ (PKCδ) is pro-apoptotic in many cell types, we investigated its potential role in the regulation of VSMC apoptosis induced by oxLDL. We found that human VSMC silenced for PKCδ exhibited a protection towards oxLDL-induced apoptosis. OxLDL triggered the activation of PKCδ as shown by its phosphorylation and nuclear translocation. PKCδ activation was dependent on the reactive oxygen species generated by oxLDL. Moreover, we demonstrated that PKCδ participates in oxLDL-induced endoplasmic reticulum (ER) stress-dependent apoptotic signaling mainly through the IRE1α/JNK pathway. Finally, the role of PKCδ in the development of atherosclerosis was supported by immunohistological analyses showing the colocalization of activated PKCδ with ER stress and lipid peroxidation markers in human atherosclerotic lesions. These findings highlight a role for PKCδ as a key regulator of oxLDL-induced ER stress-mediated apoptosis in VSMC, which may contribute to atherosclerotic plaque instability and rupture.

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Atherosclerosis is a slow degenerative process and is the underlying cause of heart attacks, strokes, and peripheral artery diseases in humans. This complex disorder is characterized by the focal accumulation of lipids and the remodeling of the arterial wall, leading to the formation of the atherosclerotic plaque. Modified lipoproteins, specially oxidized low-density lipoproteins (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.2,3 The balance between aberrant proliferation and apoptosis is responsible for mediating intense changes in the development of atherosclerosis. Apoptosis of vascular smooth muscle cells (VSMC) increases as atherosclerotic plaques develop and is sufficient to induce features of plaque vulnerability in atherosclerosis.4 Overall, loss of VSMC is detrimental for plaque stability and increases the risk of thrombotic events.

The apoptotic signaling triggered by oxLDL is mediated through a complex sequence of signaling events that lead to activation of caspase-dependent or caspase-independent apoptotic pathways. We previously reported that treatment of human VSMC with oxLDL induced a sustained rise of cytosolic calcium, leading to the activation of the intrinsic mitochondrial apoptotic pathway.5,6 More recently, we showed in human vascular endothelial cells an interaction between the deregulation of cytosolic calcium and the endoplasmic reticulum (ER) stress in triggering the apoptotic response induced by oxLDL.7 ER plays an essential role in sensing cellular stress (i.e., accumulation of misfolded proteins, potential redox or calcium deregulation) as it rapidly detects changes in cell homeostasis, and responds by eliciting UPR (unfolded protein response) via the activation of ER transmembrane sensors, PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase), IRE1α (inositol-requiring 1α) and ATF6 (activating transcription factor 6). The UPR results in a temporary downregulation of protein translation, an upregulation of ER chaperones and folding machinery, and the expression and activation of ER-associated degradation (ERAD).8 Prolonged ER stress switches towards apoptotic cell death via the activation of downstream signals like CHOP (C/EBP homologous protein), JNK and members of the Bcl-2 family.9,10 Our previous data11 and

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Abbreviations: PKCδ, protein kinase C δ; oxLDL, oxidized low-density lipoprotein; ER stress, endoplasmic reticulum stress; VSMC, vascular smooth muscle cells; UPR, unfolded protein response; ROS, reactive oxygen species

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those from Myoishi et al. demonstrated that ER stress markers are present in human advanced atherosclerotic lesions thus raising the question of the possible role of ER stress in the stability/instability of atherosclerotic plaques, since this adaptive response may influence the fate of cells to survive or die.

Protein kinase C-δ (PKCδ), a member of the PKC family of serine–threonine kinases, is known to be a critical pro-apoptotic signal in many cell types. More particularly, PKCδ-deficient mice develop exacerbated intimal hyperplasia associated with diminished SMC apoptosis in vein grafts and carotid ligation models, indicating that PKCδ is an important regulator of SMC apoptosis after vascular injury. Recently, it has been shown that PKCδ plays a crucial role in the propagation of TNFα-induced ER stress-mediated JNK activation and CHOP/GADD33 induction. Altogether, whether PKCδ contributes to oxLDL-induced vascular SMC apoptosis and ER stress is not known.

Here, we investigated the possible involvement of PKCδ in the apoptotic signaling pathway triggered by oxLDL and its role in the transmission of the pro-apoptotic signal of the ER stress in human VSMC. We found that oxLDL mediate PKCδ activation through reactive oxygen species (ROS) production and that PKCδ plays a crucial role in the regulation of oxLDL-induced apoptosis mainly through the IRE1α/JNK pathway of ER stress. Importantly, we provided evidence that activated PKCδ is colocalized with ER stress and lipid peroxidation markers in human atherosclerotic lesions.

Results

SiRNA-mediated suppression of PKCδ expression reduces oxLDL-induced human vascular smooth muscle apoptosis. We first investigated the involvement of PKCδ in the apoptosis of human vascular smooth muscle (hVSMC) cells treated with oxLDL. The expression of PKCδ was silenced by small interfering RNA (siRNA) specific to human PKCδAs shown in Figure 1a. The inhibitory effect of siRNA on PKCδ expression was obvious 48 h after transfection and was not influenced by treatment with oxLDL. To assess whether the effect of PKCδ knockdown relates to hVSMC survival, PKCδ knockdown (i) and control cells were treated with oxLDL for 24 h. PKCδ knockdown cells displayed a protection towards oxLDL-induced apoptosis as demonstrated by a significant decrease in cell death (Figure 1b).

The involvement of caspase-3 is supported by the protective effect of the multicaspase inhibitor z-VAD-fmk against oxLDL-induced apoptosis (Figure 1c). We also showed the release of cytochrome C from the mitochondria, which is accompanied by an increased expression of the pro-apoptotic protein Bak and a decreased expression of the pro-survival protein Bcl-2 in agreement with the data of Yang et al. (Supplementary Figure S1). The activation of caspase-3 is prevented in PKCδ knockdown cells as shown by the inhibition of its cleavage, compared with control cells (Figure 1d).

We further demonstrated the involvement of PKCδ in the apoptosis induced by oxLDL by the use of mouse embryonic fibroblasts (MEF) invalid for PKCδ MEF PKCδ−/− that showed a strong resistance to oxLDL-induced cell death as explored by cell viability assay, apoptotic characteristics, Bcl-2 expression, cytochrome C release and caspase-3 cleavage compared with MEF PKCδ cells (Figures 2a-c). Moreover, the central role of PKCδ in the broad regulation of apoptosis is supported by the protection of MEF PKCδ−/− cells towards the apoptotic inducer antimycin A (Figure 2a).

The resistance to apoptosis in PKCδ−/− cells likely results from the loss of PKCδ expression, to prove that apoptosis is directly dependent on PKCδ we asked if the re-expression of PKCδ is sufficient to restore the apoptotic response induced by oxLDL.

MEF PKCδ−/− cells were transduced with adenovirus expressing either GFP (AdGFP) or a PKCδ-GFP (AdPKCδ-GFP) fusion protein (Figure 3a). As shown in Figure 3b, transduction of AdPKCδ-GFP completely re-establishes their apoptotic response to oxLDL, whereas PKCδ−/− cells transduced with AdGFP remained resistant to oxLDL-induced apoptosis. Our data clearly demonstrate that re-expression of PKCδ in PKCδ−/− cells reconstitute apoptotic potential. Altogether, our results indicate that PKCδ plays a major role in the apoptosis induced by oxLDL.

PKCδ is activated in response to oxLDL stimulation in human vascular smooth muscle. The ability of PKCδ to activate an apoptotic program is regulated by key events such as phosphorylation on specific tyrosine residues and nuclear accumulation where it may be cleaved by caspase to generate a pro-apoptotic PKCδ catalytic fragment (δCF). We analyzed the phosphorylation of PKCδ on tyrosine 311 because (i) this critical residue located in the catalytic domain is phosphorylated in response to apoptotic stimuli such as oxidative stress induced by hydrogen peroxide and because (ii) oxLDL treatment generates an oxidative stress through the production of hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻). To examine the effect of oxLDL on PKCδ tyrosine 311 phosphorylation, hVSMC were treated with increasing concentrations of oxLDL (0–200 μg ApoB/ml) for 5 h or with cytotoxic concentration of oxLDL (200 μg ApoB/ml) for 1–8 h. As shown in Figure 4a, the phosphorylation level of tyrosine 311 increases with the concentration of oxLDL and with the length of time treatment being visible at 5 h stimulation. We then followed the nuclear translocation of PKCδ by two methods: cell fractionation and fluorescence microscopy. After hVSMC treatment with oxLDL, the nuclear and cytosolic fractions were separated by differential centrifugation. We found that in untreated cells, PKCδ was expressed in the cytosol and translocated to the nucleus beyond 12 h of oxLDL stimulation (Figure 4b). Using immunofluorescence microscopy, we confirmed that PKCδ was located mostly in the cytosol in untreated control cells and that oxLDL induced the translocation of PKCδ to the nucleus in about 90–95% of the cells as observed following 18 h treatment with oxLDL (Figure 4c). Collectively, these data show that oxLDL induce the activation of PKCδ to trigger hVSMC apoptosis.

ROS generated by oxLDL contribute to the activation of PKCδ. We then questioned the mechanisms involved in oxLDL-induced PKCδ activation. We previously showed that oxLDL trigger an intracellular rise in ROS in rabbit smooth muscle cells. Therefore, we investigated whether oxLDL-
induced ROS generation is involved in PKCδ tyrosine 311 phosphorylation. Incubation of hVSMC with oxLDL but not with nLDL (data not shown) resulted in a rapid increase of intracellular ROS as assessed by the rise of DCF fluorescence (Figure 5a). OxLDL-mediated ROS production is prevented by preincubation with the NADPH oxidase inhibitor 3-benzyl-7-[(2-benzoxazolyl)thio]-1,2,3-triazolo[4,5-d]pyrimidine (VAS2870, 10 μM) and with the addition of a cell permeable form of the antioxidant enzyme catalase (PEG-catalase 50 UI/ml). Indeed, addition of PEG-conjugated enzyme produces an increase in plasma membrane fluidity, thus enhancing cell association and uptake.22 These data suggest that NADPH oxidases play a major role in the generation of ROS by oxLDL and that H₂O₂ produced through the dismutation of O₂⁻ might be the mediator of ROS-induced cell signaling. We further analyzed the inhibitory efficiency of VAS2870 and PEG-catalase on the tyrosine 311 phosphorylation of PKCδ. As shown in Figure 5b, both antioxidants were able to prevent oxLDL-induced PKCδ phosphorylation in hVSMC. These findings demonstrate that oxLDL generate intracellular ROS responsible for PKCδ activation. Moreover, the inhibitory effect of VAS2870 suggests that ROS production depends on the activity of NADPH oxidase in hVSMC.

The UPR is induced upon oxLDL stimulation and the activation of the ER stress-dependent IRE1/JNK pathway is dependent on PKCδ expression. It has been
recently shown that PKCδ participates in ER stress-induced apoptosis in mouse neuroblastoma cells. To determine whether UPR is induced in hVSMC following oxLDL treatment, we investigated the activation of the three classical ER sensors: PERK, IRE1α, and ATF6. Our data showed the phosphorylation of the PERK substrate eIF2α and the nuclear translocation of ATF6 upon oxLDL stimulation in hVSMC (Figures 6a and b). Because oxLDL triggered a prolonged ER stress activation, which may have a role in apoptotic cell death through IRE1α-TRAF2-JNK pathway and CHOP expression, we checked the activation of these proapoptotic pathways in hVSMC. OxLDL elicited the activation of JNK and the expression of CHOP protein (Figure 6c), which is in agreement with our previous studies showing the contribution of these two ER stress proapoptotic mediators in oxLDL-induced human endothelial cell apoptosis. We provided further evidence for the role of the ER stress induced by oxLDL in the apoptosis of hVSMC by showing the induction of PUMA (p63 upregulated modulator of apoptosis) and BIM (BCL-2 interacting mediator of cell death), two pro-apoptotic BH3 domain-only proteins regulated by CHOP in response to ER stress (Figure 6d).

We next explored whether PKCδ plays a role in oxLDL-induced ER stress signaling. We observed the phosphorylation of the PERK substrate eIF2α and the nuclear translocation of ATF6 in MEK PKCδ−/− and PKCδ−/− cells following oxLDL treatment (Figures 7a and b). On the other side, in PKCδ−/− cells oxLDL induced the expression of IRE1α and CHOP, and JNK activation whereas PKCδ−/− cells treated with oxLDL displayed neither increased expression of IRE1α nor JNK activation but showed an increased expression of CHOP (Figures 7a and c).

We then asked whether PKCδ was necessary for the induction of the UPR. MEF PKCδ−/− and PKCδ−/− cells were stimulated by thapsigargin a potent inducer of ER stress. The activation of the UPR and the ER stress proapoptotic mediators was observed in cells expressing or not PKCδ.

Figure 2  MEF PKCδ−/− are protected from oxLDL-induced apoptosis. (a) Analysis of cell toxicity in MEF PKCδ−/− and MEF wild-type (named PKCδ+/−) was evaluated by the MTT assay. MEF PKCδ and MEF PKCδ+/+ were treated with oxLDLs (50–200 g ApoB/ml), native LDL (nLDL, 100 μg ApoB/ml) or antiinhibitor A (10 μM) for 24 h and cell toxicity was analyzed as described. Results are expressed as percentage of untreated control and represent the mean ± S.E.M. of five separate experiments. **P < 0.01 and *P < 0.05 indicate significance (comparison were made between PKCδ−/− and PKCδ+/+ treated with 100 and 200 oxLDL μg ApoB/ml or 10 μM antiinhibitor A), ns indicates no significance. (b) SYTO-13/PI staining of MEF PKCδ−/− and MEF PKCδ+/+ treated or not with oxLDL (200 μg ApoB/ml, for 24 h), the images illustrate the resistance of MEF PKCδ−/− towards oxLDL-induced apoptosis. (c) Time-course analysis of Bcl-2 expression in MEF PKCδ−/− and MEF PKCδ+/+ treated with oxLDL (200 μg ApoB/ml). Immunoblots representative of three independent experiments were performed on cell lysates using anti-Bcl-2 antibody and β-actin was used as a loading control. (d) Immunocytochemistry experiments showing the release of the cytochrome C monitored by immunofluorescence in MEF PKCδ−/− and MEF PKCδ+/+ treated with oxLDL (200 μg apoB/ml) 16 h. Cells were fixed and labeled with anti-cytochrome C antibody. The results are representative of three separate experiments. (e) Representative western blot of time-course analysis of procaspase-3 processing and cleaved caspase-3 generation in MEF PKCδ−/− and MEF PKCδ+/+ treated with oxLDL (200 μg apoB/ml, 16 h). Immunoblots representative of three independent experiments were performed on cell lysates using anti-procaspase-3, anti-cleaved caspase-3 antibodies and β-actin was used as a loading control.
lesions and colocalized with PKCδ phosphorylated and the lipid peroxidation marker 4-hydroxynonenal (4-HNE). In normal artery, there was no KDEL or 4-HNE positivity. Furthermore, we confirmed that KDEL-, 4-HNE- and activated PKCδ-positive cells were foams cells (SMC and macrophages) of the necrotic core as shown by immunostaining of serial sections with anti-CD68 antibody. These results support our in vitro data and strongly suggest that PKCδ and ER stress may be activated by oxidized lipids within the atherosclerotic lesions.

**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. In this study, we identified a novel regulatory pathway in oxLDL-induced apoptosis of VSMC. We report for the first time that PKCδ is activated by oxLDL in human VSMC and contributes to oxLDL-induced ER stress-dependent apoptotic signaling through the IRE1α/JNK pathway.

The function of PKCδ depends on the cell type and specific stimulus but currently a large number of studies are consistent with the central role of PKCδ in the regulation of cell apoptosis in response to various apoptotic stimuli. We showed that knockdown of PKCδ expression significantly reduces the effect of oxLDL-induced apoptosis in primary human VSMC and reintroduction of PKCδ into PKCδ knockout cells restores their apoptotic capacity, indicating that PKCδ is sufficient to specifically control the ability of the cells to undergo apoptosis. In addition, the modulation of the expression of pro-apoptotic and pro-survival members of the Bcl-2 family and the reduced release of cytochrome C in PKCδ knockout cells suggests that PKCδ plays a central role in the mitochondria-dependent apoptotic pathway triggered by oxLDL. Altogether our findings corroborate previous results showing that forced higher expression in normal VSMC increased their apoptotic responses to the H2O2. However, it has been demonstrated that SMC isolated from PKCδ knockout mice displayed decreased proliferation compared with wild-type SMC, thus, suggesting that PKCδ exhibits contrasting roles in cell death and cell proliferation. In our experimental conditions, we did not observe such modification in cell viability and cell number in human VSMC knockdown for PKCδ. Therefore, supporting that the proapoptotic function of PKCδ depends on the biological context.

PKCδ is activated by a large array of stimuli including mechanical stress, pro-inflammatory cytokines and oxidative stress, which are known to be associated with vascular remodeling and atherogenesis. The generation of ROS induced by oxLDL raises the question regarding how PKCδ is activated, which may include binding of second messenger diacylglycerol, phosphorylation, membrane translocation and proteolysis. One of the key event involved in the transduction of a death signal to PKCδ is the phosphorylation of PKCδ on tyrosine residues. In this study, we provided evidence that the rise in intracellular ROS triggered the phosphorylation of the tyrosine 311 residue of PKCδ, which have been linked to increased kinase activity and apoptosis in cells treated with H2O2. The inhibitory effect of VAS2870, a well-validated
specific inhibitor of NADPH oxidase (NOX). on oxLDL-induced ROS production and PKCδ translocates from the cytoplasm to the nucleus in response to specific apoptotic stimuli. Indeed, a nuclear localization sequence (NLS) has been defined in PKCδ that is required for its ability to induce apoptosis. As we observed a nuclear localization of PKCδ following oxLDL stimulation, we can hypothesize that oxLDL through the production of ROS, may induce post-translational
experiments lysates were assessed for phospho-JNK, CHOP, BIM and PUMA expression. ER stress pro-apoptotic mediators activation in human VSMC treated with oxLDL (200 μg ApoB/ml). (b) Western blot experiments were performed on total protein extracts, cell lysates were assessed for phospho-eIF2α and IRE1α expression. β-Actin was used as protein loading control. Blots are representative of three independent experiments. (b) Immunocytochemistry experiments show the cytoplasmic and nuclear translocation of ATF6 in human VSMC treated with oxLDL (200 μg ApoB/ml) for 16h. These data are representative of three separate experiments. (c, d) Time course of the ER stress pro-apoptotic mediators activation in human VSMC treated with oxLDL (200 μg ApoB/ml). Western blot experiments were performed on total protein extracts, cell lysates were assessed for phospho-JNK, CHOP, BIM and PUMA expression. β-Actin was used as protein loading control. Blots are representative of three independent experiments.

The physiological importance of PKCδ in the development of atherosclerotic lesions is evidenced by our immunohistochemical analyses as we observed the expression of tyrosine 311 phosphorylated PKCδ in advanced human atherosclerotic lesions. Interestingly, the expression of phosphorylated PKCδ colocalized in atherosclerotic areas containing 4-HNE adducts and ER stress marker such as KDEL-positive cells, thus, suggesting that oxLDL through lipid peroxidation derivatives may locally contribute to trigger PKCδ activation and ER stress. These findings indicate that a similar activation mechanism of PKCδ such as we described in human VSMC exists in the atherosclerotic lesion. Moreover, our results corroborate the work of Yamanouchi et al., which showed a robust expression of PKCδ in apoptotic cells of human restenotic lesions.

Finally, our study identify for the first time PKCδ as a major regulator of oxLDL-induced apoptosis in VSMC, we also provide evidence that ROS generated by oxLDL are responsible for PKCδ activation. Furthermore, the involvement of PKCδ in the transmission of ER stress-dependent apoptotic signaling mainly through the IRE1α/JNK pathway, points out that PKCδ is involved in the fine tuning of apoptosis and raises the question of its role in the stability of atherosclerotic plaque.

Materials and Methods

Reagents. Cell culture reagents were from Invitrogen Life Technologies (Saint Aubin, France). SYTO-13, propidium iodide and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) were from Molecular Probes (Invitrogen). VAS2870 was from Enzo Life Sciences (Villeurbanne, France), PEG-Catalase and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), antimycin A from Sigma-Aldrich (Lyon, France), z-VAD-FMK was from R&D Systems Europe (Lille, France). Thapsigargin is from Calbiochem (Millipore, Saint-Quentin-en-Yvelines, France). Following antibodies were used: anti-phospho-PKCδ, anti-IRE1α, anti-phospho-eIF2α, anti-phospho-JNK, anti-C13, anti-Caspase-3,
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Figure 7 PKCδ is involved in the activation of the pro-apoptotic ER stress IRE1/ JNK pathway but not in CHOP activation. Time course of ER stress sensors activation in MEF PKCδ+/− and MEF PKCδ+/+ treated with oxLDL (200 μg ApoB/mL). (a) Western blot experiments were performed on total protein extracts, cell lysates were assessed for phospho-eIF2α and IRE1α expression. β-actin was used as protein loading control. Blots are representative of three independent experiments. (b) Immunocytochemistry experiments show the cytoplasmic and nuclear translocation of ATF6 in MEF PKCδ−/− and MEF PKCδ+/+ treated with oxLDL (200 μg ApoB/mL) for 16 h. These data are representative of three separate experiments. (c) Time course of the ER stress proapoptotic mediators activation in MEF PKCδ+/− and MEF PKCδ+/+ treated with oxLDL (200 μg ApoB/mL). Western blot experiments were performed on total protein extracts, cell lysates were assessed for phospho-JNKs and CHOP expression. β-actin was used as protein loading control. Blots are representative of three independent experiments.

anti-cleaved caspase-3, anti-Bak, anti-Bim and anti-PUMA were from Cell Signaling Technology (Ozyme, Saint-Quentin-en-Yvelines, France), anti-ATF6 and anti-KDEL from Santa Cruz Biotechnology (Clinisiences, Nanterre, France), anti-cytochrome C from BD Biosciences (Le Pont de Clairoix, France), monclonal anti-β-actin and anti-α-actin from Sigma-Aldrich, anti-4-HNE adducts from Oxis Int (Foster City, CA, USA) and anti-CD68 was from NeoMarkers (Lab Vision, Fremont, CA, USA). Secondary antibodies anti-mouse and anti-rabbit were from Santa Cruz Biotechnology and Alexa fluor 488 was from Molecular Probes (Invitrogen). The ECL chemoluminescence kit was from Amersham Pharmacia (GE Healthcare, Ramonville Saint Agne, France). Hiperfect transfection reagent was from Qiagen (Les Ulis, France).

Cell culture. Human primary VSMC were obtained from human mesenteric arteries at postmortem examinations. All experiments were performed in 96-well plates in a humidified 5% CO2/95% air atmosphere. MEF from PKCδ−/− and wild-type MEF were a generous gift from Pr. Mary E. Reyland (University of Colorado Denver, USA).33 MEF PKCδ+/− and WT were maintained in DMEM supplemented with 10% fetal calf serum at 37 °C in a humidified, 5% CO2/95% air atmosphere.

Adenovirus expression in MEF PKCδ−/−. The generation and the use of Ad PKCδ−GFP and AdGFP have been described previously.33 MEF PKCδ−− were infected with Ad PKCδ−GFP or AdGFP at a multiplicity (focus forming units/cell) of 100. Cells were infected in serum-free DMEM overnight after which the virus containing medium was removed and replaced with normal medium. Infection was allowed to proceed for 24 h before stimulation with oxidized LDL.

LDL isolation and mild oxidation. LDL from human pooled sera were prepared by ultracentrifugation, dialyzed against PBS containing 100 μM EDTA. LDL were mildly oxidized by UV-C – copper/EDTA (5 μM) (oxLDL) as previously reported.34 OxLDL contained 4–7.4 nmol of TBARS (thiobarbituric acid – reactive substances)/μg apoB. Relative electrophoretic mobility (REM) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) reactive amino groups were 1.2–1.3 times and 85–92% of native LDL, respectively.

Nuclear and cytosolic fractionation. Cells were washed once with PBS and lysed in a buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, proteases inhibitors, 0.1% Nonidet P-40, pH 7.9 for 10 min on ice and homogenates were centrifuged at 800 × g at 4 °C for 10 min. The supernatant that contains the cytoplasmic fraction was transferred and saved for western blotting analysis. The nuclear pellet was resuspended in extraction buffer containing 20 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 100 mM NaCl, 26% glycerol (v/v), pH 7.9 for 30 min on ice with vortexing at 10-min intervals. Homogenates were centrifuged for 30 min at 14 000 × g at 4 °C and the supernatant that contains the nuclear fraction was transferred and saved for western blotting analysis.

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 sulfate, 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. In all, 40 μg of protein cell extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes (Millipore). Then membranes were probed with the indicated primary antibodies and revealed with the secondary antibodies coupled to horseradish peroxidase using the ECL chemiluminescence kit. Membranes were then stripped and reprobed with anti-α-actin antibody to control equal loading of proteins.

Evaluation of cytotoxicity, necrosis and apoptosis. For cytotoxicity experiments, cells were serum starved for 24 h and stimulated for the indicated times at 37 °C. Cytotoxicity was evaluated using the MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, as previously used.3 This method is
based on MTT reduction by the respiratory chain and other electron transport systems leading to form non-water-soluble violet formazan crystals that can be determined spectrophotometrically (OD measured at 570 nm) and serves as an estimate for the metabolic activity of living cells. Apoptotic and necrotic cells were counted after fluorescent staining by two fluorescent dyes, the permeant DNA intercalating red fluorescent probe propidium iodide (15 μM) using an inverted fluorescence microscope (Fluovert FU, Leitz, Grand Rapids, MI, USA) as previously described.7 Normal nuclei exhibit a loose green-colored chromatin. Nuclei of primary necrotic cells exhibit loose red-colored chromatin. Apoptotic nuclei exhibited condensed yellow/green-colored chromatin associated with nucleus fragmentation, whereas post-apoptotic necrotic cells exhibited the same morphological features, but were red-colored.

**siRNA transfection.** The selected siRNA specific to human PKCδ were ON-TARGET plus SMART pool siRNA human PKCδ (Dharmacon, Waltham, MA, USA). SiRNAs were transfected using the Hiperfect reagent (Quiagen, Courtaboeuf, France) according to the manufacturer’s recommendations.

**Immunofluorescence.** Human VSMC grown on cover glass slides were washed with PBS and fixed in PBS/4% paraformaldehyde for 10 min. After blocking with PBS containing 3% BSA for 30 min, cells were incubated with the indicated antibodies for 1 h and revealed with Alexa Fluor 488-conjugated secondary antibody for 1 h. The slides were visualized using a Zeiss LSM 510 fluorescence confocal microscope (Le Pecq, France).

**Quantification of intracellular ROS.** The generation of intracellular ROS was estimated using the 6-carboxy-2,7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) ROS-sensitive fluorescent probe (5 μM). Stimulated cells were incubated with the probe 30 min before determination as described.21

**Immunohistochemistry.** Human advanced carotid plaques (patients 70–75 years old) were obtained after endarterectomy (Cardiovascular Surgery Department, CHU Toulouse, France), internal mammary arteries were obtained from patients undergoing coronary artery bypass grafting. Tissues were fixed in formalin and paraffin embedded. Serial 3 μm thin sections were incubated with the anti-phosphotyrosine 311 PKCδ, anti-KDEL, anti-4-HNE-adduct, anti-CD68 and anti-α-actin antibodies, then with appropriate biotin-labeled antibodies, and revealed by using avidin–biotin horseradish peroxidase visualization system (Vectastain, ABC kit Elite, Vector Laboratories, Burlingame, CA, USA). All experiments were confirmed 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.

**Statistical analysis.** Data are given as mean ± S.E.M. Statistical comparison of the data was performed using the t-test for comparison between two groups and is explained in the figure legends (Sigma stat software, San Jose, CA, USA). Values of P < 0.05 were considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.

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1. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witzum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989; 320: 915–924.
2. Napoli C. Oxidation of LDL, atherogenesis, and apoptosis. Ann NY Acad Sci 2003; 1010: 698–709.
3. Salvayre R, Auge N, Benoist H, Negre-Salvayre A. Oxidized low-density lipoprotein-induced apoptosis. Biochim Biophys Acta 2002; 1585: 213–221.
4. Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat Med 2006; 12: 1075–1080.
5. Ingueuene C, Hyuhn-Do U, Thiers JC, Negre-Salvayre A, Salvayre R, Vindia C. Caveolin-1 sensitizes vascular smooth muscle cells to mildly oxidized LDL-induced apoptosis. Biochim Biophys Acta 2007: 1769: 889–893.
6. Ingueuene C, Hyuhn UD, Marchei B, Athias A, Gambert P, Negre-Salvayre A et al. TRPC1 is regulated by caveolin-1 and is involved in oxidized LDL-induced apoptosis of vascular smooth muscle cells. J Cell Mol Med 2009; 13: 1620–1631.
7. Muller C, Salvayre R, Negre-Salvayre A, Vindia C. HDLs inhibit endoplasmic reticulum stress and autophagic response induced by oxidized LDLs. Cell Death Differ 2011; 18: 817–828.
8. Zhang K, Kaufman RJ. From endoplasmic reticulum stress to the inflammatory response. Nature 2008; 454: 455–462.
9. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R et al. CHIP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 2004; 18: 3068–3077.

10. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mot Cell Biol* 2001; 21: 1249–1259.

11. Sanson M, Auge N, Vindis C, Muller C, Bando Y, Thiers JC et al. Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells prevention by oxygen-regulated protein 150 expression. *Circ Res* 2008; 104: 328–3102.

12. Myśliński M, Hao H, Minamino T, Watanabe K, Nishihira K, Hatakeyama K et al. Increased endoplasmic reticulum stress in atherosclerotic plaques associated with acute coronary syndrome. *Circulation* 2007; 116: 1226–1233.

13. Reyland ME. Protein kinase Cdelta and apoptosis. *Biochem Soc Trans* 2007; 35: 1001–1004.

14. Leitges M, Mayr M, Braun U, Mayr U, Li C, Pfister G et al. Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice. *J Clin Invest* 2001; 108: 1505–1512.

15. Yamanouchi D, Kato K, Ryer EJ, Zhang F, Liu B. Protein kinase Cdelta mediates arterial injury responses through regulation of vascular smooth muscle cell apoptosis. *Cardiovasc Res* 2010; 85: 434–443.

16. Greene MW, Ruhoff MS, Burrington CM, Garofalo RS, Orena SJ. TNFalpha activation of PKCdelta, mediated by NFkappaB and ER stress, cross-talks with the insulin signaling cascade. *Cell Signal* 2010; 22: 274–284.

17. Yang H, Chen S, Tang Y, Dai Y. Interleukin-10 down-regulates oxLDL induced expression of scavenger receptor A and Bak-1 in macrophages derived from THP-1 cells. *Arch Biochem Biophys* 2011; 512: 30–37.

18. Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y et al. Increased protein kinase Cdelta cleavage is necessary for apoptosis of vascular smooth muscle cells. *J Biol Chem* 2011; 286: 2634–2644.

19. Stielow C, Catar RA, Muller G, Wingerl K, Scheurer P, Schmidt HH et al. Novel NADPH oxidase of oxLDL-induced reactive oxygen species formation in human endothelial cells. *Biochem Biophys Res Commun* 2006; 344: 200–205.

20. Brodie C, Blumberg PM. Regulation of cell apoptosis by protein kinase c delta. *Apolipoprotein A-I and PKCdelta in atherosclerosis*. *Apolipoprotein A-I and PKCdelta in atherosclerosis*. *Cell Death and Disease* 2011; 2: 121–127.

21. Devries TA, Neville MC, Reyland ME. Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence. *EMBO J* 2002; 21: 6050–6060.

22. Fuji T, Garcia-Bermejo ML, Bernabo JL, Caamano J, Ohba M, Kuroki T et al. Involvement of protein kinase C delta (PKCdelta) in phorbol ester-induced apoptosis in U937 cells. *Arch Biochem Biophys* 2011; 512: 30–37.

23. Qi X, Mobily-Rosen D. The PKCdelta-Abl complex communicates ER stress to the mitochondria—an essential step in subsequent apoptosis. *J Cell Sci* 2008; 121 (Pt 6): 804–813.

24. Chu SC, Chen SP, Huang SY, Wang MJ, Lin SZ, Harnt HJ et al. Induction of apoptosis coupled to endoplasmic reticulum stress in human prostate cancer cells by n-butylidenephthalide. *PLoS One* 2011; 7: e33742.

25. Reyland ME. Protein kinase C isoforms: multi-functional regulators of cell life and death. *Front Biosci* 2009; 14: 2386–2399.

26. Lu B, Ryer EJ, Kundi R, Kamyla K, Itoh H, Faries PL et al. Protein kinase Cdelta regulates migration and proliferation of vascular smooth muscle cells through the extracellular signal-regulated kinase 1/2. *J Vasc Surg* 2007; 45: 169–178.

27. Altenhofen S, Klekers PW, Rademacher KA, Scheurer P, Rob Hermans JJ, Schiffrs P et al. The NOX toolbox: validating the role of NADPH oxidases in physiology and disease. *Cell Mol Life Sci* 2012; 69: 2327–2343.

28. Stelow C, Catar RA, Muller G, Wingerl K, Scheurer P, Schmidt HH et al. Novel NADPH oxidase of oxLDL-induced reactive oxygen species formation in human endothelial cells. *Biochem Biophys Res Commun* 2006; 344: 200–205.

29. Brodie C, Blumberg PM. Regulation of cell apoptosis by protein kinase c delta. *Apolipoprotein A-I and PKCdelta in atherosclerosis*. *Apolipoprotein A-I and PKCdelta in atherosclerosis*. *Cell Death and Disease* 2011; 2: 121–127.

30. DeVries TA, Neville MC, Reyland ME. Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence. *EMBO J* 2002; 21: 6050–6060.

31. Fuji T, Garcia-Bermejo ML, Bernabo JL, Caamano J, Ohba M, Kuroki T et al. Involvement of protein kinase C delta (PKCdelta) in phorbol ester-induced apoptosis in U937 cells. *Arch Biochem Biophys* 2011; 512: 30–37.

32. Galvani S, Trayssac M, Auge N, Uchida K, Duren H et al. Antiallergogenic effect of bisvanillyl-hydralazone, a new hydralazine derivative with antioxidant, carbonyl scavenger, and antiapoptotic properties. *Antioxid Redox Signal* 2011; 14: 2093–2106.

33. Robbelyn F, Garcia V, Auge N, Vieira O, Frisach MF, Salvayre R et al. HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kappaB activation in smooth muscle cells. *FASEB J* 2003; 17: 743–754.

34. Beckman JS, Minor RL Jr., White CW, Repine JE, Rosen GM, Freeman BA. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *J Biol Chem* 1986; 261: 6884–6892.