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Biochemical and Biophysical Research Communications 303 (2003) 313–319
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NF-κB activation in endothelial cells treated with oxidized high-density lipoprotein☆

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Received 12 February 2003

Abstract

We first determined whether oxidized high-density lipoprotein (ox-HDL) activates transcription factor nuclear factor-κB (NF-κB) in cultured human umbilical vein endothelial cells (HUVECs). Treatment for 7 h with 100 μg/ml ox-HDL elicited a marked downregulation of IκBα and upregulation of the phosphorylated form of IκBα in HUVECs in a manner dependent on the dose of ox-HDL. Electrophoretic mobility shift assay in nuclear fraction from HUVECs showed translocation of NF-κB to the nucleus and binding of NF-κB to NF-κB consensus oligonucleotides during ox-HDL exposure for 7 h, suggesting that ox-HDL brings about NF-κB activation in endothelial cells. To clarify the mechanism of NF-κB activation in HUVECs treated with ox-HDL, we investigated the effect of ox-HDL treatment on intracellular production of reactive oxygen species (ROS) in HUVECs. Ox-HDL induced a significant dose-dependent increase in ROS production during 4 h incubation and this enhanced production of ROS was inhibited in the presence of probucol or diphenylene iodonium (DPI), an inhibitor of NADPH oxidase. In addition, pretreatment with probucol or DPI suppressed the phosphorylation and degradation of IκBα protein induced by ox-HDL, demonstrating that increased generation of ROS by ox-HDL may be associated with NF-κB activation. Pretreatment with antibody against oxidized low-density lipoprotein receptor-1 (LOX-1) significantly suppressed the ox-HDL-induced downregulation of IκBα, suggesting that LOX-1 mediates NF-κB activation in endothelial cells stimulated with ox-HDL. Taking all of the above findings together, ox-HDL activates NF-κB via binding to LOX-1 on the cell surface, followed by enhancement of intracellular ROS production in endothelial cells.

Keywords: Oxidation; High-density lipoprotein; NF-κB; Endothelial cell; ROS; LOX-1

The plasma level of high-density lipoprotein (HDL) has been identified as having an inverse relationship with the onset and the progression of coronary artery disease in epidemiologic investigations [1]. HDL is generally regarded as a protective factor against several atherogenic events via reverse cholesterol transport activity [2–4], anti-thrombotic effects [5], and prostacyclin stabilizing activity [6,7]. HDL also exhibits a protective effect against the cytotoxicity of oxidized low-density lipoprotein (ox-LDL) by inhibiting LDL oxidation induced by cells [8] and inhibiting the cytotoxicity of ox-LDL in cultured cells [9]. Recently, evidence has accumulated, indicating that oxidative modification of HDL can occur in vivo followed via alteration of its conformation biologically and chemically [10–13]. In fact, clinical investigations using Cu^{2+}-ox-HDL-specific 9F5-3a antibody have indicated the presence of ox-HDL in the intima of atheromatous plaques in the human abdominal aorta [14] and in sera from patients with chronic renal failure [15]. Several investigators have demonstrated that HDL has a higher susceptibility to lipid peroxidative modification than LDL [16–19]. In addition, oxidative modification of HDL not only attenuates its beneficial properties, such as stimulation of

☆ *Abbreviations:* HUVEC, human umbilical vein endothelial cell; ox-HDL, oxidized high-density lipoprotein; NF-κB, nuclear factor κB; ROS, reactive oxygen species; DIG, digoxigenin; L-NAME, N^G-nitro-L-arginine methyl ester; DPI, diphenylene iodonium; LOX-1, lectin-like oxidized LDL receptor-1; LPC, lysophosphatidylcholine.

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doi:10.1016/S0006-291X(03)00308-5

cholesterol efflux from foam cells [10], endothelium-dependent vasoreactivity [20], and anti-oxidative activity [21–24], but also converts into a cytotoxic particle such as ox-LDL [18,25]. These reports suggest that oxidized lipoproteins including both HDL and LDL might contribute to the genesis of coronary artery spasm, while the possibility should also be considered that generation of oxidized lipoproteins may reflect an oxidative stress state predisposing the patient to coronary artery spasm.

Although lipoprotein oxidation is considered to be pivotal to the genesis of foam cells from macrophages in developing atherosclerotic lesions [26], it was recently reported that ox-HDL and its lipid peroxides inhibit the secretion of tumor necrosis factor-α from macrophages, suggesting that ox-HDL contributes to modulating the inflammatory response on macrophages as observed for ox-LDL [27]. We previously found that treatment of cultured human endothelial cells with glycoxidized HDL induces apoptosis [28] as well as increased generation of reactive oxygen species (ROS) which are thought to play an important role in the pathophysiology of atherosclerosis and diabetes mellitus [29]. Moreover, it is possible that inductions of ROS generation and endothelial apoptosis may be potentiated by additional oxidative modification of glucose-binding HDL. Activation of the transcription factor nuclear factor-κB (NF-κB) is suggested to be a vital signaling factor for several vascular events such as endothelial apoptosis, ROS generation, and inflammatory responses. In this study, to clarify the role of oxidative modification of HDL in endothelial function, we investigated whether NF-κB activation is observed in human endothelial cells stimulated with ox-HDL.

Materials and methods

Materials. An enhanced chemiluminescence substrate system was obtained from Amersham (Arlington Heights, IL, USA), a NE-PER nuclear and cytoplasmic extraction kit was from Pierce (Rockford, IL, USA), a digoxigenin (DIG) luminescent detection kit was from Roche (Mannheim, Germany), and polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA, USA). For cell culture, normal human umbilical vein endothelial cells (HUVECs) and endothelial cell growth medium (EBM-2) were from BioWhittaker (Walkersville, MD, USA). The fluorogenic reagent, 6-carboxy-2′,7′-dichlorohydrofluorescein diacetate, di(acetoxymethyl ester), was from Molecular Probes (Eugene, OR, USA). For Western blot analysis, antibodies for IκBα, phosphorylated IκBα, p65, and oxidized-LDL receptor-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). κ-Carrageenan and polyinosinic acid were from Sigma (St. Louis, MO, USA). All other chemicals were reagents of the highest purity (above 99%) and were from Wako Pure Chemical Industry (Osaka, Japan).

Preparation of oxidized lipoproteins. Native lipoproteins were isolated from fresh normolipidemic human serum by sequential ultracentrifugation as described previously [30]. Each fraction of HDL and LDL was pooled and dialyzed extensively against 50 mM phosphate-buffered saline (PBS), pH 7.5, containing 1 mM EDTA. To prepare oxidized HDL (ox-HDL), HDL at a final concentration of 0.5 mg/ml was incubated at 37 °C for 16 h in the presence of 30 μM $CuSO_4$. Ox-LDL was prepared by the incubation of 0.1 mg/ml LDL with 10 μM $CuSO_4$ for 16 h. These products were dialyzed extensively against PBS, pH 7.4, containing 1 mM EDTA to prevent further oxidation, and each lipoprotein was stored in a dark environment at 4 °C until required for use.

Cell culture. HUVECs were cultured in EBM-2 growth medium containing 2% heat-inactivated fetal bovine serum, antibiotics, and several growth factors [29]. In all experiments, cells were used at passages 4 and 5, and endothelial cobblestone morphology was confirmed microscopically before their use. HUVECs were seeded at a density of 2×10^6 cells per dish on type-I collagen-coated 100-mm dishes and incubated for 2 days at 37 °C in a 5% CO_2 and 95% air environment. After replacement of the culture medium with a growth supplement-free medium (EBM-2 plus 1 mg/ml heat-inactivated lipoprotein-deficient human serum), the cells were incubated for 48 h and then treated with 100 μg/ml of each modified lipoprotein.

Western blot analysis. Western blotting of HUVECs was performed as previously described [31]. After treatment with the lipoproteins, adherent cells were gently harvested and washed twice with ice-cold PBS containing 0.3 mM phenylmethylsulfonyl fluoride (PMSF). Forty μg sample of the protein was subjected to SDS–polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. After blocking for 12 h, the membrane was incubated with 1 μg/ml of antibodies against human p65, human IκBα, and human phosphorylated IκBα. After incubation with 1 μg/ml anti-immunoglobulin G antibody conjugated to horseradish peroxidase, peroxidase activity on the membrane was visualized using an enhanced chemiluminescence substrate system.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay was performed according to the method described by Aikawa et al. with minor modification [32]. After HUVECs were gently harvested and washed twice with ice-cold PBS containing 0.3 mM PMSF, the nuclear fraction was extracted from the cell pellet of the cells using a NE-PER nuclear and cytoplasmic reagent. Ten μg of the nuclear fraction was incubated at 37 °C for 30 min in the presence of 1 μg poly(dI–dC) with double-stranded NF-κB consensus oligonucleotides labeled at the 3′ terminal by DIG. The following consensus probe for NF-κB was used: 5′-ACCACAGTCCATGCCATCAC-3′. The samples were separated on 5% PAGE and then transferred to a PVDF membrane by electroblotting. The DIG on the membrane was detected using a DIG luminescent detection kit.

Determination of reactive oxygen species production. The reactive oxygen species content in HUVECs was fluorometrically measured using 6-carboxy-2′,7′-dichlorohydrofluorescein diacetate, di(acetoxymethyl ester) as a fluorogenic substrate [33]. The fluorogenic substrate solution was added to the conditioned medium and incubated for 1 h at 37 °C in a 5% CO_2 incubator. The adherent cells collected by trypsinization were disrupted with 0.1% sodium dodecyl sulfate and fluorescence intensities of the solutions were measured on a spectrofluorometer with an excitation wavelength of 510 nm and an emission wavelength of 534 nm.

Statistical analysis. Statistical evaluation of the data was performed using the unpaired Student's t test and ANOVA followed by Fisher's test. A p value < 0.05 was considered statistically significant.

Results

Incubation for 7 h with ox-HDL attenuated the level of IκBα protein in HUVEC homogenates in a manner dependent on the dose of ox-HDL, as shown in Fig. 1A. One hundred μg/ml ox-HDL was found to induce a significant decrease in IκBα protein ($63 \pm 10\%$ of untreated control), but was less than that with ox-LDL

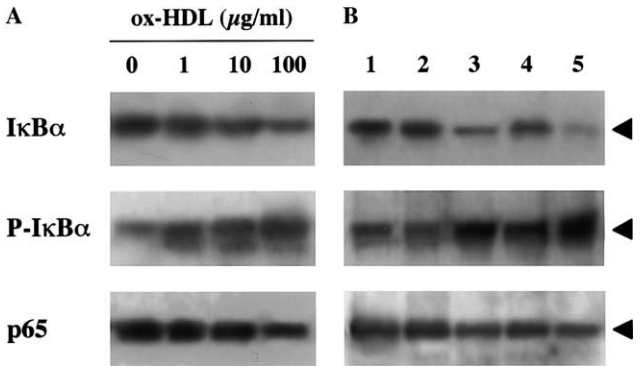

Fig. 1. Alterations of IκBα, phosphorylated IκBα, and p65 in ox-HDL-treated HUVECs. (A) Dose–response effects of ox-HDL on protein amounts of IκBα, phosphorylated IκBα, and p65 in HUVECs treated with ox-HDL. (B) Western blotting of IκBα, phosphorylated IκBα, and p65 in HUVECs stimulated with modified lipoprotein. These typical photographs are from a single representative experiment of four independent replications. The lane labels are (1) untreated control; (2) native HDL; (3) ox-HDL; (4) native LDL; and (5) ox-LDL.

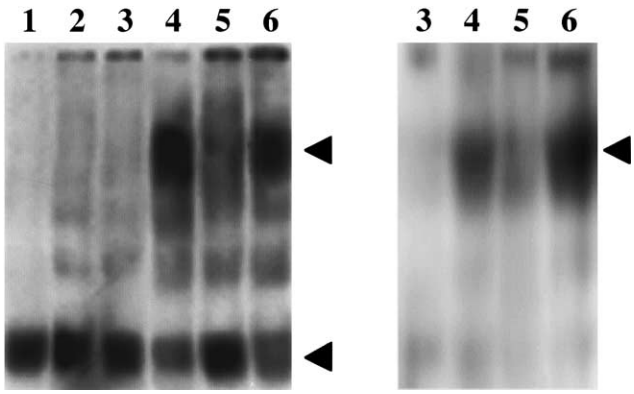

Fig. 2. Electrophoretic mobility shift assay of ox-HDL-treated HUVECs. Each photograph on the left and right shows the results of an electrophoretic mobility shift assay and Western blotting of the membrane using an anti-p65 antibody, respectively. These two typical photographs are from a single representative experiment of three independent replications. The lane labels are (1) DIG-labeled oligonucleotides alone and mixtures of DIG-labeled oligonucleotides with each nuclear fraction of HUVECs treated with (2) untreated control; (3) native HDL; (4) ox-HDL; (5) native LDL; and (6) ox-LDL.

(34 ± 16% of control). In contrast, native HDL showed a significant enhancement of IκBα protein (123 ± 8% of control). When the alterations in HUVECs treated with modified lipoproteins were compared, stimulation of HUVECs for 7 h with 100 μg/ml ox-HDL or ox-LDL significantly increased in the phosphorylated form of IκBα protein (186 ± 12% and 231 ± 11% of untreated

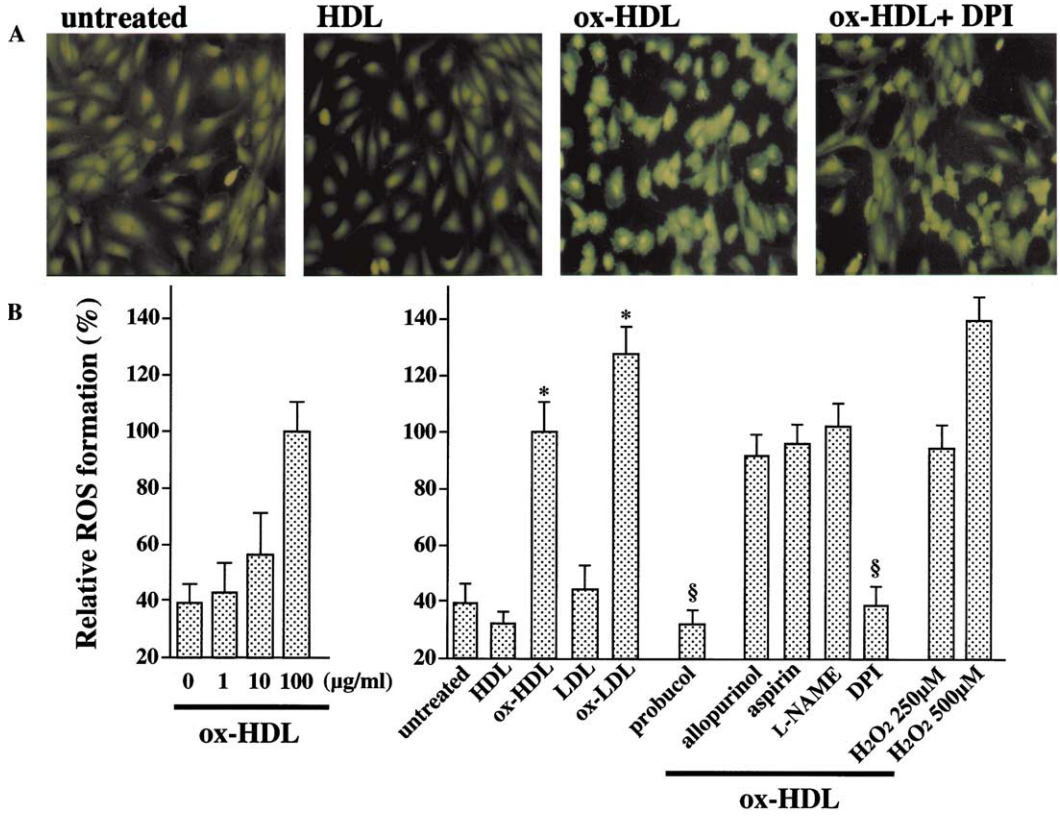

Fig. 3. ROS production in HUVECs treated with ox-HDL. (A) Fluorometrical photographs of ROS in HUVECs treated with modified HDL. Original magnification, 200×. (B) ROS amount in HUVEC treated with ox-HDL. HUVECs were preincubated with either 50 μM probucol, 200 μM L-NAME, 500 μM allopurinol, 100 μM aspirin, or 5 μM DPI for 1 h at 37 °C in the presence of 5 mM L-arginine and 3 μM TB_4. HUVECs were then incubated for 7 h with 100 μg/ml modified HDL. Hydrogen peroxide was used as a stimulator of ROS in HUVECs. Photographs show typical results from a single representative experiment of three independent replications. Each bar is the means ± SD of four determinations in two independent experiments. *Significant difference from the untreated control, $p < 0.05$.

control, respectively) (Fig. 1B). The oxidized lipoproteins also induced a slight decrease in the level of p65 protein, a subunit of NF-κB, in the cytosol fraction of HUVECs. Electrophoretic mobility shift assay detected an increased band upward to an unbound band in a mixture of DIG-labeled NF-κB consensus oligonucleotides with the nuclear fraction of HUVECs treated with 100 μg/ml oxidized lipoproteins, although incubation of the cells with either native HDL or LDL failed to alter this translocation (Fig. 2). When the PVDF membrane was reprobed with anti-p65 antibody, marked increase in p65 protein in the groups of oxidized lipoprotein was detected at the position of the shifted band, demonstrating that incubation with ox-HDL releases a transcriptionally active NF-κB caused by IκB phosphorylation in HUVECs. These data provide evidence that NF-κB activation may be a signal for ox-HDL-induced alteration of endothelial function.

To clarify the mechanism of NF-κB activation in ox-HDL-treated HUVECs, we next investigated whether ox-HDL induces ROS production in those cells. As shown in Fig. 3, the ROS production in HUVECs stimulated for 4 h with 100 μg/ml ox-HDL was significantly higher than that in cells stimulated with native HDL, and the ox-HDL-induced ROS production was dose-dependent on ox-HDL. Preincubation with probucol, known to work as a free radical scavenger, significantly reduced ox-HDL-induced ROS production in HUVECs (33 ± 6% of that in the group treated with ox-HDL alone). Pretreatment with DPI, an inhibitor of NADPH oxidase and endothelium nitric oxide synthase (eNOS), markedly inhibited ox-HDL-induced formation of ROS (38 ± 8% of ox-HDL alone), whereas aspirin, allopurinol, or N^G-nitro-L-arginine methyl ester (L-NAME) in excess of L-arginine and tetrahydrobiopterin failed to induce the ROS production by ox-HDL. As shown in Fig. 4, attenuation of IκBα protein was also

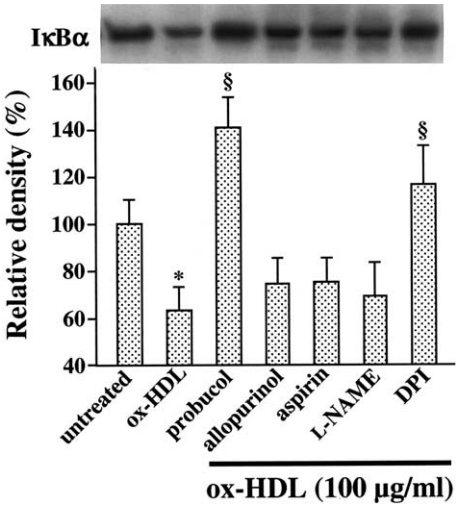

Fig. 4. Effects of pretreatments with scavengers and inhibitors of ROS on IκBα degradation in ox-HDL-treated HUVECs. HUVECs were prepared as described in Fig. 3. Upper photograph and lower graph show a representative result on Western blotting of IκBα protein in HUVEC homogenates and a result on densitometrical analysis of the band of IκBα protein, respectively. Photograph shows a typical result from a single representative experiment of three independent replications. Values are means ± SD of three determinations. *Significant difference from untreated control, $p < 0.05$. §Significant difference from ox-HDL alone, $p < 0.05$.

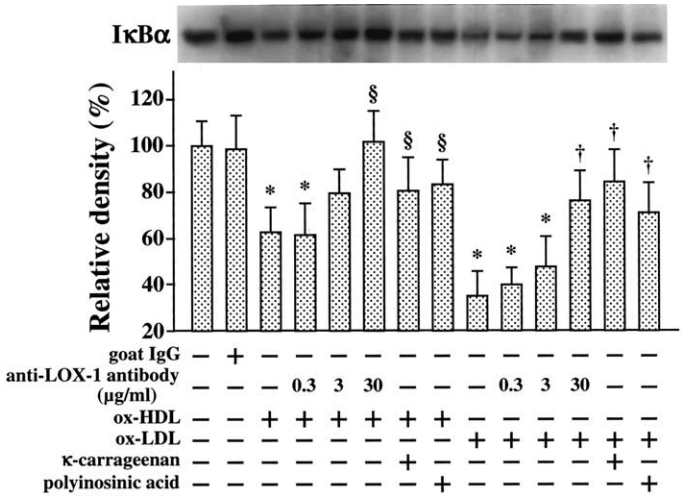

Fig. 5. Effect of anti-human LOX-1 antibody on IκBα degradation in ox-HDL treated HUVECs. HUVECs were preincubated with either of nonimmune goat IgG (30 μg/ml), anti-LOX-1 antibody (indicated concentration), κ-carrageenan (250 μg/ml), or polyinosinic acid (250 μg/ml) for 1 h at 37 °C. HUVECs were then incubated for 7 h with 100 μg/ml oxidized lipoprotein. Upper photograph and lower graph show a representative result on Western blotting of IκBα protein in HUVEC homogenates and a result on densitometrical analysis of the band of IκBα protein, respectively. Photograph shows a typical result from a single representative experiment of three independent replications. Values are means ± SD of three determinations. *Significant difference from untreated control, $p < 0.05$. §Significant difference from ox-HDL alone, $p < 0.05$. †Significant difference from ox-LDL alone, $p < 0.05$.

associated with the amounts of ROS in HUVECs treated with these oxidized lipoproteins or inhibitors, demonstrating that ox-HDL-induced NF-κB activation may be mediated by ROS generation in endothelial cells in the early stage of ox-HDL signaling.

To clarify whether ox-HDL-induced NF-κB activation is mediated by its binding to lectin-oxidized LDL receptor-1 (LOX-1), i.e., probably to ox-LDL, we investigated the effect of pretreatment with anti-human LOX-1 antibody on altered attenuation of IκBα in HUVECs stimulated with ox-HDL. As shown in Fig. 5, pretreatment for 1 h with 30 μg/ml anti-LOX-1 antibody caused a 44% inhibition of the ox-LDL-induced reduction of IκBα protein in the cells. In contrast, the ox-HDL-reduction of the protein was essentially blocked by the pretreatment with anti-LOX-1 or LOX-1 chemical inhibitors such as κ-carrageenan and polyinosinic acid [34] suggested that ox-HDL-induced NF-κB activation is associated with LOX-1 binding to the endothelial cell surface.

Discussion

The purpose of the present study was to determine whether ox-HDL-induced alteration of endothelial function is attributable to the NF-κB signaling pathway. The present study provides direct evidence that a marked activation of NF-κB occurs via enhanced generation of ROS in HUVECs during exposure to ox-HDL. In resting cells, the NF-κB complex, which consists of proteins such as p65 and p50, localizes as a complex bound to IκBs in the cytoplasm. Upon functional alteration of the cells due to various inflammatory or apoptotic stimuli, IκBs are detached from the complex via its signaling, which is followed by phosphorylation and subsequent ubiquitination in the cytoplasm. The transcriptionally active NF-κB translocates rapidly to the nucleus and binds to a κB site in DNA. Several researchers have demonstrated that NF-κB activation caused by ox-LDL stimulation may contribute to apoptotic signaling in various cells [34,35]. We recently found that glycoxidative modification of HDL induces apoptosis in cultured human aortic endothelial cells and that additional oxidation of glycated HDL may play an important role in this apoptosis [28]. We therefore hypothesized that ox-HDL may bring about endothelial apoptosis via NF-κB activation in the cells. Since endothelial cells in atherosclerotic intimas are exposed for a long time to oxidatively modified lipoproteins, enhanced concentrations of advanced ox-HDL molecules were adopted to observe the effects of short term-exposure and to clarify the acute role of ox-HDL on endothelial functions in these experiments. The findings obtained in our study indicate that ox-HDL induces the downregulation of IκBα and upregulation of its phosphorylated form in HUVECs in a dose-dependent manner. Further investigation using an electrophoretic mobility shift assay showed that the nuclear fraction of HUVECs treated with ox-HDL can bind to a NF-κB consensus probe, and suggested that stimulation with ox-HDL may activate the NF-κB signaling pathway via degradation of IκBα and subsequent production of transcriptionally active NF-κB in HUVECs.

Since several investigations have provided evidence that the enhancement of intracellular ROS induced by various stimuli acts as a signaling factor leading to NF-κB activation [36–38], the alterations of ROS generation in HUVECs treated with ox-HDL were investigated to identify causal factors for NF-κB activation in these cells. Xanthine oxidase, cyclooxygenases, eNOS [39], and NADPH oxidase [40] are generally recognized to be responsible for progressive production of endothelial superoxide anion as potential sources of ROS. As shown in Figs. 3 and 4, preincubation with DPI, a selective inhibitor of NADPH oxidase [41] and eNOS [42], or probucol, a ROS scavenger, significantly inhibited both ROS generation and degradation of IκBα protein induced by treatment with ox-HDL in HUVECs. In contrast, treatment with aspirin, allopurinol, or L-NAME in excess of L-arginine and tetrahydrobiopterin slightly attenuated ROS formation and promoted degradation of IκBα by ox-HDL. Since exposing endothelial cells to L-NAME in excess of L-arginine and tetrahydrobiopterin reportedly inhibits the release of superoxide anion from eNOS [43], ROS formation induced by ox-HDL may be released mainly via NADPH oxidase in cells. These data raise the possibility that intracellular ROS enhanced by ox-HDL might participate in the signaling pathway underlying a NF-κB activation. Although the enhanced production of ROS from endothelial cells exposed to oxidized lipoproteins is well known for association with the onset and the progression of cardiovascular diseases, clinical trials using anti-oxidative vitamins indicated little scavenging effect for suppression of the vascular diseases [44,45]. We have already reported that reductions of antioxidant enzymes such as superoxide dismutase and catalase are observed in endothelial cells treated with ox-HDL [29]. These alterations of endothelial function caused by oxidized lipoproteins may play an important role in the progression of cardiovascular diseases.

During oxidation of HDL particles, compositional alterations, such as enhanced lipid peroxides, disappearance of lipophilic antioxidants, and reduced paraoxonase activity, as well as structural alterations, such as lowered density and cross-linking of apolipoproteins, are detectable in the particles. Oxidative modification of HDL particles reduces ligand activity toward native HDL receptors [46,47]. Sakai et al. also demonstrated the significant loss of HDL ligand activity toward the HDL receptor upon Cu^{2+}-mediated

oxidation to be due to cross-linking of HDL apolipoproteins, particularly of apolipoprotein A-I [46]. When HUVECs were exposed to native HDL, the amount of IκBα was increased as compared to the untreated control, as shown in Fig. 1. Since HDL reportedly suppresses apoptosis of endothelial cells [33], enhancement of IκBα by HDL may be due to inactivation of the NF-κB signaling pathway leading to apoptosis. This finding suggests that HDL oxidation lowers an affinity for the native HDL receptor and prompts oxidized HDL to bind to another receptor which has an affinity for ox-HDL. Furthermore, cross-competition binding assay in our preliminary study yielded data, indicating that specific binding of ox-LDL to LOX-1 was slightly displaced by the addition of excess ox-HDL. Since ox-LDL activates the NF-κB pathway via specific binding to LOX-1 on the endothelial cell surface, we investigated whether ox-HDL-induced NF-κB activation is also mediated by LOX-1 as observed in ox-LDL. As shown in Fig. 5, preincubation of HUVECs with anti-LOX-1 antibody blocked ox-HDL-induced degradation of IκBα. This finding demonstrates that ox-HDL-induced NF-κB activation may be initiated by the binding of ox-HDL to LOX-1. As it was recently reported that LOX-1 is involved in binding of HOCl-HDL_3 to endothelial cells [48], oxidation of HDL by copper ions may bring about a similar structural change in HOCl-HDL_3 within the particles. Lysophosphatidylcholine (LPC) formed during oxidative modification of lipoproteins is suggested to play a key role in the binding of ox-LDL to LOX-1 [49] and to stimulate superoxide anion production in endothelial cells via a NADH/NADPH oxidase-dependent mechanism [50]. Indeed, we have found that raising the level of LPC in the particles instead of eliminating PC is observed during the oxidation of HDL. Although these lines of evidence suggest that increased formation of LPC might contribute to ox-HDL binding to LOX-1, the mechanism of the binding of oxidized HDL to LOX-1 remains obscure.

In conclusion, we found that human vascular endothelial cells exposed to ox-HDL activate the NF-κB signaling pathway via an enhanced generation of ROS in these cells, and that this pathway may be triggered by the binding of ox-HDL to LOX-1. These findings indicate that ox-HDL may also participate in a pathobiological mechanism involving LOX-1 probably via ox-LDL.

Acknowledgments

This work was supported in part by a Grand-in-Aid for Scientific Research (No. 14770067) from the Ministry of Education, Science and Culture, Japan, and a grant from Mitsubishi Pharma, Co., Tokyo, Japan.

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