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Oxidized low density lipoprotein inhibits phosphate signaling and phosphate-induced mineralization in osteoblasts. Involvement of oxidative stress

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ABSTRACT

Background: It is well admitted that oxidized LDL (OxLDL) plays a major role in the generation and progression of atherosclerosis. Since atherosclerosis is often accompanied by osteoporosis, the effects of OxLDL on phosphate-induced osteoblast mineralization were investigated.
Methods: Calcium deposition, expression of osteoblast markers and inorganic phosphate (Pi) signaling were determined under OxLDL treatment.
Results: OxLDL, within the range of 10–50 μg protein/ml, inhibited Pi-induced UMR106 rat osteoblast mineralization. In parallel, the expression of Cbfa1/Runx2 transcription factor was decreased, and the intracellular level of the osteoblast marker osteopontin (OPN) was reduced. The extracellular level of another marker, receptor activator of nuclear factor kappa B ligand (RANKL), was also diminished. OxLDL inhibited Pi signaling via ERK/JNK kinases and AP1/CREB transcription factors. OxLDL triggered the generation of reactive oxygen species (ROS), either in the absence or presence of Pi. Furthermore, the effects of OxLDL on Pi-induced mineralization, generation of ROS and extracellular level OPN were reproduced by the lipid extract of the particle, whereas the antioxidant vitamin E prevented them.
Conclusions: This work demonstrates that OxLDL, by generation of an oxidative stress, inhibits of Pi signaling and impairs Pi-induced osteoblast differentiation.
General significance: This highlights the role of OxLDL in bone remodeling and in degenerative disorders other than atherosclerosis, especially in osteoporosis.

1. Introduction

Bone mass results from an active remodeling process which involves an equilibrium between mineralization, mediated by osteoblasts, and resorption, mediated by osteoclasts. During osteoblast differentiation, the expression of osteoblast marker genes such as alkaline phosphatase and osteocalcin [1], or osteopontin OPN [2,3] are enhanced. A crosstalk between osteoblasts and osteoclasts exists in that osteoblast differentiation is accompanied by expression of RANKL (Receptor Activator of Nuclear Factor kappa B Ligand) [4], a cytokine involved in osteoclast differentiation. Conversely, inorganic phosphate (Pi) released by bone resorption is a signaling molecule in the events preceeding mineralization of osteoblasts [5]. In MC3T3-E1 mouse osteoblasts, it has been demonstrated that Pi induced OPN gene expression via ERK and PKC kinases [6]. Other kinases have also been demonstrated to mediate osteoblast differentiation. In this regard, PKD activation mediates Bone Morphogenetic Protein 2 BMP2 and Insulin-like Growth Factor 1 IGF1 treatment [7], and p38 mediates diosmetin treatment [8]. JNK is also involved in late-stage osteoblast differentiation [2].

Concerning the transcription factors, Runx2/Cbfa1/Osf2 plays a crucial role in bone mineralization [9]. The activity of this factor is regulated by both gene expression and post-translational regulation. The expression of Runx2 was demonstrated to be under the control of PKC [10] and p38 [11]. In addition, the binding activity of Runx2 is enhanced by phosphorylation via Ras/Raf/ERK1/2 pathway [12]. Besides Runx2, AP1 activation has also been described in BMP2-induced bone formation [13,14]. In addition, CREB (cyclic AMP Response Element Binding Protein) phosphorylation and transactivation has been demonstrated in TNFα-induced mineralization [15]. Direct interactions between Runx2 and other transcription factors

Abbreviations: Pi, Inorganic phosphate; OxLDL, Cu^{2+}-oxidized low density lipoprotein; OPN, osteopontin; RANKL, Receptor Activator of NFkappaB ligand; ERK, Extracellular signal-regulated kinase; JNK, Jun kinase; AP1, Activator Protein 1; CREB, cAMP Response Element Binding protein; ROS, Reactive oxygen species

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such as AP1 and Smads, which results in binding to separate sites of DNA, were also described [16].

It is well established that oxidized LDL (OxLDL) plays a major role in the generation and progression of the atherosclerotic plaque (Rev in [17]). It has been further observed that atherosclerosis is often accompanied by osteoporosis [18]. We recently reported a role for OxLDL in bone remodeling in that these particles impair RANKL signaling and RANKL-induced osteoclast differentiation [19]. In addition, it was reported that minimally oxidized LDL and lipid oxidation products, such as oxidized phosphatidyl-choline and isoprostane8-iso prostaglandin E2, inhibit bone cell differentiation [20]. This effect of OxLDL has been attributed to generation of an oxidative stress [21]. However, the molecular mechanism of this inhibitory effect was not yet described. We previously demonstrated that OxLDL inhibits insulin signaling and that this effect might be ascribed to the generation of an oxidative stress [22]. Several reports mentioned the sensitivity of osteoblasts towards oxidative stress [23,24], and especially, osteoblasts signaling pathways such as Wnt pathway might be impaired [25]. In the current study, we demonstrate that OxLDL prevented Pi-induced differentiation of osteoblasts and inhibited Pi signaling through ERK/JNK kinases, and AP1/CREB transcription factors. Furthermore, this phenomenon might be ascribed to the generation of reactive oxygen species ROS, since the antioxidant vitamin E prevented it.

2. Materials and methods

2.1. Cell culture

UMR106 rat osteoblasts were purchased from the American Type Culture Collection. Cells were cultured in DMEM containing 10% foetal calf serum. For induction of mineralization, cells were shifted to medium supplemented with 5% foetal calf serum and 4 mM Pi (NaH_2PO4 in 0.2 M stock solution equilibrated to pH 7.4), taking into account the phosphate content of the medium (1 mM). OxLDL 10–50 μg protein/ml, and in some experiments 50 μg/ml OxLDL lipid extract and 50 μM of the antioxidant vitamin E (α-tocopherol from Sigma) were introduced simultaneously with Pi for 4 days.

Elisa kits for rat OPN was from IBL, Hambourg, Deutchland, and for rat RANKL from Biomedica, Vienne, Austria. Determination of intracellular OPN was performed on 2 μg protein. Secreted OPN was determined after 1/100 dilution of culture medium and secreted RANKL was measured after concentration by a factor 5 using Microcon tubes from Millipore.

For the study of Pi signaling, cells were preincubated 24 h in medium devoid of serum and supplemented with 0.1% bovine albumin in order to downregulate all signaling pathways. OxLDL were then introduced for 4 h and 4 mM Pi was added for 45 min. Cellular or nuclear extracts were then performed for determination of kinase phosphorylation by western blot, and transcription factor DNA binding activities by Elisa.

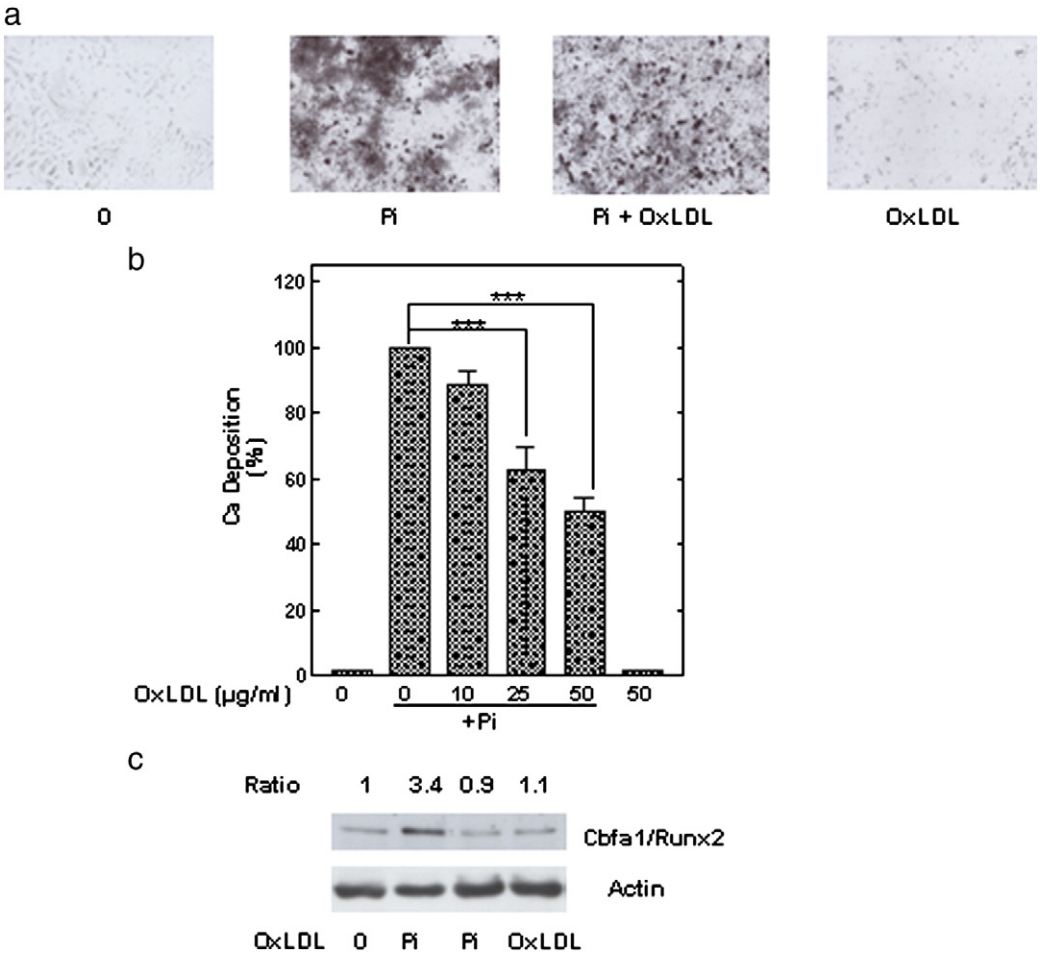

Fig. 1. OxLDL inhibited Pi-induced mineralization of UMR106 rat osteoblasts. (a) Von Kossa staining. (b) Calcium deposition. (c) Cbfa1/Runx2 expression. In a–b, the cells were incubated in 48-well plates in DMEM supplemented with 5% foetal calf serum, 4 mM Pi and 10–50 μg OxLDL protein/ml for 8 days. Von Kossa staining was performed as described in [26] and calcium deposition by spectrophotometry [27]. 100% calcium: 20–25 μg/well. In c, the cells were incubated in 6 wells-plates in DMEM supplemented with 5% foetal calf serum, 4 mM Pi and 50 μg/ml OxLDL protein for 4 days. (b) Means of 3 independent experiments ± SD. ***$p<0.001$ by Student's *t*-test.

2.2. Visualization and determination of calcium deposition

Cells were treated in 48 well-plates and calcification was visualized using the Von Kossa staining technique in the presence of $AgNO_3$ for phosphate deposition [26]. Cells were fixed with 95 % ethanol during 15 min and treated with 5% AgN03 for 30 min in the dark, with 5% $Na_2S_2O_3$ for 5 min and then dried at room temperature. For determination of calcium deposition, cells were treated with 300 µl of 0.6 N HCl for 4 h. Supernatants were then collected and calcium content was colorimetrically determined by spectrophotometry by the o-cresolphthalein complexone method from Ray Sarkar [27]. Results are expressed in %, taking as 100% the value obtained with 4 mM Pi.

2.3. LDL preparation and oxidation

LDL (d 1.024–1.050) was prepared from normal human serum by sequential ultracentrifugation according to Havel et al. [28], and dialyzed 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 was performed by incubation at 37 °C of 1 mg LDL protein/ml Ham F10 with 5.10^{-6} M $CuSO_4$ for 48 h. The level of LDL oxidation was checked by determination of thiobarbituric acid reactive substances (TBARS) according to Yagi [29] and amounted to 38–42 nmol equivalent malondialdehyde/mg LDL protein. Lipid extraction of OxLDL was performed according to Bligh and Dyer [30].

2.4. Determination of intracellular OPN, Cbfa1/Runx2 expression and ERK/JNK phosphorylation by immunoblot analysis

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 during 15 min at 4 °C and sonicated. After centrifugation at 14,000*g* for 2 min, the supernatants were taken as cell lysates. Equal amounts of proteins (40–50 µg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with specific antibodies. Equal loading was checked by neutral red staining. Immunoblots were visualized by enhanced chemiluminescence detection kit from Amersham (Saclay, France). The OPN antibody was from Santa Cruz (CA, USA), Cbfa1/Runx2 antibody from Calbiochem (CA, USA) and ERK/JNK antibodies were from Cell Signaling (MA, USA).

2.5. Determination of AP1/CREB DNA binding activities by ELISA

The binding activity assays were performed using the Trans-AM ELISA-based kits from Active Motif (Carlsbad, CA) according to the manufacturer's protocol. Nuclear extracts were prepared using Active Motif kits. Equal amounts of proteins were incubated in 96-well plates coated with oligonucleotides containing the AP1 or CREB consensus binding sites. Activated transcription factors bound to the respective immobilized oligonucleotides were detected using the antibodies directed against c-jun or CREB, and a secondary antibody conjugated to horseradish peroxidase. The developing solution was then added for 10 min at room temperature and OD were determined by a microplate reader from Molecular Devices. Results are expressed in % of the OD obtained with non treated cells.

2.6. Determination of reactive oxygen species (ROS) with chloro-methyl-dichlorofluorescein

The increase in fluorescence in living cells in the presence of this probe allows the quantitation of ROS such as superoxide anion, hydrogen peroxide and the hydroxyl radical [31].

Cells in 3.5 cm Petri dishes were incubated for 15 min with 10^{-5} M chloro-methyl-2'7'dichloro-fluorescein (Molecular Probes) in PBS, washed three times with PBS, solubilized in H_2O and sonicated. The fluorescence was determined at 503/529 nm, normalized on a protein basis and expressed as % of control.

3. Results

3.1. OxLDL inhibited Pi-induced osteoblast mineralization

We first studied the effect of OxLDL on mineralization of UMR106 rat osteoblasts, 12 days after 4 mM Pi addition. It was observed that OxLDL, within the range of 10–50 µg protein/ml, reduced calcification

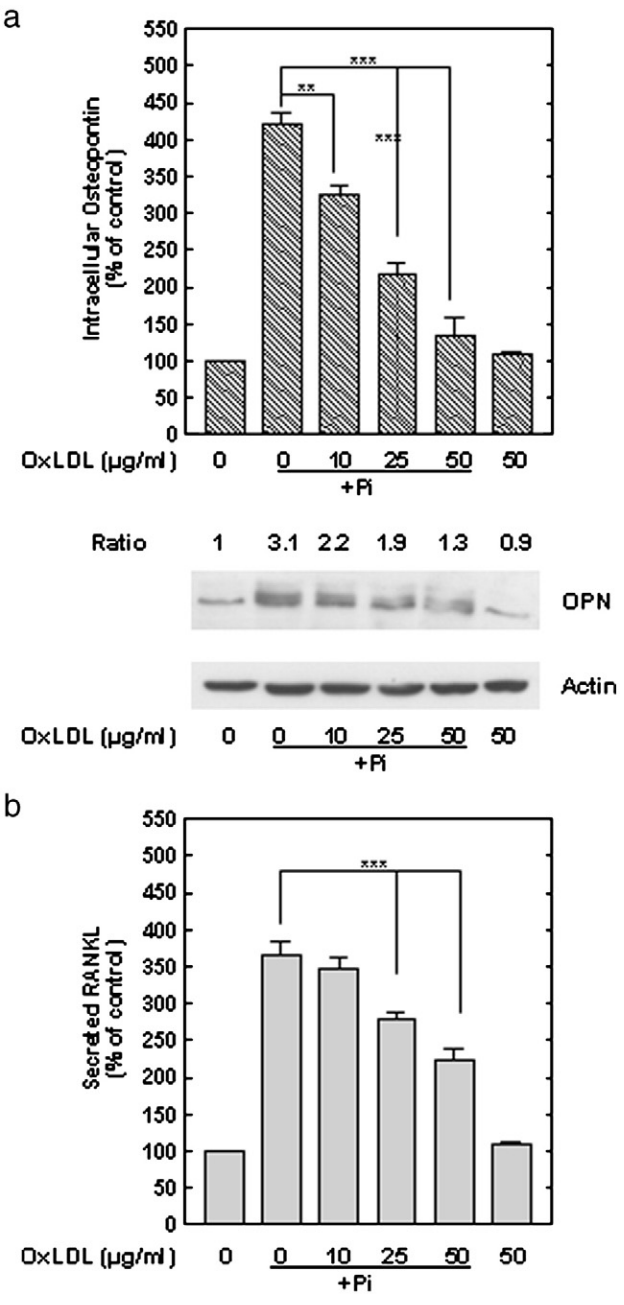

Fig. 2. OxLDL inhibited Pi-induced expression of OPN and RANKL. (a) Intracellular OPN. (b) Secreted RANKL. UMR106 cells were incubated in 12 wells plates in DMEM supplemented with 5% foetal calf serum, 4 mM Pi and 10–50 µg OxLDL protein/ml for 4 days. RANKL and OPN were determined by Elisa kits. Determination of intracellular OPN was performed on 2 µg protein. Secreted OPN was determined after 1/100 dilution of culture medium and secreted RANKL was measured after concentration by a factor 5 using Microcon tubes from Millipore. 100% intracellular OPN: 0.5–2.5 ng/µg protein; secreted RANKL: 35–50 fmol/µg protein. Means of 3 independent experiments ± SD. $^{**}p<0.01$ and $^{***}p<0.001$ by Student's *t* test.

in a dose-dependent manner (Fig. 1a), and decreased Pi-induced Cbfa1/Runx2 intracellular level (Fig. 1b). OxLDL by itself, in the absence of Pi, exhibited no effect. Furthermore, native LDL did not inhibit Pi-induced mineralization (result not shown).

3.2. OxLDL reduced Pi-induced expression of OPN and RANKL

We then investigated the effects of OxLDL on the expression of specific osteoblast markers. The data from Fig. 2a show that Pi, as previously described in mouse osteoblasts [6], enhanced intracellular OPN by about 4 fold. In addition, in the presence of OxLDL, the Pi-induced augmentation of OPN was inhibited in a dose-dependent manner. Concerning RANKL, we were unable to determine its intracellular level, but extracellular RANKL level could be assessed after a 5 fold concentration of the culture medium. It was found that OxLDL also prevented Pi-induced augmentation in extracellular RANKL in a dose-dependent manner (Fig. 2b).

3.3. OxLDL impaired Pi signaling via ERK/JNK and AP1/CREB

The next experiment was designed to investigate the effects of OxLDL on Pi signaling. It was found that a 4 h preincubation of osteoblasts with 10–50 μg/ml OxLDL enhanced ERK and JNK phosphorylation (Fig. 3). Pi induced a 2.3- and 1.6-fold increase in ERK and JNK phosphorylation, respectively, and finally, OxLDL inhibited Pi-induced phosphorylation of both kinases.

OxLDL also induced an increase in AP1 binding activity, but had no effect on CREB (Fig. 4). Pi 4 mM enhanced AP1 and CREB activity by 2.8- and 1.8-fold, respectively and a pre-treatment with OxLDL prevented this effect, and thus in a dose-dependent manner.

3.4. OxLDL enhanced the oxidative stress initiated by Pi

In the next experiment, we determined the intracellular level of ROS by means of the fluorescent probe dichloro-fluorescein under Pi and

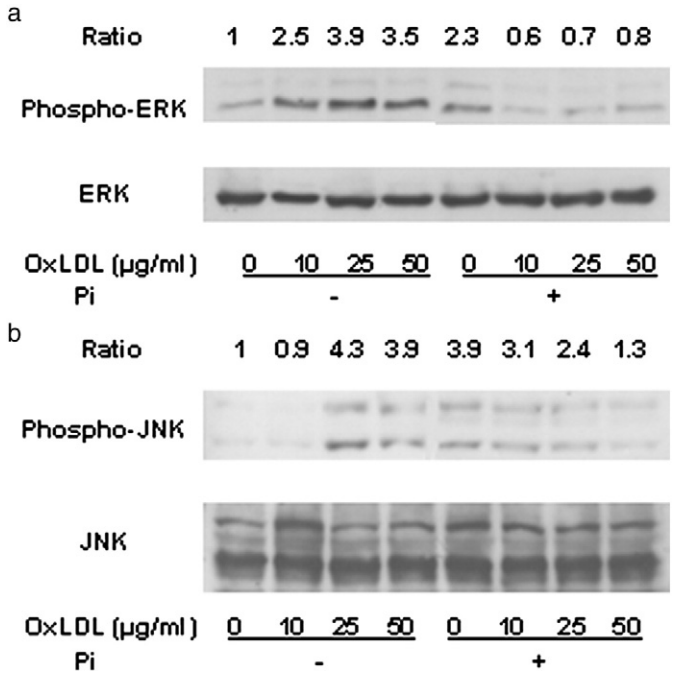

Fig. 3. OxLDL impaired Pi signaling via ERK (a) and JNK (b). UMR106 cells in 6-well plates were preincubated in DMEM supplemented with 0.1% bovine albumin for 24 h for downregulation of signaling pathways, 10–50 μg OxLDL protein/ml were added for 4 h before a further incubation with 4 mM Pi during 45 min. The phosphorylation state of ERK/JNK was determined by immunoblot analysis. Results are from a typical experiment. This experiment was performed 3 times with similar results.

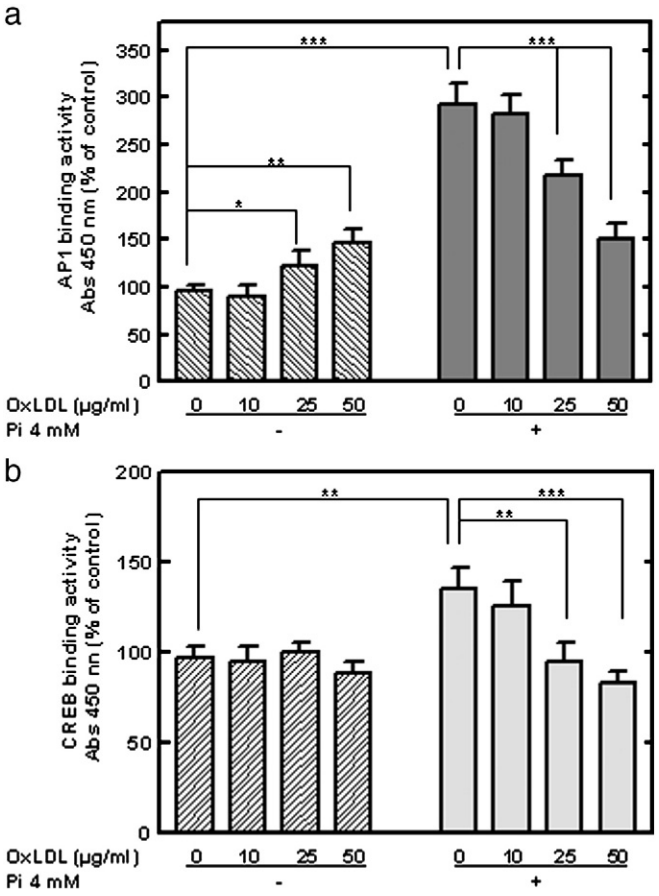

Fig. 4. OxLDL impaired Pi signaling via AP1 and CREB. UMR106 cells in 6 wells-plates were preincubated in DMEM supplemented with 0.1% bovine albumin for down-regulation of signalling pathways, 10–50 μg OxLDL protein/ml were added for 4 h before a further incubation with 4 mM Pi during 45 min. AP1/CREB DNA binding activity was determined with ELISA kits from Active Motif. Results (OD at 450 nm) are expressed in % of control. Means of 3 independent experiments ± SD. ***$p<0.001$ by Student's t test.

OxLDL treatment. The results from Fig. 5 indicate that a 4-h incubation with 4 mM Pi initiated a slight but significant increase (×1.4) in ROS. Furthermore, OxLDL 10–50 μg/ml increased the generation

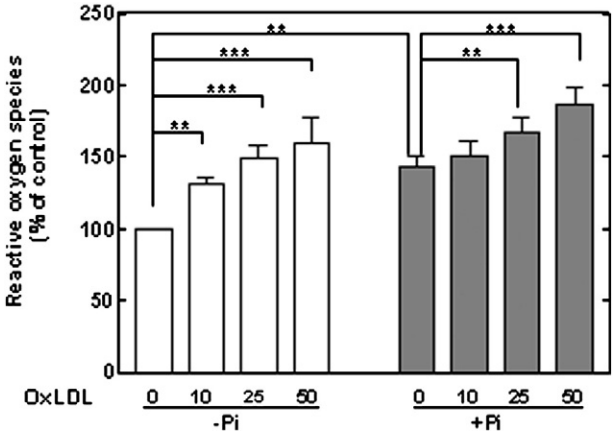

Fig. 5. OxLDL enhanced Pi-induced generation of ROS. UMR106 cells were incubated in 12-well plates in DMEM supplemented with 0.1% bovine serum albumin for 24 h, 10–50 μg OxLDL protein/ml were then added for 4 h before a further incubation with 4 mM Pi for 45 min. Determination of ROS was then performed with the fluorescent probe chloro-methyl-2'7'dichlorofluorescein. Results, normalized on the protein basis, are expressed in % of control. Means of 3 independent experiments ± SD.*$p<0.05$; **$p<0.01$ and ***$p<0.001$ by Student's t test.

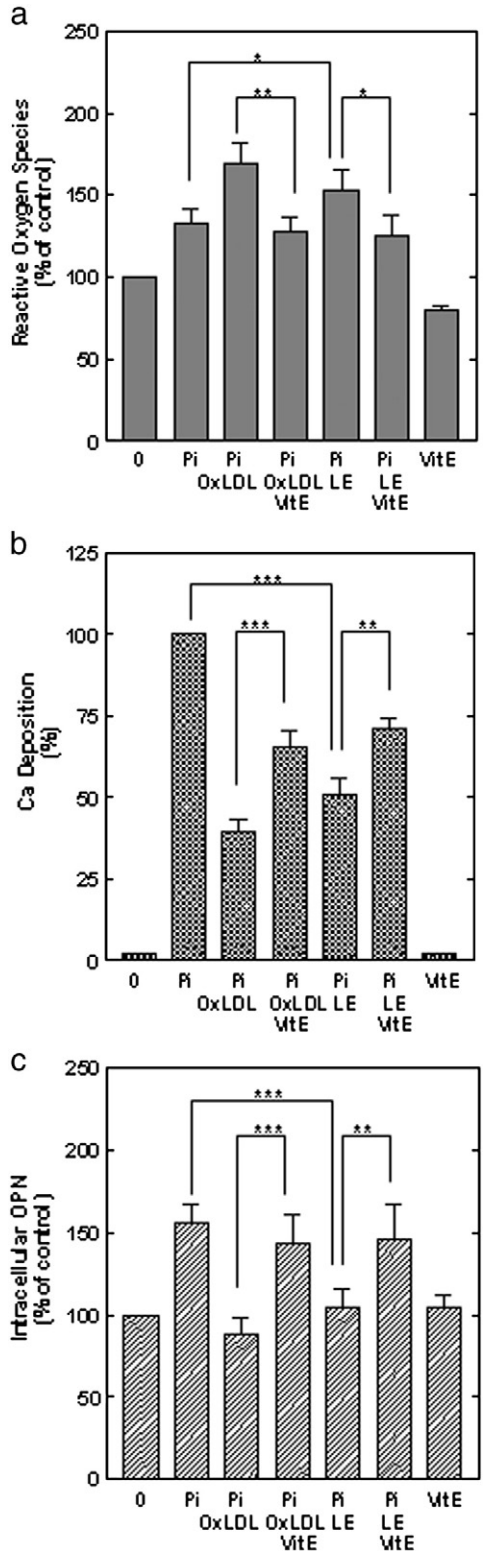

Fig. 6. The lipid extract (LE) of OxLDL reproduced whereas Vitamin E prevented the effects of OxLDL. (a) Reactive oxygen species, (b) Calcium deposition, (c) Intracellular osteopontin. In a, the cells were preincubated in medium supplemented with 0.1% albumin and 10 μM vitamin E for 24 h before addition of 50 μg OxLDL protein/ml or its lipid extract for 4 h. After addition of 4 mM Pi for 45 min, determination of ROS was performed with the fluorescent probe chloro-methyl-2'7'dichlorofluorescein. In b–c, cells were incubated in medium supplemented with 5% foetal calf serum, 4 mM Pi, 50 μg OxLDL protein/ml or its lipid extract (LE) and 10 μM vitamin E for 8 days (b) or 4 days (c). Means of 3 independent experiments ± SD.*$p<0.05$; **$p<0.01$ and ***$p<0.001$ by Student's t test.

of ROS in a dose-dependent manner, both in the absence or presence of Pi.

3.5. The lipid extract of OxLDL reproduced whereas vitamin E prevented the effects of OxLDL

We then investigated the effects of the lipid peroxidation products included in OxLDL. The data from Fig. 6 indicate that the lipid extract from OxLDL, like the particle itself, increased Pi-induced generation of ROS (6A) and simultaneously reduced mineralization (6B) and OPN level (6C). By contrast, the antioxidant vitamin E partially but significantly reversed these effects of OxLDL and its lipid extract.

4. Discussion

In this study, we first demonstrated that OxLDL inhibited Pi-induced mineralization of UMR rat osteoblasts (Fig. 1a). OxLDL also prevented Pi-induced expression of Cbfa1/Runx2 transcription factor (Fig. 1b), and decreased Pi-induced expression of two osteoblastic markers, OPN and RANKL (Fig. 2). This is in accordance with the report from Parhami et al. [20], which described an inhibitory effect of minimally-oxidized LDL and of oxidized phosphatidyl-choline on bone cell differentiation. These authors utilized minimally iron-oxidized LDL at the concentration of 200 μg LDL protein/ml, which is equivalent, in terms of lipid peroxidation products (TBARS), to the highest concentration used in our experimental model (50 μg LDL protein/ml). They further demonstrated that minimally oxidized LDL inhibits osteogenic differentiation of marrow stromal cells by directing these cells to undergo adipogenic differentiation by activation of PPAR alpha [32]. However, these authors only determined alkaline phosphatase as osteoblast marker, and furthermore, the effect of OxLDL on osteoblast signaling pathways was not yet reported.

The current studies demonstrate that in rat osteoblasts, Pi signaling involves ERK/JNK kinases (Fig. 3), and AP1/CREB transcription factors (Fig. 4). Pi transduction signal via ERK in mouse osteoblasts [6] or via AP1 in BALB/c mouse JB6 epidermal cells [33] was previously reported. Furthermore, OxLDL alone exhibited the same stimulatory effect, in accordance with previous reports concerning ERK [34], JNK [35] and AP1 [36]. However, preincubation with OxLDL prevented Pi signaling, thus demonstrating a crosstalk between both activation mechanisms.

It is well admitted that ERK controls AP1 [37] and Cbfa1/Runx2 [38] activities. In addition, the expression of OPN gene was also demonstrated to be under the control of ERK in smooth muscle cells [39] or cardiac fibroblasts [40]. It is also of note that ERK mediates OPN expression in arterial smooth muscle cells by activation of AP1 [41] and CREB [42]. Concerning the role of JNK in osteoblast differentiation, it was recently reported that it is essential in the late stage of this phenomenon [8], and mediates OPN gene expression [42]. Finally, it has been demonstrated that CREB is one of the transcription factors involved in RANKL expression [43]. Thus, it is reasonable to assume that the inhibitory effect of OxLDL on OPN and RANKL gene expression is related to an impairment of Pi signaling pathways.

Brodeur et al. [44] reported that low concentrations of OxLDL (10–50 μg/ml) induced proliferation of osteoblasts whereas high concentrations (above 150 μg/ml) were cytotoxic. In our experimental model, generation of ROS and impairment of Pi signaling were observed after short-term (4 h) incubation with OxLDL, and thus cannot be ascribed to a cytotoxic effect of these particles. Furthermore, even after 4 days of incubation with Pi and 10–50 μg/ml OxLDL, the experimental conditions chosen for the study of OPN and RANKL expression, no cytotoxicity could be detected in our model, as assessed by the Neutral Red uptake method (data not shown).

It was further demonstrated that Pi induced a significant increase in ROS (Fig. 5), which is in accordance with the report from Shuto et

al. on aortic endothelial cells [45]. Furthermore, OxLDL or its lipid extract triggered this effect, whereas the antioxidant vitamin E prevented it (Fig. 6). In this regard, it can be noted that oxidized phosphatidylcholine, a lipid component of OxLDL, has been reported to inhibit spontaneous osteogenic differentiation of marrow stromal cells [32] and to decrease osteogenic signaling induced by Bone Morphogenetic Protein 2 and parathyroid hormone in osteoblasts [46]. Concerning the mechanism whereby OxLDL initiate generation of ROS, a recent report pointed at the role of CD36 scavenger receptor and NADPH oxidase [47] in macrophages, whereas the contribution of lipoxygenase and of the mitochondrial pathways has been described in vascular smooth muscle cells [48]. Finally, in osteoblasts, Hamel et al [49] reported the role of NADPH oxidase in OxLDL-induced ROS generation.

Our studies demonstrate that a moderate generation of ROS, initiated by a single agent, in our case by OxLDL or Pi, activates signaling kinases and transcription factors. By contrast, a too high level of ROS, initiated by OxLDL and 4 h later by Pi, might induce a negative effect by generation of an "overoxidized" state. In this regard, it is of note that the oxidative stress caused by hydrogen peroxide also inhibits Pi-induced mineralization in our experimental system (data not shown).

It is well known that OxLDL might inhibit hormone and growth factor signaling pathways, most probably by generation of ROS. In this regard, it was demonstrated that these particles prevent insulin signaling in fibroblasts [22], PDGF transduction in vascular cells [50] or RANKL transduction signal in osteoclasts [19]. It can thus be supposed that OxLDL might impair not only Pi signaling but also other transduction pathways crucial for osteoblast differentiation. It must be also noted that osteoblasts have the capacity to oxidize LDL [44], and that OxLDL are thus generated inside bone matrix.

In conclusion, our studies demonstrate that OxLDL inhibits Pi signaling and Pi-induced mineralization of osteoblasts via generation of an oxidative stress. OxLDL finally downregulates the expression of osteoblast specific genes such as Cbfa1/Runx2, OPN and RANKL. This highlights the negative role of OxLDL in bone formation, and points at its harmful effect in degenerative pathologies such as osteoporosis.

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