Remnant Lipoprotein Particles Induce Apoptosis in Endothelial Cells by NAD(P)H Oxidase–Mediated Production of Superoxide and Cytokines via Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Activation Prevention by Cilostazol

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Background—Remnant lipoprotein particles (RLPs), products of lipolytic degradation of triglyceride-rich lipoprotein derived from VLDL, exert atherogenesis. In this study, we observed how RLPs induced cytotoxicity in human umbilical vein endothelial cells (HUVECs) and cilostazol prevented cell death.

Methods and Results—RLPs were isolated from the plasma of hyperlipidemic patients by use of an immunoaffinity gel mixture of anti–apolipoprotein A-1 and anti–apolipoprotein B-100 monoclonal antibodies. RLPs (50 μg/mL) significantly increased superoxide formation in HUVECs associated with elevated gp91phox mRNA and protein expression and Rac1 translocation, accompanied by increased production of tumor necrosis factor (TNF)-α and interleukin-1β, DNA fragmentation, and cell death. Cilostazol (1 to 100 μmol/L) significantly suppressed not only NAD(P)H oxidase–dependent superoxide production but also TNF-α and interleukin-1β release and restored viability. RLPs activated a lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which was not inhibited by cilostazol. Treatment of HUVECs with monoclonal antibody for LOX-1 attenuated RLP-mediated production of superoxide, TNF-α, and interleukin-1β and DNA fragmentation.

Conclusions—RLPs stimulated NAD(P)H oxidase–dependent superoxide formation and induction of cytokines in HUVECs via activation of LOX-1, consequently leading to reduction in cell viability with DNA fragmentation, and cilostazol exerts a cell-protective effect by suppressing these variables. (Circulation. 2004;109:1022-1028.)

Key Words: apoptosis ■ atherosclerosis ■ lipoproteins ■ superoxide

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A growing body of evidence has suggested that remnant lipoprotein particles (RLPs), derived from VLDL and chylomicrons, play a role as an atherogenic factor. Remnant lipoprotein particles (RLPs) are known to be one of the most significant risk factors for sudden cardiac death. Kugiyama et al emphasized the importance of remnant lipoprotein level of fasting serum in predicting future coronary events in patients with coronary artery disease independently of other risk factors. Nakajima et al have developed a simple, rapid assay method for determination of RLP-cholesterol (RLP-C), ie, chylomicron and VLDL remnants. RLP-C is composed of lipoproteins remaining unbound to the immunoaffinity gel mixture of an anti–apolipoprotein (apo) B-100 and an anti–apoA-1 monoclonal antibodies and is rather enriched in apoE and cholesteryl esters. Inoue et al have documented the impairment of acetylcholine-induced coronary artery relaxation in patients with high serum RLPs, suggestive of an association between high serum RLP-C and coronary vascular endothelial cell dysfunction. Doi et al and Kugiyama et al have shown that RLPs isolated by an immunoseparation method have a causative role in endothelial vasomotor dysfunction in human coronary arteries, and RLPs directly induce endothelial dysfunction in the isolated rabbit aorta. Doi et al have further emphasized that endothelium-derived reactive oxygen species may initiate and propagate chains of free radical reactions in polyunsaturated fatty acid in RLPs, thereby leading to production of highly reactive intermediates that could transfer from RLPs to endothelial cells. Conversely, cilostazol was introduced to increase intracellular cAMP by blocking its hydrolysis by type III phosphodiesterase. Recently, Kim et al addressed the in vitro inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells (HUVECs), in that the lipopolysaccharide-induced decrease in Bcl-2 protein and...
increase in Bax protein and cytochrome c release were reversed by cilostazol. Reactive oxygen species and tumor necrosis factor (TNF)-α are critically implicated in the development and progression of atherosclerotic lesions in humans12 and in the induction of endothelial apoptosis.13,14 Thus, therapeutic drugs suppressing the generation of superoxide and cytokines can be a potential strategy for prevention of atherosclerosis.

In the present study, we (1) examined the effect of RLPs/oxidized RLPs on cell viability in HUVECs in comparison with the effect of ox-LDL, (2) determined RLP-induced NAD(P)H oxidase–dependent superoxide generation, (3) assessed the signal transduction pathway underlying superoxide generation, (4) tested whether RLPs caused production of TNF-α and interleukin-1β (IL-1β) in HUVECs, and (5) identified the involvement of lectin-like ox-LDL receptor-1 (LOX-1) in the action of RLPs. We also assessed the inhibitory effects of cilostazol on these variables.

**Methods**

**Isolation of RLPs**

EDTA-plasma (1 mg/mL EDTA) was obtained at 5 hours after the test meal from 10 hyperlipidemic diabetic subjects (n=10; mean age, 47.2±6.5 years; BMI, 22.5±0.7 kg/m²; plasma triglyceride, <150 mg/dL; plasma cholesterol, <200 mg/dL). The energy content of the test meal was 490 kcal/m² body surface area. The subjects had no serious diseases and had not taken cardiovascular medications, pharmacological doses of antioxidants, or estrogen for ≥7 days. RLPs were routinely prepared with columns packed with immunoaffinity gel containing anti-apoA-1 and anti–apoB-100 monoclonal antibodies (donated by Dr Katsuyuki Nakajima). The unbound fractions containing apoE-enriched lipoproteins and albumin were eluted with PBS (mmol/L: 138 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.1 KH₂PO₄, pH 7.4), and the unbound fractions were ultracentrifuged (d<1.006) to isolate RLPs. According to SDS-PAGE, the unbound fraction isolated consisted primarily of VLDL remnants and small amounts of chylomicron remnants (determined by densitometric analysis on SDS-PAGE, the ratio of the amount of apoB-48 relative to apoB-100 was 0.13±0.01).

**Cell Cultures**

HUVECs (CRL-1730, endothelial cell line derived from the vein of normal human umbilical cord; American Type Culture Collection) were cultured in Kaighn’s F-12K medium with 1% FBS 3 hours before stimulation. Cells were exposed to RLPs for 24 hours. MTT solution (20 μL/well for 2 hours) was added. The medium was aspirated and replaced with 150 μL/well of ethanol/dimethyl sulfoxide solution (1:1). Optical density was measured at 570 to 630 nm using ELIZA (Bio-Tek Instruments, Inc).

**Measurement of Superoxide Anion**

Endothelial homogenates (100 μg protein/well) were placed into the luminometer (Microlumat LB96P, EG & G Berthold). Immediately before recording chemiluminescence, NADH and NADPH (final concentration, 100 μmol/L each) were added, and dark-adapted lucigenin (bis-N-methylacridinium nitrate, 5 μmol/L) was added via an autodispenser. Each experiment was performed in triplicate.

**p22phox/gp91phox mRNA Expression**

Expression of the NAD(P)H oxidase subunits p22phox and gp91phox was determined by reverse transcription–polymerase chain reaction (RT-PCR). Primers for amplification of p22phox and gp91phox were designed from published human phagocyte sequences to amplify fragments of 316 and 403 bp, respectively. Primer sequences were (1) p22phox: sense, 5′-GTTTGTGTCCTGCTGGAGT-3′; antisense, 5′-TGGGCCGCTGCTTGATGGT-3′; (2) gp91phox: sense, 5′-GCTGTCTA-ATGC- TTGTTGCT-3′; antisense, 5′-TCTCTCTCATC-ATGCACA-3′; β-Actin cDNA as an internal control was amplified by use of sense, 5′-TCATGAAGTGGCAGTGACATCCGT-3′; and anti-sense, 5′-CTTAGAAGCATTGCCTGCAGATG-3′ primers. Cycles comprised 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 90 seconds, followed by extension at 72°C for 10 minutes. A total of 25 cycles were used for p22phox and 35 cycles for gp91phox.

**Western Blot Analyses**

After the cells were lysed and centrifuged at 12 000 rpm, 50 μg of total protein of each sample was loaded into 12% SDS-polyacrylamide electrophoresis gel and transferred to nitrocellulose membrane (Amersham Biosciences, Inc). The blocked membrane was then incubated with antibodies to gp91phox (generously donated by Dr Mark T. Quinn, Montana State University, Bozeman, Mont) and oxidized low-density lipoprotein receptor-1 (LOX-1) (generously donated by Dr Tatsuya Sawamura, National Cardiovascular Center Research Institute, Suita, Osaka, Japan). The immunoreactive bands were visualized with chemiluminescent reagent of the Supersignal West Dura Extended Duration Substrate Kit (Pierce). The signals of the bands were quantified with a Calibrated Imaging Densitometer (GS-710, Bio-Rad Laboratories).

**Rac Activation Assay**

Rac1 activation was determined with a Rac activation assay kit (Upstate Biotechnology). Selective precipitation of GTP-bound Rac1 was performed by addition of cell lysate to GST-PBD–bound agarose beads for 2 hours at 4°C. Samples were then centrifuged at 2500g for 5 minutes, followed by 3 washes with lysis buffer. After samples were boiled at 100°C for 5 minutes in SDS-PAGE sample buffer followed by centrifugation, samples were loaded onto a 12% SDS-polyacrylamide gel for Western blotting against anti-Rac1 antibodies.

**Enzyme-Linked Immunosorbent Assay**

The amounts of TNF-α and IL-1β in HUVEC supernatants were measured with ELISA kits (Quantikine, R&D) according to the manufacturer’s protocol.

**DNA Fragmentation Assay**

After incubation in the absence and presence of the drug for 3 hours, cells were exposed to RLPs (50 μg/mL) for 24 hours. Equivalent amounts of DNA (15 to 20 μg) were loaded into wells of 1.6% agarose beads for 2 hours at 4°C. Samples were then centrifuged at 2500g for 5 minutes, followed by 3 washes with lysis buffer. After samples were boiled at 100°C for 5 minutes in SDS-PAGE sample buffer followed by centrifugation, samples were loaded onto 12% SDS-polyacrylamide gel for Western blotting against anti-Rac1 antibodies.

**Drugs**

Cilostazol (OPC-13013) (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinoilnone) was donated by Otsuka Pharmaceutical Co Ltd and dissolved in dimethyl sulfoxide as a 10 mM stock solution. LDL, MTT, lucigenin, β-nicotinamide
adenine dinucleotide (phosphate) reduced form, superoxide dismutase (SOD), catalase, N-acetyl cysteine, diphenyleneiodonium (DPI), and genistein were purchased from Sigma Chemical Co. TNF-α antibody was from Santa Cruz Biotechnology, and Clostridium difficile toxin B and Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) were from Calbiochem.

**Statistical Analysis**

The results are expressed as mean±SEM. The comparison of changes in cell viability between groups was analyzed by repeated-measures ANOVA, followed by Tukey’s multiple comparison tests as a post hoc comparison. Student’s t test was used for analyzing values between the data of vehicle- and cilostazol-treated groups of other results. A value of \( P < 0.05 \) was considered to be significant.

**Results**

**Cell Death and DNA Fragmentation**

The lipoproteins, including RLPs, oxidized RLPs, LDL, and ox-LDL, showed concentration- and time-dependent cytotoxicity in HUVECs. The percent death of HUVECs at 24 hours of incubation was significantly reduced to 26.8±2.3% \((P<0.01)\) and 35.9±3.5% \((P<0.01)\) in response to 50 \( \mu \)g/mL RLPs and 50 \( \mu \)g/mL ox-RLPs, respectively. Both LDLs (50 \( \mu \)g/mL) showed less potency than RLPs or oxidized RLPs (Figure 1).

Exposure of HUVECs to RLPs induced concentration-dependent oligonucleosomal DNA fragmentation, and pretreatment with cilostazol (0.01 to 100 \( \mu \)mol/L) or MnTBAP (50 \( \mu \)mol/L) strongly suppressed the DNA laddering feature evoked by RLPs (Figure 2).

**RLP-Induced NAD(P)H Oxidase-Dependent Superoxide Production**

When measured by recording chemiluminescence, superoxide production from HUVECs in response to NAD(P)H (control, 10.4±0.5 counts \( \cdot \) s \( ^{-1} \) \cdot mg protein \( ^{-1} \)) was significantly elevated when incubated in medium containing 50 \( \mu \)g/mL ox-LDL (12.8±0.4 counts \( \cdot \) s \( ^{-1} \) \cdot mg protein \( ^{-1} \), \( P<0.01 \)) and 50 \( \mu \)g/mL RLPs (16.3±0.5 counts \( \cdot \) s \( ^{-1} \) \cdot mg protein \( ^{-1} \), \( P<0.01 \)) for 24 hours (Figure 3, inset). RLP-induced superoxide level (18.8±0.6 counts \( \cdot \) s \( ^{-1} \) \cdot mg protein \( ^{-1} \)) was markedly reduced by application of...
mean ± SEM of 4 experiments. **P<0.01; ***P<0.001 vs control.

Expressions of p22phox and gp91phox mRNA of NAD(P)H oxidase subunits analyzed by RT-PCR in HUVECs. A, Representative findings of agarose gel electrophoresis showing 316- and 403-bp PCR product of p22phox and gp91phox mRNA. Both results were subjected to densitometric analysis representing mean ± SEM of 4 experiments. *P<0.01 vs control, **P<0.01 vs vehicle.

RLP-Induced Superoxide Formation

Regulation of LOX-1 by RLPs

Incubation of HUVECs with RLPs for 24 hours significantly increased LOX-1 protein in a concentration-dependent manner (Figure 7A), which was not affected by cilostazol and MnTBAP (Figure 7B).

Pretreatment of HUVECs with a monoclonal antibody for LOX-1 (3 and 10 µg/mL) concentration-dependently suppressed the RLP-mediated NAD(P)H oxidase–dependent superoxide production, secretion of TNF-α and IL-1β, and RLP-evoked DNA fragmentation (Figure 7C).

Figure 4. Expressions of p22phox and gp91phox mRNA of NAD(P)H oxidase subunits analyzed by RT-PCR in HUVECs. A, Representative findings of agarose gel electrophoresis showing 316- and 403-bp PCR product of p22phox and gp91phox mRNA. Both results were subjected to densitometric analysis representing mean ± SEM of 4 experiments. *P<0.01 vs control, **P<0.01 vs vehicle.
Discussion

In the present study, we showed that (1) RLPs caused a significant increase in NAD(P)H oxidase–dependent superoxide production as well as secretion of TNF-α and IL-1β via activated LOX-1, which was associated with DNA fragmentation, and (2) cilostazol effectively protected the endothelial cells by suppressing these variables.

NAD(P)H oxidase is one of the major sources of superoxide formation in endothelial cells and plays an essential role in TNF-α–induced superoxide generation. Dimmeler et al. have reported that ox-LDL triggers apoptotic cell death by elevation of reactive oxygen species. In line with this study, our results showed RLP-evoked superoxide formation associated with DNA fragmentation in endothelial cells. In the present results, the enzymatic source of superoxide by RLPs in HUVECs was ascribed to the activation of NAD(P)H oxidase, because DPI strongly suppressed superoxide production but not by allopurinol and rotenone. These results were further supported by evidence that endothelial cells showed increased expression of gp91phox and translocation of Rac1 protein in response to RLPs in association with constitutively expressed p22phox. Ox-LDL and angiotensin II have been reported to induce NAD(P)H oxidase expression and superoxide anion formation in human endothelial cells. Rac1 protein, which is inhibited by C. difficile toxin B, is required for superoxide formation in response to inflammatory stimuli in a variety of cell types, including endothelial cells. Tyrosine phosphorylation was demonstrated to be implicated in depolarization-induced endothelial superoxide production. In agreement with these reports, gp91phox mRNA and protein expression was inhibited by genistein (protein tyrosine kinase inhibitor) and C. difficile toxin B (Rac1 inhibitor). These findings further strengthened the hypothesis that RLPs caused a significant increase in NAD(P)H oxidase–dependent superoxide production in HUVECs. However, it goes beyond the scope of our in vitro study to illustrate the mechanism(s) of how genistein controls gp91phox expression and membrane translocation of Rac1 at the present time.

TNF-α production was early demonstrated to be sensitive to redox system. It has been documented that TNF-α causes cell death via induction of nitric oxide or oxygen free radicals in various cells and induces apoptosis. NAD(P)H oxidase plays an essential role in TNF-α–induced superoxide generation. Consistent with these reports, our results demonstrated RLP-evoked cell death with increased production of superoxide and cytokine releases (TNF-α and IL-1β). In the present study, exposure of HUVECs to RLPs induced a significant increase in superoxide formation in association with increases in TNF-α and IL-1β secretion. Alternatively, elevation of TNF-α and IL-1β by RLPs was suppressed by radical scavengers, such as SOD plus catalase and MnTBAP.
(a metalloporphyrin-based SOD mimic) as well as DPI, indicating that superoxide and TNF-α act reciprocally for their productions.

The endothelial receptor for ox-LDL (LOX-1) is a protein that is expressed in vivo in the vascular endothelium. In endothelial cells, LOX-1 protein was demonstrated to mediate apoptotic cell death. Our present data showing that RLPs caused LOX-1 receptor protein expression, and that increased production of both superoxide and cytokines and enhanced DNA fragmentation by RLPs was significantly inhibited by monoclonal antibody for LOX-1 receptor provide strong evidence that NAD(P)H oxidase-dependent superoxide production stimulated by RLPs is dependent on the activation of LOX-1 receptors.

Evidence has accumulated that apoptosis of endothelial cells contributes to vascular injury and atherosclerosis. Cilostazol was introduced to increase intracellular cAMP by blocking its hydrolysis by type III phosphodiesterase. Gonzalez and Montminy emphasized the importance of cAMP stimulation of somatostatin transcription by CREB phosphorylation at Ser-133. Franke et al also documented that dibutyryl cAMP enhances the survival-promoting effect of brain-derived neurotrophic factor or neurotrophin-3. On the basis of these reports, it was considered that activation of cAMP-dependent protein kinase might constitute a signal transduction pathway in RLP-evoked superoxide formation and TNF-α secretion. However, it is inappropriate at the present time to directly correlate the suppressive effect of cilostazol on NAD(P)H oxidase-dependent superoxide formation with its property of increasing intracellular cAMP.

Taken together, RLPs stimulate NAD(P)H oxidase-dependent superoxide formation and the induction of proinflammatory cytokines via activation of LOX-1 in association with DNA fragmentation and apoptotic cell death in HUVECs, and cilostazol has a protective effect by suppressing these variables.

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**References**

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