Characterization of oxidized low-density lipoprotein-induced hormesis-like effects in osteoblastic cells

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Epidemiological studies indicate that patients suffering from atherosclerosis are predisposed to develop osteoporosis. Atherogenic determinants such as oxidized low-density lipoprotein (oxLDL) particles have been shown both to stimulate the proliferation and promote apoptosis of bone-forming osteoblasts. Given such opposite responses, we characterized the oxLDL-induced hormesis-like effects in osteoblasts. Biphasic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reductive activity responses were induced by oxLDL where low concentrations (10–50 μg/ml) increased and high concentrations (from 150 μg/ml) reduced the MTT activity. Cell proliferation stimulation by oxLDL partially accounted for the increased MTT activity. No alteration of mitochondria mass was noticed, whereas low concentrations of oxLDL induced mitochondria hyperpolarization and increased the cellular levels of reactive oxygen species (ROS). The oxLDL-induced MTT activity was not related to intracellular ROS levels. OxLDL increased NAD(P)H-associated cellular fluorescence and flavoenzyme inhibitor diphenyleneiodonium (DPI) enhanced MTT activity, suggesting an enhancement of NAD(P)H-dependent cellular reduction potential. Low concentrations of oxLDL reduced cellular thiol content and increased metallothionein expression, suggesting the induction of compensatory mechanisms for the maintenance of cell redox state. These concentrations of oxLDL reduced osteoblast alkaline phosphatase activity and cell migration. Our results indicate that oxLDL particles cause hormesis-like response with the stimulation of both proliferation and cellular NAD(P)H-dependent reduction potential by low concentrations, whereas high concentrations lead to reduction of MTT activity associated with the cell death. Given the effects of low concentrations of oxLDL on osteoblast functions, oxLDL may contribute to the impairment of bone remodeling equilibrium.

osteoblasts; atherosclerosis; oxysterol

ELEVATED LEVELS OF serum low-density lipoprotein (LDL) particles are considered as the most important atherogenic risk factor. LDL particles are thought to become atherogenic after undergoing oxidative modifications, and key roles of oxidized LDL (various oxidized species collectively designated oxLDL) in atherosclerosis have been largely reviewed by Steinberg (46). OxLDL and oxysterols have been shown to increase or decrease proliferation and trigger the apoptosis process depending on the cell types related to vasculature alterations, nature of oxLDL and oxysterols, and on the concentrations used (52), thus supporting numerous deleterious effects that sustain the development of atherosclerosis. Also, a number of clinical studies suggest an association between cardiovascular disease and the development of osteoporosis, independently of age and hormonal deficiency (2, 3, 48–50). Positive relationships with LDL particles and LDL-associated apoB both in men and in women have been reported for osteoporosis (1, 37, 41). Lipid accumulation has been observed in osteoporotic and aging bone (36, 42, 47). The presence of oxidized lipids was revealed in the bone marrow of hyperlipidemic mice (47). Furthermore, Maggio et al. (33) have reported a marked decrease in plasma antioxidants in aged osteoporotic women. Therefore, these studies suggest the existence of one or several contributory factors in the parallel development of atherosclerosis and osteoporosis.

The bone is a dynamic tissue that is continuously being remodeled following two opposite and coordinated processes. Under normal conditions, specialized cells called osteoclasts transiently break down old bone (resorption process) at multiple sites as other cells known as osteoblasts are replacing it with new tissue (bone formation). Following differentiation from mesenchymal stem cells, osteoblastic cells assure bone formation and mineralization through the secretion of bone matrix components (type I collagen and noncollagenous proteins) and also play a central role in the regulation of bone resorption by providing essential factors such as macrophage colony-stimulating factor and receptor activator of NF-κB ligand for the differentiation of osteoclasts (32). In this context, alteration of osteoblastic proliferation, differentiation, secretary functions, or apoptosis rate are thought to compromise the maintenance of bone remodeling equilibrium. Parhami et al. (40) reported a reduction of bone mineralization in mice fed with an atherogenic high-fat diet, with a decreased expression of osteoblastic marker osteocalcin by marrow cells, suggesting an inhibition of osteoblastic differentiation. Accordingly, oxLDL particles have been reported to promote in vitro cell proliferation and to inhibit the differentiation of murine osteoprogenitor cell line MC3T3-E1 and of bone marrow osteoblastic precursor cells (38, 39). Liu et al. (29) showed that low concentrations of oxysterol cholestane-3b,5a,6b-triol increased cell viability and that high concentrations inhibited osteoblastic differentiation and promoted the apoptosis of primary rat bone marrow stromal cells. Klein et al. (20) reported an inhibition of the osteoblastic phenotype marker alkaline phosphatase activity and cell death by oxLDL in human osteoblastic SaOS cells. We have reported that high concentrations of oxLDL cause cell death through the apoptosis of human osteoblastic MG-63 cells (6), which may contribute to the imbalance of bone remodeling equilibrium.

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Altogether, the studies have reported paradoxical stimulation and loss of osteoblastic viability by oxLDL, which have highlighted oxLDL as a contributory factor in the parallel development of atherosclerosis and osteoporosis.

Given that the effects of oxLDL appear to be dependent on the concentrations, we speculated that the effects of oxLDL on cell viability may not correspond to typical monophasic dose response but rather be associated with biphasic responses related to hormesis. Determination of dose-response effects is general procedure in toxicology for risk evaluation and the establishment of exposure guidelines in view of monophasic responses. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide (MTT) assay has been widely used toward this end and has also permitted to reveal in some cases biphasic hormesis responses (13, 14, 43, 51). Hormesis has been defined as a dose-response relationship in which a stimulatory response occurs at low doses, and an inhibitory response takes place at high doses, resulting in a U- or inverted U-shaped dose response (10). Biphasic relationships have been described for various end-point functions such as growth (metabolism, proliferation, survival, and longevity) and deleterious effects (disease, cancer, etc). Hormesis is considered as an evolutionarily conserved process, and the mechanisms underlying hormesis remain an enigma. The induction of biphasic hormesis-like relationships has been described for mild heat stress, radiation, and exposure to environmental toxic agents such as heavy metals (11). In some cases, hormesis has been considered as an adaptive or conditioning response that increases the resistance of the cell or organism to moderate to severe levels of stress. Such observations of biphasic dose-response relationships have changed the general conception of the risk evaluation since the stimulation effect of low concentrations may result in the loss of regulation and equilibrium of cell functions, especially in view of bone remodeling.

Given that opposite effects by oxLDL such as stimulation of cell viability and promotion of cell death have been reported in osteoblastic cells, we have characterized the hormesis-like effects induced by oxLDL in osteoblastic cells and focused on the effects of low concentrations of oxLDL on osteoblastic functions.

MATERIALS AND METHODS

Cell culture. Human osteoblastic MG-63, SaOS, U2 OS, and murine osteoprogenitor MC3T3 cells (ATCC, Rockville, MD) were grown in a 1:1 mixture of phenol-free DMEM/Ham’s F12 medium (DMEM/F12; Sigma, Oakville, ON, Canada) for MG-63, in McCoy’s medium (Hyclone, Logan, UT) for SaOS and U2 OS cells, and in α-MEM medium (Sigma) for MC3T3 cells. All media were supplemented with 10% fetal bovine serum (FBS; Camera, Etobicoke, ON, Canada), l-glutamine (Invitrogen, Burlington, ON, Canada), and penicillin/streptomycin (Invitrogen). Cells were cultured in 5% CO₂ at 37°C and were harvested weekly with trypsin-EDTA solution (Invitrogen). Bone marrow stromal cells and primary mouse osteoblasts (mOB) from C57BL/6 mice were isolated as described previously (12, 23). Briefly, mice were euthanized according to institutional procedure for the use of experimental animals, and the protocol was approved by the Institutional Animal Care and Use Committee of Université du Québec à Montréal. The femur and tibia were removed under aseptic conditions. Bones were broken in half and centrifuged for the collection of bone marrow stromal cells. Following a short spin, the cell pellets were resuspended, seeded in 100-mm dishes (Sarstedt, Montreal, QC, Canada), and allowed to adhere for 2 days in α-MEM medium (osteoblastic differentiation medium) supplemented with 15% FBS. The cells remaining in suspensions were washed out, and adherent cells were cultured for 1 to 2 wk. For mOB, bone fragments were subjected to three consecutive digestions with collagenase A (Sigma), and digested fragments were plated with α-MEM medium in 100-mm dishes (Sarstedt) until cell outgrowth was performed and confluence was reached.

Isolation and modification of lipoproteins. Lipoprotein particles were isolated from human plasma obtained from Bioreclamation (Hicksville, NY). Before isolation, the plasma was adjusted to 0.01% EDTA, 0.02% sodium azide, and 10 μM phenylmethylsulfonyl fluoride. Human LDL (density: 1.025–1.063 g/ml) was prepared as described by Brissette et al. (5). Lipoprotein particles contained no detectable amount of apoE as assessed by immunoblotting.

LDL preparations were dialyzed against Tris-buffered saline to remove EDTA before oxidation. OxLDL particles were prepared as described by Lougheed and Steinbrecher (31). LDL particles (200 μg protein/ml in Tris-buffered saline) were incubated with 5 μM CuSO₄ for 20 h at 37°C. Oxidation was stopped by the addition of EDTA (final concentration of 100 μM), and butylated hydroxytoluene (40 μM final) and the oxLDL particles were concentrated to 15–20 mg/ml using Centriplus-100 ultrafiltration devices (Amicon, Oakville, ON, Canada). OxLDL typically resulted in a 2.8-fold increase in the electrophoretic mobility relative to native LDL (nLDL) on 0.5% agarose/barbitral gels.

MTT reduction assay. For measurement of cell proliferation or viability, cells were seeded in 96-well plates (Sarstedt). After 5 days of culture in media containing 10% FBS, the cells were further incubated in DMEM/F12 without FBS in the absence or presence of native LDL, oxLDL, or oxysterols 7-ketocholesterol and 7β-hydroxycholesterol (Sigma). Two hours before the end of treatments, the media were replaced with DMEM/F12 containing 0.5 mg/ml MTT (Sigma). Cellular reduction of the tetrazolium ring of MTT resulted in the formation of a dark-purple water-insoluble deposit, the formazan crystals. At the end of the incubation, media were aspirated and formazan crystals were dissolved in DMSO. Absorbance was measured at 575 nm with a spectrophotometer, and data were expressed as relative MTT activity corresponding to the ratio of absorbance of liprotein-treated cells versus control cells incubated with DMEM/F12 alone. In certain experiments, the cells were pretreated with chloroquine or diphenylelenedionium (DPI) 1 h before the addition of MTT or with N-acetylcysteine or l-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, 24 h before treatment with oxLDL. Chloroquine diffuses into acidic compartments and becomes protonated, thereby destroying the acidic environment and inactivating the acid-dependent lysosomal enzymes. DPI phenylates and inhibits a variety of flavoenzymes, such as the mitochondrial NADH dehydrogenase (complex I) and the NADPH oxidase.

Flow cytometry and confocal microscopy analysis. For cell division analysis, carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) was used. This cell-permeable dye is deesterified by intracellular enzymes, creating a charged molecule trapped inside the cells. Upon division, daughter cells get one-half of the fluorescent marker, and therefore reduction of fluorescence may be used to monitor cell division. CFSE (5 mM stock solution in DMSO) was added (final concentration of 2 μM) to the cells for 10 min at 37°C. Labeling was stopped by the addition of 10% FBS for 15 min. CFSE-labeled cells were cultured in vitro under different conditions. Cells were therefore trypsinized and analyzed by flow cytometry with a logarithmic detection of green fluorescence (CFSE). For cell counts, internal calibrator microspheres were added immediately before flow cytometric analysis. Using the cytometer and forward scatter and side scatter parameters, the interference of apoptotic cells and debris was excluded. Cell size was determined by the forward side scatter function (SSC). Data were acquired in a FACScan flow cytometer (Becton Dickinson) using Cell Quest software.
For the determination of mitochondria mass, the cells were incubated with 200 nM MitoTracker Green FM (Invitrogen) in DMEM/F12 for 30 min at 37°C, washed twice, and then analyzed by a FACScan flow cytometer. This dye accumulates in the mitochondria regardless of the membrane potential, which allows the quantification of the amount of mitochondria. The mitochondrial membrane potential of intact cells was measured by flow cytometry with the lipophilic cationic probe 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Invitrogen). According to the mitochondrial potential, monomeric forms of JC-1 emit green fluorescence upon depolarization, whereas their aggregation leads to red fluorescence with the hyperpolarization of mitochondria. Hence, the red/green fluorescence ratio is a way to measure the polarization, or potential energy, of the mitochondria. Following treatments, the cells were trypsinized, and the cell pellets were resuspended in 500 µL PBS and incubated with 10 µM JC-1 for 20 min at 37°C. The cells were subsequently washed once with cold PBS, suspended in a total volume of 350 µL, and analyzed by flow cytometry. The production of intracellular reactive oxygen species (ROS) was measured with the hydrophobic, peroxido-sensitive fluorescent dye 5-chloromethylcarboxyethyl dichlorofluorescin diacetate (CM-H2DCF-DA; Invitrogen). This probe is nonfluorescent until cleavage by intracellular esterases, and its oxidation by intracellular hydrogen peroxide increases the fluorescence. Cells were incubated in culture media with CM-H2DCF-DA for 1 h at a final concentration of 10 µM. Thereafter, cells were incubated at 37°C for various periods of time with oxLDL, washed with PBS, harvested, and analyzed immediately by flow cytometry. Cell migration. A wound scratch assay was performed with a 10× (0.25 numerical aperture) objective linked to a CoolSnap Es CCD camera for 24 h. This time interval has been chosen because it is shorter than MG-63 doubling time in these conditions. For lysosomal staining, MG-63 cells were vitally stained with an acridine orange (AO; Sigma) solution at 5 µg/mL in complete medium for 30 min at 37°C. AO is a lysosomotropic weak base and a metachromatic fluorochrome showing red fluorescence at high concentrations and green fluorescence at low concentrations. The intensities of red and green AO fluorescence were then examined with a laser-scanning confocal (Bio-Rad) microscope (Nikon TE300) using a Plan-Apochromatic ×60 oil (numerical aperture 1.4) objective lens. For NAD(P)H-associated cellular fluorescence dye carboxymethyl dichlorofluorescein diacetate (CM-H2DCF-DA) for 20 min at 37°C. The cells were subsequently washed once with cold PBS, suspended in a total volume of 350 µL, and analyzed by flow cytometry. This dye accumulates in the mitochondria F12 for 30 min at 37°C, washed twice, and then analyzed by a FACScan flow cytometer. This dye accumulates in the mitochondria F12 for 30 min at 37°C, washed twice, and then analyzed by a FACScan flow cytometer. The optical density of the yellow product para-nitrophenol produced in nmol·1 h⁻¹·mg protein⁻¹.

Cellular protein quantification. Cellular protein contents were quantified by MicroBCA protein assay (Pierce, Rockford, IL) using BSA as a standard.

Statistical analysis. Statistical differences were analyzed by ANOVA or Student’s t-test using GraphPad Prism3 software. A level of P < 0.05 was considered significant.

RESULTS

Effects of oxLDL on MTT activity of MG-63 cells. The levels of circulating oxLDL have been mentioned as a predictive and sensitive marker of coronary artery disease. Holvoet et al. (17–19) have reported circulating concentrations of oxLDL ranging from 6–14 µg/mL for patients showing no risk equivalent for coronary artery disease to 13–75 µg/mL for patients who have a greater prevalence of coronary artery disease. To investigate both stimulation and inhibition responses corresponding to hormesis and to reveal the effects of oxLDL at concentrations physiologically significant, we evaluated the effects of oxLDL by monitoring the MTT activity of osteoblastic cells exposed to concentrations ranging from 10 to 250 µg/mL. The MTT activity was determined in various models of human osteoblastic cells (MG-63, SaOS, and U2 OS), murine osteoblastic MC3T3 cells, primary cultures of murine bone marrow stromal cells, and osteoblasts from long bones (mOB) incubated with increasing concentrations of oxLDL. As shown in Fig. 1A, oxLDL increased or decreased the MTT activity by 10.22±0.32 on October 29, 2017 http://ajpcell.physiology.org/ Downloaded from
MC3T3 cells. As shown in Fig. 1B, oxLDL (20 μg/ml) increased the MTT activity (P < 0.001, ANOVA), leading to a significant augmentation at 9 h to 48 h (P < 0.01, Dunnett’s), whereas the MTT activity declined thereafter and was not significantly different from controls at 72 h. A similar time-dependent decline of MTT activity was observed with MC3T3 cells (Fig. 1B). As oxLDL induced comparable hormesis-like biphasic responses in all the osteoblastic models analyzed, subsequent experiments were performed with MG-63, which was the most responsive cell line.

**Effects of oxysterols on MG-63 cells.** We further evaluated the effects of 7β-hydroxycholesterol and 7-ketocholesterol, two oxysterols, detected in atherosclerotic lesions (7), formed by the copper-catalyzed oxidation of LDL on the MTT activity by MG-63 cells. As shown in Fig. 2A, low concentrations of 7β-hydroxycholesterol (from 5 μM) increased the MTT activ-
The increase in MTT activity by 7-hydroxycholesterol was shown to be time dependent. Lysophosphatidylcholine, another main component of oxLDL, did not alter the MTT activity of MG-63 cells (data not shown).

Effects of oxLDL on cell division of MG-63 cells. Given that an increased MTT activity is usually regarded as representative of cell proliferation, the effect of nLDL and oxLDL on division was further determined by estimation of total cell counts and measurement of the decrease in CFSE fluorescence, associated with cytokinesis. As shown in Fig. 3, A and B, the number of cells and the relative cell division, determined by the decrease in CFSE fluorescence, occurred in the presence of 200 μg/ml nLDL, which confirms the stimulation of cell proliferation by nLDL as suggested by the increased MTT activity. In addition, low concentrations of oxLDL (10 and 20 μg/ml) also increased significantly the cell number and relative cell division. As expected, concentrations of oxLDL from 50 μg/ml significantly reduced the cell number (P < 0.01, Dunnett’s) and reduced cell division evaluated by CFSE fluorescence when compared with low cell proliferation rate in control condition with the culture medium alone (Fig. 3, A and B). It should be noted that cell number with 50 μg/ml oxLDL was not different compared with basal condition before the 48-h period of incubation, which indicates the absence of cell proliferation. Furthermore, when the relative MTT activity was normalized according to the relative cellular protein content (Fig. 3C, P < 0.0001, ANOVA) or cell number (Fig. 3D), ratio above 1 was revealed for cells treated with 20 and 50 μg/ml oxLDL, which argues for a significant discrepancy between the MTT activity and cell proliferation at these concentrations. Nevertheless, for concentrations of oxLDL above 100 μg/ml, ratio of MTT activity on cellular protein or cell number was ~1, confirming osteoblastic cell death as we have previously reported (6). Therefore, our results indicate that part of the increased MTT activity induced by low concentrations of oxLDL corresponds to cell proliferation and that high concentrations of oxLDL induced cell death, which are characteristic of the oxLDL-induced hormesis-like effect. Because a discrepancy between the MTT activity and cell proliferation at low concentrations of oxLDL was observed, the nature of the increased MTT activity promoted by 10, 20, and 50 μg/ml oxLDL was studied.

MTT activity and lysosomal function. Because oxLDL particles are known to disturb lysosomal function (27) and because MTT activity has been associated with endosome/lysosome (30), the correspondence between oxLDL-induced increased MTT activity and lysosome activity was evaluated. As shown in Fig. 4A, incubation of MG-63 cells with low concentrations of oxLDL for 48 h led to an increase in cell autofluorescence, which suggests the presence of undegradable lipofuscin/ceroid-like materials. The mitochondrial respiratory chain inhibitor sodium azide had no effect on basal or oxLDL-induced cellular autofluorescence, suggesting that the latter fluorescence may be associated with an accumulation of lipofuscin/neroid material (data not shown). We hypothesized that a compensatory increase of cell endosome/lysosome activity could account for the increased MTT activity. As shown in Fig. 4B, no increase in the cellular content of acidic compartments was shown in cells incubated for 24 h with low concentrations of oxLDL following the staining of acidic organelles with AO. Moreover, the inhibition of lysosomal function by the addition of chloroquine before MTT activity assays did not alter the MTT activity of the cells either in the absence or presence of low concentrations of oxLDL (Fig. 4B).

Effects of oxLDL on mitochondrial mass and cell size. Cellular hypertrophy has been reported in human umbilical vein endothelial cells exposed to oxLDL (44) and could account for the discrepancy between the MTT activity due to increased cell volume and organelle mass without an increase in cell number. Therefore, we evaluated the effect of oxLDL on mitochondrial mass and cell size. Results in Fig. 5A show that low concentrations of oxLDL do not increase the mitochondrial mass of MG-63 cells (P = 0.1680, ANOVA). Similar results were obtained with 7β-hydroxycholesterol and therefore increased mitochondrial mass excluding that it could account for the increased MTT activity. Moreover, oxLDL and 7β-hydroxycholesterol did not affect the cell size of MG-63 cells (data not shown).

Effects of oxLDL on the mitochondrial membrane potential and the production of ROS. Because the increase in the MTT activity of MG-63 cells by oxLDL may result from an imbalance in mitochondrial activity, the mitochondria membrane...
potential was measured using JC-1. As shown in Fig. 5B, low concentrations of oxLDL increased the mitochondrial membrane potential in MG-63 cells. For comparison, incubation of MG-63 cells with the known mitochondria uncoupler FCCP was associated with mitochondria depolarization as revealed by reduced ratio of red/green fluorescence (Fig. 5B). Because the hyperpolarization of mitochondria has been associated with the production of ROS, the levels of ROS in MG-63 cells incubated with low concentrations of oxLDL were further measured. As shown in Fig. 5C, the experimental concentrations of oxLDL used induced the production of ROS in MG-63 cells. Similarly to oxLDL, low concentrations of 7β-hydroxycholesterol induced mitochondria hyperpolarization (Fig. 5B) and the production of ROS (Fig. 5C).

Relationship between ROS levels and MTT activity. Because part of the intracellular reduction of MTT has been associated with superoxide production (9), we determined whether the production of ROS induced by oxLDL was associated with increased MTT activity by MG-63 cells. As shown in Fig. 6A, incubation of cells with the antioxidant N-acetylcysteine before treatments with low concentrations of oxLDL did not prevent the increase in MTT activity. Moreover, depletion of the cellular ROS scavenger glutathione by incubation with BSO, which increased basal and oxLDL-induced ROS levels (Fig. 6C), neither altered the basal MTT activity nor further increased the MTT activity induced by oxLDL in MG-63 cells (Fig. 6B), but rather reduced the MTT activity induced by oxLDL (*P < 0.001, two-way ANOVA).

Involvement of flavoenzyme and NAD(P)H on MTT activity. Both mitochondrial and nonmitochondrial NAD(P)H-dependent MTT activity have been reported (30). Therefore we hypothesized that the effect of oxLDL on MTT activity by MG-63 cells was associated with flavoenzyme activity and cellular NAD(P)H levels. As shown in Fig. 7A, the addition of the flavoenzyme inhibitor DPI for 3 h reduced both basal (P < 0.003, ANOVA; P < 0.05 from 10 μM, Dunnett’s) and oxLDL-induced MTT activity (P < 0.0001, ANOVA; P < 0.01 from 5 μM, Dunnett’s) in a dose-dependent manner. Therefore, our results indicate that DPI reduced in a comparable manner the MTT activity both in control cells as well as in cells treated with oxLDL, which suggests that the latter increase the flavoenzyme activity. Moreover, NAD(P)H-associated cellular fluorescence was also increased by oxLDL (Fig. 7B), and the fluorescence specificity for NAD(P)H was demonstrated by the reduction of fluorescence with FCCP, which promotes NADH oxidation.
Effect of oxLDL on the cellular amount of thiol-containing proteins. Because oxidative stress was shown to be induced by oxLDL in MG-63 cells (Fig. 5C), we postulated that mechanisms involving thiol-containing ROS scavenger proteins may be associated with the maintenance of cellular redox state. Members of the thiol-containing family of proteins undergo reversible oxidation/reduction catalyzed by proteins of the NADPH-dependent thioredoxin-fold family and thereby contribute to maintain the redox state of cells (4). As shown in Fig. 8A, thiol content monitored by CMFDA fluorescence measurement was decreased in MG-63 cells following incubation with oxLDL, which indicates increased oxidation of thiol-containing proteins. Moreover, the expression of thiol-containing metallothionein in cells exposed to oxLDL was increased by 2.5-fold (Fig. 8B), which further suggests the induction of processes to maintain the redox state of cells.

The effects of low concentrations of oxLDL on osteoblastic functions. To determine the significance of the effects of low concentrations of oxLDL with respect to bone metabolism, we monitored osteoblastic functions under conditions of incubation with low concentrations of oxLDL. Because exposure to oxLDL has been associated with increased osteoblastic proliferation, with a concomitant reduction of differentiation (39), we determined the alkaline phosphatase activity in MG-63 cells exposed for 48 h to low concentrations of oxLDL. As shown in Fig. 9A, the treatment of MG-63 cells with low concentrations of oxLDL reduced the alkaline phosphatase activity, indicating that osteoblastic functions are altered by low concentrations of oxLDL. In addition, we observed that 50 μg/ml oxLDL reduced the basal migration of MG-63 cells, whereas the same concentration of nLDL increased by 2.5-fold the migration (Fig. 9B).

DISCUSSION

Determination of hormesis-like effects induced by oxLDL and oxysterol. Incubation of osteoblastic cell lines with increasing concentrations of oxLDL and 7β-hydroxycholesterol, an oxysterol usually formed along copper-mediated LDL oxidation, resulted in biphasic MTT activity dose responses. Such nonmonotonic dose-response relationship agrees with the qualitative characteristic of hormesis where low concentrations of oxLDL were incubated in culture medium (CTL) or with 20 μg/ml oxLDL for 48 h. Left: representative cellular autofluorescence monitored as described in MATERIALS AND METHODS with the corresponding phase contrast image. Right: data are expressed as means ± SE of the relative autofluorescence from at least 3 independent experiments. One-way ANOVA, Dunnett’s: *P < 0.05, **P < 0.01, ***P < 0.001 compared with control condition without lipoprotein.

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a stressful stimulus trigger a stimulatory response, whereas an inhibitory response occurs at high concentrations (10). Furthermore, quantitative features of hormesis as established by Cabalrese and Baldwin (10) were also observed: the average maximum amplitude of the stimulatory response was between 130% and 160% of the control, and the range of the hormetic zone was 10- to 20-fold. Moreover, the increase in MTT activity induced by oxLDL was time dependent with a significant augmentation observed at 9 h incubation, a maximal effect occurring at 48 h that was not evident at 72 h. Therefore we show for the first time that oxLDL and 7β-hydroxycholesterol induce hormesis-like effects in osteoblastic cells. Similar dose- and time-dependent induction of biphasic MTT activity responses by oxLDL has been reported in macrophage cells (16) according to their degree of oxidation. Moreover, similar hormesis-like response of MTT activity and time-dependent response have been shown for marrow stromal cells incubated with the oxysterol cholestane-3b,5a,6b-triol (29).

Both direct stimulation hormesis (DSH) and overcompensation stimulation hormesis (OCSH) have been described with...
distinct temporal features (10). A disruption of homeostasis characterized as an initial reduction of end-point function monitored, a modest overcompensation, the reestablishment of homeostasis, and the adaptive nature of the process are all key conceptual features of OCSH. In contrast, DSH does not result from a disrupted homeostasis but represents a direct stimulatory response to initial stimuli, operating within normal maintenance functions without initial reduction of end-point function. In the current study, MTT activity was the endpoint function monitored and did not show any initial reduction from 4 h. Therefore, our results more likely correspond to DSH.

Correspondence of MTT activity with cell proliferation and death. As the stimulatory MTT response triggered by low concentrations of oxLDL seems at first glance associated with cell proliferation, we further determined the correspondence of the increased MTT activity with cell division. Our results indicate that part of the increase in MTT activity reflects a stimulation of osteoblastic cell proliferation by low concentrations of oxLDL. Accordingly, Parhami et al. (39) have reported that minimally oxLDL particles promote cell proliferation and inhibit the differentiation of MC3T3 bone cells based on evidence of increased [3H]-thymidine incorporation and inhibition of the induction of alkaline phosphatase as a marker for osteoblastic differentiation. On the other hand, our
HORMESIS-LIKE EFFECT INDUCED BY oxLDL IN OSTEOBLASTS

Fig. 8. Evaluation of the content of thiol-containing proteins in cells exposed to oxLDL. A: cells were incubated in culture medium (CTL) or in the presence of 200 µg/ml nLDL or 20 µg/ml oxLDL for 24 h. Thereafter, cells were loaded with 5-chloromethylfluorescein diacetate (CMFDA) and the fluorescence was analyzed by confocal microscopy. Values are expressed as means ± SE of the relative CMFDA fluorescence compared with values of the control conditions from at least 3 independent experiments. Two-tailed Student’s t-test: **P < 0.01 compared with the control condition. B: cells were incubated in culture medium or in the presence of 20 µg/ml oxLDL for 24 h. Total RNA was isolated and subjected to RT-PCR using specific primers for human metallothionein 1/2 (MT) and GAPDH. Densitometric determinations were analyzed and expressed as the relative MT expression normalized against GAPDH expression when compared with the control condition of 3 independent experiments. Two-tailed Student’s t-test: *P < 0.05.

data afford arguments that suggest potential discrepancies between the increased MTT activity and cell proliferation of MG-63 cells. First, a significant increase in MTT activity was seen as soon as 9 h after the addition of oxLDL to the incubation media. Under high cell proliferation rate in the presence of serum, MG-63 cells rather showed doubling time of 28 h (22). Therefore completion of cell cycle that would be associated with increased cell number and MTT activity within 9 h is unlikely. Moreover, a ratio above 1 was shown when the relative MTT activity was normalized by relative cell number or cellular protein content. Therefore, part of the increased MTT activity by osteoblastic cells incubated with oxLDL was of other nature.

Our data also indicate that high concentrations of oxLDL particles promote an inhibitory response evidenced by the reduction of MG-63 cell viability, indicated by the loss of MTT activity and the reduction of cell number. Accordingly, we (6) and others (20, 29) have reported that oxLDL particles induce the apoptosis of osteoblastic cells followed by annexin V staining, DNA fragmentation, loss of lysosomal particles, and appearance of pro-apoptotic proteins. Furthermore, increasing concentrations of oxysterols such as 7β-hydroxycholesterol and 7-ketocholesterol resulted in the reduction of MG-63 cell viability (from 20–30 µM) as indicated by the loss of MTT activity after 48 h of incubation. Our results agree with studies of Liu et al. (29), which showed that concentrations above 15 µM of oxysterol cholestan-3b,5α,6b-triol promote cell death of primary rat bone marrow cells after 2 days of culture.

OxLDL and lysosome activity. Given that a discrepancy was shown between MTT activity and cell proliferation for osteoblastic cells incubated with oxLDL, we further characterized the nature for oxLDL-induced increase of MTT activity. Inhibition of lysosomal activity by chloroquine and acidic compartment staining with AO indicated that the increased osteoblastic MTT activity induced by oxLDL was not associated with enhanced endosomal/lysosomal activity. Nevertheless, we observed that incubation of MG-63 cells with low concentrations of oxLDL was associated with an increase of cell autofluorescence, which suggests the presence of lipofuscin/ceroid-like materials. Lipofuscin/ceroid formation results from the progressive accumulation of biological “garbage” material, such as defective mitochondria, cytoplasmic protein aggregates as an intralysosomal undegradable material, with bright, wide-spectrum autofluorescence (8). It has been shown that, after its uptake into macrophage lysosomes by receptor-mediated endocytosis, oxLDL particles are poorly degraded, resulting in ceroid-containing foam cells (27). Moreover, oxLDL-induced cytotoxicity in macrophages (26) and in osteoblast (6) has been associated with lysosomal rupture. Our results suggest for the first time that oxLDL particles may promote lipofuscin/ceroid accumulation in osteoblastic cells.

Effects of oxLDL on mitochondria of osteoblastic cells. We suspected that the increase of MTT activity could correspond to increased mitochondria mass or metabolic activity. Such an increase of mitochondrial mass under oxidative stress condition has been reported (24, 25). However, our results showed that the mitochondria mass was not increased by oxLDL and therefore cannot account for the oxLDL-induced increase of MTT activity. However, low concentrations of oxLDL induced mitochondria membrane hyperpolarization in osteoblastic cells as has been reported in Caco-2 intestinal cells exposed to oxLDL (15). Mitochondrial hyperpolarization has been described as an early apoptotic event (34) and has been associated with an exponential increase in ROS production (21) being a major contributor to the oxidative signal induced by oxLDL (53). Accordingly, our results indicate that oxLDL particles stimulate the production of ROS in MG-63 cells. Therefore, mitochondrial hyperpolarization induced by low concentrations of oxLDL in osteoblastic cells may be part of the hormesis stimulatory response necessary to compensate and maintain the cellular metabolic homeostasis, disrupted by the stressful stimulus. However, a higher metabolic rate is also associated with the production of ROS, which may subsequently culminate in apoptosis. Since MTT activity has been associated with cellular superoxide production (9), the oxLDL-induced ROS production could result in the augmentation of MTT activity. However, the use of antioxidant N-acetylcyste-
ine or BSO did not modify the oxLDL-induced increase of MTT activity in osteoblastic cells, suggesting that the increase of MTT activity is not associated with intracellular ROS levels.

**Association of the increased MTT activity with NAD(P)H-dependent mechanisms for the reduction of cellular thiols.** As we report that oxLDL particles promote the production of ROS in osteoblastic cells, we suspected that pathways involved in ROS scavenging, such as thiol proteins, may be triggered. Accordingly, the cellular content of reduced thiol was decreased by exposure to oxLDL and the expression of thiol-containing metallothionein was increased. Moreover, the depletion of cellular ROS scavenger glutathione by incubation with BSO increased the levels of ROS induced by oxLDL and reduced the MTT activity induced by oxLDL, suggesting that, under these conditions, the levels of ROS may not be regulated, which lead to an accentuation of loss of cell viability. Flavoenzymes use flavin as coenzyme in a variety of electron transfer reactions required for energy producing, biosynthesis, and more particularly in detoxification and electron scavenging pathways. Key flavoenzymes in defense against oxidative stress are members of the thioredoxin-fold family of proteins (thioredoxin and glutaredoxin), which catalyze the NADPH-dependent reduction of protein thiols to maintain the redox state of cells. Our results showed that oxLDL particles increase NAD(P)H cellular fluorescence. We also reported that the increased MTT activity induced by oxLDL was inhibited by the flavoenzyme inhibitor DPI. Therefore, oxLDL-induced increased MTT activity in osteoblastic cells may correspond to compensation mechanisms afforded by to maintain the redox state of cellular thiols.

**OxLDL-induced hormesis in osteoblastic cells and bone metabolism.** As shown by our results, induction of hormesis-like response by oxLDL in osteoblastic cells is associated with the stimulation of cell proliferation and ROS production by low concentrations of oxLDL. It is generally accepted that the stimulation of osteoblastic proliferation may compromise their differentiation into competent bone-forming cells (28, 38, 39). In accordance, our results indicate that low concentrations of oxLDL reduced the alkaline phosphatase activity, a marker of osteoblastic maturity. In addition, we showed that oxLDL compromises the migration of osteoblastic cells. Both functions have been shown to play a critical role in bone formation,
remodeling, and fracture repair (45). Therefore, our current study indicates that low concentrations of oxLDL may alter the bone metabolism by reducing osteoblastic differentiation in favor of uncontrolled cell proliferation and by affecting cell migration. On the other hand, high concentrations of oxLDL cause osteoblastic cell death that will result in reduced bone formation. In summary, our results indicate that oxLDL particles alter osteoblastic cell proliferation, migration and apoptosis rate, and thereby may contribute to alteration of bone metabolism equilibrium and may be responsible for the reduction of bone mass associated with atherogenic conditions.

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