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Oxidized low density lipoprotein increases RANKL level in human vascular cells. Involvement of oxidative stress

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ABSTRACT

Receptor Activator of NFκB Ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) have been shown to play a role not only in bone remodeling but also in inflammation, arterial calcification and atherosclerotic plaque rupture. In human smooth muscle cells, Cu^{2+}-oxidized LDL (CuLDL) 10–50 μg/ml increased reactive oxygen species (ROS) and RANKL level in a dose-dependent manner, whereas OPG level was not affected. The lipid extract of CuLDL reproduced the effects of the whole particle. Vivit, an inhibitor of the transcription factor NFAT, reduced the CuLDL-induced increase in RANKL, whereas PKA and NFκB inhibitors were ineffective. LDL oxidized by myeloperoxidase (MPO-LDL), or other pro-oxidant conditions such as ultraviolet A (UVA) irradiation, incubation with H_2O_2 or with buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, also induced an oxidative stress and enhanced RANKL level. The increase in RANKL in pro-oxidant conditions was also observed in fibroblasts and endothelial cells. Since RANKL is involved in myocardial inflammation, vascular calcification and plaque rupture, this study highlights a new mechanism whereby OxLDL might, by generation of an oxidative stress, exert a deleterious effect on different cell types of the arterial wall.

1. Introduction

RANKL is a cytokine of the TNF family which was first demonstrated to stimulate osteoclast activation and differentiation in bone [1]. Most of the biological effects of RANKL are inhibited by its soluble decoy receptor OPG, which reacts with RANKL and subsequently inhibits the binding of the cytokine to its cell surface receptor RANK [Rev in 2]. Thus, the RANKL/OPG ratio must be taken into account when considering the role of RANKL in various physiological and pathological processes. Interestingly, many authors reported inverse relationship between osteoporosis and vascular calcification, which suggests that common mediators may adversely regulate bone and vascular mineralization, and a role of the RANKL/OPG system in vascular mineralization has been proposed [Rev in 3]. Indeed, the ratio RANKL/OPG is increased in calcified arteries [4]. In addition, besides bone physiology, this ratio also has essential roles in the immune response, especially in lymph node formation and establishment of the thymic microenvironment [Rev in 5]. Furthermore, in view of the role of inflammation in atherosclerosis, several reports pointed at the inflammatory properties of RANKL, such as chemotactism for human monocytes [6] or induction of cytokine and chemokine secretion by monocytes [7].

Oxidative stress is believed to be involved in several aspects of inflammation, especially concerning the response of vascular cells [Rev in 8]. OxLDL was demonstrated to play an important role in inflammatory genes expression [Rev in 9]. We previously reported that OxLDL enhanced the expression of osteopontin, a cytokine with inflammatory properties, in different vascular cell types [10], and that this effect was mediated by an oxidative stress. We also demonstrated that OxLDL activates the transcription factor NFAT [11], which is in accordance with the fact that NFAT is involved in the expression of inflammatory cytokines such as TNFα [12].

The aim of this study was to investigate the effects of OxLDL on RANKL and OPG expression in cultured human coronary aortic smooth muscle cells HCASMC. It was demonstrated that CuLDL induced an increase in ROS and RANKL levels without affecting OPG level. The involvement of the transcription factor NFAT was suggested by the inhibitory effect of its inhibitor Vivit. Other pro-oxidants such as MPO-LDL, H_2O_2, BSO or UVA, had similar effects

Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized LDL; CuLDL, Cu^{2+}-oxidized LDL; MPO-LDL, myeloperoxidase-oxidized LDL; RANKL, receptor activator of NFkappaB ligand; OPG, osteoprotegerin; ROS, reactive oxygen species; NFAT, nuclear factor of activated T cells; H_2O_2, hydrogen peroxide; BSO, buthionine sulfoximine; HCASMC, human coronary artery smooth muscle cells.

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on RANKL levels. The increase in RANKL expression under pro-oxidants conditions was also observed in other vascular cell types such as fibroblasts and endothelial cells.

2. Materials and methods

2.1. Cell culture and chemicals

Human coronary artery smooth muscle cells HCASMC primary cultures were purchased from Cascade Biologics, Portland, Oregon, USA. Before starting experiments, cells were differentiated to smooth muscle cells during 6 days with the appropriated medium and then treated with CuLDL. MRC5 human fetal lung fibroblasts and the SK-HEP1 human endothelial cell line were purchased from the European Collection of Cell Cultures (UK) and maintained in DMEM and EMEM medium respectively with 10% fetal calf serum from Gibco (Grand Island, NY, USA). All chemicals were from Sigma–Aldrich, and the inhibitors Vivit, H89 and Ro 106–9920 were from Calbiochem (San Diego, CA, USA).

2.2. LDL preparation and oxidation

LDL (d 1.024–1.050) was prepared from normal human serum by sequential ultracentrifugation according to Havel et al. [13], and dialysed against 0.005 M Tris, 0.05 M NaCl, 0.02% EDTA pH 7.4 for conservation. Prior to oxidation, EDTA was removed by dialysis. Oxidation by Cu^{2+} was performed by incubation at 37 °C of 1 mg LDL protein/ml with 5 μM CuSO4 for 48 h. Oxidation by myeloperoxidase-generated reactive nitrogen species was conducted as previously described [14].

The degree of LDL oxidation was checked by determination of lipid peroxidation end products (thiobarbituric acid reactive substances TBARS), determined using the Yagi's method [15], and by electrophoresis. CuLDL and MPO-LDL contained 18–24 and 12–16 nmoles equivalent malondialdehyde/mg ApoB respectively, and their relative electrophoretic mobilities were 2.2 and 2.0, respectively.

2.3. Generation of oxidative stress

For generation of oxidative stress, cells were treated with 10–50 μg/ml CuLDL or MPO-LDL, 10–50 μM H_2O_2 or 5–10 μM BSO for 2 days. UVA irradiation was performed with a Vilber–Lourmat table equipped with a TF-20 L tube, emitting maximally at 365 nm, at a dose rate of 3 ± 0.2 mW/cm^2. Cells were irradiated in PBS at 4.5–9 J/cm2 (15–30 min in our conditions), then incubated at 37 °C for 4 h for amplification of oxidative stress, before the shift to 1% serum-supplemented medium for 2 days.

2.4. Determination of RANKL and OPG

Cells were lysed in 50 mM Tris, 50 mM NaF, 20 mM p-nitrophenyl phosphate, 1 mM EGTA, 0.05 mM Na-Vanadate, 5 mM benzamidine and 1% TX100 for 15 min at 4 °C and sonicated. After centrifugation at 14 ,000*g* for 2 min, the supernatants were taken as cell lysates. RANKL and OPG were determined with ELISA kits from Biomedica, Vienna, Austria. Results are expressed as % of the control.

Alternatively, equal amounts of proteins were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with RANKL antibody from R&D, MN, USA. Immunoblots were visualized by the enhanced chemiluminescence detection kit from Amersham.

2.5. Determination of reactive oxygen species with Chloro-Methyl-Dichlorofluorescein

Cells in 3.5 cm Petri dishes were incubated for 15 min with 0.1 μM chloro-methyl-2'7'dichlorofluorescein (Molecular Probes) in PBS, washed three times, resuspended in H_2O and sonicated. The fluorescence was determined at 503/529 nm, normalized on a protein basis and expressed as % of control.

2.6. Determination of NFAT transcription factor binding activity by ELISA

The nuclear extracts were prepared with the Active Motif kit. The DNA binding activity was determined using the TransAM ELISA kit NFAT-c1 from Active Motif (Carlsbad, CA, USA). Results are expressed as % of OD of controls.

3. Results

3.1. CuLDL increased intracellular RANKL and induced an oxidative stress in HCASMC

The intracellular levels of RANKL and of its decoy receptor OPG were determined in HCASMC cells. CuLDL within the range of 10–25 μg/ml exhibited a dose-dependent stimulating effect on RANKL level, whereas OPG level was not affected (Fig 1a). Concerning RANKL, the results obtained by ELISA were in accordance with those obtained from western-blot. In parallel, ROS production was also enhanced in a dose-dependent manner (Fig 1b). Native non-oxidized LDL had no significant effect on RANKL and ROS levels. It is of note that the lipid extract of CuLDL had similar effects on ROS and RANKL levels as compared to the whole particle, albeit to a somewhat lesser extent (Fig 1).

3.2. The NFAT inhibitor Vivit reduced the effect of CuLDL whereas the PKA inhibitor H89 and the NFκB inhibitor Ro 106–9920 were ineffective

To determine whether the transcription factor NFAT is involved in CuLDL-stimulated RANKL expression, we utilized its cell-permeable inhibitor Vivit. The data from Fig 2 show that CuLDL stimulated NFAT DNA binding activity by about 2fold (Fig 2a). In parallel, Vivit significantly reduced RANKL level in the presence of CuLDL (Fig 2b). In addition, whereas cyclic AMP + Theophylline exhibited a stimulatory effect, H89 and Ro 106–9920 had no effect in the presence of CuLDL, ruling out the involvement of the transcription factors CREB and NFκB.

3.3. MPO-LDL, H_2O_2, BSO and UVA induced an increase in ROS and RANKL in HCASMC

We then investigated the effects of other pro-oxidant factors such as MPO-LDL, hydrogen peroxide H_2O_2, BSO, an inhibitor of glutathione synthesis, and UVA irradiation. It was demonstrated that incubation of HCASMC cells in all these ROS-generating conditions was accompanied by a significant and dose-dependent elevation in RANKL level (Fig 3).

3.4. CuLDL, H_2O_2, BSO and UVA induced an increase in RANKL in human fibroblasts and endothelial cells

We then investigated if the oxidative stress generated by CuLDL, H_2O_2, BSO and UVA was effective on RANKL expression in other human vascular cell types, such as MRC5 fibroblasts and SK-HEP1 endothelial cells (Fig 4). It was found i/ that RANKL gene was

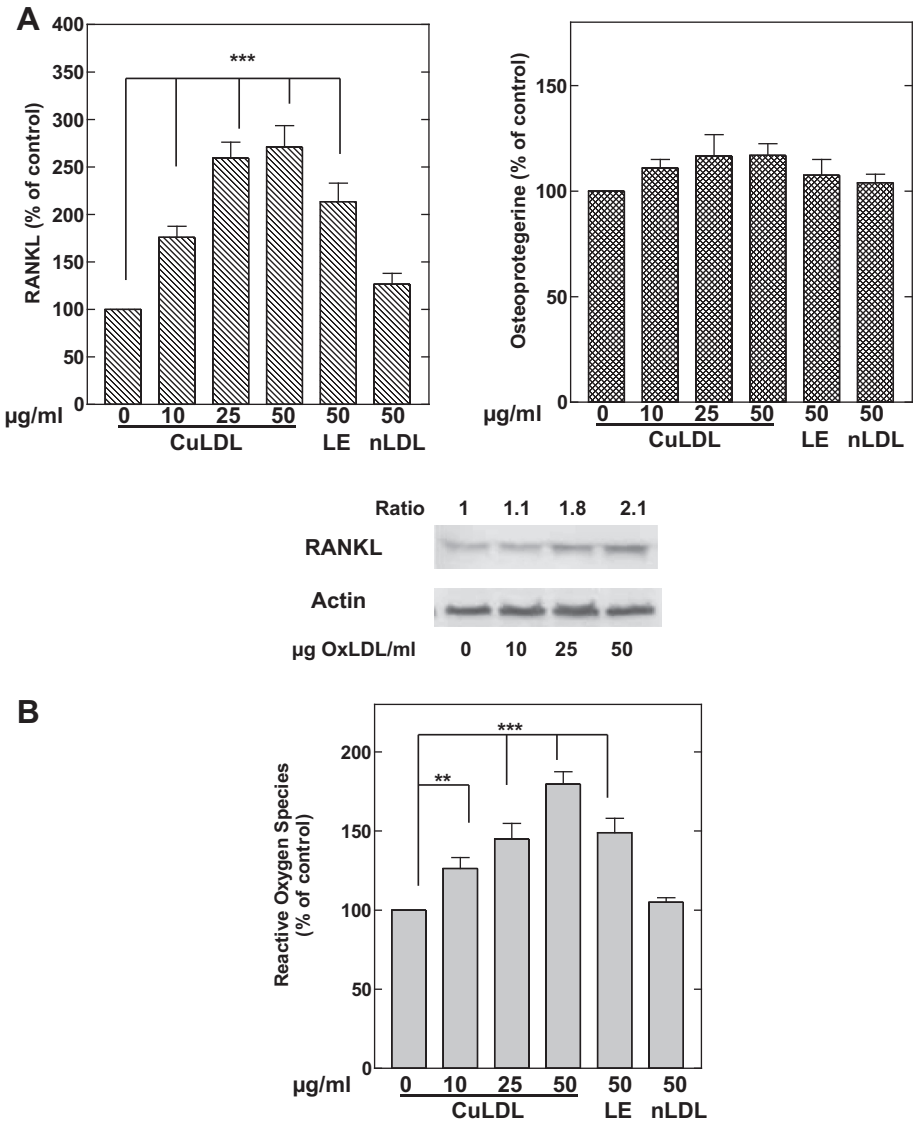

Fig. 1. CuLDL increased RANKL (1A) and induced an oxidative stress (1B) in HCASMC. (1A) Cells in 6 cm Petri dishes were incubated for 2 days in differentiation medium in the presence of 10–50 µg /ml CuLDL protein or the lipid extract LE of 50 µg/ml CuLDL. 100%: 2.7 ± 0.3 and 320 ± 35 pmol/mg protein for RANKL and OPG respectively. nLDL: native LDL. (1B) Cells in 12-well plates were incubated for 24 h in medium supplemented with 0.5% bovine albumin before a 4 h treatment with 10–50 µg/ml OxLDL protein. Results, normalized on a protein basis, were expressed as % of control. Means of 4 experimental values + s.d. $^{**}p < 0.01$; $^{***}p < 0.001$ by the Student's *t* test.

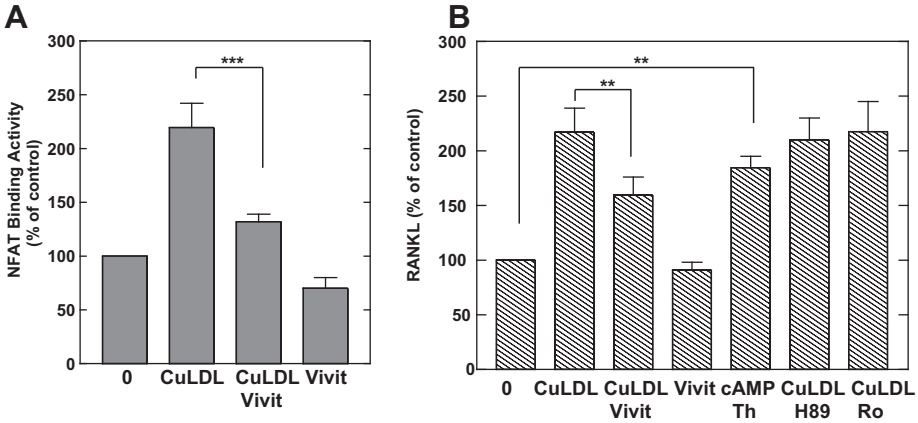

Fig. 2. The NFAT inhibitor Vivit reduced the effect of CuLDL on RANKL expression, whereas PKA and NFkappaB inhibitors had no effect. (2A) Cells were incubated for 24 h in medium supplemented with 0.1% bovine serum albumin, then treated for 1 h with 50 µg/CuLDL in the presence or absence of 1 µM Vivit. (2B) Cells in differentiation medium were incubated for 2 days with 50 µg/ml CuLDL, 0.1 mM cyclic AMP + 1 mM Theophylline Th, 1 µM Vivit, 5 µM H89 or 1 µM Ro 106–9920. Results are means from 3 experimental values ± s.d. $^{**}p < 0.01$; $^{***}p < 0.001$ by the Student's *t* test.

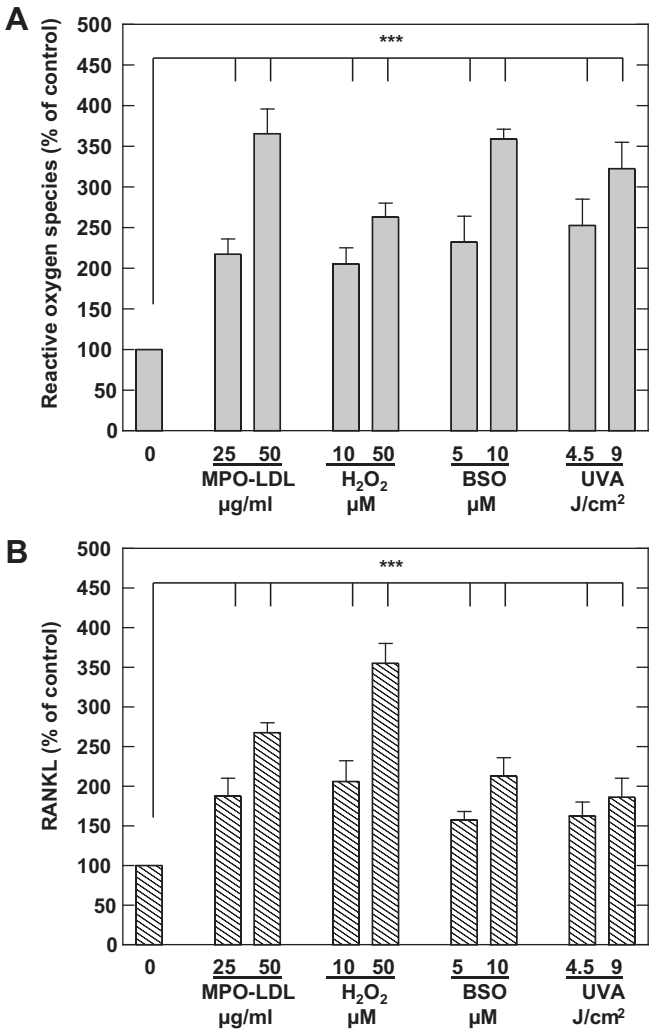

Fig. 3. MPO-LDL, BSO, H_2O_2 and UVA induced an oxidative stress (3A) and increased RANKL (3B) in HCASMC. (3A) Cells were incubated for 24 h in medium supplemented with 0.5% bovine albumin, then treated with MPO-LDL or H_2O_2 for 4 h. Alternatively, cells were treated for 24 h with BSO in the same medium. For UVA treatment, cells were exposed to UVA and then left in PBS for 4 h before measurement of ROS. (3B) Cells were incubated for 2 days in Smooth Muscle Differentiation Supplemented-medium in the presence of the indicated concentrations of MPO-LDL, H_2O_2 and BSO. Alternatively, cells were irradiated with UVA in PBS and left in PBS for a further 4 h incubation, before the shift to 1% serum-supplemented medium for 2 days. Means of 3 experimental values + s.d. $^{***}p < 0.001$ by the Student's t test.

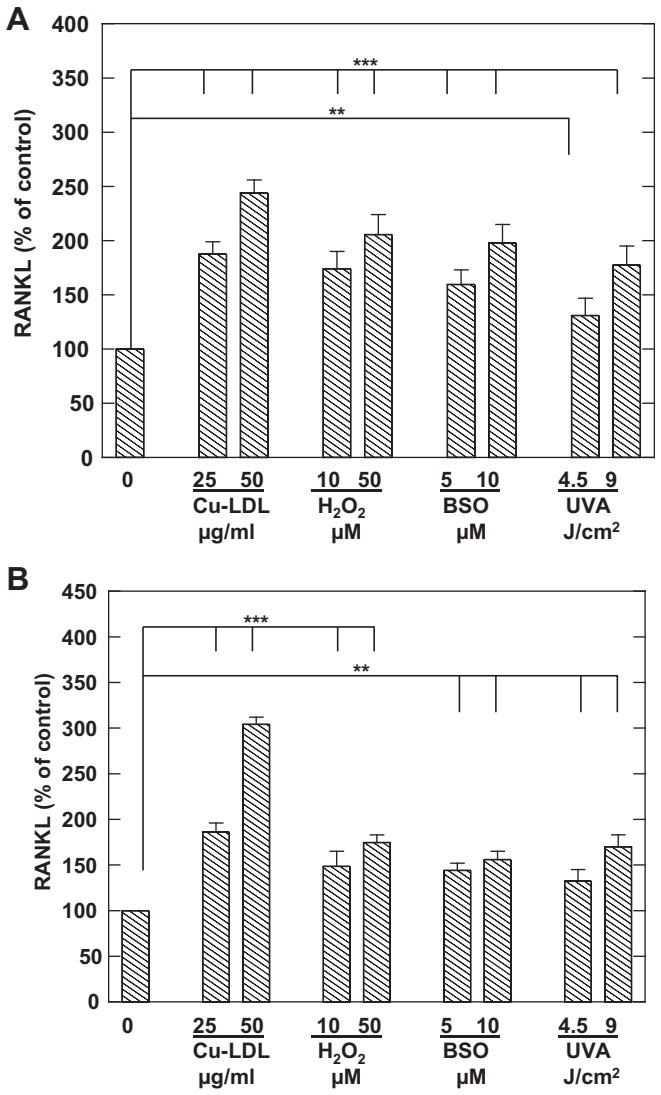

Fig. 4. CuLDL, H_2O_2, BSO and UVA increased RANKL level in human fibroblasts (4A) and endothelial cells (4B). Cells were incubated for 2 days in 1% serum-supplemented medium in the presence of the indicated concentrations of Cu-LDL, H_2O_2 and BSO. Alternatively, cells were irradiated with UVA and left in PBS for a further 4 h incubation, then shifted to 1% serum-supplemented medium for a further 2 days incubation. 100%: 15.3 + 2.1 and 4.6 ± 0.4 pmol/mg protein for fibroblasts and endothelial cells respectively. Means from 3 experimental values + s.d. $^{**}p < 0.01$; $^{***}p < 0.001$ by the Student's t test.

expressed in these cells, and ii/ that, under our experimental conditions and whatever the oxidative stress inducing agent considered, a dose-dependent increase in RANKL levels was observed.

4. Discussion

This work demonstrates that CuLDL significantly increases in a dose-dependent manner ROS and RANKL levels in cultured HCASMC, whereas the OPG level was not affected (Fig 1). More physiologically oxidized LDL such as MPO-LDL had a similar effect (Fig 3). The involvement of the lipid peroxidation products included in the CuLDL particle was suggested by the fact that the lipid extract from CuLDL also increased both RANKL and ROS levels (Fig 1). Our results are in accordance with the report from Graham et al. [16], who demonstrated that oxidized lipids and minimally-oxidized LDL enhanced RANKL secretion in circulating human T lymphocytes, and these authors pointed at the involvement of this phenomenon in bone remodeling.

NFAT is a transcription factor involved in the expression of inflammatory cytokine genes, and the present work strongly suggests that it mediates the effect of CuLDL on RANKL gene expression in HCASMC, since its inhibitor Vivit antagonized the CuLDL-induced increase in RANKL (Fig 2). Since NFAT is activated by cytosolic Ca^{2+}, the second messenger responsible for dephosphorylation of NFAT by calcineurin [17], we measured intracellular Ca^{2+} with the fluorescent probe Fluo 3 and found a 1.3 fold increase ($p < 0.05$) after addition of 50 µg/ml CuLDL. This result is also in accordance with our previous report concerning activation of NFAT by CuLDL in several cell types such as T lymphocytes, fibroblasts, endothelial cells and macrophages [11].

Since Tseng et al. [18] reported that the PKA agonist forskolin stimulated RANKL expression in murine smooth muscle cells, we also checked the effect of cAMP and of the PKA inhibitor (and thus CREB inhibitor) H89. It was found that if cAMP was able to enhance RANKL expression in our experimental model HCASMC, H89 did not prevent the effect of CuLDL, suggesting that CREB is not

involved in CuLDL signaling pathway (Fig 2b). We also tested the effect of the NFκB inhibitor Ro 106–9920 on CuLDL-induced RANKL expression, but this compound had no significant effect, thus ruling out the hypothesis of an involvement of NFκB in this experimental model (Fig 2b).

Besides OxLDL treatment, other pro-oxidant conditions, such as UVA irradiation or incubation with H_2O_2 and BSO, also increased RANKL in a dose-dependent manner in HCASMC (Fig 3), as well as in other vascular cell types such as fibroblasts and endothelial cells (Fig 4). In these two last cell types, a parallel increase in ROS was also observed (results not shown). Thus, oxidative stress-regulated RANKL expression appears to be a general phenomenon, and not cell specific. Bai et al. [19] also demonstrated that ROS generated by H_2O_2 or by the xanthine/xanthine oxidase system stimulated RANKL expression in mouse and human osteoblasts. If OxLDL have been shown to stimulate RANKL expression in osteoblasts [20], this study demonstrates the involvement of OxLDL in the regulation of RANKL expression in vascular cells. In view of the fact that a role for the RANKL/OPG system in vascular calcification has been proposed [3,4], the observation that OxLDL stimulated RANKL expression in different vascular cell types points to one of the mechanisms whereby coronary and aortic calcification occurred in patients with elevated OxLDL level [21], as well as in familial hypercholesterolemia [22]. These results might also be related to the fact that OxLDL enhanced mineralization of vascular smooth muscle cells [23].

It is of note that the RANKL and OPG have been localized in atherosclerotic plaques [24], where accumulation of OxLDL and generation of ROS were observed. These authors also reported enhanced expression of the OPG/RANKL/RANK system both in clinical and experimental atherosclerosis, especially within thrombus material obtained at the site of plaque rupture. Soluble RANKL serum level has emerged as a highly significant predictor of plaque destabilization and rupture [25]. Our study suggests a new mechanism whereby OxLDL might exert a harmful effect on different cell types of the arterial wall. It also points at the fact that anti-oxidant strategies might have benefit effects not only in osteoporosis and vascular calcification, but also in the prevention of atherosclerosis plaque rupture.

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