Native low-density lipoprotein-induced superoxide anion contributes to proliferation of human aortic smooth muscle cells

Department of Anesthesiology and Pain Medicine, Yonsei University Wonju College of Medicine, Wonju,
*Biology Program, Division of Life Sciences, Kangwon National University, Chuncheon, Korea

Hyun Kyo Lim[#], Woosung Shin^{*,#}, Ji Yeon Lee, and Sungwoo Ryoo^{*}

Background: Native low-density lipoprotein (nLDL) was one of the modifiable risk factors contributed directly to cardiovascular diseases development. We investigated that nLDL stimulation induced NADPH oxidase activation and superoxide production that was an important factor on human aortic smooth muscle cells (hAoSMC) proliferation.

Methods: Superoxide generation was recorded with fluorescent-staining of dihydroethidine or by measuring lucigenin-induced chemiluminescence for 5 minutes. We examined cell proliferation with 4[-3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) reagent and analyzed the change of gene expression by northern blot analysis.

Results: nLDL stimulation increased superoxide anion production in hAoSMC that confirmed through dihydroethidine staining and lucigenin-induced chemiluminescence methods. nLDL-induced proliferation abolished with preincubation of superoxide scavengers or NADPH oxidase inhibitor. NADPH as a substrate of NADPH oxidase increased superoxide generation in both nLDL-stimulated and unstimulated cell homogenate, which was completely blocked at the diphenylene iodonium (DPI)- or apocynin-pretreated hAoSMC homogenates. Furthermore, superoxide generation was only observed at the fraction of cellular precipitate, but not in soluble fraction. Expression of p22phox in mRNA level increased with nLDL treatment as early as 30 minutes and transfection of anti-sense oligonucleotide of p22phox completely abolished nLDL-induced proliferation of hAoSMC.

Conclusions: The above results have shown that nLDL-induced proliferation in hAoSMC depends on superoxide production through NADPH oxidase activation. (Korean J Anesthesiol 2009; 57: 622~8)

Key Words: Human aortic smooth muscle cells, NADPH oxidase, Native low-density lipoprotein, Superoxide anion.

INTRODUCTION

Elevated plasma level of native low-density lipoproteins (nLDL) contributes to numerous vascular diseases including hypercholesterolemia, atherosclerosis, hypertension, heart failure, and diabetes that is closely linked to vascular proliferation [1-3] and oxidative stress [4]. Vascular smooth muscle cell

(VSMC) proliferation is particularly important to the pathophysiological conditions of in-stent restenosis, transplant vasculopathy, and vein bypass graft failure that are associated with inflammation, apoptosis, and matrix alternation through endothelial dysfunction and VSMC activation [5].

Reactive oxygen species (ROS) produced from all vascular cell types have been considered deleterious to cell function and suggested that play a role in the pathophysiology of cardiovascular system [6-8]. Besides their deleterious effect, ROS are also being recognized as important regulators of cell function and modulators of cell signaling pathway. In VSMC, ROS generation affords an important regulatory system to growth and hypertrophy through extracellular signal regulated kinase (Erk) 1/2 activation [9]. Atherosclerosis is associated with chronic oxidative stress [10] as well as increased activity, abundance expression, and localization of Erk 1/2 [11].

Previous studies have shown that nLDL-mediated VSMC proliferation is multiple lines of signaling pathway such as phosphoinositide-dependent protein kinase C (PKC) activation

Received: March 31, 2009.

Revised: 1st, May 4, 2009; 2nd, May 18, 2009.

Accepted: June 22, 2009.

Corresponding author: Sungwoo Ryoo, Ph.D., Biology Program, Division of Life Sciences, Kangwon National University, 1, Kangwondae-gil, Chuncheon 200-701, Korea. Tel: 82-33-250-8534, Fax: 82-33-251-3990, E-mail: ryooosw08@kangwon.ac.kr

[#]The following authors contributed equally to this work.

Copyright © Korean Society of Anesthesiologists, 2009

© This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

and proto-oncogen induction [12], intracellular calcium and pH changes [13], pertussis-toxin sensitive Erk1/2 MAPK activation and intracellular calcium increases [14], protein kinase C (PKC)-zeta activation [15], redox-sensitive Erk1/2 activation [9], up-regulation of cyclin D1/cyclin-dependent kinase (CDK)2 and cyclinE/CDK4 complexes and down-regulation of p21 (Cip21) [16]. However, the enzymatic source of oxygen-free radical production in nLDL-stimulated human aortic smooth muscle cells (hAoSMC) and its activation mechanisms are unknown although it is demonstrated that oxidative stress is associated to proliferation in nLDL-stimulated VSMC.

Therefore, we investigated whether the production of superoxide was elevated by nLDL stimulation and whether superoxide from nLDL stimulation involved in hAoSMC proliferation.

MATERIALS AND METHODS

Materials

Dihydroethidine (DHE), Lucigenin, 1,2-bis (o-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) were purchased from Calbiochem Co. (Gibbstown, NJ, USA). Polymer chain reaction (PCR) primers, PCR premix were obtained from Bioneer Co. (Daejeon, Korea). All other reagents were purchased from Sigma (Saint Louis, MO, USA) unless otherwise stated.

Isolation of nLDL and cell culture

nLDL isolation from normocholesterolemic plasma by ultracentrifugation [17] and the maintenance of hAoSMC (Cascade biologics, Salem, OR, USA) were followed previously described method [17].

Intracellular superoxide measurement

The serum-starved cells in phenol-red free DMEM (Invitrogen, San Diego, CA, USA) were labeled with DHE (10 μ g/ml) for 10 min and incubated with nLDL (100 μ g/ml) another for 30 min in phenol-red free HBSS (Invitrogen, San Diego, CA, USA). The cells were washed three times with PBS and then imaged under epifluorescence microscope equipped with krypton/argon laser (excitation 488 nm, emission 610 nm). Lucigenin (5 μ M/L)-induced chemiluminescence was recorded by luminometer (Monolight^{TM} 3010, BD Biosciences, San Diego, CA, USA) in Krebs-HEPES (pH 7.4). All experiments were measured chemiluminescence for 5 minutes.

Proliferation assay

Cell proliferation was assessed with WST-1 (4[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) following the supplier's protocol (Roche Co., Nutley, NJ, USA). Briefly, approximately 1×10^5 cells were incubated in 96-well plates and starved for 24 hours with serum-free DMEM. After cells were treated with appropriate inhibitors and/or nLDL, each well was added 10 μ l of WST-1 reagent and absorbance was read at 450 nm.

Cellular fractionation

Treated cells were homogenized in Tris buffer (25 mM, pH 7.4, protease inhibitors (Roche Co.), 250 mM NaCl, 0.1% β -mercaptoethanol, and 3 mM EDTA) and centrifuged at 1,000 g for 10 minutes to remove cell debris and unbroken cells. The supernatant were applied to centrifugation at 21,000 g for 45 minutes at 4°C. The cytosolic and membrane fractions containing 20 μ g protein were used to examine superoxide generation and western blot analysis for translocation of cytosolic NADPH components.

Northern blot analysis

Northern blot analysis follows previously described method [18]. Probes prepared by gel-extraction of PCR products were radiolabeled with ³²P- α dCTP. The primer sequences of p22phox, gp91, and GAPDH were as follows: *p22phox* forward 5'-CAG TGT CCC AGC CGG GTT CGT GTC-3', reverse 5'-CTT CGC TGC GTT TAT TGC AGG TGG-3', product size is 670 bp. *gp91*, forward 5'-AGA AAG ATG TGA TCA CAG GCC TG-3', reverse 5'-ATC TTT TCC CTA ACT TCC ACT GAC-3', product size is 478 bp. *GAPDH* forward 5'-AAG AAT TCA TGG GGG ACA CCT TCA TCC GTC AC-3', reverse 5'-AAG TCG ACT CAG ACG GCA GAC GCC AGG AA-3', product size is 475 bp.

Transient transfection

The oligonucleotide of p22phox antisense (Bioneer Co. Daejeon, Korea), sense and scrambled were used to transfection using Superfect (Qiagen, Valencia, CA, USA) reagent according to supplier's protocol: antisense, 5'-GAT CTG CCC CAT GGT GAG GAC C-3', sense, 5'-GGT CCT CAC CAT GGG GCA GAT C-3', scrambled, 5'-TAG CAT AGC CCT CCG CTG GGG A-3'. Briefly, cells were seeded on 24-well plates and grown to 70% confluency. 3 μ g of p22phox oligonucleotides

Fig. 1. nLDL stimulated superoxide production in hAoSMC. (A) nLDL (100 μ g/ml, 10 min) increased dihydroethidine (10 μ M/L, 10 min) fluorescence that was abolished with preincubation of PEG-SOD (500 U/ml). Representative image from 3 experiments (B), Lucigenin-induced chemiluminescence was increased with nLDL stimulation at time-dependent manner. OxLDL (10 μ g/ml) as a positive control was used. nLDL: native low-density lipoprotein, hAoSMC: human aortic smooth muscle cells, PEG-SOD: polyethylene glycol-superoxide dismutase, RLU: relative lucigenin unit, OxLDL: oxidized low-density lipoprotein. *P < 0.01 vs. untreated, n = 5.

were incubated with 100 μ l DMEM and 15 μ l Superfect reagent for 10 minutes, followed by addition of 2,300 μ l complete growth medium. Cells were incubated with 200 μ l of this mixture per well for 8 hours, followed by incubation for 16 hours with growth medium. Cells starved with serum for 4 hours treated with nLDL for 48 hours and measured growth at 450 nm.

Statistical analysis

Values were presented as mean $\pm$ standard deviation (SD). Statistical significant was assessed by unpaired student's t-test (GraphPad Prism 4.02). A P value of <0.05 was used as the criterion for statistical significance.

RESULTS

nLDL increased superoxide generation in hAoSMC

We first wished to determine the effect of nLDL on superoxide production in cultured hAoSMC. nLDL (100 μ g/ml) stimulation increased in DHE fluorescence and preincubation of polyethylene glycol-superoxide dismutase (PEG-SOD, 500 U/ml) abolished nLDL-induced DHE fluorescence (Fig. 1A). In addition, nLDL induced a time-dependent increase lucigenin chemiluminescence at 4 minutes (Fig. 1B) from $1,434 \pm 132$ to $2,446 \pm 195$ a.u. (n = 4, P < 0.01). Oxidized low-density lipoprotein (OxLDL) also increased chemiluminescence at 2

Fig. 2. Superoxide anion induced cell proliferation. Superoxide dismutase (500 U/ml) and DPI (10 μ mol/L) completely blocked the proliferation, but not catalase (500 U/ml). WST-1: 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, nLDL: native low-density lipoprotein, PEG-SOD: polyethylene glycol-superoxide dismutase, DPI: diphenylene iodonium. *P < 0.01 vs. untreated, †P < 0.01 vs. nLDL, n = 5.

minutes after stimulation.

Superoxide scavengers abolished nLDL-induced cell proliferation

Given recent data suggesting that superoxide promote VSMC proliferation, we tested whether a superoxide was associated with nLDL induction in hAoSMC proliferation. As demon-

strated Fig. 2, nLDL-induced proliferation (1 ± 0.04 a.u. vs. 1.25 ± 0.02 a.u.) completely abolished with preincubation of PEG-SOD (0.95 ± 0.03 a.u.) or diphenylene iodonium (DPI, 0.97 ± 0.11 a.u.), but catalase treatment still increased cell proliferation by nLDL stimulation (1.16 ± 0.04 a.u.).

nLDL-dependent superoxide producing enzyme

Because several enzymatic reactions had been demonstrated to produce superoxide anion, we wished to determine which enzyme was responsible for the production in hAoSMC. Superoxide production investigated with nLDL-stimulated or unstimulated hAoSMC in response to a variety of substrates. NADPH was greater substrate to produce superoxide than other substrate in both nLDL-stimulated ($4,040 \pm 158$ a.u./min/mg protein) and unstimulated ($2,284 \pm 97$ a.u./min/mg protein) hAoSMC homogenates (Fig. 3A). In the homogenates, superoxide

production in response to nLDL ($4,040 \pm 158$ a.u./min/mg protein) was 2.27-fold higher than substrate-untreated control cells ($1,775 \pm 83$ a.u./min/mg protein). Furthermore, we investigated subcellular localization of superoxide producing enzyme by separating the homogenates into cytosolic and membrane fractions. As demonstrated in Fig. 3B, NADPH-dependent superoxide producing enzyme was predominantly localized at precipitated fraction, and NADPH-driven superoxide production in precipitate fraction was higher in the stimulated ($3,227 \pm 72$ a.u./min/mg protein) than in the unstimulated ($2,619 \pm 112$ a.u./min/mg protein) precipitate fraction.

nLDL increased mRNA level of p22phox and gp91phox and p22phox antisense inhibited nLDL-induced proliferation in hAoSMC

We wished to determine whether nLDL changes in p22phox

Fig. 3. nLDL activated NADPH oxidase. (A) NADPH ($100 \mu\text{M/L}$) increased superoxide production at both nLDL-stimulated and unstimulated homogenates of hAoSMC. * $P < 0.01$ vs. untreated control (-), $n = 5$. (B) NADPH-stimulated precipitates increased superoxide generation. * $P < 0.01$ vs. NADPH-treated supernatant, $n = 5$. (C) Preincubation of NADPH oxidase inhibitors, DPI ($10 \mu\text{M/L}$) and apocynin ($100 \mu\text{M/L}$) blocked nLDL-induced superoxide production. * $P < 0.01$ vs. untreated control, $n = 4$, † $P < 0.01$ vs. nLDL, $n = 4$. nLDL: native low-density lipoprotein, RLU: relative lucigenin unit, Allop: allopurinol ($100 \mu\text{M/L}$), Indo: indomethacin ($10 \mu\text{M/L}$), L-NMMA: NG-monomethyl-L-arginine ($10 \mu\text{M/L}$), Roten: rotenone ($100 \mu\text{M/L}$), DPI: diphenylene iodonium.

Fig. 4. nLDL stimulation increased mRNA levels of p22phox and gp91phox, and p22phox antisense blocked nLDL-induced hAoSMT proliferation. (A) nLDL (100 μ g/ml) increased mRNA levels of p22phox and gp91phox as early as 30 minutes. (B) Transfection of p22phox antisense inhibited hAoSMTs proliferation by nLDL stimulation after 48 hours incubation, but sense and scrambled oligonucleotides had no effect. *P < 0.01 vs. untreated control, †P < 0.01 vs. nLDL, n = 12 from 3 independent experiments. nLDL: native low-density lipoprotein, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, WST-1: 4[-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate, S: sense, AS: antisense, SM: scramble.

and gp91phox (NOX-2) expression levels in nLDL-stimulated hAoSMT. Therefore, we analyzed mRNA levels by Northern blot analysis with specific probe to p22phox or gp91phox mRNA. nLDL stimulation quickly increased mRNA level of p22phox and gp91phox in hAoSMT after 30 minutes (Fig. 4A) suggested that increased NADPH oxidase mediate superoxide generation in early stage of atherosclerosis. In order to determine whether inhibition of expression with antisense against p22phox regulates nLDL-induced proliferation, anti-sense oligonucleotide was transiently transfected and measured the effect of proliferation in nLDL-stimulated hAoSMT. As shown in Fig. 4B, there is a significant decrease in proliferation at transfected cells with p22phox-antisense compared to nLDL-stimulated cells (nLDL vs. nLDL + AS, 1.15 ± 0.09 a.u. vs. 0.54 ± 0.01 a.u.).

Thus, in summary, nLDL-induced proliferation in hAoSMT depends on superoxide production through NADPH oxidase activation.

DISCUSSION

We have demonstrated that nLDL stimulation increases superoxide generation in hAoSMT that contributes to cellular

proliferation. NADPH as a substrate potentiates superoxide production and NADPH oxidase inhibitors abolish increased cell growth in nLDL-stimulated hAoSMT. Furthermore, nLDL treatment upregulates p22phox expression in mRNA level and specific anti-sense to p22phox prevents nLDL-induced cell proliferation. The proliferation of smooth muscle cells during necrotic core formation, a complex of foam cell and died muscle cells directly caused to vascular stiffness during atherogenesis. With together, NADPH oxidase activation in nLDL-induced hAoSMT may play an important role in superoxide generation participating directly in nLDL modification to OxLDL. Oxidative modification of nLDL to OxLDL that induced proliferation [18], apoptosis [19], and eventual plaque rupture [20] was a critical step in atherogenesis.

nLDL causes to endothelial dysfunction by increasing the adhesiveness with respect to leukocytes or platelet, as well as its permeability and uptakes [21] and inducing interleukin-8 and monocyte chemotactic protein secretion [22], although the mechanism is not clear, into the subendothelial space and subsequent modified in response to reactive oxygen species (superoxide and hydrogen peroxide) to oxidized LDL revealed to induce proinflammatory cytokines, vascular cell proliferation, and apoptosis [23]. With together, nLDL also induces vaso-

constriction [13], impairment of endothelium-dependent relaxation [24], upregulation of endothelin receptor [25] and VSMC proliferation [12,26]. Increased nLDL-induced VSMC proliferation is the prime feature of atherosclerosis [5] and vascular oxidative stress also contributed to development of the pathogenesis [27]. Our previous study indicates that nLDL induces a chemokine, interleukin-8, production via p38 MAPK, H₂O₂, and transcription factor AP-1 [17]. Our present observation in the smooth muscle cells supports again a pathophysiological importance of reactive oxygen species because nLDL enhances interleukin-8 production and increases superoxide generation.

Among various sources for ROS production, such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide synthase, and mitochondrial electron transport, NAD(P)H oxidase had been considered to be a major source of superoxide generation in both endothelial and smooth muscle cells [28]. Several stimulants increased ROS generation via NADPH oxidase activation, which is one of the major target molecules regulating redox balance in vascular system. Also, increased NADH oxidase-mediated superoxide production directly contributes to atherogenesis in the nLDL-receptor defeat rabbit model [29]. In this line, the preferred substrate (NADH or NADPH) for NAD(P)H oxidase is still need to be investigated under different physiological conditions because analysis with electron spin resonance spectroscopy indicates that NADH or NADPH are equally good substrates in VSMC but that NADH-driven superoxide production predominates in endothelial cells.

A representative activation mechanism of NADPH oxidase established that cytosolic components of the enzyme, p47phox and p67phox, was activated by multiple phosphorylation and translocated to membrane fraction for assembling with p22phox and mitogenic oxidase (mox) components. In particular, p22phox forming the complex with mox is integral for the oxidase activation to produce superoxide anion. Therefore, nLDL-induced upregulation of p22phox can promote superoxide production in hyperlipidemic condition. Given that there is a possibility that Erk1/2 MAPK and PKC activation in nLDL-treated VSMC is closely related to proliferation and its inhibition abolish the cell growth [30], although we do not understand the detail activation mechanism of NADPH oxidase in nLDL-stimulated hAoSMC, these kinases may involve in phosphorylated activation of the cytosolic components.

It is worthwhile to note that nLDL at 100 μ g apoB/ml, which is a border line of normocholesterolemia (less than 100 μ g

apoB/ml) and hypercholesterolemia (more than 160 μ g apoB/ml), induces a significant increases in superoxide anion production. This result indicate that increasing amount of nLDL in blood plasma could locally initiate activation of NADPH oxidase to generate of superoxide anion that directly increasing the modification of nLDL to OxLDL, a pivotal molecule in initiating and propagating the atherosclerotic process.

In conclusion, nLDL-induced proliferation in hAoSMC depends on superoxide production through NADPH oxidase activation. Furthermore, inhibition of superoxide generation with NADPH oxidase inhibitor or its scavenger (PEG-SOD) blocks completely nLDL-induced cell proliferation. This mechanism importantly provides a potential target for antioxidant therapy in conditions of associated with hypercholesterolemia.

ACKNOWLEDGEMENTS

This study was supported by 2008 Research Grant from Kangwon National University (to S.R.) and Grant from Yonsei University Wonju College of Medicine. Native low-density lipoprotein-induced superoxide anion contributes to proliferation of human aortic smooth muscle cells.

REFERENCES

1. The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease. *JAMA* 1984; 251: 351-64.
2. Grundy SM. Cholesterol and coronary heart disease. Future directions. *JAMA* 1990; 264: 3053-9.
3. Steinberg D, Witztum JL. Lipoproteins and atherogenesis. Current concepts. *JAMA* 1990; 264: 3047-52.
4. Aviram M. Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. *Free Radic Res* 2000; 33 Suppl: S85-97.
5. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med* 2002; 8: 1249-56.
6. Grech ED, Dodd NJ, Jackson MJ, Morrison WL, Faragher EB, Ramsdale DR. Evidence for free radical generation after primary percutaneous transluminal coronary angioplasty recanalization in acute myocardial infarction. *Am J Cardiol* 1996; 77: 122-7.
7. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 1993; 328: 1450-6.
8. White CR, Brock TA, Chang LY, Crapo J, Briscoe P, Ku D, et al. Superoxide and peroxynitrite in atherosclerosis. *Proc Natl Acad Sci U S A* 1994; 91: 1044-8.

9. Locher R, Brandes RP, Vetter W, Barton M. Native LDL induces proliferation of human vascular smooth muscle cells via redox-mediated activation of ERK 1/2 mitogen-activated protein kinases. *Hypertension* 2002; 39: 645-50.
10. Griending KK, Harrison DG. Dual role of reactive oxygen species in vascular growth. *Circ Res* 1999; 85: 562-3.
11. Hu Y, Dietrich H, Metzler B, Wick G, Xu Q. Hyperexpression and activation of extracellular signal-regulated kinases (ERK1/2) in atherosclerotic lesions of cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol* 2000; 20: 18-26.
12. Scott-Burden T, Resink TJ, Hahn AW, Baur U, Box RJ, Buhler FR. Induction of growth-related metabolism in human vascular smooth muscle cells by low density lipoprotein. *J Biol Chem* 1989; 264: 12582-9.
13. Sachinidis A, Mengden T, Locher R, Brunner C, Vetter W. Novel cellular activities for low density lipoprotein in vascular smooth muscle cells. *Hypertension* 1990; 15: 704-11.
14. Sachinidis A, Seewald S, Epping P, Seul C, Ko Y, Vetter H. The growth-promoting effect of low-density lipoprotein may be mediated by a pertussis toxin-sensitive mitogen-activated protein kinase pathway. *Mol Pharmacol* 1997; 52: 389-97.
15. Carlin S, Yang KX, Donnelly R, Black JL. Protein kinase C isoforms in human airway smooth muscle cells: activation of PKC-zeta during proliferation. *Am J Physiol* 1999; 276: L506-12.
16. Heo KS, Kim DU, Ryoo S, Nam M, Baek ST, Kim L, et al. PPARgamma activation abolishes LDL-induced proliferation of human aortic smooth muscle cells via SOD-mediated down-regulation of superoxide. *Biochem Biophys Res Commun* 2007; 359: 1017-23.
17. Ryoo SW, Kim DU, Won M, Chung KS, Jang YJ, Oh GT, et al. Native LDL induces interleukin-8 expression via H₂O₂, p38 Kinase, and activator protein-1 in human aortic smooth muscle cells. *Cardiovasc Res* 2004; 62: 185-93.
18. Yang CM, Chien CS, Hsiao LD, Pan SL, Wang CC, Chiu CT, et al. Mitogenic effect of oxidized low-density lipoprotein on vascular smooth muscle cells mediated by activation of Ras/Raf/MEK/MAPK pathway. *Br J Pharmacol* 2001; 132: 1531-41.
19. Jovinge S, Crisby M, Thyberg J, Nilsson J. DNA fragmentation and ultrastructural changes of degenerating cells in atherosclerotic lesions and smooth muscle cells exposed to oxidized LDL in vitro. *Arterioscler Thromb Vasc Biol* 1997; 17: 2225-31.
20. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994; 94: 2493-503.
21. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; 340: 115-26.
22. De Castellarnau C, Sanchez-Quesada JL, Benitez S, Rosa R, Caveda L, Vila L, et al. Electronegative LDL from normolipemic subjects induces IL-8 and monocyte chemotactic protein secretion by human endothelial cells. *Arterioscler Thromb Vasc Biol* 2000; 20: 2281-7.
23. Dimmeler S, Haendeler J, Galle J, Zeiher AM. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. *Circulation* 1997; 95: 1760-3.
24. Persegol L, Sementa V, Athias A, Lecerf J, Lallemand C, Gamber P. Co-incubation of native and oxidized low-density lipoproteins: potentiation of relaxation impairment. *Eur J Pharmacol* 2000; 406: 429-37.
25. Muller G, Catar RA, Niemann B, Barton M, Knels L, Wendel M, et al. Upregulation of endothelin receptor B in human endothelial cells by low-density lipoproteins. *Exp Biol Med* 2006; 231: 766-71.
26. Libby P, Miao P, Ordovas JM, Schaefer EJ. Lipoproteins increase growth of mitogen-stimulated arterial smooth muscle cells. *J Cell Physiol* 1985; 124: 1-8.
27. Alexander RW. Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* 1995; 25: 155-61.
28. Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etayo JC, et al. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* 2000; 35: 1055-61.
29. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, et al. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation* 1999; 99: 2027-33.
30. Heo KS, Kim DU, Kim L, Nam M, Baek ST, Park SK, et al. Activation of PKCbeta(II) and PKCtheta is essential for LDL-induced cell proliferation of human aortic smooth muscle cells via Gi-mediated Erk1/2 activation and Egr-1 upregulation. *Biochem Biophys Res Commun* 2008; 368: 126-31.