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

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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 iodinium (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.

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...
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 μg/ml) for 10 min and incubated with nLDL (100 μg/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 (exitation 488 nm, emission 610 nm). Lucigenin (5 μM(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-tetrazolo]-1,3-benzene disulfonate) following the supplier’s protocol (Roche Co., Nutley, NJ, USA). Briefly, approximately 1 × 10⁵ 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 μl 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 was applied to centrifugation at 21,000 g for 45 minutes at 4°C. The cytosolic and membrane fractions containing 20 μg 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 T G - 3 ' ,  r e v e r s e  5 ' - A T C  T T T  T C C  C T A  A C T  T C C  A C T G A C - 3 ' ,  p r o d u c t  s i z e  i s  4 7 8  b p . GAPDH forward 5'-AAG AAT TCA TGG GGG ACA CCT TCA ACT TCC ACT GAC-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 ACT TCC ACT GAC-3', product size is 478 bp. 623

**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'-GTT CCT CAT CAC CTT CAC CAC CTC CTC GGG A-3', scrambled, 5'-TAG CAT AGC CTT CAC CAG GGG A-3', product size is 475 bp.
were incubated with 100 μl DMEM and 15 μl Superfect reagent for 10 minutes, followed by addition of 2,300 μl complete growth medium. Cells were incubated with 200 μl 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 ± 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 μg/ml) stimulation increased DHE fluorescence and preincubation of polyethylene glycol-superoxide dimutase (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 ± 132 to 2,446 ± 195 a.u. (n = 4, P < 0.01). Oxidized low-density lipoprotein (OxLDL) also increased chemiluminescence at 2 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 ± 158 a.u./min/mg protein) and unstimulated (2,284 ± 97 a.u./min/mg protein) hAoSMC homogenates (Fig. 3A). In the homogenates, superoxide production in response to nLDL (4,040 ± 158 a.u./min/mg protein) was 2.27-fold higher than substrate-untreated control cells (1,775 ± 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 ± 72 a.u./min/mg protein) than in the unstimulated (2,619 ± 112 a.u./min/mg protein) precipitate fraction.

**nLDL increased mRNA level of p22phox and gp91phox**

We wished to determine whether nLDL changes in p22phox production in response to nLDL (4,040 ± 158 a.u./min/mg protein) was 2.27-fold higher than substrate-untreated control cells (1,775 ± 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 ± 72 a.u./min/mg protein) than in the unstimulated (2,619 ± 112 a.u./min/mg protein) precipitate fraction.

We wished to determine whether nLDL changes in p22phox antisense inhibited nLDL-induced proliferation in hAoSMC.

Fig. 3. nLDL activated NADPH oxidase. (A) NADPH (100 μM/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. untreated control, n = 5. (C) Preincubation of NADPH oxidase inhibitors, DPI (10 μM/L) and apocynin (100 μM/L) blocked nLDL-induced superoxide production. *P < 0.01 vs. untreated control, n = 4. nLDL: native low-density lipoprotein, RLU: relative lucigenin unit, Allop: allopurinol (100 μM/L), Indo: indomethacin (10 μM/L), L-NMMA: NG-nomethyl-L-arginine (10 μM/L), Roten: rotenone (100 μM/L), DPI: diphenylene iodonium.
and gp91phox (NOX-2) expression levels in nLDL-stimulated hAoSMC. 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 hAoSMC 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 hAoSMC. 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 hAoSMC depends on superoxide production through NADPH oxidase activation.

**DISCUSSION**

We have demonstrated that nLDL stimulation increases superoxide generation in hAoSMC that contributes to cellular proliferation. NADPH as a substrate potentiates superoxide production and NADPH oxidase inhibitors abolish increased cell growth in nLDL-stimulated hAoSMC. 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 hAoSMC 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 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 NADPH 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 translocated to membrane fraction for assembling with p22phox and p67phox, was activated by multiple phosphorylation and established that cytosolic components of the enzyme, p47phox, 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 μg apoB/ml, which is a border line of normocholesterolemia (less than 100 μg apoB/ml) and hypercholesterolemia (more than 160 μg 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.

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