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Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells

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Abstract

Objective: Death of vascular smooth muscle cell (VSMC) induced by oxidized LDL (oxLDL) can occur by both necrosis and apoptosis which may contribute to plaque instability and rupture. Reactive oxygen species (ROS) induces apoptosis in VSMC and is involved in oxLDL action, we tested the hypothesis here that a coupling exists between ROS generation and apoptosis of oxLDL-treated VSMC. **Methods:** Cultured VSMC from rat aorta were treated with oxLDL, apoptosis and necrosis were distinguished by using FITC-annexin V label and propidium iodide stain, analyzed by flow cytometry. ROS generation of VSMCs was detected by the fluorescence intensity of DCF. Apoptosis was also determined by cleavage of procaspase-3. **Results:** OxLDL induced apoptosis (3 h) in a dose-dependent manner and reached maximum (with near-basal necrosis) at a concentration of 300 μg/ml. At this and lower (100 μg/ml) concentration, oxLDL, but not native LDL, stimulated ROS production rapidly (≤5 min) and ROS level remained elevated for at least 45 min. Catalase and deferoxamine reduced both oxLDL-induced apoptosis and ROS generation. Superoxide dismutase and benzoic acid neither reduced the oxLDL-induced ROS generation nor inhibited apoptosis. Since oxLDL-induced ROS generation were inhibited by nordihydroguaiaretic acid and rotenone, lipoxygenase and mitochondrial pathways could be involved. In addition, catalase, deferoxamine, and *N*-acetylcysteine inhibited oxLDL-induced cleavage of procaspase-3 as well. **Conclusions:** ROS generation and apoptosis are tightly coupled in oxLDL-treated VSMCs. Antioxidants that reduced ROS level inhibited apoptosis, those that did not reduce ROS level were ineffective. Both mitochondrial and lipoxygenase activities may be involved.

Keywords: Apoptosis; Atherosclerosis; Free radicals; Lipoproteins; Smooth muscle

1. Introduction

There is an increasing body of evidence showing that apoptosis of vascular smooth muscle cells (VSMCs) participates in the pathogenesis of atherosclerosis [1–3]. In early atherosclerotic lesions, VSMCs and macrophages accumulate in the subendothelial space. As the disease progresses most of these cells die, generating fatty streaks that develop into plaques [4,5]. Apoptotic VSMCs are also observed in advanced atherosclerotic plaques [6] and may contribute to the reduced synthesis and secretion of extracellular matrix proteins and thus a weakened intimal plaque texture, leading to plaque instability. This may increase the risk of plaque rupture and subsequent thrombosis [7–9]. OxLDL is known to accumulate in atherosclerotic plaques and its presence increases as the plaque advances in severity [10–12]. In early atherosclerotic lesions, the amount of oxLDL is likely low and exerts mainly proinflammatory effects [13–15]. In more advanced atherosclerotic lesions, increased accumulation of oxLDL containing more peroxidation products may generate a cytotoxic effect contributing to plaque degeneration and rupture. Jovinge et al. [16] demonstrated that oxLDL induces degenerative changes in cultured VSMCs that are

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similar to those observed in dying VSMC in human atherosclerotic lesions. These findings suggest that oxLDL contributes in plaque cell death.

Recent studies have demonstrated that oxLDL can induce apoptosis of cultured VSMCs [17–19], and that reactive oxygen species (ROS) may modulate the programmed death of VSMCs [20–22]. Since oxLDL may be a source of free radical insult in arterial wall [23,24], we ask that whether if changes of ROS production is necessary for oxLDL-induced apoptosis. We thus compared the time course of several events including ROS generation, phosphatidylserine (PS) exposure (an early indicator of apoptosis), and procaspase-3 cleavage in cultured VSMCs and checked that whether apoptosis is blocked by ROS scavenging agents effective in preventing oxLDL-stimulated ROS generation.

2. Methods

2.1. Chemicals

FITC-annexin V apoptosis detection kit, bovine erythrocyte catalase (EC 1.11.1.6), and lipid peroxidation assay kit, nordihydroguaiaretic acid (NDGA) were purchased from Calbiochem-Novabiochem Biosciences, Inc.(San Diego, CA). Medium MCDB-107 was from JRH Biosciences (Lenexa, Kansas). nBODIPY-FL conjugated acetylated LDL (FL-AcLDL) and 2′,7′-dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes, Inc. (Eugene, OR). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), and phenol red-free DMEM were from Life Technologies (Grand Island, NY). Benzoic acid (BA), L-buthionine-[*S,R*]-sulfoximine (BSO), desferrioxamine mesylate (deferoxamine), diphenyleneiodonium chloride (DPI), mannitol, ethylenediamine tetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-acetylcysteine (NAC), penicillin–streptomycin lyophilized powder, superoxide dismutase (SOD, EC1.15.1.1), *tert*-butyl hydroperoxide (t-BuOOH), and lipoprotein deficient serum (LPDS) were from Sigma Chemical Co. (St. Louis, MO). Affinity purified, phycoerythrin (PE)-conjugated anti-active caspase-3 (17/11 kDa subunits) antibody was from PharMingen International (San Diego, CA).

2.2. Cell culture

VSMCs were isolated from thoracic aortas of 20-week-old male Wistar–Kyoto rats as described previously [25]. Cells were grown in medium consisting of DMEM, antibiotics (100 Units/ml penicillin, 100 μg/ml streptomycin), and 10% (v/v) heat-inactivated FCS. Cells at passage 4 through 10 were used for experiments. Endothelial cells were isolated from human umbilical cord veins (HUVEC) and cultured as described previously [26].

2.3. Isolation and oxidative modification of LDL

Native LDL (nLDL; density: 1.019 to 1.063 g/ml) was prepared by sequential ultracentrifugation of plasma from healthy donors and characterized as described previously [27]. The study has been approved by Medical Ethics Committee and conformed with the principles outlined in the Declaration of Helsinki (*Cardiovascular Research* 1997;35:2–3). Oxidized LDLs were prepared by dialyzing nLDL against isotonic saline (pH 7.4, 4°C) containing 50 μM ferrous sulfate until the nLDL progressed through a predictable color change as described by Coffey and coworkers [28,29]. The oxidation was terminated by filtering (0.45 μm) the sample and then dialyzing oxLDL against phosphate buffered saline (PBS: NaCl, 136 mM; KCl, 2.6 mM; KH_2PO_4, 1.5 mM; Na_2HPO_4, 7.9 mM; pH 7.4) containing EDTA (2.5 mM, 5 l) for 6 days with two exchanges daily. OxLDL was further dialyzed against EDTA-free PBS for 24 h to remove EDTA and sterilized by passing through a 0.22-μm filter. Lipid peroxidation products of oxLDL preparations were determined by measuring thiobarbituric acid reactive substance (TBARS) using lipid peroxidation assay kit. Values are expressed as nanomolar equivalents of MDA per milligram LDL protein (nmol MDA/mg LDL prot.). The level of TBARS in oxLDL was 14.7±0.9 nmol MDA/mg LDL prot. Relative electrophoretic mobility (REM) of oxLDL was measured by polyacrylamide gels system (Lipofilm kit; Sebia, France). The REM value which was determined as the ratio of the migrating distance of oxLDL to that of nLDL and was 1.7–2.0. Two concentrations of oxLDL, 100 and 300 μg/ml, were chosen because these concentrations were employed earlier and shown to affect VSMC [30] as well as to mimic in vivo situation [31,32].

2.4. Determination of cytotoxicity and apoptosis

2.4.1. MTT test

VSMCs (1×10^5 cells/well) were subcultured onto 24-well plates and maintained in low serum (0.5% LPDS)/phenol red-free DMEM (16–18 h). The cells then underwent treatments of oxidative enzyme inhibitors for 4 h or ferrous sulfate (37°C, 18-h oxidation) for 24 h. After treatments, mitochondrial dehydrogenase activity was used as an index of cell viability and was assessed using MTT assay [33]. We found no significant cytotoxic effect in VSMCs treated with ferrous sulfate (1, 5, 10, or 50 μM).

2.4.2. Detection of cell surface phosphatidylserine

Phosphatidylserine (PS) translocation from the inner to the outer leaflet of plasma membrane is one of the earliest apoptotic features. VSMCs were incubated with low (100 μg/ml) or high (300 μg/ml) amount of oxLDL for 24 or 3 h, respectively, and the binding of annexin V to cell surface PS was detected with a commercially available annexin V kit. Flow cytometry (Model: FACScan, Becton-

Dickinson, CA) analysis was performed immediately after staining. Typically, untreated viable cell (Fig. 1, panel A) did not bind annexin V or uptake propidium iodide as shown in the lower left-hand quadrant of the dot plot. There were no significant difference between the dot plots of untreated- and nLDL (400 μg/ml, panel B)-treated VSMCs. When VSMCs were exposed to the same concentration of oxLDL (Panel C), apoptotic- and necrotic-VSMCs increased as shown in the lower and upper-right hand quadrants, respectively.

2.5. Measurement of reactive oxygen species

VSMCs (1×10^6 cells/ml of culture medium) were first incubated for 30 min with 10 μM DCF-DA in 0.5% LPDS-DMEM without phenol red at 37°C [34]. Positive control was performed with t-BuOOH at concentration of 0.5 mM [35],which caused rapid oxidation of mitochondrial pyridine nucleotides followed by mitochondrial production of ROS. The DCF fluorescence (Ex: 488 nm; Em: 530±15 nm) resulting from the oxidation of DCF-DA was measured in 1×10^4 cells on a logarithmic scale of fluorescence with a flow cytometry. The change of DCF fluorescence of living cells allows the quantitation of intracellular ROS [36]. We had also evaluated the influence of oxLDL itself on the detection of DCF fluorescence by fluorescence spectrophotometer (Hitachi, Ltd., F-2000). OxLDL alone caused an immediate response in fluorescence which is relatively small (~5% of the later effect with VSMCs) and transient (~3 min) and could reflect that a small amount of ROS was derived from the oxLDL in the absence of cells (see Results section). However, a larger and more sustained fluorescence followed with a pattern similar to other report [37] that was attributed to oxLDL-induced ROS production. Furthermore, it could be eliminated by some antioxidants/enzymes whose actions are associated with cells (e.g. Fig. 7). Oxidative enzyme inhibitors and mitochondrial electron transport chain inhibitor, rotenone, did not interfere with the observations of ROS content of VSMCs in the presence of oxLDL (see Results below).

2.6. Detection of active caspase 3-like protein

To further investigate the effects of ROS on caspase-3 activation, VSMCs were treated with oxLDL (300 μg/ml) with or without catalase, deferoxamine, or NAC for 7 h. Following oxLDL-incubation, the cells were fixed with 4% formaldehyde–PBS solution for 5 min at room temperature. Fixed cells were permeabilized with 0.05% Nonidet P-40–PBS solution, and then stained with PE-conjugated anti-active caspase-3 antibody which was effective to rat cells [38]. After staining, VSMCs were washed once with PBS and PE-fluorescence intensity of each sample was measured by flow cytometry (Ex: 488 nm; Em: 585±20

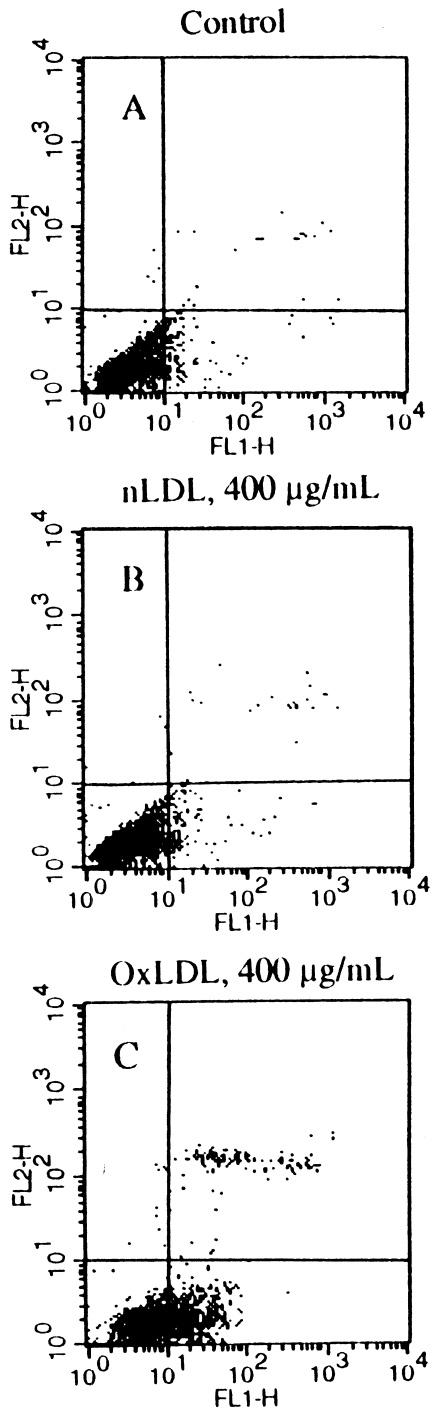

Fig. 1. Apoptotic and necrotic death of oxLDL-treated VSMCs were distinguished by using of FITC-annexin V(AV) label and propidium iodide (PI) stain. VSMCs were treated as indicated above each panel for 3 h and then analyzed by flow cytometry. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for AV binding (AV^-/PI^-). The lower right quadrants represent the apoptotic cells, AV positive and PI negative (AV^+/PI^-). The upper right quadrants, contain the necrotic cells, positive for AV binding and for PI uptake (PI^+). FL1-H: the fluorescence height of AV; FL2-H: the fluorescence height of PI.

nm). Activation of caspase-3 of VSMCs was expressed as cleavage of pro-caspase-3.

2.7. Detection of AcLDL uptake

VSMCs were grown in 10% FCS-DMEM and trypsinized 2 days before the assay. Cultured VSMCs or HUVECs (1×10^6 cells) were washed with PBS and incubated with 10 μg/ml FL-AcLDL in 2% LPDS-DMEM at 37°C for 5 h. Uptake of AcLDL by HUVECs was used as a positive control, and uptake by VSMCs in the presence of 100 μg/ml oxLDL was a negative control. Immediate after the incubations, the cells were placed on ice and washed twice with ice-cold PBS. Cells were harvested by trypsinization and suspended in 2% FCS-PBS solution.

2.8. Statistical analyses

All data are expressed as mean±S.E.M. Differences between mean values of multiple groups were analyzed by analysis of variance (ANOVA) with a Newman–Keuls post-hoc analysis [39]. Values of $P<0.05$ were considered to be statistically significant. All comparisons were computed using GraphPad Instat 2.0 program (GraphPad software, CA). All results collected from flow cytometry analyses were analyzed using the program CellQuest.

3. Results

3.1. OxLDL induced apoptosis of VSMCs

Vascular smooth muscle cells (VSMC) were exposed to oxLDL for 4 h and the percentages of apoptotic and necrotic cell population were determined by flow cytometry. We found that oxLDL (from 100 to 300 μg/ml) caused apoptosis of VSMCs in a dose-dependent manner (Fig. 2). Cell death was predominantly apoptotic (AV^+/PI^-) with small percentage (<5%, control was 3%) of necrosis (AV^+/PI^+). At the highest concentration (400 μg/ml) employed, the apoptotic cell population decreased from maximal response to 24.3% (Fig. 2, column 5) while the necrotic cell population increased from near baseline to 15.3%. T-BuOOH was employed as a positive control for ROS production. After 1.5 h exposure to t-BuOOH (0.5 mM), cell death was predominantly apoptotic (18.9±0.4%, $n=6$) with 5.1±0.1% ($n=6$) of necrosis. Prolonged treatment (3 h) increased both apoptosis (~26%) and necrosis (~70%), similar to the results of Li and co-workers [20,21] where H_2O_2 was directly added. Apoptosis determined by phosphatidylserine exposure (AV^+/PI^-) was confirmed by activation of caspase-3 (see below) and DNA fragmentation ladder pattern at 24 h (data not shown).

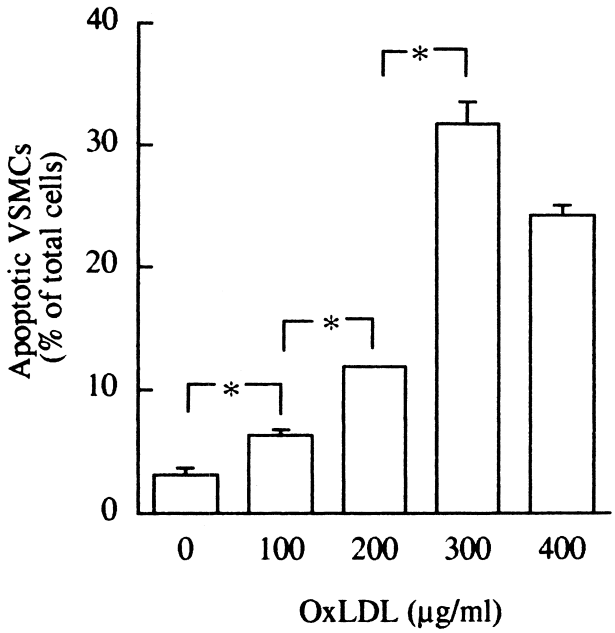

Fig. 2. Apoptotic effects of various concentrations of oxLDL on VSMCs were exposed to oxLDL for 4 h and the percentage of apoptotic cell population was determined by flow cytometry with annexin V and propidium iodide double staining. Each bar represents the mean±S.E.M. of six determinations. * Indicates $P<0.001$.

3.2. OxLDL stimulated ROS production in VSMCs

We first evaluated the influence of oxLDL itself on the detection of DCF fluorescence. In the absence of VSMCs, background DCF fluorescence in the experimental culture medium (0.5% LPDS-DMEM) was measured prior to oxLDL (300 μg/ml) addition and subtracted from values obtained from the medium after oxLDL addition. Fluorescence values of 0.38±0.04 (4) in arbitrary units (AU) were measured in experimental culture medium at 1 min, and increased to a maximum of 0.68±0.02 (4) AU at 3 min. After 3 min, DCF fluorescence intensity of experimental culture medium decreased and remained steady (0.41–0.49 AU) over the entire experimental period (60 min). DCF fluorescence values of experimental culture medium remained steady (0.41–0.48 AU) after oxLDL (100 μg/ml) addition. We have also determined DCF fluorescence intensity at the same time point (30 min post-oxLDL treatment) in the absence or presence of VSMCs and found the fluorescence intensity to be 0.49±0.03 and 12.73±0.10 AU, respectively. Thus the influence of oxLDL itself is insignificant.

VSMCs were exposed to oxLDL (100 and 300 μg/ml) and intracellular ROS generation was determined by flow cytometry with DCF-DA over 45-min. The time course of a typical experiment is shown in Fig. 3. The intensity of DCF fluorescence of oxLDL (300 μg/ml)-treated VSMCs increased with time and reached maximal (more than 10-fold of control) at 30 min, then decreased thereafter (Fig. 3, filled squares). At lower concentration of oxLDL

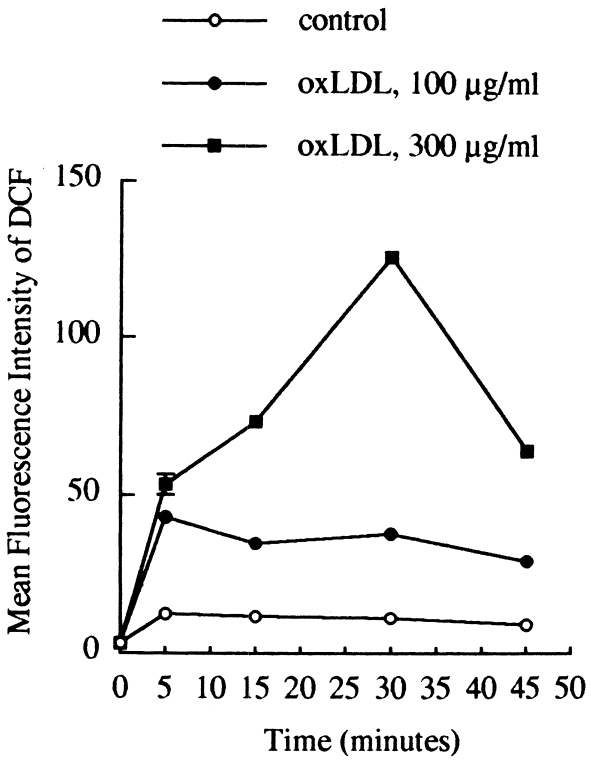

Fig. 3. Time course of ROS production of VSMCs in the presence of oxLDL. ROS production of VSMCs treated for 5, 15, 30, and 45 min with oxLDL (100 or 300 μg/ml) were measured using a flow cytometer. Data shown is a typical result (n=3 per group) from three representative experiments.

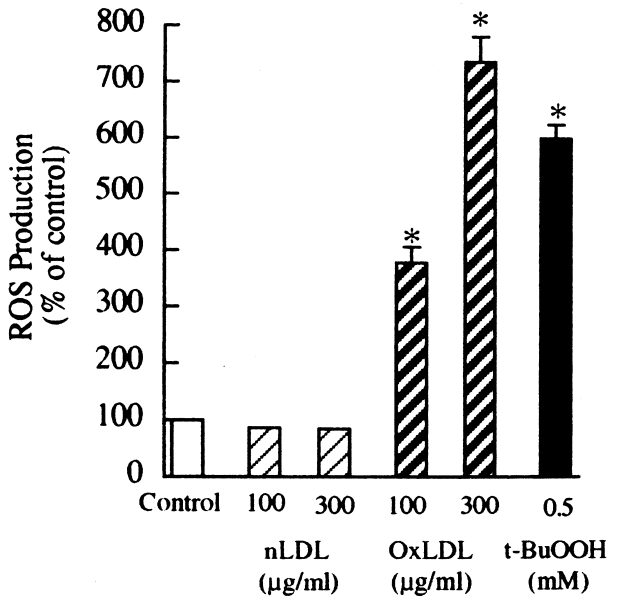

Fig. 4. OxLDL stimulated ROS production of VSMCs in an dose-dependent manner. DCF fluorescence was measured 30 min after lipoproteins addition using flow cytometry. A positive control was performed with t-BuOOH used at 0.5 mM. Each bar represents the mean±S.E.M. of n=6–12 separate determinations. * P<0.001 compared with control.

(100 μg/ml), a significant increase in fluorescence of VSMCs was observed within 5 min, and remained elevated up to 45 min (filled circles). The fluorescence intensity of control cells remained low over the entire experimental period (open circles).

Furthermore, oxLDL-stimulated ROS production of VSMCs also exhibited a dose-dependent increase (Fig. 4). Compared to control cells (open column), 100 μg/ml oxLDL caused an average 3–4-fold increase (P<0.001, n=12) in ROS content of VSMCs from three experiments. At 300 μg/ml, oxLDL induced 7–8-fold (P<0.001, n=12) increase in ROS production. T-BuOOH stimulated approximate 6-fold (n=6) increase in ROS content. There was no change in the ROS content of cells following nLDL (100 or 300 μg/ml) treatment.

3.3. Role of ROS in oxLDL-induced apoptosis

Since oxLDL rapidly stimulated ROS accumulation (<5 min) and caused apoptosis at 3 h in VSMC, the association between these two events were further examined. Benzoic acid, catalase, mannitol [40], or superoxide dismutase (SOD) were added simultaneously with oxLDL, whereas deferoxamine was preincubated with cells for 2 h before oxLDL treatment. Flow cytometry with annexin V and propidium iodide double staining was used to determine the apoptosis of VSMCs. The untreated control demonstrated low (<5%) level of spontaneous apoptosis (open columns Fig. 5A and B). There was a significant increase (P<0.05) in apoptotic cells (15.4%, n=12) when VSMCs were exposed to 100 μg/ml oxLDL for 24 h. Catalase dose-dependently alleviated this apoptosis (Fig. 5A) and could fully inhibit oxLDL-induced apoptosis at 500 U/ml (Fig. 5A, column 4 versus 1). Pretreatment with deferoxamine (0.25–0.5 mM) also caused a significant and dose-dependent prevention of oxLDL-induced apoptosis (Fig. 5A, column 5–7). SOD (250–1000 U/ml), benzoic acid (0.1 μM–1 mM), or mannitol (0.1 μM–1 mM) did not reduce oxLDL-induced apoptosis (data not shown). The effectiveness of catalase and deferoxamine persisted at higher oxLDL concentration (300 μg/ml) where apoptosis reached 30% (n=6) of the VSMC population. Similarly, catalase (500 U/ml) or deferoxamine (0.5 mM) could each provide near-complete protection of VSMCs (Fig. 5B).

3.4. Decrease of oxLDL-stimulated ROS production of VSMCs with agents that prevent apoptosis

Since not all agents prevented apoptosis, we further examined those agents effective in preventing oxLDL-induced apoptosis. ROS content was measured by flow cytometry with DCF-DA at 30 min as before. Oxidation of the intracellular DCF-DA to fluorescent DCF was observed in oxLDL-treated VSMC as shown by the greater right shift of mean fluorescence value, which was higher in oxLDL (300 μg/ml)-treated cells (second panel, Fig. 6A

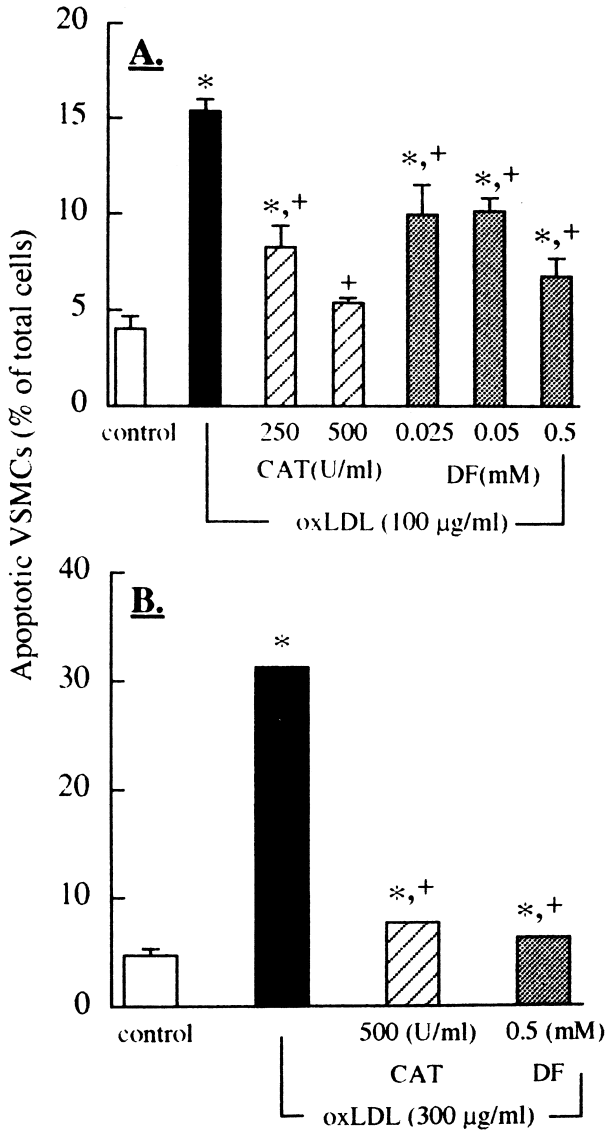

Fig. 5. Protective effects of catalase (CAT) and deferoxamine (DF) on oxLDL-induced apoptosis. (A) VSMCs were exposed to oxLDL (100 μg/ml) for 24 h in the presence of CAT, or DF. (B) VSMCs were exposed to oxLDL (300 μg/ml) for 4 h in the presence of CAT, or DF. Each bar represents the mean±S.E.M. of $n=6$–9 separate determinations. Significance of the differences between control and oxLDL-treated cells (* $P<0.05$); significance of the differences between oxLDL-treated cells and (oxLDL plus CAT or DF)-treated cell ($^{+}$ $P<0.05$).

and B) than untreated cells (top panel, Fig. 6A and B), indicating the stimulation of production of ROS. At 30 min, ROS production was reduced (left shift) by catalase and deferoxamine significantly (3rd and 4th panel, Fig. 6A). Statistical analysis of data from four separate experiments illustrated that catalase and deferoxamine blocked ROS production of oxLDL-treated VSMCs by 30.0±4.4% ($P<0.001$, $n=12$) and 84.6±3.0% ($P<0.001$, $n=12$), respectively. In contrast, the administration of SOD (third panel, Fig. 6B) enhanced oxLDL-induced ROS production by 66±6% ($n=6$, $P<0.001$, compared with oxLDL alone) while benzoic acid did not alter the oxLDL-induced stimulation of ROS significantly (bottom panel, Fig. 6B).

3.5. Possible sources of ROS induced by oxLDL

Several potential sources of ROS production in cultured VSMCs exist, including xanthine/xanthine oxidase (XO) system, NADPH oxidase, cyclooxygenase (COX), and lipoxygenase (LOX) and we used allopurinol [41], DPI [42], indomethacin [43], and NDGA [44] as inhibitors, respectively. These inhibitors were preincubated with cells for 1 h before oxLDL addition. We found that DPI caused VSMCs death at concentrations ≥25 μM while indomethacin and NDGA were also toxic at concentrations ≥50 μM (data not shown). Non-cytotoxic concentrations of inhibitors (assessed by MTT test for 4 h) were chosen for this studies.

Allopurinol (100 μM), DPI (10 μM), and NDGA (25 μM) did not alter basal ROS production of control cells, whereas indomethacin (25 μM) caused a slight but significant (8.8%, $n=6$) increase in basal ROS content. In Fig. 7A, oxLDL-stimulated ROS production of VSMCs was blocked by NDGA (91% inhibition, $P<0.001$, $n=6$) and allopurinol (21% inhibition, $P<0.05$, $n=6$), but not by indomethacin. In contrast, DPI enhanced oxLDL-stimulated ROS production of VSMCs by 30% ($P<0.01$, $n=9$). The inhibitory effect of NDGA was dose-dependent (Fig. 7B).

In addition, pretreatment with rotenone [45] resulted a significant inhibition of oxLDL-treated VSMCs. At the concentrations of 10 and 100 μM, rotenone reduced oxLDL stimulated ROS production by 55 and 80% ($n=6$, $P<0.001$), respectively. Low dose (10 μM) of rotenone did not alter basal ROS level of control cells, whereas higher dose (100 μM) caused a slight but insignificant increase.

3.6. ROS also mediated oxLDL-induced caspase-3 cleavage

Caspase-3, a member of the interleukin-1β-converting enzyme (ICE) like family, has been identified as one of the key enzymes responsible for apoptosis. On activation, proteolytic cleavage of procaspase-3 (CPP32/Apopain/Yama; 32 kDa) releases two subunits as the active form (17/11 kDa or 20/11 kDa). We employed anti-active caspase-3 antibody to evaluate the proteolytic cleavage of procaspase-3 in oxLDL-treated VSMCs with flow cytometry. The proteolytic cleavage of procaspase-3 of oxLDL-treated VSMCs increased progressively between 3 and 7 h (2.5-fold compared with control cells at 7 h) and was reduced to control level by catalase, deferoxamine, and NAC (Fig. 8, columns 3–5 versus 2).

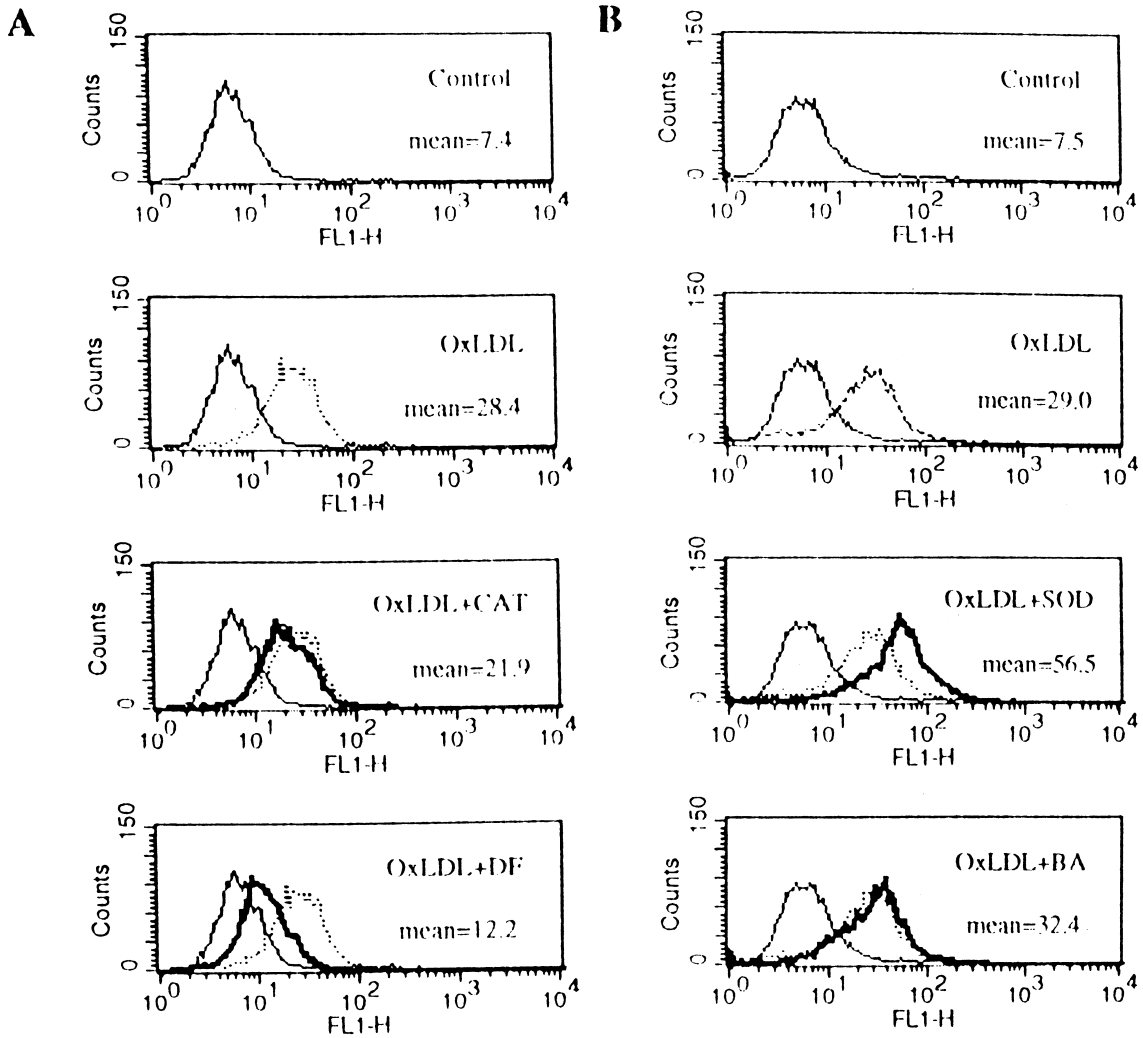

Fig. 6. Analysis of ROS production in untreated VSMCs (top panel, A and B), oxLDL (300 μg/ml) treated cells (second panel, A and B, dashed curves), oxLDL plus catalase (CAT, 500 U/ml)-treated cells (third panel, A, bold curve), oxLDL plus deferoxamine (DF, 0.5 mM)-treated cells (fourth panel, A, bold curve), oxLDL plus superoxide dismutase (SOD, 500 U/ml)-treated cells (third panel, B, bold curve), oxLDL plus benzoic acid (BA, 1 mM)-treated cells (fourth panel, B, bold curve). At the end of the incubation time (30 min), ROS production was quantified by flow cytometry. FL1-H: the fluorescence height of DCF of VSMCs.

3.7. Uptake of acetylated LDL by vascular smooth muscle cells

Pitas et al. [46] had previously demonstrated that rabbit VSMC internalized and degraded fluorescent labeled lipoproteins and became intensely fluorescent. The fluorescence intensity is proportional to the amount of lipoprotein internalized and degraded. To determined whether cultured VSMCs bind and internalize AcLDL, VSMCs were incubated with FL-AcLDL (10 μg/ml) for 5 h at 37°C and then analyzed by flow cytometry. As Fig. 9 showed, the mean relative fluorescence intensity (MFI) of VSMCs incubated with FL-AcLDL increased from 3.1 (trace A) to 9.0 (trace C). Preincubation of cultured VSMCs with 10-fold excess of unlabeled oxLDL (100 μg/ml) caused a 75% loss of FL-AcLDL uptake (MFI=4.5, trace B). HUVECs are known to possess scavenger receptors and were used as positive control, a high level (MFI=318.6) of FL-AcLDL uptake was observed (trace D). These studies demonstrated that oxLDL was taken up by cultured VSMCs, consistent with the view that these cells express the acetyl LDL (scavenger) receptor [47,48].

4. Discussion

We demonstrated that the exposure of VSMCs to oxLDL led to rapid generation of ROS (≤5 min). Correlation existed between the ROS generation and the oxidative level, or the amount of oxLDL. In contrast, native LDL exhibited no stimulation of ROS production. The oxLDL-stimulated ROS production was inhibited after treating

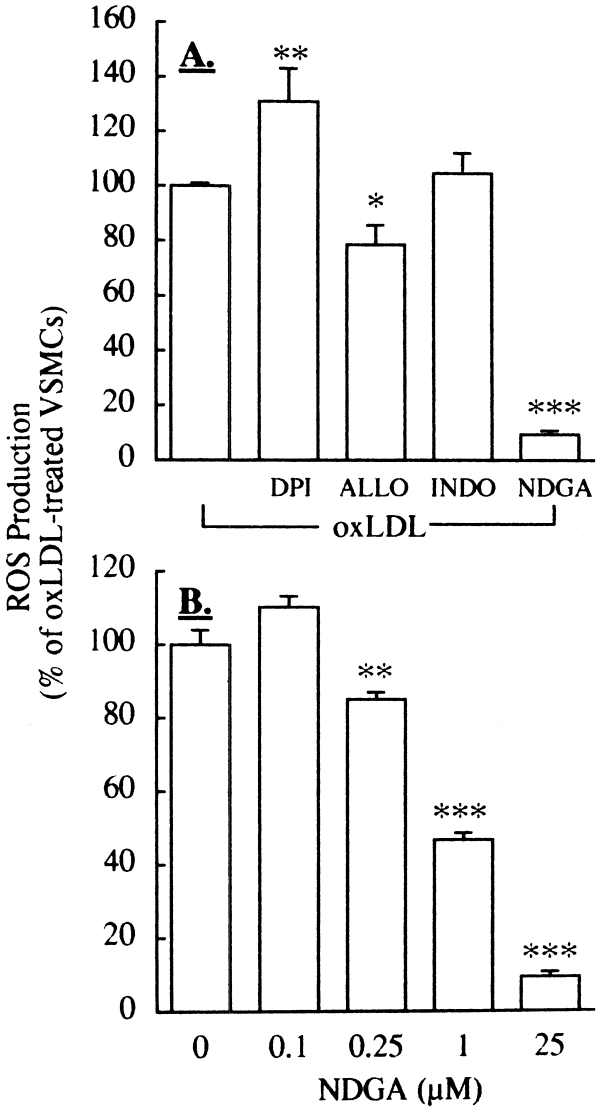

Fig. 7. (A) Effects of oxidative enzymatic inhibitors on the oxLDL-stimulated ROS generation in VSMCs. VSMCs were exposed to oxLDL (300 μg/ml) in the presence of diphenyleneiodonium chloride (DPI, 10 μM), allopurinol (ALLO, 100 μM), indomethacin (INDO, 25 μM), or nordihydroguaiaretic acid (NDGA, 25 μM) for 30 min. (B) Lipoxygenase inhibitor, NDGA, abolished ROS generation of oxLDL-treated VSMCs in an dose-dependent manner. Each bar represents the mean±S.E.M. of n=6–9 separate determinations, * P<0.05, ** P<0.01, *** P<0.001 compared to oxLDL-treated VSMCs.

VSMCs with catalase (500 U/ml) or deferoxamine (0.5 mM) but not SOD, benzoic acid, or mannitol. Because H_2O_2 is stable and permeable to membrane, intracellular H_2O_2 could be removed by exogenous catalase. Catalase may also enter cells and leads to a decrease of intracellular ROS levels as shown in platelet-derived growth factor treated VSMCs [49]. In a careful study, Brown et al. [50] have shown that rat VSMC transfected with human catalase exhibited an COX-2 dependent increase in apoptosis as well as a decrease in ROS fluorescence for H_2O_2. Interestingly, Santanam et al. [51] also used stable over-

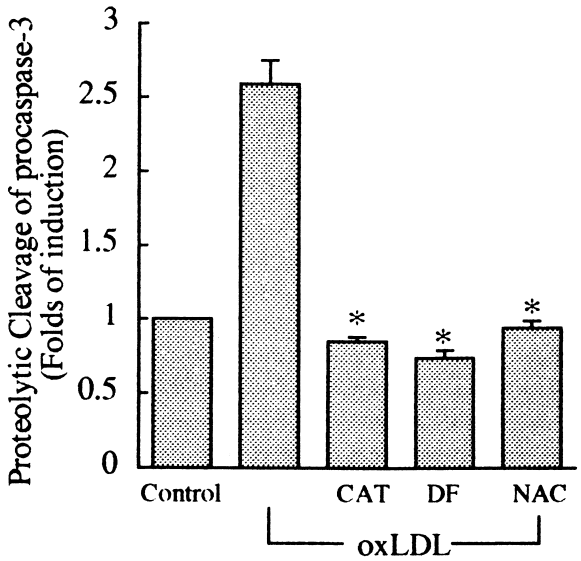

Fig. 8. Inhibitory effects of ROS scavenging agents on the activation of caspase-3 in oxLDL-treated VSMCs. VSMCs were treated with oxLDL (300 μg/ml) in the presence of catalase (CAT, 500 U/ml), deferoxamine (DF, 0.5 mM), or *N*-acetylcysteine (NAC, 5 mM). After 7 h, the PE-fluorescence intensity of each sample (1×10^4 cells) was measured by flow cytometry. Each bar represents the mean±S.E.M. for three independent experiments in duplicate. * Indicate P<0.001 compared to oxLDL-treated VSMCs.

expression of human catalase in rabbit VSMC and found that it inhibits oxLDL-induced cytotoxicity. In addition to the different origins of cells, Brown et al. [50] examined a 'basal' state regarding the role of catalase in ROS economy while Santanam et al. [51] examined a 'challenged' state. It thus appears that under different situations catalase may play different roles depending on the redox state of the cell. On the other hand, superoxide anion

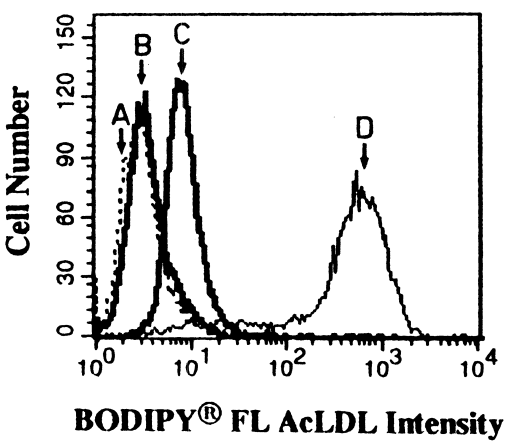

Fig. 9. Flow cytometry analysis of uptake of fluorescent-labeled acetylated LDL (FL-AcLDL, 10 μg/ml) by VSMCs (traces A–C) and HUVECs (trace D). The cells were incubated with (solid traces) or without (broken trace) FL-AcLDL for 5 h at 37°C. Uptake of FL-AcLDL was inhibited when VSMCs were incubated with 10-fold excess of unlabeled oxLDL. Data shown represents results from two similar experiments each with identical duplicates.

produced inside a cell has short half life and is in general not accessible to extracellular SOD [45]. Our findings that catalase but not SOD, significantly reduced oxLDL-stimulated ROS production and inhibited apoptosis of VSMCs (Figs. 5 and 6) are consistent with this view. In the presence of oxLDL, SOD was found to enhance ROS production (Fig. 6B) and the mechanisms were unknown. The lack of correlation with the extent of apoptosis may reflect that ROS–apoptosis relationship is not a linear one without limitation and that ROS-independent pathway, e.g. Fas-mediated apoptosis [52], also operates in vascular cell [53]. These questions require further investigation. In addition to catalase, deferoxamine protects VSMCs from oxLDL-induced apoptosis as well (Fig. 5). Furthermore, superoxide anion, or H_2O_2 has been shown to directly induce VSMC apoptosis which can be similarly inhibited by pretreating VSMCs with antioxidants [20,21].

The increase in ROS of oxLDL-treated VSMCs was inhibited by pretreatment with rotenone indicating that mitochondria could be a sources of ROS in VSMCs, consistent with ceramide's involvement [54,55]. In addition, pretreatment of VSMCs with lipoxygenase inhibitor (NDGA), but not COX inhibitor, reduced oxLDL-stimulated ROS production dose-dependently (Fig. 7) suggesting that lipoxygenase pathway might be also involved in the oxLDL signal transduction pathway [56,57]. Li et al. [44] had also shown that oxidized products of arachidonic acid from lipoxygenase pathway are required for mitochondrial ROS production and cell death. However, there was no significant protection (<5%) of NDGA (25 μM) on oxLDL-induced VSMC death, suggesting that additional signaling pathways [58] and/or other effects of NDGA [59], may be involved.

Studies of Lizard et al. [60] have extensively examined major oxysterols such as 7β-hydroxycholesterol and 7-ketocholesterol as active components of human oxLDL and as inducers for cell death [61,62]. Furthermore, their data demonstrated that glutathione is implicated in the control of 7-ketocholesterol-induced apoptosis of U937 cells associated with the production of ROS [63]. We also found that *N*-acetylcysteine (NAC, a scavenger of free radicals or a precursor of glutathione) [64] contribute to the observed protection against VSMC death induced by oxLDL-treatment (Fig. 8). In contrast, VSMCs depleted of glutathione by the γ-glutamylcysteine synthetase inhibitor, L-buthionine-[*S,R*]-sulfoximine, exhibited an enhanced cytotoxicity to oxLDL (unpublished observation).

Our result that oxLDL was taken up by VSMC (Fig. 9) revealed a possible pathway for oxLDL in exerting its cytotoxic effects. Interaction of oxLDL with scavenger receptors leads to uptake of oxLDL into the cells, and that subsequent oxidative damage of lysosomal membranes by several oxLDL peroxidation products could occur [65]. However, oxLDL is recently reported to activate p38 mitogen-activated protein kinase (MAPK) and this activation is not mediated by scavenger receptor [66] and that activation of p38 MAPK cascades can also trigger apoptosis [67]. Therefore, the site(s) of actions of oxLDL relating to ROS production is (are) not clear. Considering that oxLDL also induce a rapid Ca^{2+} increase in vascular cells [68], interactions among Ca^{2+}, ROS, and other mediators may all contribute to the oxLDL-induced cell death.

In conclusion, oxidized LDL induced VSMCs apoptosis and that this induction was prevented by pretreating the cells with only those radical-scavenging agents (catalase, deferoxamine, NAC) effective in the reduction of ROS generation. There may be multiple sources for ROS generation and multiple mechanisms for ROS signal transduction in oxLDL-induced apoptosis. The existence of a link between ROS generation and oxLDL-induced apoptosis would imply that antioxidants or radical-scavenging agents have as yet an unexplored therapeutic effects in this regard.

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