Reactive Oxygen Species Mediate Oxidized Low-Density Lipoprotein-Induced Inhibition of Oct-4 Expression and Endothelial Differentiation of Bone Marrow Stem Cells

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Abstract

This study was to test the hypothesis that oxidized low-density lipoprotein (ox-LDL) modified the behavior of bone marrow stem cells, including proliferation, Oct-4 expression, and their endothelial differentiation through reactive oxygen species (ROS) formation *in vitro*. Rat bone marrow multipotent adult progenitor cells (MAPCs) were treated with ox-LDL with or without the antioxidant N-acetylcysteine (NAC). Ox-LDL generated a significant amount of ROS in the culture system as measured with electron paramagnetic resonance spectroscopy, and substantially inhibited the proliferation, Oct-4 expression, and endothelial differentiation of MAPCs. ROS production from ox-LDL in the culture system was completely prevented by NAC (1 mM). NAC treatment completely restored endothelial differentiation potential of MAPCs that was diminished by low-dose ox-LDL. NAC also significantly, but not completely, reversed the inhibitory effect of ox-LDL on proliferation and Oct-4 expression in MAPCs. NAC treatment only slightly restored Akt phosphorylation impaired by ox-LDL in the cells. ROS formation was important in the action of ox-LDL on MAPCs, including Oct-4 expression, proliferation, and endothelial differentiation. However, other mechanism(s) like Akt signaling and apoptosis might also play a critical role in mediating the effect of ox-LDL on these cells. *Antioxid. Redox Signal.* 13, 1845–1856.

Introduction

ENDOTHELIAL DYSFUNCTION OR INJURY is one of the major factors that contribute to the development of atherosclerosis and coronary heart disease (12, 21). Bone-marrow-derived circulating endothelial progenitor cells (EPCs) play a key role in angiogenesis, vascular re-endothelialization, and prevention of neointima formation after vascular injury (38, 44, 47, 56). It is well known that the number and function of EPCs are significantly reduced in patients with atherosclerosis and coronary artery disease, as well as hyperlipidemia (6, 49). However, the exact mechanism(s) for the deficiency of EPCs remains largely unknown in hyperlipidemic patients.

Oxidized low-density lipoprotein (ox-LDL), a key component in hyperlipidemic state, has been implicated in the formation of atherosclerotic plaques. Ox-LDL has been shown to inhibit proliferation and endothelial differentiation of EPCs, and suppress their function, including inhibition of cell migration, adhesion, and *in vitro* vasculogenesis, as well as

ischemia-induced neovascularization *in vivo* (23, 24, 30, 53, 59). Since EPCs originate from bone marrow stem cells, the number and function of EPCs could be intimately associated with the status of bone marrow stem cells and their differentiation potential into EPCs. Indeed, we recently observed that ox-LDL dramatically suppressed the proliferation of bone marrow stem cells, significantly inhibited expression of stem cell marker Oct-4, and blocked endothelial differentiation of these cells (8).

Ox-LDL interferes with the function of EPCs through multiple mechanisms, including inhibition of endothelial nitric oxide synthase, downregulation of E-selectin and integrin $\alpha(v)\beta(5)$ expression, inactivation of telomerase, and acceleration of cell senescence (24, 30, 39). Recently, we showed that ox-LDL promoted apoptosis, and inhibited Oct-4 expression and proliferation of bone marrow stem cells, and impaired their endothelial differentiation *via* suppression of Akt signaling (8). It is believed that oxidative stress is also an important mechanism responsible for the actions of ox-LDL

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on a variety of biological system (29, 39). Reactive oxygen species (ROS)/reactive nitrogen species regulate tissue redox status that is directly related to tissue oxidative stress, and the development and progression of cardiovascular diseases (17). It is reported that the rate of ROS generation in the peripheral blood monocytes is increased in hyperlipidemic patients along with elevated plasma ox-LDL level (50), and ox-LDL increases the intracellular formation of ROS in cultured endothelial cells (11).

In the present study, experiments were designed to test the hypothesis that ox-LDL modified the behavior of bone marrow stem cells, including proliferation and Oct-4 expression as well as their endothelial differentiation through the production of ROS *in vitro*. Rat bone marrow multipotent adult progenitor cells (MAPCs), a well-characterized bone marrow mesenchymal stem cells (MSCs) with high level of Oct-4 expression, were used as the source of bone marrow stem cells, and treated with ox-LDL with or without the antioxidant N-acetylcysteine (NAC). We found that ox-LDL produced a significant amount of ROS in the culture system, and substantially inhibited the proliferation and Oct-4 expression as well as endothelial differentiation of MAPCs. NAC effectively prevented the release of ROS from ox-LDL, and significantly reversed the effects of ox-LDL on MAPCs, including proliferation, Oct-4 expression, and endothelial differentiation. These data supported our hypothesis that ox-LDL negatively regulated the behavior of bone marrow stem cells *via* ROS formation *in vitro*.

Materials and Methods

Preparation of LDL and ox-LDL

Plasma was obtained from healthy human subjects. Native LDL was prepared from the plasma by sodium bromide stepwise density gradient centrifugation as described (1, 10). Ox-LDL was prepared by exposure of native LDL to $CuSO_4$ ($5\,\mu M$) at $37^{\circ}C$ for 3 h. The reaction was stopped by adding EDTA (final concentration, $0.25\,mM$). The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid reactive substances (TBARS) as described (10). The value for TBARS in ox-LDL was determined to be 44 ± 6 nmol malondialdehyde/mg protein. No TBARS were detectable in native LDL as expected.

Cell culture and endothelial differentiation

Rat MAPCs were produced and characterized in Dr. Verfaillie's laboratory in the Stem Cell Institute at the University of Minnesota, Minneapolis, MN, as described (4, 46). Phenotypically, these cell were positive for Oct-4, Rex-1, c-Kit, and Pdgfr-α, and negative for Sca-1, CD34, CD45, Sox-2, and Nanog (46). The cells were cultured at a density of 100–200 cells/cm^2 in expansion medium at $37^{\circ}C$ with 5% O_2, 5% CO_2, and 90% N_2 with the method as described (8). To investigate the effect of ox-LDL on MAPCs, the cells were cultured at a density of 500 cells/cm^2 in the presence of ox-LDL (from 0 to $20\,\mu g/ml$) for 24, 48, and 72 h. Native LDL was used as control. The cells were counted in each group, and collected for real-time polymerase chain reaction (RT-PCR) and Western blot analysis. To evaluate the effect of ox-LDL on endothelial differentiation, the cells were induced to differentiate into endothelial cells with or without ox-LDL as described (4, 8, 46).

To determine the involvement of ROS in the actions of ox-LDL on MAPCs and their endothelial differentiation, experiments were repeated with the cells that were pretreated with the antioxidant NAC ($1\,mM$, final concentration). Cells were collected at days 1, 7, and 14 of differentiation for RT-PCR, Western blot analysis, immunofluorescence staining, and flow cytometry analysis to determine expression of endothelial markers vWF, Flk-1, and CD31, as well as stem cell marker Oct-4.

Measurement of ROS formation

Production of ROS from ox-LDL in the culture system was quantitatively determined using electron paramagnetic resonance (EPR) spectroscopy as described (60). A total of 2 million cells were mixed with different concentrations of ox-LDL (from 0 to $20\,\mu g/ml$) in a total volume of $100\,\mu l$ detection solution with or without NAC (final concentration of $1\,mM$). Media alone and media with NAC were used as background. Native LDL was used as control. For NAC groups, NAC was added into the mixture 5 min before adding ox-LDL or native LDL.

Quantitative RT-PCR analysis

Total RNA was extracted from undifferentiated and differentiating MAPCs at different time points with or without ox-LDL and NAC using the RNeasy RNA isolation kit (Qiagen, Inc.) as per manufacturer's instruction, and quantified by spectrophotometry at 260 nm. The mRNA was reversely transcribed using a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems) as described (4, 46). The primers used for amplification were as follows: for vWF, CCCA CCGGATGGCTAGGTATT (forward) and GAGGCGGATC TGTTTGAGGTT (reverse); for Flk-1, CCAAGCTCAGCACA CAAAAA (forward) and CCAACCACTCTGGGAACTGT (reverse); for CD31, GGACTGGCCCTGTCACGTT (forward) and TTGTTCATGGTGCCAAAACACT (reverse); for GAPDH, CAGTGCCAGCCTCGTCTCAT (forward) and AGGGGCC ATCCACAGTCTTC (reverse). The mRNA levels were normalized using GAPDH as housekeeping gene. Each experiment was repeated for at least three times.

Western blot analysis

Cell lysates were prepared from the undifferentiated and differentiating MAPCs at different time points with or without ox-LDL and NAC for Western immunoblot analysis to determine the expression levels of endothelial markers vWF, Flk-1, and CD31, as well as stem cell marker Oct-4, and Akt (both total and phosphorylated). The supernatant was collected for analysis after centrifugation at $14,000\,g$ for 15 min at $4^{\circ}C$. Protein concentration was determined by the bicinchoninic acid protein assay (Piece). A total of $20\,\mu g$ protein was loaded on 10% sodium dodecyl sulfate–polyacrylamide gel, and immunoblotting was conducted as described (8, 9). The dilution factor for the primary antibodies against vWF, Flk-1, and Oct-4 (Santa Cruz), as well as CD31, Akt, and β-actin (Abecam), was 1:500, 1:500, 1:1000, 1:1000, and 1:1000, respectively, as recommended by the manufacturers. The protein levels were determined using horseradish peroxidase-linked secondary antibodies (1:2000 dilution) and ECL System (Amersham Biosciences), and quantified using ImageJ software

(National Institutes of Health). All Western blot experiments were repeated for at least three times.

Immunofluorescence staining

Undifferentiated or differentiating MAPCs were plated in FN-coated chamber slides. The cells were collected at different time points of differentiation (from day 1 to 14), and fixed with 4% paraformaldehyde (Sigma) for 30 min at room temperature. The cells were further prepared for immunofluorescence staining as described (8, 46). To avoid possible experimental artifacts, the nonspecific binding was blocked with 1.5% species-specific serum in phosphate-buffered saline for 1 h at room temperature. The dilution factor for the primary antibodies against vWF, Flk-1, and CD31 was 1:200, and for the secondary antibodies (species-specific fluorescein isothiocyanate-conjugated IgG; Sigma) was 1:200. Cells exposed to the secondary antibodies only were used as negative controls in addition to the nuclear stain DAPI (Molecluar Probe) at a dilution factor of 1:1000. The cells were then examined with confocal fluorescence microscopy (Zeiss Axiovert; Carl Zeiss, Inc.).

Vascular tube formation assay

In vitro vascular tube formation from MAPC-derived cells was evaluated in growth factor-reduced Matrigel (10 mg/ml; Sigma) as described (8, 9). Briefly, the extracellular matrix gel (Sigma) was thawed at 4°C overnight, and placed on a six-well culture plate at 37°C for 1 h to allow solidification. The cells were harvested, and replated (10,000 cells/well) on the top of the solidified extracellular matrix gel in EBM-2 endothelial basal medium supplemented with 0.5% bovine serum albumin and vascular endothelial growth factor (100 ng/ml). The cultures were maintained at 37°C for 12 h.

Tube formation was defined as a structure exhibiting a length four times its width. The networks of tubes were photographed from six randomly chosen fields with a microscope.

Flow cytometry assay

Expression of endothelial-specific marker vWF was also evaluated after differentiation with or without ox-LDL and NAC treatment using flow cytometry following the manufacturer's protocol. Undifferentiated MAPCs and the cells at week 2 of differentiation were harvested, washed, and suspended in ice-cold phosphate-buffered saline with a cell density of ~ 1–5×10^6 cells/ml. The preparations were then incubated with the primary antibody against vWF at 4°C for 30 min, followed by exposure to the fluorescein isothiocyanate-conjugated secondary antibody for 20 min. The cells were readily to be fixed with 4% paraformaldehyde for flow cytometry analysis using FACS Calibur (Becton-Dickinson).

Statistical analysis

The data were expressed as mean ± standard deviation for all experiments, and statistically analyzed by independent sample t-test or one-way analysis of variance when appropriate. Differences were considered statistically significant when $p < 0.05$.

Results

Ox-LDL inhibited proliferation of MAPCs and downregulated Oct-4 expression

To test the hypothesis, we first determined the effect of ox-LDL on proliferation of MAPCs and Oct-4 expression. Under normal condition, the doubling time for MAPCs was estimated to be 12.5 h that was similar to our previous
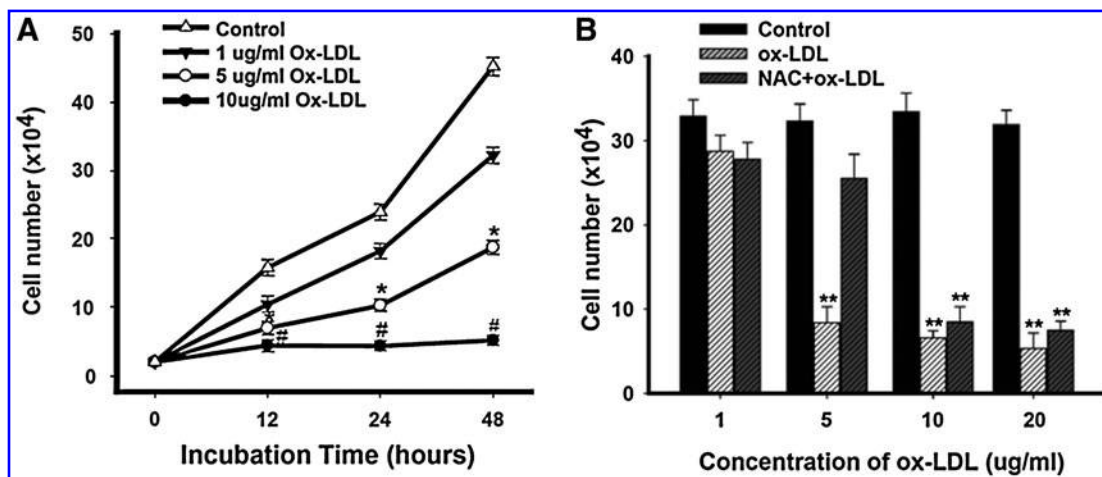

FIG. 1. Effect of ox-LDL on the proliferation of MAPCs with and without NAC. When MAPCs were exposed to ox-LDL (from 0 to 20 μg/ml) for up to 48 h, the cell number was significantly decreased in a dose- and time-dependent manner. Pretreatment of the cells with the antioxidant NAC reversed the inhibitory effect of ox-LDL on the proliferation when ox-LDL concentration was low (5 μg/ml or less). No protective effect of NAC was observed when ox-LDL concentration was above 5 μg/ml. *$p < 0.05$ *versus* control ($n = 3$) and #,**$p < 0.01$ *versus* control ($n = 3$). **(A)** Ox-LDL time and dose dependently (from 0 to 10 μg/ml) inhibited cell proliferation of MAPCs. The antioxidant NAC itself (1 m*M*) had no effect on the proliferation of the cells (data not shown for clarity of the figure). **(B)** Cell proliferation of MAPCs was dramatically decreased dose dependently by ox-LDL (from 5 to 20 μg/ml) after 24 h of culture. Treatment with NAC only rescued the cell growth in the group when ox-LDL concentration was 5 μg/ml. MAPCs, bone marrow multipotent adult progenitor cells; NAC, N-acetylcysteine; ox-LDL, oxidized low-density lipoprotein.

observation (8). When incubated with ox-LDL, the population of MAPCs in culture was dramatically decreased in a concentration- and time-dependent manner (Fig. 1A, B). When ox-LDL concentration was increased to $20\,\mu g/ml$, the cell number was reduced to <20% of control ($p < 0.01$; $n = 3$).

A significant level of Oct-4 expression was observed in MAPCs under normal condition as expected (Fig. 2). When MAPCs were exposed to ox-LDL for 48 h, transcriptional expression of Oct-4 was significantly decreased with a dramatic reduction of mRNA level by 88% as compared to control (data not shown). Western blot analysis showed that Oct-4 protein content in MAPCs was also substantially decreased in the cells treated with ox-LDL by 82% as compared to control. These data were consistent with our previous observation (8). Immunofluorescence staining showed that both Oct-4 expression level in the individual cells and Oct-4-positive cell population were decreased in ox-LDL-treated group (data not shown).

Ox-LDL suppressed endothelial differentiation of MAPCs

To investigate the effect of ox-LDL on endothelial differentiation of MAPCs, the cells were induced to differentiate into endothelial cells in the presence of ox-LDL. As expected, the differentiating MAPCs started to express endothelial markers vWF, CD31, and Flk-1 by day 7 of differentiation

FIG. 2. **Treatment of MAPCs with NAC significantly prevented the inhibitory effect of ox-LDL on expression of Oct-4 protein.** A significant amount of the stem cell marker Oct-4 was expressed in MAPCs cultured in normal condition as determined using Western blotting analysis. When MAPCs were incubated with ox-LDL ($5\,\mu g/ml$) for 24 h, expression of Oct-4 was significantly decreased in the cells. Pretreatment of the cells with NAC ($1\,mM$) effectively prevented the downregulation of Oct-4 expression by ox-LDL in MAPCs. **$p < 0.01$ *versus* control ($n = 3$). Control, cells cultured in normal condition; NAC+ox-LDL, cells were pretreated with NAC before exposure to ox-LDL; ox-LDL, cells exposed to ox-LDL.

under normal condition as determined using real-time-PCR, Western blotting, immunofluorescence staining, and flow cytometry (Figs. 3–7). The differentiated cells also stained positive for vWF, CD31, and FlK-1, and formed capillary structures on growth factor-reduced Matrigel by day 14 of differentiation (Figs. 5 and 7), suggesting that the MAPC-derived cells were indeed functional endothelial cells. When treated with ox-LDL, expression of vWF, CD31, and Flk-1 was all significantly decreased in the cells with dramatic reduction in their mRNAs and proteins (Fig. 3), indicating that ox-LDL significantly impaired the endothelial differentiation of MAPCs. There was no vascular structure formation by ox-LDL-treated cells after 2 weeks of differentiation on the Matrigel (Fig. 7), further suggesting that the cells were not endothelial cells.

Spontaneous production of ROS from ox-LDL

To evaluate the role of ROS in mediating the effect of ox-LDL on MAPCs, we determined if ROS were produced from ox-LDL *in vitro* using EPR. A significant amount of ROS was generated from ox-LDL in the culture system within a few seconds after addition of ox-LDL into the mixture in a dose- and time-dependent manner (Fig. 8). ROS generation increased over time, and reached the maximum by 80 s, and stayed at high level during the measuring period for up to 30 min. No difference in the peak level of ROS formation was observed in the presence or absence of MAPCs, indicating that ROS production from ox-LDL was spontaneous and independent from the cells. ROS formation from ox-LDL in the culture system was completely prevented by the antioxidant NAC. Of note, there was a small detectable amount of ROS generated from native LDL in the media (Fig. 8C).

NAC reversed the inhibitory effect of ox-LDL on proliferation and Oct-4 expression of MAPCs

To further test the hypothesis, we then evaluated the effect of ox-LDL on MAPCs in the presence of the antioxidant NAC. Treatment of the cells with NAC ($1\,mM$) significantly reversed the inhibitory effect of ox-LDL on Oct-4 expression in MAPCs as shown in Figure 2. The Oct-4 protein content in ox-LDL-treated MAPCs was dramatically decreased to 14% of control, and was substantially recovered to 79% of the control level when the cells were pretreated with NAC, suggesting that ox-LDL downregulated Oct-4 expression in MAPCs mainly due to ROS formation.

The population of MAPCs in culture was dramatically decreased in the presence of ox-LDL in a concentration- and time-dependent manner (Fig. 1A, B). There was minimal increase in cell population after 48 h of culture with ox-LDL in the media at the concentration of $5\,\mu g/ml$. The cell number in ox-LDL-treated group was only 22% of that in control group after 48 h of culture ($p < 0.01$, $n = 3$), and was significantly increased to 78% of the control when NAC was added in the media, indicating that ox-LDL at $5\,\mu g/ml$ inhibited the proliferation of MAPCs largely due to ROS formation. There was no change in the cell number after 48 h of culture in the NAC-treated group when ox-LDL concentration was increased to $10\,\mu g/ml$ or above although ROS generation was completely blocked by NAC in these conditions as determined using EPR (Fig. 8). Of note, NAC itself did not affect the proliferation of MAPCs or Oct-4 expression in culture (data not shown). These
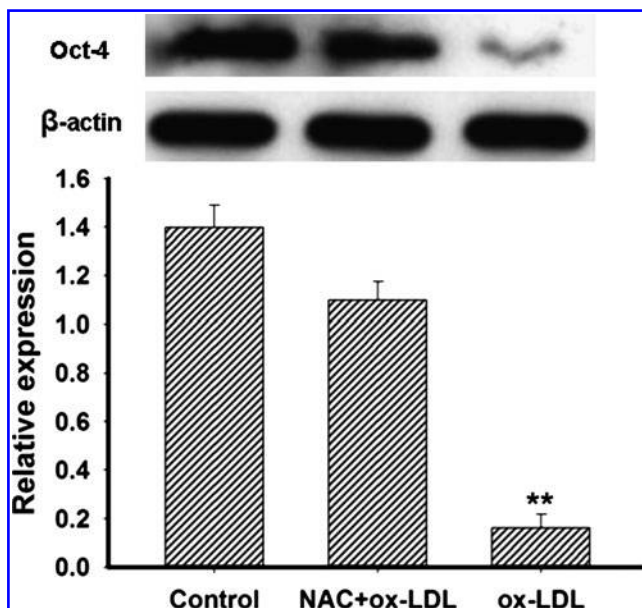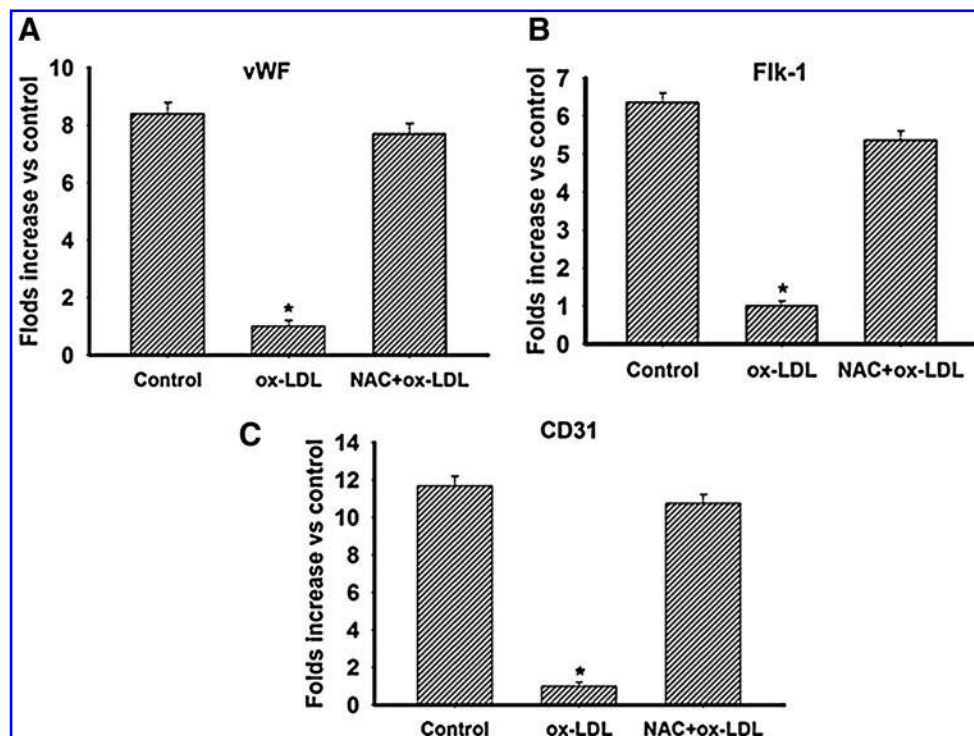
FIG. 3. Treatment with NAC completely restored the diminished endothelial differentiation of MAPCs by ox-LDL. When MAPCs were induced to differentiate into endothelial cells, the transcriptional expression of endothelial markers, including vWF, Flk-1, and CD31, were increased significantly by 2 weeks of differentiation as reflected by increased mRNA levels as analyzed by real-time–polymerase chain reaction. Endothelial differentiation of MAPCs was substantially inhibited in the presence of ox-LDL (5 µg/ml) with dramatically decreased transcriptional expression of endothelial markers, including vWF, Flk-1, and CD31. Treatment of the cells with the antioxidant NAC (1 mM) completely restored the suppressed endothelial differentiation potential of MAPCs by ox-LDL with recovery of mRNA levels for the endothelial markers. *$p < 0.05$ *versus* control ($n = 3$). Control, cells cultured in normal condition; ox-LDL, cells exposed to ox-LDL; NAC+ox-LDL, cells were pretreated with NAC before exposure to ox-LDL. **(A)** Transcriptional expression of vWF (mRNA) in MAPCs during their endothelial differentiation was dramatically inhibited by ox-LDL, and was normalized when treated with NAC. Cells before the induction of differentiation were used as the baseline. **(B)** Ox-LDL substantially suppressed Flk-1 expression in the differentiating cells as analyzed by real-time–polymerase chain reaction that was reversed by treatment of the cells with NAC by 2 weeks of differentiation. **(C)** CD31 expression in the differentiating cells was dramatically reduced by ox-LDL, and returned to normal when treated with NAC at week 2 of differentiation.

data suggested that NAC provided protection against the inhibitory effect of ox-LDL on MAPCs only when low concentration of ox-LDL was present in the media.

NAC effectively prevented the inhibition of endothelial differentiation by ox-LDL

When MAPCs were induced to differentiate into endothelial cells under normal conditions, the cells started to exhibit endothelial-like phenotype both structurally and functionally with expression of endothelial markers such as vWF, CD31, and Flk-1 by day 7 of differentiation as evaluated with real-time-PCR, Western blotting, immunofluorescence staining, and flow cytometry, as well as vascular tube formation (Figs. 3–7). When MAPCs treated with ox-LDL, expression of vWF, CD31, and Flk-1 was significantly decreased in the cells with a dramatic reduction in their mRNAs and proteins (Fig. 3), indicating that ox-LDL significantly impaired the endothelial differentiation of MAPCs. There was no vascular structure formation by ox-LDL-treated cells after 2 weeks of differentiation on the Matrigel (Fig. 7), further suggesting that the cells were not endothelial cells. Treatment of the cells with NAC

completely restored the potential of MAPCs to differentiate into functioning endothelial cells impaired by ox-LDL as reflected by normalization of diminished expression of endothelial markers, including vWF, CD31, and Flk-1, as well as *in vitro* vascular structure formation (Figs. 3–5 and 7). Flow cytometry analysis demonstrated that 46% of cells were positive for endothelial-specific marker vWF by 2 weeks of differentiation under normal condition, whereas only 19% of cells treated with ox-LDL were positive for vWF that was significantly increased to 45% for the cells exposed to ox-LDL in the presence of NAC (Fig. 6). These data suggested that ox-LDL attenuated endothelial differentiation of MAPCs mediated through production of ROS.

NAC minimally restored Akt phosphorylation suppressed by ox-LDL in MAPCs

Interruption of serine/threonin protein kinase Akt (Akt) signaling was recently shown to be involved in the actions of ox-LDL on MAPCs and their endothelial differentiation (13). In the present study, a detectable level of Akt phosphorylation was observed in MAPCs cultured in normal condition.
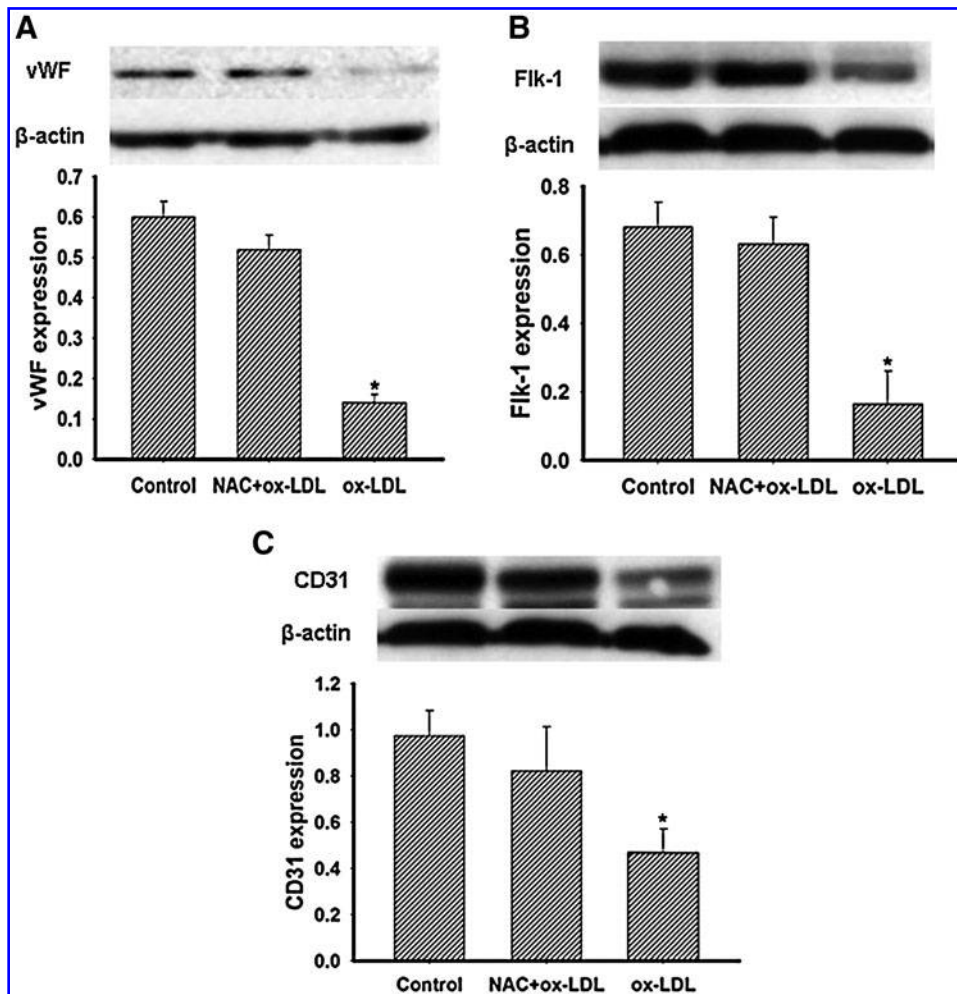
FIG. 4. NAC treatment effectively prevented ox-LDL-induced suppression of endothelial differentiation of MAPCs. By week 2 of differentiation, a significant amount of proteins for endothelial markers vWF, Flk-1, and CD31 was expressed in the cells in normal condition as determined using Western blot analysis. Endothelial differentiation of MAPCs was markedly inhibited by ox-LDL with dramatically decreased protein content of the endothelial markers vWF, Flk-1, and CD31. Pretreatment of the cells with NAC sufficiently restored the endothelial differentiation potential of MAPCs attenuated by ox-LDL with recovery of the endothelial protein expression after 2 weeks of differentiation. $*p < 0.05$ *versus* control ($n = 3$). Control, cells cultured in normal condition; ox-LDL, cells exposed to ox-LDL; NAC+ox-LDL, cells were pretreated with NAC before exposure to ox-LDL. **(A)** vWF protein content in the differentiating MAPCs was significantly decreased by ox-LDL that was normalized when treated with NAC at week 2 of differentiation. **(B)** Ox-LDL substantially suppressed Flk-1 expression in the differentiating cells that was prevented when NAC was present in the media at week 2 of differentiation. **(C)** CD31 expression in the differentiating cells was dramatically reduced by ox-LDL, and was recovered when the cells were treated with NAC at week 2 of differentiation.

However, the level of Akt phosphorylation was dramatically decreased to undetectable level in MAPCs when incubated with ox-LDL for 24 h (Fig. 9). Ox-LDL-impaired Akt phosphorylation was only slightly restored in the cells when pretreated with NAC with the Akt phosphorylation level close to 20% of the control. These data indicated that ox-LDL impaired the population and function of MAPCs *via* multiple mechanisms, including ROS generation and inhibition of Akt signaling.

Discussion

In the present study, we demonstrated that ox-LDL significantly downregulated Oct-4 expression in MAPCs, inhibited the proliferation, and suppressed the endothelial differentiation of MAPCs. A significant amount of ROS was generated spontaneously from ox-LDL in the culture media. The antioxidant NAC completely blocked ROS formation from ox-LDL, and effectively prevented the effects of ox-LDL on MAPCs. These data were consistent with our hypothesis that ox-LDL modified the behavior of bone marrow stem cells, including proliferation and Oct-4 expression, as well as their endothelial differentiation *via* the production of ROS *in vitro*.

The outcomes of ox-LDL on its target cells are variable and complex depending on individual cell type. Ox-LDL promotes proliferation of macrophages and vascular smooth muscle cells, and inhibits apoptosis of macrophages and monocytes (16, 20, 22, 31, 32, 41, 58). On the other hand, ox-LDL inhibits proliferation and promotes apoptosis of vascular endothelial cells and EPCs (5, 7, 55). Recently, we demonstrated that ox-LDL inhibited cell proliferation, induced apoptosis of MAPCs, and attenuated their endothelial differentiation (8). Ox-LDL interacts with a variety of cells through multiple mechanisms, including (but not limited to) mitogen-activated protein kinase activation, downregulation of E-selectin, and integrin $\alpha(v)\beta(5)$ expression, activation of E-cadherin/β-catenin/Tcf signaling pathway, and inhibition of PI3K/Akt signaling (2, 8, 14, 15).

Another important mechanism for the actions of ox-LDL is ROS formation and oxidative stress. Ox-LDL is a rich source of ROS that are directly related to tissue oxidative stress, and the development and progression of cardiovascular diseases like hypertension and atherosclerosis (18, 43, 45, 51). ROS and oxidative stress also play an important role in regulation of stem cells, including bone marrow stem cells. Mouse
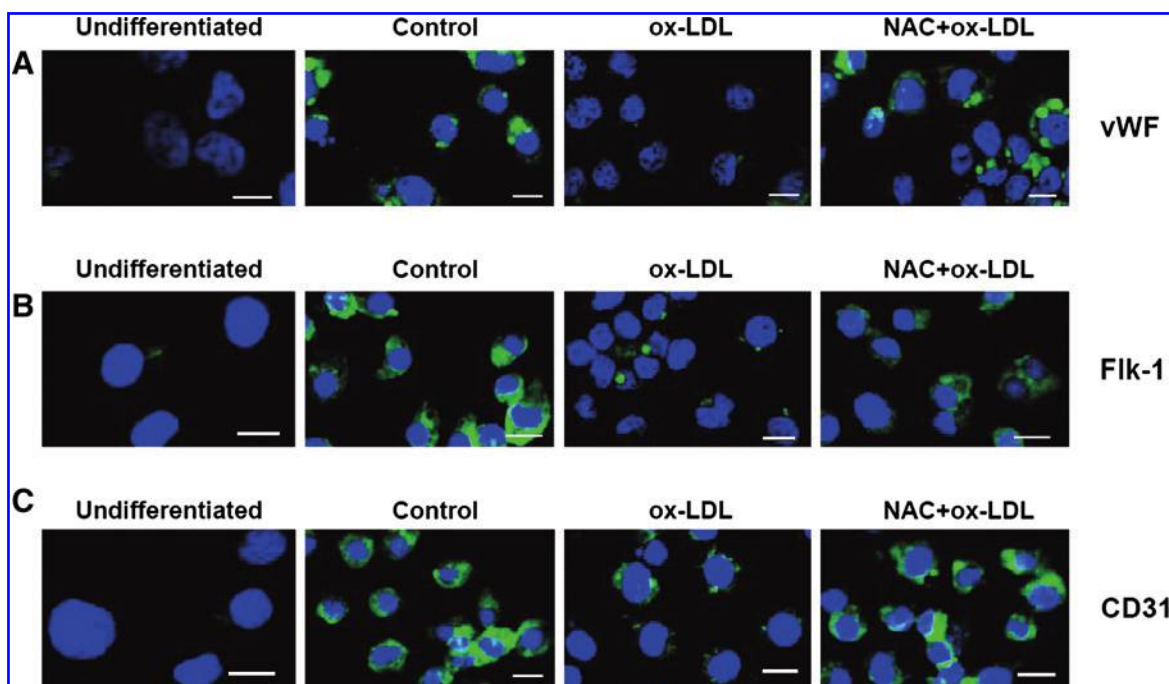
FIG. 5. Immunofluorescence staining of endothelial proteins. Immunofluorescence staining demonstrated that a significant level of endothelial proteins vWF, Flk-1, and CD31 was present in the cells in the control group by 2 weeks of differentiation **(A–C)**. Only minimal immunofluorescence staining for these proteins was observed in the cells treated with ox-LDL after 2 weeks of differentiation. When the cells were pretreated with the antioxidant NAC before exposure to ox-LDL, the cells exhibited a similar level of immunofluorescence staining for vWF, Flk-1, and CD31 after 2 weeks of differentiation. The green fluorescence presented proteins for vWF, Flk-1, or CD31. The nuclei stained blue with DAPI. Scale bar: 20 μm. Undifferentiated, undifferentiated MAPCs; Control, cells cultured in normal condition; Ox-LDL, cells exposed to ox-LDL; NAC+Ox-LDL, cells were pretreated with NAC before exposure to ox-LDL.

embryonic stem cells (ESCs) are sensitive to H_2O_2-induced apoptosis. The cells enter a transient cell cycle arrest when exposed to sublethal concentration of H_2O_2 for a short period (19). Similarly, exposure of rat MSCs to hydrogen peroxide resulted in a concentration-dependent decrease in cell viabil-

ity (52). Overexpression of Hsp20 protects MSCs against cell death triggered by oxidative stress *in vitro* in association with enhanced Akt activation and increased secretion of growth factors (vascular endothelial growth factor, fibroblast growth factor-2, and insulin-like growth factor-1) (54). *In vivo*
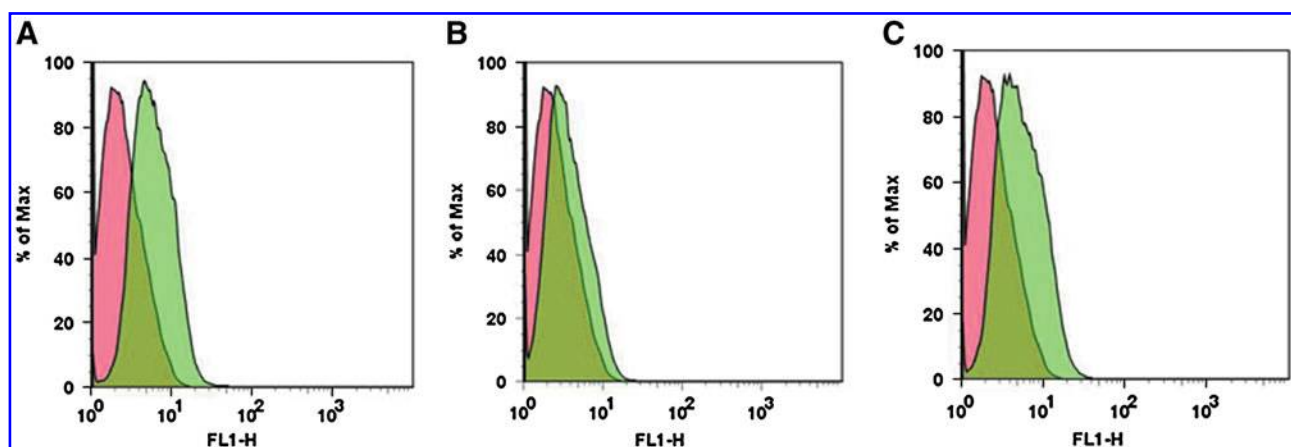

FIG. 6. Flow cytometry analysis of endothelial-specific marker vWF expression after 2 weeks of differentiation of MAPCs. The expression profile for vWF was analyzed using flow cytometry in the cells after 2 weeks of differentiation. There was a clear shift of cell populations that expressed vWF in the control group **(A)**. The vWF-positive cell population diminished when incubated with ox-LDL **(B)**. The vWF expression profile was similar to the control in the cells that were pretreated with NAC before exposure to ox-LDL **(C)**, indicating that NAC reversed the inhibitory effect of ox-LDL on endothelial differentiation of MAPCs. *Pink curves*: flow cytometry profile for the cells before differentiation; *light blue curves*: flow cytometry profile for the cells after 2 weeks of differentiation.
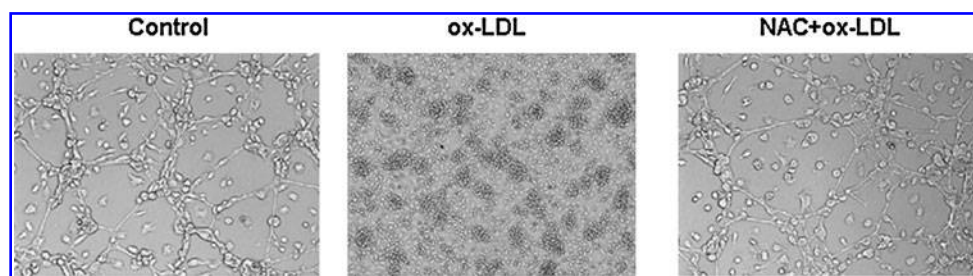
FIG. 7. Vascular structure formation by MAPC-derived cells in different groups. Vascular structure formation by MAPC-derived cells at week 2 of differentiation was observed on Matrigel under normal condition (*left panel*). When MAPCs were treated with ox-LDL, no vascular structures were generated at week 2 of differentiation (*middle panel*). When MAPCs were pretreated with the antioxidant NAC, the diminished capability of the MAPC-derived cells to form vascular structures on Matrigel by ox-LDL was completely recovered (*right panel*). The experiment was repeated for three times (×200 with inverted phase-contrast microscopy).

experiment shows that exposure of mice to a sublethal dose of total body irradiation induces a persistent increase in ROS production, leading to the induction of hematopoietic stem cell senescence (54). In the present study, we observed for the first time that ROS formation from ox-LDL mediated the actions of ox-LDL on MAPCs, including Oct-4 expression, proliferation, and endothelial differentiation.

Oct-4 is a transcription factor, and is expressed at high level in ESCs. It is critical to the pluripotency, self-renewal, and differentiation of stem cells (3, 27, 34). Like ESCs, MAPCs exhibit remarkable self-renewal capability and express Oct-4 abundantly (4, 8, 46). Recently, we demonstrated that nitric oxide enhanced Oct-4 expression and promoted endothelial differentiation of mouse MAPCs *in vitro* (9). In the present
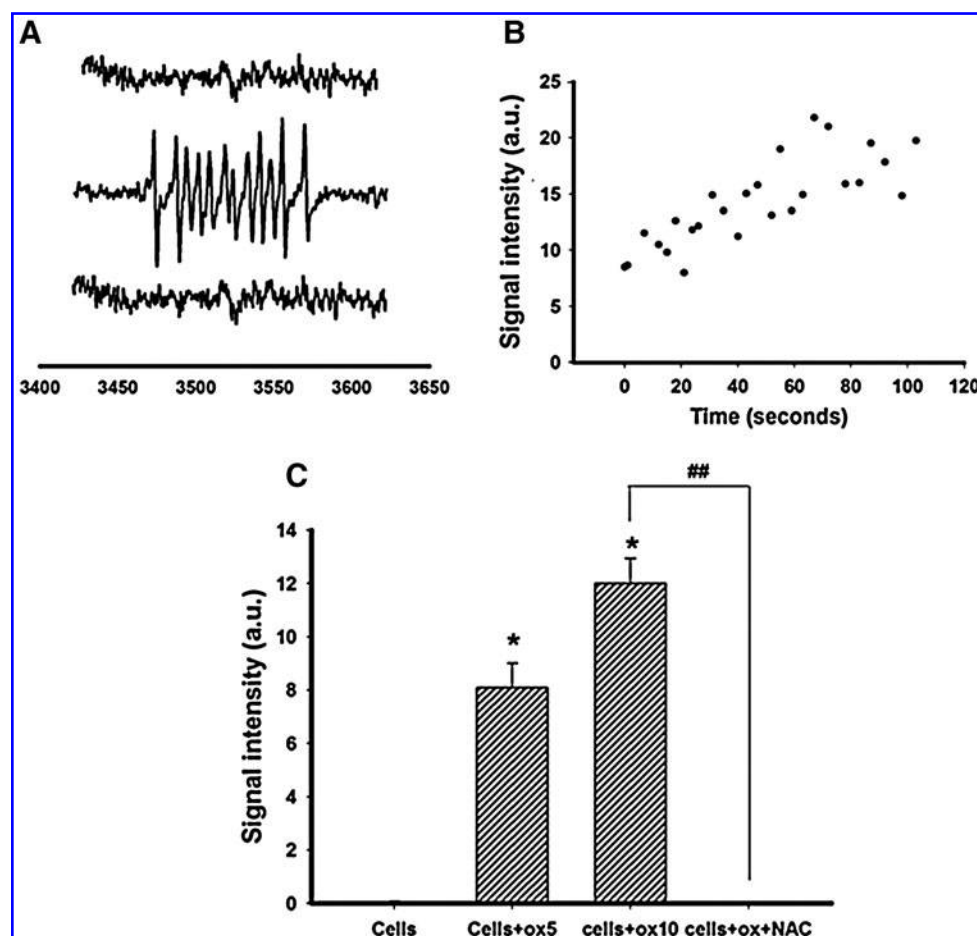

FIG. 8. ROS formation from ox-LDL with and without NAC. A significant amount of ROS was generated from ox-LDL in a dose- and time-dependent manner when mixed with MAPCs as determined quantitatively with EPR. ROS formation from ox-LDL was completely suppressed in the mixture when the antioxidant NAC was present. **(A)** Representative EPR signals from ox-LDL mixed with MAPCs. The top curve in **A** showed no EPR signals (only baseline noise) in the control group (MAPCs with detection media). The middle curve in **A** demonstrated the large EPR signals for ROS in the culture system of MAPCs with 5 μg/ml ox-LDL. The bottom curve in **A** showed no EPR signals for ROS in the culture system of MAPCs with 5 μg/ml ox-LDL when NAC (1 mM) was present. **(B)** Time-depend formation of ROS from ox-LDL. EPR signals occurred rapidly when ox-LDL was mixed with MAPCs. The signal amplitudes reached a plateau after 60 s, and stayed stable afterward. **(C)** The EPR signal amplitude was dependent on ox-LDL doses. Cells, MAPCs in culture media with negligible EPR signals; Cells+OX 5, MAPCs with 5 μg/ml ox-LDL; Cells+OX 10, MAPCs with 10 μg/ml ox-LDL; Cells+OX+NAC, MAPCs with 10 μg/ml ox-LDL plus 1 mM NAC. *$p < 0.05$ as compared with control "cells" group ($n = 4$); ##$p < 0.01$ as compared with 10 μg/ml ox-LDL group ($n = 4$). ROS, reactive oxygen species.
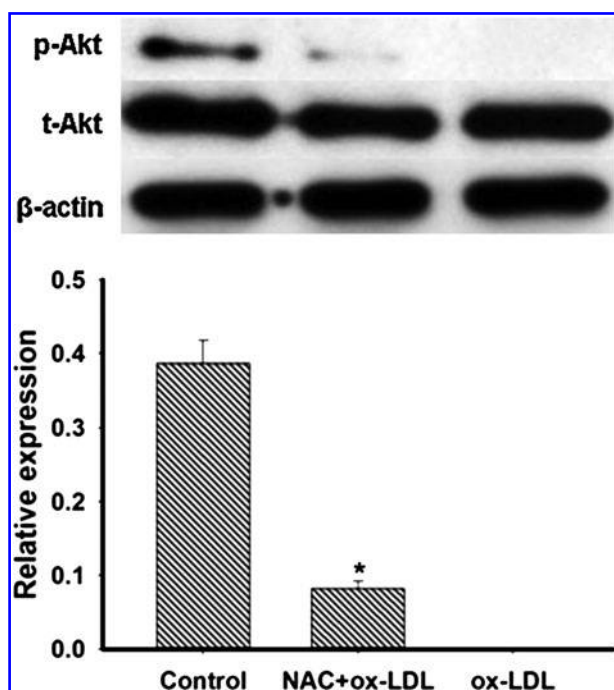
FIG. 9. Akt phosphorylation in MAPCs. There was a detectable level of Akt phosphorylation in MAPCs cultured in normal condition. When MAPCs were incubated with ox-LDL (5 μg/ml) for 24 h, Akt phosphorylation was dramatically decreased to almost nondetectable level in the cells. Ox-LDL-impaired Akt phosphorylation was only slightly restored in the cells treated with NAC with the Akt phosphorylation level close to 20% of the control. Control, cells cultured in normal condition; NAC+ox-LDL, cells were pretreated with NAC before exposure to ox-LDL; ox-LDL, cells exposed to ox-LDL. *$p < 0.01$ *versus* control group ($n = 3$). p-Akt, phosphorylated Akt; t-Akt, total Akt.

study, we showed that ROS generated from ox-LDL significantly suppressed Oct-4 expression, and attenuated endothelial differentiation of MAPCs that was reversed by treating the cells with the antioxidant NAC. This was the first time to report that oxidative stress was involved in the regulation of Oct-4 expression. Oxidative stress also regulates the selectivity of Oct-4 protein for specific DNA sequences (28), providing another mechanism for the actions of oxidative stress on Oct-4 and cell differentiation. Increased ROS beyond their basal level promotes hematopoietic progenitors to differentiate into mature blood cells in *Drosophila* (36). Clearly, the regulatory roles of oxidative stress in stem cell function, including differentiation, are complex and require further investigation (35, 37).

One of the interesting findings in the present study was that the antioxidant NAC completely blocked ROS formation from ox-LDL at a concentration of up to 20 μg/ml, and restored the endothelial differentiation potential of MAPCs. However, NAC only reversed the inhibitory effect of ox-LDL at a low concentration (5 μg/ml) on the proliferation of MAPCs, suggesting that other mechanisms were also important in mediating the effects of ox-LDL on these cells. Apoptosis is involved in the toxic effects of ox-LDL on various types of cells, including endothelial cells and the

development of atherosclerosis (33, 40, 42). In fact, we recently observed that impaired Akt signaling and apoptosis were involved in the actions of ox-LDL on MAPCs (8). Ox-LDL-induced apoptosis of MAPCs was effectively prevented by overexpression of active Akt, suggesting that the apoptotic event was due to impaired Akt signaling. In the present study, we confirmed that ox-LDL dramatically suppressed Akt phosphorylation in MAPCs. However, ox-LDL-impaired Akt phosphorylation was only slightly restored in the cells when treated with NAC. These data strongly supported the concept that ox-LDL impaired the population and function of MAPCs *via* multiple mechanisms, including oxidative stress, inhibition of Akt signaling, and apoptosis. Very likely, these mechanisms played a different role in the actions of ox-LDL at different concentrations on MAPCs. It appeared that both oxidative stress and impaired Akt signaling were important when ox-LDL concentration was low (5 μg/ml or less), whereas Akt signaling was the dominant mechanism when ox-LDL concentration was above 5 μg/ml. Further investigation is needed to define the relationship between these mechanisms in mediating the effects of ox-LDL on MAPCs.

Although the present study was conducted in cell culture system, the data had close clinical relevance. On the basis of recently published human data, the blood serum ox-LDL concentration was 0.7 mg/dl (7 μg/ml) in healthy individuals. The serum ox-LDL level was 1.72 mg/dl (17.2 μg/ml), and 2.36 mg/dl (23.6 μg/ml) for the patients with stable coronary artery disease (with average LDL 125.8 mg/dl) and acute coronary syndrome (with average LDL 143.0 mg/dl), respectively (25, 26, 57). The ox-LDL concentrations used in the present study was 1–20 μg/ml that were within the range of serum ox-LDL levels in both healthy persons and in patients with coronary artery disease. However, future studies are warranted to investigate the effect of ox-LDL and hyperlipidemia on bone marrow stem cells and their endothelial differentiation as well as the underlying mechanism(s) *in vivo*. However, it is important to point out that ox-LDL injected intravenously is cleared extremely rapidly (within a few minutes) by the reticular endothelial system (13, 48) that may create a very challenging situation for *in vivo* studies.

In conclusion, the data from the present study demonstrated that ROS formation was important in the action of ox-LDL on bone marrow stem cells, including Oct-4 expression, proliferation, and endothelial differentiation. However, other mechanisms like Akt signaling and apoptosis might also play a critical role in mediating the effect of ox-LDL on these cells. These data may have important clinical impact on the treatment of hyperlipidemic patients, and on patient selection for cell therapy with bone marrow stem cells especially for those with poorly controlled hyperlipidemia (for both recipients and donors) in view of the critical role of bone-marrow-derived EPCs in maintaining the integrity of endothelial structure and function.

Author Disclosure Statement

No competing financial interests exist for any of the authors.

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Abbreviations Used

EPCs = endothelial progenitor cells
EPR = electron paramagnetic resonance
ESCs = embryonic stem cells
MAPCs = bone marrow multipotent adult
progenitor cells
MSCs = mesenchymal stem cells
NAC = N-acetylcysteine
ox-LDL = oxidized low-density lipoprotein
ROS = reactive oxygen species
RT-PCR = real-time polymerase chain reaction
TBARS = thiobarbituric acid reactive substances

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