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Oxidized low-density lipoprotein alters endothelial progenitor cell populations

Yuqi Cui[1], Chandrakala A Narasimhulu[2], Lingjuan Liu[1], Xin Li[1], Yuan Xiao[1], Jia Zhang[1], Xiaoyun Xie[1], Hong Hao[1], Jason Z Liu[1], Guanglong He[1], Peter J Cowan[3], Lianqun Cui[4], Hua Zhu[1], Sampath Parthasarathy[2], Zhenguo Liu

[1]Dorothy M. Davis Heart and Lung Research Institute, Division of Cardiovascular Medicine, Department of Internal Medicine, Wexner Medical Center, The Ohio State University, Columbus, OH, USA; [2]Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, Orlando, FL, USA, [3]Department of Medicine, University of Melbourne, and Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia; [3]Department of Cardiology, Shandong Provincial Hospital, Shandong University, Jinan, China

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1. ABSTRACT

Oxidized low-density lipoprotein (ox-LDL) is critical to atherosclerosis in hyperlipidemia. Bone marrow (BM)-derived endothelial progenitor cells (EPCs) are important in preventing atherosclerosis, however these cells are significantly decreased in number in hyperlipidemia. This study aimed to determine whether ox-LDL and hyperlipidemia exert similar effects on EPC populations, and to investigate the involvement of reactive oxygen species (ROS). ROS production in BM and blood was significantly increased in male C57BL/6 mice treated with intravenous ox-LDL, and in hyperlipidemic LDL receptor knockout mice fed with a 4-month high-fat diet. ROS formation was effectively blocked by overexpression of antioxidant enzymes or N-acetylcysteine treatment. In both hyperlipidemic and ox-LDL-treated mice, the number of c-Kit$^+$/CD31$^+$ cells in BM and blood and of Sca-1$^+$/Flk-1$^+$ cells in blood significantly decreased, whereas the number of blood CD34$^+$/Flk-1$^+$ cells increased. In contrast, the number of blood CD34$^+$/CD133$^+$ cells increased in ox-LDL-treated mice but decreased in hyperlipidemic mice. Only the changes in CD34$^+$/Flk-1$^+$ cell number were prevented by inhibiting ROS production. These data suggested that ox-LDL produced significant changes in BM and blood EPC populations, largely similar to chronic hyperlipidemia, via predominantly ROS-independent mechanism(s).

2. INTRODUCTION

Endothelial dysfunction or injury is considered one of the major factors that contribute to the development of atherosclerosis and coronary heart disease (1,2). Endothelial progenitor cells (EPCs) play a critical role in vascular re-endothelialization, angiogenesis, and prevention of neointima formation after vascular injury (3-6). It is well known that the number and function

of circulating EPCs are significantly decreased in patients with atherosclerosis, coronary artery disease (CAD) and hyperlipidemia (7,8). However, the exact mechanism(s) for the deficiency of EPCs remains largely unknown.

Oxidized low-density lipoprotein (ox-LDL), a key component in the hyperlipidemic state, has been implicated in the formation of atherosclerotic plaques. The serum ox-LDL level is significantly increased in the patients with stable CAD and further increased in patients with acute coronary syndrome (9-11). There are extensive interactions between ox-LDL and a variety of cells through multiple mechanisms. The outcomes of the interactions between ox-LDL and its target cells are highly variable and complex, depending on the individual cell types. Ox-LDL promotes proliferation of macrophages and vascular smooth muscle cells, and inhibits apoptosis of macrophages and monocytes through activation of the PI-3 kinase/PKB and mitogen-activated protein kinase (MAPK) pathways (12-16). In contrast, ox-LDL inhibits proliferation and promotes apoptosis of vascular endothelial cells (7,17). Ox-LDL also inhibits EPC proliferation and differentiation, and suppresses EPC function, including inhibition of migration, adhesion, *in vitro* vasculogenesis, and ischemia-induced neovascularization *in vivo* (17-21). However, it is not known whether ox-LDL has similar effects on EPCs *in vivo* as hyperlipidemia.

Oxidative stress with reactive oxidative species (ROS) formation plays a critical role in the development of atherosclerosis (3, 22-26). ROS generation in monocytes is increased in hyperlipidemic patients with an elevated plasma ox-LDL level. Exposure to ox-LDL increases intracellular ROS formation in cultured endothelial cells (27, 28). *In vitro* studies demonstrated that a significant amount of ROS was produced spontaneously from ox-LDL at clinically relevant concentrations, and played an important role in the action of ox-LDL on bone marrow stem cells, including expression of the stem cell specific marker Oct-4, cell proliferation, and endothelial differentiation (29, 30).

The present study was to determine whether ox-LDL produced changes similar to hyperlipidemia in EPC populations *in vivo,* at least partially through ROS formation. One of the challenges in this type of study is to correctly identify EPCs (31-33), as there are currently no unified criteria to define them. We used four cell marker combinations (c-Kit$^+$/CD31$^+$, Sca-1$^+$/Flk-1$^+$, CD34$^+$/CD133$^+$, and CD34$^+$/Flk-1$^+$) that have been commonly used to identify and characterize EPCs in the literature. We observed that ox-LDL treatment and hyperlipidemia produced complex changes in these EPC populations in BM and blood. In both hyperlipidemic and ox-LDL-treated mice, the number of c-Kit$^+$/CD31$^+$ cells was significantly decreased in both BM and blood, and the number of Sca-1$^+$/Flk-1$^+$ cell was significantly decreased in blood only. Interestingly, CD34$^+$/Flk-1$^+$ cells were significantly increased in blood in both hyperlipidemic and ox-LDL-treated mice. However, there were also differences between hyperlipidemic and ox-LDL treated mice, with blood CD34$^+$/CD133$^+$ cells decreased in the former and increased in the latter. Using either a transgenic mouse model (TG) with concomitant global overexpression of an antioxidant network (AON) of human copper/zinc superoxide dismutase (SOD1), extracellular SOD3, and glutathione peroxidase (Gpx-1) or treatment with N-acetylcysteine (NAC) to block ROS formation, we observed that the changes in EPC populations induced by ox-LDL and hyperlipidemia were predominantly ROS-independent in both peripheral blood and BM.

3. MATERIALS AND METHODS

3.1. Ox-LDL preparation

Following Institutional Review Board approval, blood was collected in heparinized tubes from consented adult healthy donors, and stored on ice. Plasma was obtained from the blood after centrifuging at 1500g for 20 min. Lipoproteins were isolated from the plasma using sequential ultracentrifugation with a Beckman TL-100 tabletop ultracentrifuge (Beckman, Palo Alto, CA) as described (34). The isolated lipoproteins were dialyzed against 0.3 mM EDTA in 1x phosphate buffered saline (PBS, pH 7.4) overnight and subsequently sterilized with 0.22 μM filter. The protein concentration in the lipoproteins was determined using the Lowry method. Ox-LDL was produced from the native LDL immediately after dialysis using 5 μM copper sulfate and was stopped by adding EDTA (0.25 mM, final concentration) as described (35).

3.2. Animal models and ox-LDL treatment

All animal experiments were performed in accordance with the “Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health”. The experimental protocols for the present study were reviewed and approved by the Institutional Animal Care and Use Committee of the Ohio State University Wexner Medical Center, Columbus, OH, USA. Wild-type male C57 BL/6 mice (4-6 weeks old, Jackson Lab, ME, USA) were given 50 μg ox-LDL via tail vein injection (once daily for 3 days). To evaluate the role of ROS formation in the action of ox-LDL, the mice were pre-treated with NAC (in the drinking water) for 24 hours prior to ox-LDL administration.

To further evaluate the role of ROS production in mediating the effects of ox-LDL, a triple-transgenic (TG) mouse model (on C57 BL/6 background; 4-6 weeks old, male) with concomitant global overexpression of an antioxidant network (AON) composed of SOD1, SOD3,

and glutathione peroxidase (GSHPx)-1 was used to repeat the experiment, with littermate WT male mice as the control (36). TG mice were confirmed by genotyping and increased protein expression of the enzymes (using Western blot analysis) as well as increased enzyme activities as shown in Figure 5. To determine whether NAC treatment or AON expression affected the distribution and clearance of ox-LDL *in vivo*, the ox-LDL levels in the serum and bone marrow (BM) were measured at different time points after a single ox-LDL (50 μg) injection using a human ox-LDL ELISA kit (Mercodia Inc. Winston Salem, NC). To determine if ox-LDL could produce the effects on EPCs similar to hyperlipidemia, homozygous LDL receptor knockout ($LDLR^{-/-}$) mice (6-8 weeks old, on C57 BL/6 background, Jackson Lab, ME, US) were fed with a high fat diet (HFD) (17% AMF; 0.2% cholesterol) for 4 months to induce hyperlipidemia, with or without NAC treatment (in the drinking water) (37). Age-matched $LDLR^{-/-}$ C57BL/6 mice with normal diet were used as control. The mouse serum lipid profile was quantitatively determined using a lipid profile analyzer and cassette (Cholestech LDX, CA, US) to confirm the establishment of hyperlipidemic state.

3.3. Intracellular and extracellular ROS detection

Blood was collected from the mice, and red blood cells (RBCs) were eliminated using RBC lysis as described (38). The level of intracellular ROS formation in blood cells after injection of ox-LDL was determined using the ROS Detection Reagents-FITC DCF (Invitrogen) as described (39). The cells were incubated with the reagent at the concentration of 5 μg/ml for 10 min at 37°C. The labeled cells were washed twice with PBS and then suspended in warm PBS for analysis using flow cytometry. The fluorescence-positive cells were quantitatively evaluated using an LSRII (BD Bioscience, CA, USA) at the wavelength of 525nm as described (40). Extracellular ROS formation in the serum was quantitatively determined using the *ex vivo* electron paramagnetic resonance (EPR) spectroscopy with 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) (Santa Cruz) as the spin trap as described. ROS production was measured using EPR spectroscopy through a mixture of 50 mM DEPMPO (final concentration) with serum as described (29).

3.4. Murine EPC analysis

To determine the effect of ox-LDL or hyperlipidemic state on the populations of BM and blood EPCs, BM and blood cells were harvested in the mice after injection of 50 μg ox-LDL for 3 days, and in the hyperlipidemic $LDLR^{-/-}$ mice after 4 months of HFD with and without NAC treatment. After elimination of RBC with RBC lysis buffer, multicolor analysis for BM and blood EPCs was performed using an LSRII system (Becton Dickinson Biosciences, NJ, USA). A variety of cell surface markers and their combinations for identification of BM and blood EPCs were used as described (41-47), including $CD34^+/Flk\text{-}1^+$, $Sca\text{-}1^+/Flk\text{-}1^+$, $c\text{-}Kit^+/CD31^+$ and $CD34^+/CD133^+$. All antibodies were obtained from Biolegend (San Diego, CA, U.S) except Flk-1 APC-Cy7 from Becton Dickinson Biosciences (NJ, USA), and CD34 FITC from eBioscience (San Diego, CA, U.S.).

3.5. Statistical analysis

All the data were presented as means ± standard deviation (SD), and statistically analyzed using unpaired Student t-test (two-sided) for two groups of data or one way ANOVA (analysis of variance) (PRISM Version 4.0.; GraphPad Software, Inc., San Diego, CA) followed by post hoc conservative Tukey's test for three or more groups of data to minimize type I error as appropriate. The differences were considered statistically significant when a two-tailed $p < 0.05$.

4. RESULTS

4.1. Measurable level of ox-LDL in BM and blood

To evaluate the effect of ox-LDL on EPC populations, we first determined if ox-LDL was present in the BM and blood after IV administration. As shown in Figure 1A, after a single intravenous injection of human ox-LDL in WT C57BL/6 mice, a significant level of ox-LDL was detected in the blood within 2 min of injection, and declined rapidly thereafter. No detectable level of ox-LDL was present in the blood after 10 min. In the BM, the ox-LDL level increased within 2 min, peaked at 5 min, remained stable for 10 min, then declined rapidly thereafter (Figure 1B).

4.2. Ox-LDL significantly altered EPC populations in BM and blood

BM and blood cells were collected for EPC analysis after ox-LDL treatment. Flow cytometry analysis showed that ox-LDL significantly decreased the population of $c\text{-}Kit^+/CD31^+$ cells in BM (Figure 2A and Tables 2 and 4). In the peripheral blood, the populations of $Sca\text{-}1^+/Flk\text{-}1^+$ and $c\text{-}Kit^+/CD31^+$cells were significantly reduced in ox-LDL-treated mice (Figure 2D, 2E and Tables 3 and 4). On the other hand, the BM population of $CD34^+/CD133^+$ cells was significantly increased with ox-LDL treatment without changes in $CD34^+/Flk\text{-}1^+$ or $Sca\text{-}1^+/Flk\text{-}1^+$ cell populations (Figure 2B and Tables 2 and 4). The populations of blood $CD34^+/Flk\text{-}1^+$ and $CD34^+/CD133^+$ cells were significantly increased in ox-LDL-treated mice (Figure 2C, 2F and Tables 3-4).

4.3. Ox-LDL produced mostly similar changes in EPC populations as hyperlipidemia

Next, we compared the changes in EPC populations in the BM and blood in ox-LDL-treated and

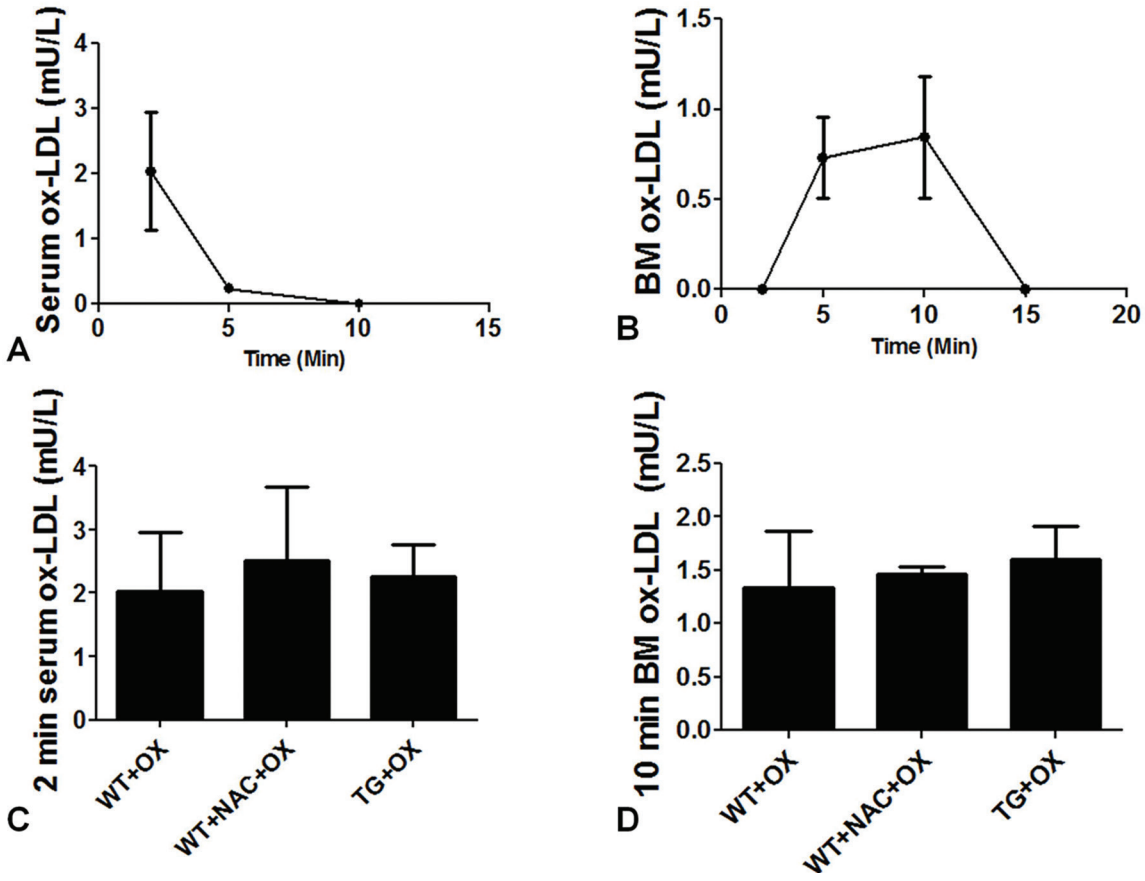

Figure 1. Dynamics of ox-LDL in the BM and serum. Concentrations of human ox-LDL in the serum (A) and BM (B) were determined in 6-8 weeks old C57BL/6 mouse at different time points after a single injection of human ox-LDL. The ox-LDL level reached the peak level in the serum in 5 min and stayed at a significantly elevated level for 10 min, then decreased to undetectable level in 15 min (B). On the other hand, the serum ox-LDL level reached its peak in 2 min after injection, and became undetectable in 10 min (A). Treatment with NAC or overexpression of AON had no significant difference in the peak BM and serum concentration of ox-LDL after intravenous administration in the mice (C, D). WT+OX: C57BL/6 mouse with ox-LDL injection; WT+OX+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG+OX: Triple transgenic mice with AON overexpression with ox-LDL injection. * $P < 0.05$, $n = 5$.

Table 1. Mouse serum lipid profile

	WT+ND (Mean±SD, n=8)	KO+ND (Mean±SD, n=8)	KO+HFD (Mean±SD, n=12)	KO+HFD+NAC (Mean±SD, n=8)
TC (mg±dl)	<100	280.4±12.3*	1468.67±218.35**	1395.4±204
HDL (mg±dl)	79.4±2.07	81±1.58	90±17.48**	90.2±15.82
TRG (mg±dl)	118.8±8.5	178.2±13.65*	476±242.49**	287.2±66.9
LDL (mg±dl)	<100	153.4±15.73*	1295.33±174.91**	1198±172.81
Non-HDL (mg±dl)	<100	191.2±14.87*	1372.67±210.1**	1198±172.81
TC±HDL	<2	3.46±0.21*	16.61±2.53**	16.11±4.88

C57BL/6 and $LDLR^{-/-}$ mice were fed with normal diet or high fat diet for 4 months as indicated with or without NAC. Serum lipid profile was determined. WT+ND: WT C57BL/6 mouse with normal diet for 4 months; KO+ND: $LDLR^{-/-}$ mice with normal diet for 4 months; KO+HFD: $LDLR^{-/-}$ mice with high fat diet for 4 months; KO+HFD+NAC: $LDLR^{-/-}$ mice with high fat diet and NAC for 4 months. HDL: high density lipoprotein; LDL: low density lipoprotein; TRG: triglycerides; TC: total cholesterol,*p<0.001 (KO+ND vs WT+ND); **p<0.001 (KO+HFD vs KO+ND)

hyperlipidemic mice. Hyperlipidemia was confirmed by serum analysis (Table 1). In both groups, the number of c-Kit^+/$CD31^+$ cells was significantly decreased in both BM and blood, and the number of Sca-1^+/Flk-1^+ cells was significantly decreased in blood but not in BM (Figure 2A, 2D, 2E, and Tables 2-4). In addition, blood $CD34^+$/Flk-1^+ cells increased in both groups (Figure 2C, and Tables 3-4). However, there were also some differences between

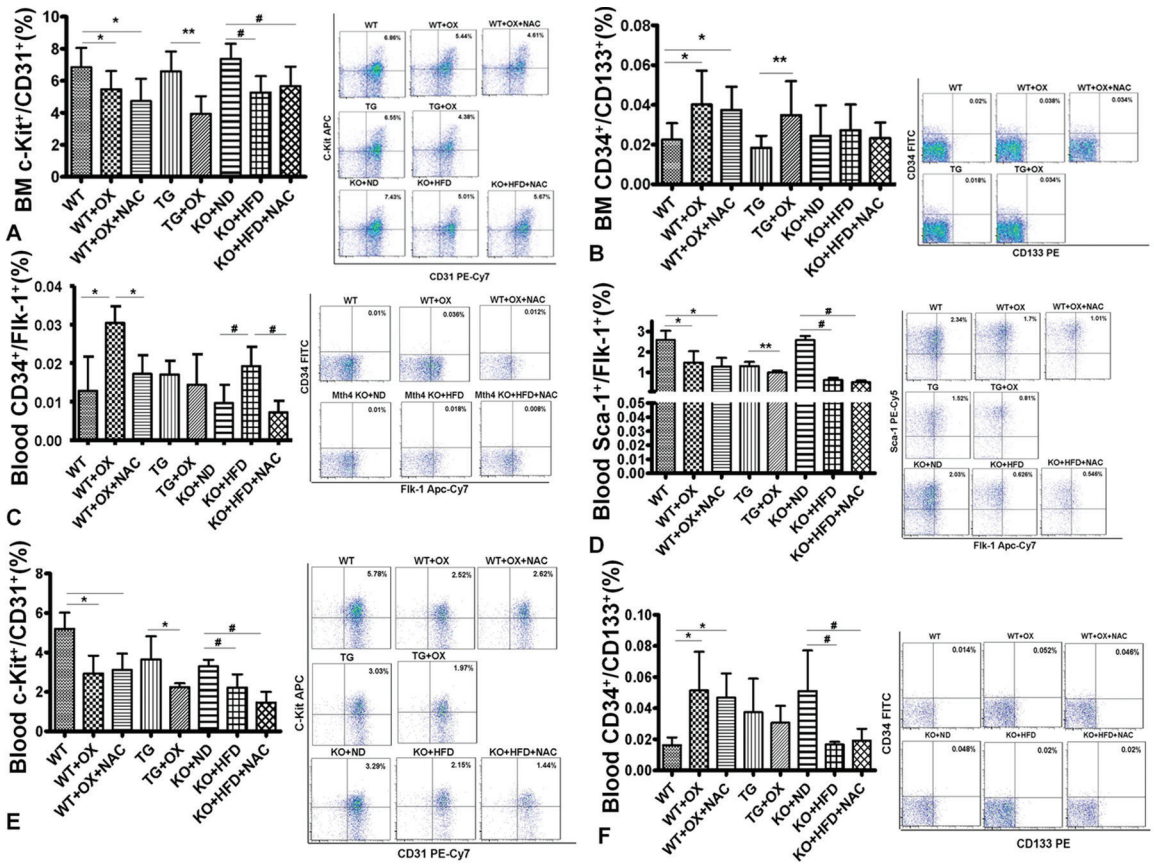

Figure 2. The role of ROS in mediating the effects of ox-LDL and hyperlipidemia on murine BM and circulating EPCs. After 3 days of ox-LDL injection or with HFD treatment, the BM and peripheral blood cells were collected and the RBC were eliminated for flow cytometry analysis. The populations of the cells expressing CD34+/Flk-1+, or Sca-1+/Flk-1, or c-Kit+/CD31+ or CD34+/CD133+ in both BM and blood were analyzed as shown (A-F). Experiments were repeated with mice overexpressing AON or treated with antioxidant NAC to block ROS production. WT: C57BL/6 mouse with PBS injection; WT+OX: C57BL/6 mouse with ox-LDL injection; WT+OX+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG: Triple transgenic mice with AON overexpression with PBS injection; TG+OX: Triple transgenic mice with AON overexpression with ox-LDL injection; KO+ND: LDLR-/- mice with normal diet for 4 months; KO+HFD: LDLR-/- mice with high fat diet for 4 months; KO+HFD+NAC: LDLR-/- mice with high fat diet and NAC for 4 months. * WT+ox-LDL and WT+ox-LDL+NAC vs WT, $p<0.05$, n=8; ** TG+ox-LDL vs TG, $p<0.05$, n=8; # KO+HFD and KO+HFD+NAC vs KO+ND, $p<0.05$, n=8-12.

the groups, including blood $CD34^+/CD133^+$ cells which were elevated with ox-LDL treatment but decreased in hyperlipidemic mice (Figure 2F and Tables 3-4).

4.4. Ox-LDL and hyperlipidemia increased extracellular ROS production in BM and blood, but only increased intracellular ROS production in blood

Ox-LDL treatment induces significant ROS generation *in vitro* (29). We observed that both extracellular and intracellular ROS levels were significantly increased in the blood within 10 min of IV injection of ox-LDL (Figure 3B and 4B). The extracellular ROS level in the BM of ox-LDL-treated animals were also significantly increased (Figure 3A), while there was no difference in intracellular ROS formation in the BM (Figure 4A). Hyperlipidemia induced similar changes to ox-LDL treatment (Figure 3A and 3B, Figure 4B).

4.5. NAC treatment or overexpression of antioxidant enzymes blocked ox-LDL-induced ROS production

To determine the role of ROS production in mediating the effects of ox-LDL and hyperlipidemia on EPC populations in BM and blood, mice were treated with NAC to inhibit ROS formation. We also used a triple-transgenic (TG) mouse model that over-expressed a network of antioxidant enzymes (SOD-1, SOD-3 and Gpx-1); the protein levels and activities of these enzymes were significantly increased in the transgenic mice (Figure 5). As expected, the blood intracellular and extracellular ROS and BM extracellular ROS production induced by ox-LDL were effectively blocked in the NAC-treated mice and in the transgenic mice (Figure 3 and Figure 4B). The distribution and clearance of ox-LDL were not affected in either group (Figure 1C and 1D). Similarly, extracellular and intracellular ROS formation

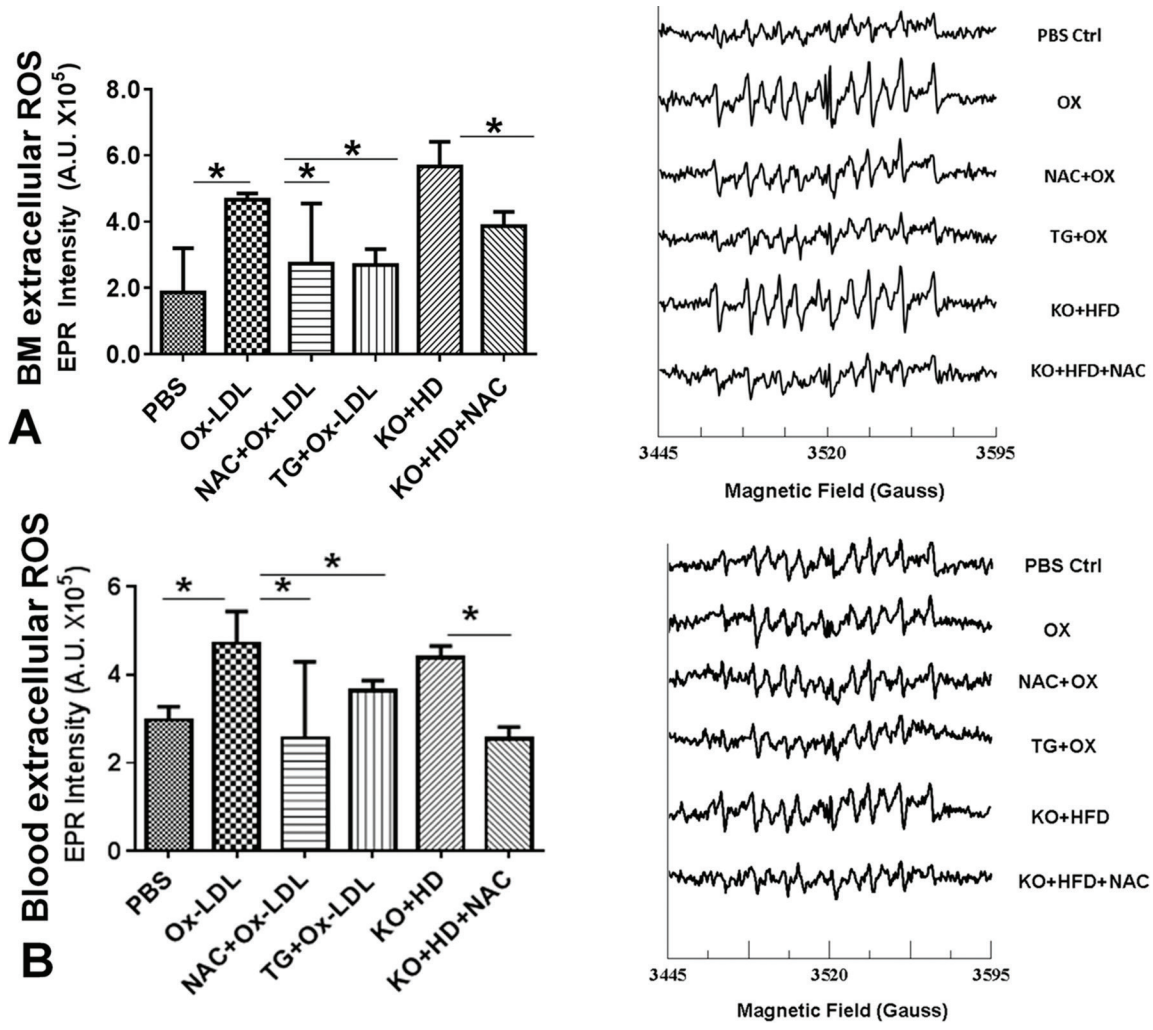

Figure 3. Ox-LDL and hyperlipidemia increased extracellular ROS production that was effectively blocked by AON expression or NAC treatment in BM and blood. Serum ROS production was significantly increased in the mice with ox-LDL injection, and in the hyperlipidemic LDLR-/- mice (B). Similarly, BM extracellular ROS formation was significantly increased in the WT C57BL/6 mice with ox-LDL injection and the hyperlipidemic LDLR-/- mice (A). Increased ROS production (both BM and serum) was completely blocked by NAC treatment or overexpression of AON in the mice received ox-LDL, and in the hyperlipidemic LDLR-/- mice (A and B). No significant change in ROS production was observed in the animals treated with PBS (control, A and B). PBS: C57BL/6 mouse with PBS injection; ox-LDL: C57BL/6 mouse with ox-LDL injection; ox-LDL+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG+ox-LDL: TG mouse with ox-LDL injection; KO+ND: LDLR-/- mice with normal diet for 4 months; KO+HFD: LDLR-/- mice with high fat diet for 4 months; KO+HFD+NAC: LDLR-/- mice with high fat diet and NAC for 4 months. * P<0.05, n=8-12.

was blocked by NAC treatment of hyperlipidemic LDLR$^{-/-}$ mice (Figure 3 and 4B).

4.6. Inhibition of ROS production only partially reversed the effects of ox-LDL and hyperlipidemia on EPC populations

Next, we examined if inhibition of ROS production could prevent the effects of ox-LDL and hyperlipidemia on EPC populations. Some changes in blood EPC populations induced by ox-LDL or hyperlipidemia were reversed by NAC treatment. As shown in Figure 2C and Tables 3-4, the increase in blood CD34$^+$/Flk-1$^+$ cells induced by ox-LDL was prevented by NAC treatment or AON overexpression. Similarly, NAC treatment prevented the increase in blood CD34$^+$/Flk-1$^+$ cells in hyperlipidemic mice (Figure 2C and Tables 3-4).

However, NAC treatment or overexpression of AON had no impact on ox-LDL-induced or hyperlipidemia-associated changes in the c-Kit$^+$/CD31$^+$, Sca-1$^+$/Flk-1$^+$, and CD34$^+$/CD133$^+$ populations (Figure 2A, 2B, 2D-2F, and Table 2-4). These data suggested that ox-LDL and hyperlipidemia-induced changes in EPCs in BM and blood were mediated by both ROS-dependent and ROS-independent mechanisms depending on the individual cell populations.

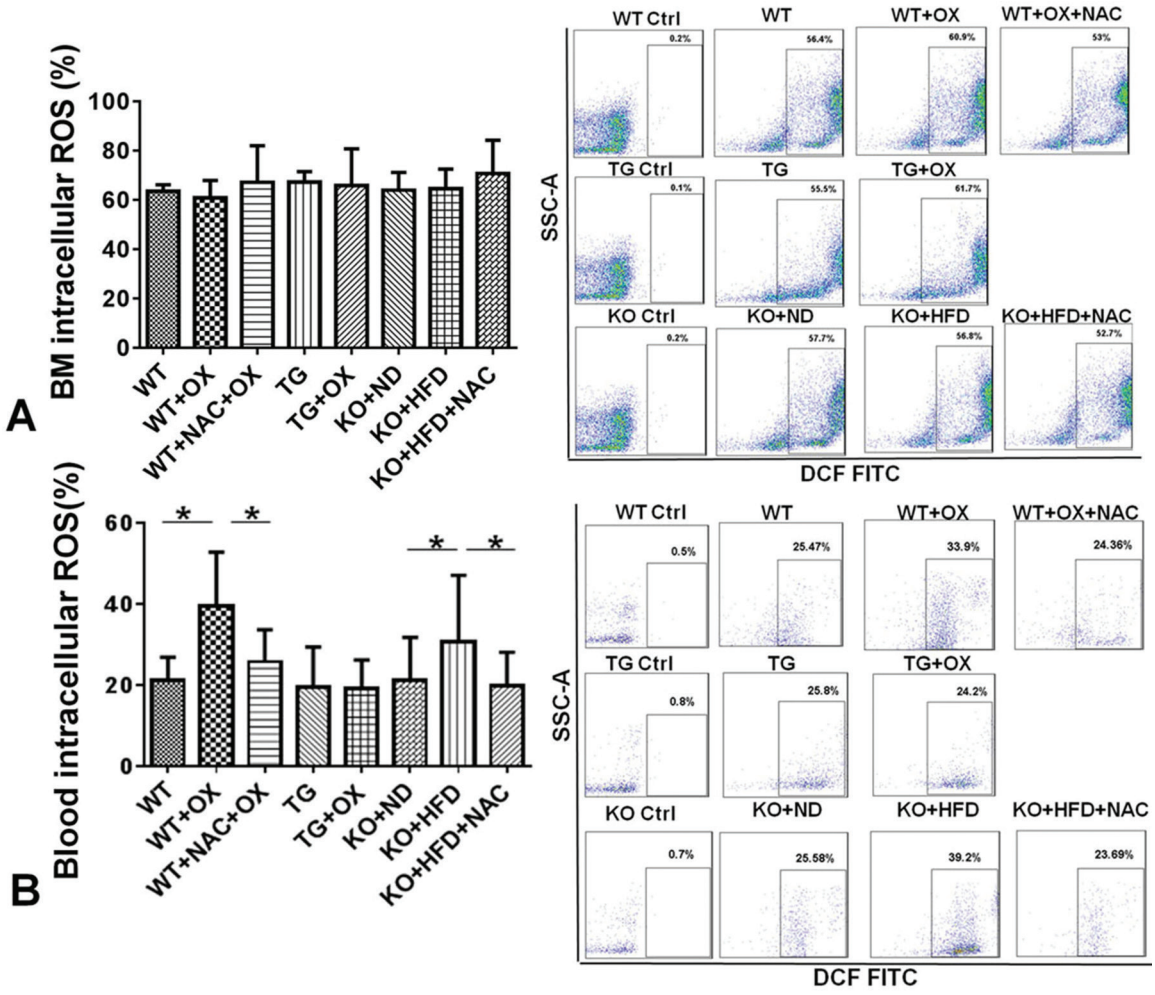

Figure 4. Different effect of ox-LDL treatment and hyperlipidemia on BM and blood intracellular ROS formation. Intracellular ROS production was significantly increased in the blood cells in the mice with ox-LDL injection and in the hyperlipidemic LDLR-/- mice (B). However, there was no change in the intracellular ROS formation in the BM in all groups of mice (A). Increased intracellular ROS production in blood cells was completely blocked by NAC treatment or overexpression of AON in the mice received ox-LDL, and in the hyperlipidemic LDLR-/- mice (B). No significant change in intracellular ROS production was observed in the animals treated with PBS (control, B). WT: C57BL/6 mouse with PBS injection; WT+ox-LDL: C57BL/6 mouse with ox-LDL injection; WT+ox-LDL+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG: TG mouse with PBS injection; TG+ox-LDL: TG mouse with ox-LDL injection; KO+ND: LDLR-/- mice with normal diet for 4 months; KO+HFD: LDLR-/- mice with high fat diet for 4 months; KO+HFD+NAC: LDLR-/- mice with high fat diet and NAC for 4 months. * P<0.05, n=8-12.

5. DISCUSSION

In the present study, we demonstrated that ox-LDL treatment induced generation of ROS in the BM and blood, and significantly altered the populations of EPCs in the BM and blood in a similar manner to chronic hyperlipidemia. Our data suggested that these changes involved both ROS-dependent and predominantly ROS-independent mechanisms *in vivo*. This was the first demonstration that ox-LDL was at least partially responsible for the altered number of EPCs in hyperlipidemic states, and support the concept that ox-LDL is a critical component of hyperlipidemia.

The outcomes of the interactions between ox-LDL and its target cells are variable and complex depending on the individual cell type. Ox-LDL promotes proliferation of macrophages and vascular smooth muscle cells, and inhibits apoptosis of macrophages and monocytes (12-16, 48, 49). Low level of ox-LDL has been reported to stimulate the proliferation, migration, and adhesion of mouse BM mesenchymal stem cells (50). On the other hand, ox-LDL inhibits proliferation and promotes apoptosis of vascular endothelial cells and EPCs (7,17,51). Recently, we have demonstrated that ox-LDL inhibits cell proliferation, induces apoptosis of rat BM multipotent adult progenitor cells (MAPCs), and attenuates their endothelial differentiation (30). Similarly, in the present study, ox-LDL produced a complex change in the cell populations in both BM and blood: some cell populations were selectively increased, while others decreased.

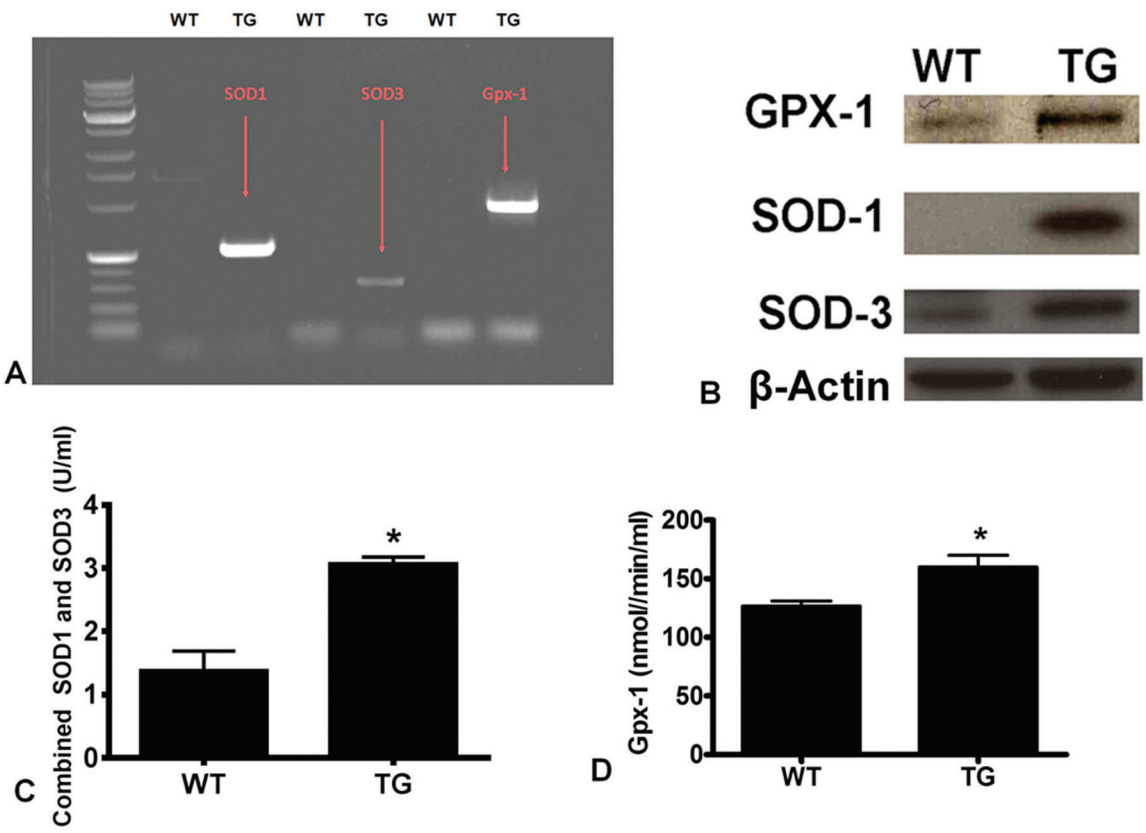

Figure 5. Identification of SOD1 and 3 and Gpx-1 triple transgenic mice. Successful creation of the transgenic mice with expression of human antioxidant enzyme network was confirmed with both genotyping (A) and protein expression (using western blot analysis with 20ug total protein (B). The SOD and Gpx-1 enzyme activities in TG mouse were significantly increased as compared to the WT control (C, D). WT: C57BL/6 mouse; TG: triple transgenic mice with overexpression of antioxidant network composed of SOD1, SOD3, and glutathione peroxidase (Gpx-1). * TG vs WT, p<0.001, n=8.

Table 2. The effects of ox-LDL and hyperlipidemia on BM EPC populations

	BMCs No. (Million)	($CD34^+/Flk\text{-}1^+$)%	($Sca\text{-}1^+/Flk\text{-}1^+$)%	($C\text{-}Kit^+/CD31^+$)%	($CD34^+/CD133^+$)%
WT	33.54±12.38	0.48±0.25	1.60±0.45	6.85±1.03	0.02±0.01
WT+OX	26.24±7.81	0.33±0.16	1.59±0.42	5.46±0.96*	0.04±0.02*
WT+OX+NAC	29.09±11.47	0.40±0.15	1.75±0.47	4.73±1.39*	0.04±0.01*
TG	38.15±3.79	0.45±0.15	1.82±0.50	6.58±1.25	0.02±0.01
TG+OX	31.59±11.16	0.36±0.13	1.47±0.24	3.93±1.11**	0.03±0.02**
KO+ND	29.50±11.26	0.46±0.15	2.24±0.87	7.37±0.94	0.02±0.02
KO+HFD	23.70±5.60	0.45±0.17	2.06±0.71	5.27±1.02#	0.03±0.01
KO+HFD+NAC	28.30±15.04	0.43±0.11	2.22±0.40	5.66±1.22#	0.02±0.01

The total number of murine BM cell and EPC percentile were determined in the mice with different treatments as mean±SD. BMCs No.: the total number of BM cells after red blood cell (RBC) elimination. WT: WT C57BL/6 mouse with PBS injection; WT+OX: C57BL/6 mouse with ox-LDL injection; WT+OX+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG: Triple transgenic mice with overexpression of antioxidant network (AON) composed of SOD1, SOD3, and glutathione peroxidase (Gpx-1) with PBS injection; TG+OX: Triple transgenic mice with AON overexpression with ox-LDL injection; KO+ND: $LDLR^{-/-}$ mice with normal diet for 4 months; KO+HFD: $LDLR^{-/-}$ mice with high fat diet for 4 months; KO+HFD+NAC: $LDLR^{-/-}$ mice with high fat diet and NAC for 4 months. * WT+ox-LDL and WT+ox-LDL+NAC vs WT, p<0.05, n=8; **TG+ox-LDL vs TG, p<0.05, n=8; # KO+HFD and KO+HFD+NAC vs KO+ND, p<0.05, n=8-12

Ox-LDL interferes with the function of EPCs through multiple mechanisms, including inhibition of endothelial nitric oxide synthase (eNOS), downregulation of E-selectin and integrin alpha(v)beta(5) expression,

Table 3. The effects of ox-LDL and hyperlipidemia on blood EPC populations

	(CD34$^+$/Flk-1$^+$)%	(Sca-1$^+$/Flk-1$^+$)%	(C-Kit$^+$/CD31$^+$)%	(CD34$^+$/CD133$^+$)%
WT	0.010±0.006	2.58±0.46	5.75±1.41	0.02±0.01
WT+OX	0.031±0.009*	1.74±0.15*	2.87±0.84*	0.05±0.02*
WT+OX+NAC	0.014±0.004	1.04±0.43*	2.87±1.92*	0.05±0.02*
TG	0.017±0.004	1.52±0.29	3.55±1.30	0.04±0.02
TG+OX	0.014±0.008	0.81±0.07**	1.98±0.25**	0.03±0.01
KO+ND	0.01±0.005	2.63±0.22	3.30±0.38	0.05±0.03
KO+HFD	0.02±0.01#	0.62±0.12#	2.22±0.67#	0.02±0.002#
KO+HFD+NAC	0.01±0.003	0.48±0.11#	1.48±0.62#	0.02±0.01#

The murine blood EPC percentile was determined with flow cytometry as mean±SD. WT: C57BL/6 mouse with PBS injection; WT+OX: C57BL/6 mouse with ox-LDL injection; WT+OX+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG: Triple transgenic mice with overexpression of antioxidant network (AON) composed of SOD1, SOD3, and glutathione peroxidase (Gpx-1) with PBS injection; TG+OX: Triple transgenic mice with AON overexpression with ox-LDL injection; KO+ND: LDLR$^{-/-}$ mice with normal diet for 4 months; KO+HFD: LDLR$^{-/-}$ mice with high fat diet for 4 months; KO+HFD+NAC: LDLR$^{-/-}$ mice with high fat diet and NAC for 4 months. * WT+ox-LDL and WT+ox-LDL+NAC vs WT, $p<0.05$, n=8; ** TG+ox-LDL vs TG, $p<0.05$, n=8; # KO+HFD and KO+HFD+NAC vs KO+ND, $p<0.05$, n=8-12

Table 4. EPC changes summary

BM EPC	WT+OX	WT+OX+NAC	TG+OX	KO+HFD	KO+HFD+NAC
CD34$^+$/Flk-1$^+$					
Sca-1$^+$/Flk-1$^+$					
C-Kit$^+$/CD31$^+$ # ↓↓	↓	↓	↓	↓	↓
CD34$^+$/CD133$^+$ #	↑	↑	↑		
Circulating EPC					
CD34$^+$/Flk-1$^+$ * ↑↑	↑	B		↑	B
Sca-1$^+$/Flk-1$^+$ # ↓↓	↓	↓	↓	↓	↓
C-Kit$^+$/CD31$^+$ # ↓↓	↓	↓	↓	↓	↓
CD34$^+$/CD133$^+$	↑	↑		↓	↓

Summary of all murine EPC changes. WT+OX: C57BL/6 mouse with ox-LDL injection; WT+OX+NAC: C57BL/6 mouse with NAC treatment and ox-LDL injection; TG+OX: Triple transgenic mice with overexpression of antioxidant network (AON) composed of SOD1, SOD3, and glutathione peroxidase (Gpx-1) with ox-LDL injection; KO+HFD: LDLR$^{-/-}$ mice with high fat diet for 4 months; KO+HFD+NAC: LDLR$^{-/-}$ mice with high fat diet and NAC for 4 months. ↑: increased EPC percentile compared to control. ↓: decreased EPC compared to control. ↑↑: increased EPC in both C57BL/6 mouse with ox-LDL injection and LDLR$^{-/-}$ mice with high fat diet for 4 months compared to their control; ↓↓: decreased EPC in both C57BL/6 mouse with ox-LDL injection and LDLR$^{-/-}$ mice with high fat diet for 4 months compared to their control; B: reversion of the effects of ox-LDL on EPCs population by NAC. *ROS-dependent EPC change; #ROS-independent EPC change

inactivation of telomerase, and acceleration of cell senescence (19, 21, 52). An 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 (53-56). Recently, we demonstrated that ox-LDL induced the generation of ROS spontaneously *in vitro* that was completely prevented when the antioxidant NAC was present (29). In the present study, we showed that intravenous injection of ox-LDL also produced a significant level of ROS in BM and blood *in vivo* similar to hyperlipidemia. ROS and oxidative stress are involved in EPC dysfunction in many diseases, including hyperlipidemia, diabetes, coronary artery disease and renal ischemia-reperfusion injury (57-59). We observed here that in both hyperlipidemic and ox-LDL-treated mice, the c-Kit$^+$/CD31$^+$ cell population was significantly decreased in both BM and blood, and the number of Sca-1$^+$/Flk-1$^+$ cells was significantly decreased in blood without change in BM. Blocking ROS formation using a transgenic antioxidant mouse model or treatment with NAC had no significant impact on the changes in the populations of these cells. These

data suggested that ox-LDL and hyperlipidemia-induced reduction in EPCs in BM and blood is mediated largely by ROS-independent mechanisms. Further investigations are needed to evaluate these other mechanisms.

Interestingly, the increase in blood $CD34^+$/$Flk\text{-}1^+$ cell number in both hyperlipidemic and ox-LDL-treated mice was reversed by NAC treatment or AON overexpression, suggesting a ROS-dependent mechanism. The clinical significance of this finding is unclear at this point, since the number of $CD34^+$/$Flk\text{-}1^+$ cells is usually decreased in hyperlipidemic patients (60). This might reflect a differential response to hyperlipidemia in mice and humans for this particular cell population.

The mechanisms for the alterations of BM and blood EPCs in hyperlipidemia are complex and remain largely unknown. Although several similarities in the changes of EPC populations in ox-LDL-treated mice and mice with hyperlipidemia were observed in the present study, there were also differences, notably in the blood $CD34^+$/$CD133^+$ population. NAC treatment or AON overexpression had no effect on changes in this population induced by ox-LDL or hyperlipidemia, suggesting a role for factors other than ox-LDL. This finding is perhaps not surprising, since the oxidative modification of LDL is very complex in hyperlipidemic animals, with generation of many modified lipoprotein ligands in addition to ox-LDL (61,62).

ROS-independent mechanisms played a critical role in the overall changes in EPC populations in BM and blood in response to ox-LDL exposure and in the setting of hyperlipidemia. This may provide an explanation for the controversy and dilemma in view of the critical role of BM-derived EPCs in maintaining the integrity of endothelial structure and function. It is believed that oxidative stress and excessive ROS production are important to the development of cardiovascular diseases in hyperlipidemia. However, despite strong pre-clinical evidence and promising data in animal studies, most clinical trials on antioxidants including vitamin E or β-carotene have failed to demonstrate a significant clinical benefit in patients with hyperlipidemia and cardiovascular diseases (63). The data from the present study showed that blocking ROS production from ox-LDL or hyperlipidemia either had no effect on restoring the decreased EPC number or preventing the increase of EPC subpopulation like circulating $CD34^+$/$Flk\text{-}1^+$ cell. Clearly, the mechanisms for the alteration of EPC populations in response to ox-LDL exposure and hyperlipidemia are complex and require further investigation (22, 64).

In conclusion, we have shown that ox-LDL and hyperlipidemia produced similar but not identical changes in the EPC populations in BM and blood, predominantly through mechanism(s) independent of ROS formation. Further studies are needed to investigate these mechanisms and to characterize the functional status of the individual EPC subgroups that change in response to ox-LDL and hyperlipidemia.

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Send correspondence to: Zhenguo Liu, Davis Heart and Lung Research Institute, The Ohio State University, Wexner Medical Center, Room 200 DHLRI; 473 West 12th Ave**,** Columbus, OH 43210, USA**,** Tel: 614-247-8435; Fax: 614-293-5614**,** E-mail: zhenguo.liu@osumc.edu