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Modulation of Reactive Oxygen Species in Endothelial Cells by Peroxynitrite-Treated Lipoproteins

Toshiyuki Matsunaga,*,[1] Takanori Nakajima,* Masaru Sonoda,† Iwao Koyama,‡ Shin-ichiroh Kawai,* Ikuo Inoue,§ Shigehiro Katayama,§ Kazuyuki Hirano,¶ Shigeru Hokari,* and Tsugikazu Komoda*

The First Department of Biochemistry and §The Fourth Department of Internal Medicine, Saitama Medical School, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, †Department of Food Science and Nutrition, Kyoritsu Women's University, 2-2-1 Hitotsubashi, Chiyoda-ward, Tokyo 101-8433, ‡Department of Medical Technology, Junior College, Saitama Medical School, 38 Morohongo, Moroyama, Iruma-gun, Saitama, 350-0495, and ¶Department of Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585

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Peroxynitrite has been implicated in the oxidative modification of low-density lipoprotein (LDL) particles, and nitrotyrosine residues in the LDL have been detected in atherosclerotic plaques. Studies have suggested that lipoproteins modified by peroxynitrite lead to the onset of atherosclerotic vascular disease. We therefore prepared *in vitro* lipoproteins oxidatively modified by peroxynitrite (NO_2-lipoprotein) and investigated the effect of NO_2-lipoprotein on the viability of cultured endothelial cells. After exposure of a high-density lipoprotein (HDL) to peroxynitrite, some intermolecular complexes of apolipoproteins in HDL were detected on immunoblotting with monoclonal antibodies against apolipoprotein AI and AII, suggesting that nitration of HDL by peroxynitrite causes intermolecular cross-linking of the apolipoproteins in the particles. Treatment with 1 mM peroxynitrite increased the 3-nitrotyrosine level to 28.5 mmol/mol of tyrosine residues in the prepared NO_2-HDL, as quantitated by HPLC, and the amount in NO_2-lipoprotein depended on the peroxynitrite concentration. HDL exhibited a shorter lag phase and the reaction plateaued more rapidly than that with LDL. To clarify whether or not NO_2-lipoproteins affect the function of endothelial cells, we first examined the viability of cultured human aortic endothelial cells (HAECs) exposed to NO_2-lipoproteins. Incubation with either NO_2-HDL or NO_2-LDL significantly reduced the HAEC viability at 72 h. The results of RT-PCR and Western blotting showed that NO_2-HDL markedly suppressed at 48 h not only the expressed levels of mRNA and protein but also the activity of catalase in HAECs. In contrast, NO_2-LDL significantly reduced the expression and activity of Cu^{2+},Zn^{2+}-superoxide dismutase (CuZn-SOD) in the cells. Neither NO_2-HDL nor NO_2-LDL interfered with nitric oxide production or expression of cyclooxygenases and NADPH oxidase in HAECs. Increased radical production in NO_2-lipoprotein-treated HAECs implied that reactive oxygen species such as superoxide anions and hydroxyl radicals may contribute to the mechanism of the toxic effect induced in endothelial cells by NO_2-lipoprotein. Overall, NO_2-lipoprotein may lead to deterioration of the vascular function through these endothelial cell responses.

Key words: antioxidant enzyme, endothelial cell, lipoprotein, peroxynitrite, reactive oxygen species.

Nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) radicals are released by endothelial and inflammatory cells, and can react with each other to form the strong oxidant and nitrating species peroxynitrite ($ONOO^-$). Peroxynitrite can be directly cytotoxic, and intermediates, including the hydroxyl radical ($OH^{\cdot}$), and nitronium ions (NO_2^{+}), are formed during its decomposition. The peroxynitrite reaction with proteins yields nitrotyrosine, a stable product formed through the addition of a nitro group to the ortho position of tyrosine, without the need for transition metals. Nitrotyrosine has been determined in many diseases, and is a useful marker for peroxynitrite detection, for example in the serum and synovial fluid of rheumatoid arthritis patients (*1*), in chronic renal failure patients with septic shock (*2*), and in human atherosclerosis (*3*). In addition, Buttery *et al.* demonstrated that stimulated expression of inducible nitric oxide synthase (iNOS) is associated with atherosclerosis, and that the activity of this enzyme preferentially promotes the formation and activity of peroxynitrite (*4*). These findings suggest that peroxynitrite may be important in the

[1] To whom correspondence should be addressed. Phone: +81-492-76-1155, Fax: +81-492-76-1155, E-mail t_matsu@saitama-med.ac.jp

Abbreviations HAEC, human aortic endothelial cell, NO_2-HDL, nitrated high-density lipoprotein; apoAI, apolipoprotein AI, NOS, nitric oxide synthase, CuZn-SOD, Cu^{2+},Zn^{2+}-superoxide dismutase; COX, cyclooxygenase; TBARS, thiobarbituric-acid-reactive substance, RT-PCR, reverse transcription-polymerase chain reaction; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

pathology of atherosclerosis, which contributes to lipid peroxidation and to vascular damage.

Lipoprotein oxidation is considered to be pivotal in the development of foam cells from macrophages in developing atherosclerotic lesions (*5*), and low-density lipoprotein (LDL) particles containing modifications consistent with *in vivo* oxidation have been isolated from human atheromatous lesions (*6–8*). Several potential mechanisms for the *in vivo* oxidation of lipoproteins have been proposed (*9*). Heinecke *et al* previously reported the detection of nitrotyrosine residues in LDL in atherosclerotic plaques (*10*).

In addition to the role of high-density lipoprotein (HDL) in cholesterol reverse transport (removal of excess cholesterol from peripheral tissues), HDL exhibits a protective effect against the cytotoxicity of oxidized LDL by inhibiting LDL oxidation induced by cells (*11*) and inhibiting the cytotoxicity of oxidized LDL toward cultured cells (*12*). Several reports have suggested that HDL is more susceptible to oxidation than LDL *in vitro* (*13, 14*). However, oxidatively modified HDL isolated from *in vivo* sources has never been reported. Recently, we reported finding that oxidized HDL is localized in atheromatous plaques and binds to human aortic endothelial cells (*15*). Although this observation suggests that oxidatively modified HDL is associated with the progression of atherosclerosis, little is known about the effect of the modified HDL on the functions of endothelial cells. To investigate this suggestion, we characterized nitrated lipoproteins prepared *in vitro* and determined their effect on the viability of endothelial cells. We also focused on the production of reactive oxygen species as a pivotal factor for endothelial dysfunction in cells, and observed the expression of antioxidant enzymes as well as the production of reactive oxygen species in endothelial cells stimulated by nitrated lipoproteins

MATERIALS AND METHODS

Materials—An enhanced chemiluminescence substrate system was obtained from Amersham (Arlington Height, IL, USA). The polyvinylidene difluoride (PVDF) membrane was from Millipore (Tokyo). Block Ace (a casein digestor) was from Dai-Nippon Pharmaceutical (Osaka). Normal human aortic endothelial cells and endothelial cell growth medium (EBM-2) were from BioWhittaker (MD, USA). The bicinchoninic acid Protein Assay System was from Pierce (Rockford, IL, USA) The Titan™ One Tube RT-PCR system and DNA molecular weight marker XIII were from Boehringer Mannheim (Germany). The oligo $(dT)_{15}$ cellulose column (Type 3) was from Collaborative Research (Bedford, USA). For Western blot analysis, anti–apo A-I, anti–human Cu^{2+},Zn^{2+}-superoxide dismutase, and anti–human catalase antibodies were purchased from The Binding Site (Birmingham, England). Anti-inducible NOS and anti–endothelium NOS antibodies were obtained from Transduction Laboratories (Lexington, KY), and anti–COX-I and anti–COX-II antibodies were from Cayman Chemical (Ann Arbor, MI). Anti–apo A-II and anti-nitrotyrosine antibodies were products of ICN Biomedical (Aurora, OH) and Upstate Biotechnology (Lake Placid, NY), respectively. Anti-p22phox antibodies were kindly provided by Dr. I. Nagaoka (Juntendo University School of Medicine, Tokyo) (*16*). All other chemicals were reagents of the highest purity (above 99%) from Wako Pure Chemical Industry (Osaka). Milli-Q water was used throughout this study.

Preparation of Modified Lipoproteins—Native HDL and LDL were isolated from fresh normolipidemic human serum by sequential ultracentrifugation, as described previously (*17*) The HDL fraction ($1.063 < d < 1.210$) and LDL fraction ($1.019 < d < 1.063$) were pooled and then dialyzed extensively against 0.05 M phosphate-buffered saline (PBS), pH 7.5, containing 1 5 mM EDTA. Peroxynitrite was prepared as described by Kaur *et al.*, and the concentration of peroxynitrite was determined spectrophotometrically at 302 nm (ε = 1,670 M^{-1} cm^{-1}) (*18*). Residual H_2O_2 in the prepared peroxynitrite solution was removed with prewashed granular MnO_2. The native lipoprotein was nitrated by three additions of a peroxynitrite solution in Tris-HCl buffer, pH 9.0, containing 1.5 mM EDTA, at 4°C. Nitration was terminated by extensive dialysis against PBS buffer, pH 7.5, containing 1 mM EDTA. The nitrated lipoprotein was stored in the dark at 0–4°C until analysis. The relative amount of 3-nitro-L-tyrosine in the lipoprotein was determined by HPLC on a C_{18} column (*1*). The eluant was 500 mM KH_2PO_4-H_3PO_4 (pH 3.01) with 10% methanol (v/v), the flow rate was 1 ml/min, with an isocratic pump, and the ultraviolet detector was set at 274 nm. Identification of the peak was carried out on the basis of the retention time of authentic 3-nitro-L-tyrosine.

Cell Culture—Human aortic endothelial cells (HAECs) were maintained on EBM-2 containing heat-inactivated fetal bovine serum (FBS) (2%), antibiotics, and several growth factors (hydrocortisone, bFGF, VEGF, IGF, EGF, etc.) in a 37°C, 5% CO_2, 95% air incubator. HAEC cultures were serially passaged on type-I collagen–coated 100-mm dishes after digestion with trypsin (0.125%)–EDTA and propagated in the growth medium. In the majority of experiments, cells were used at passages 4 and 5, and the endothelial cobblestone morphology was confirmed microscopically before use.

Measurement of Cell Viability—Cell viability was assessed by means of a colorimetric method with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium mono sodium salt (WST-1) (Wako Pure Chemical Industries) as previously described (*19*). Briefly, HAECs were plated into a type-I collagen-coated-96-well plate at a density of 1×10^4 cells per well. Samples were added to the culture medium and the plate was incubated at 37°C in a CO_2 incubator. After incubation, a tetrazolium salt solution consisting of 5 mM WST-1, 0.2 mM 1-methoxy phenazine methosulfate (Wako Pure Chemical Industries), and 20 mM HEPES, pH 7.4, was added to the medium, and the plate was then incubated for an additional 3 h. The absorbance of the solution was measured at 405 nm with a microplate reader.

Cell Treatments—HAECs were plated on the growth medium on a 100-mm dish at a density of 5×10^5 cells per dish. One day after confluence had been reached, the medium was replaced with a growth-supplement-free medium (EBM-2 plus 5 mg/ml heat-inactivated lipoprotein-deficient human serum), and the cells were incubated for an additional 48 h. After treatment with the lipoproteins, the cell monolayers were gently washed three times with 4 ml of PBS and then the cells were harvested from the dish with a rubber policeman. They were then centrifuged at 400 ×*g* for 5 min and washed twice with 10 ml of ice-cold PBS, the resulting pellet being stored at –80°C until analysis.

Western Blot Analysis—The pellet of HAECs treated with lipoproteins was resuspended in PBS containing 1 mM phenyl methionyl sulfonyl fluoride and then homogenized with a Polytron homogenizer. The protein content of the homogenate was determined by the bicinchoninic acid protocol with bovine serum albumin as a standard

A 20 μg sample of the protein was lysed in 0.1% SDS and then subjected to SDS–polyacrylamide gel electrophoresis (7.5 and 12% acrylamide, 1.5-mm thick slab gel) under reducing conditions. Proteins were then transferred to a PVDF membrane by electroblotting. The membrane was blocked for 12 h at 4°C in Block Ace and then incubated with 1 μg/ml of primary antibodies. After washing, the membrane was incubated with 1 μg/ml of anti-immunoglobulin G antibodies conjugated to horseradish peroxidase. Peroxidase activity on the membrane was visualized by means of an enhanced chemiluminescence substrate system (*15*).

RNA Preparation and RT-PCR Analysis—RT-PCR was performed to estimate the mRNA levels of eNOS, iNOS, catalase, Cu^{2+},Zn^{2+}-superoxide dismutase (CuZn-SOD), p22 phox, cyclooxygenase-I (COX-I), and COX-II in the lipoprotein-treated HAECs. Total RNA was isolated from the pellet of HAECs by the acid guanidium-phenol-chloroform method Single-stranded cDNA was prepared from 0.6 μg of total RNA with AMV reverse transcriptase as a template for PCR, and PCR was carried out with Taq DNA polymerase and Pwo DNA polymerase in a Titan™ One Tube RT-PCR system kit using a thermal cycler. The RT reaction was performed at 50°C for 30 min to maximize cDNA synthesis. The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 4 min, followed by cooling to 4°C. The PCR products were analyzed on a 5% acrylamide gel with staining with ethidium bromide, with DNA molecular weight marker XIII. The marker polyadenylated RNAs were further purified with an oligo $(dT)_{15}$ cellulose column (Type 3) kit

The PCR primer sequences were

iNOS: sense: 5′-TAGAAACAACAGGAACCTACCA-3′, antisense: 5′-ACGGGGGTGATGCTCCCAGACA-3′

eNOS: sense: 5′-GAAGAGGAAGGAGTCCAGTA-3′, antisense· 5′-GACTTGCTGCTTTGCAGGTT-3′

catalase: sense: 5′-CCATTTGAATATTGTAGCTG-3′, antisense: 5′-GAAATCAATTTAAGTCAACA-3′

p22 phox: sense: 5′-GTTTGTGTGCCTGCTGGA-3′, antisense: 5′-TGGGCGGCTGCTTGATGGT-3′

CuZn-SOD: sense: 5′-GGCGTCATTCACTTCGAGCAGAAG-3′, antisense: 5′-GGCAATCCCAATCACACCACAAGC-3′

COX-I: sense: 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′, antisense: 5′-GTGCATCAACACAGGCGCCTCTTC-3′

COX-II: sense: 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, antisense: 5′-AGATCATCTCTGCCTGAGTATCTT-3′

GAPDH: sense: 5′-ACCACAGTCCATGCCATCAC-3′, antisense: 5′-TCCACCACCCTGTTGCTGTA-3′

Determination of Enzyme Activity—Catalase activity was measured as the decrease in extinction at 240 nm based on H_2O_2 decomposition (*20*). A 50 μg sample of a HAEC homogenate was added to a 30 mM H_2O_2 solution at 20°C, and then the decrease in extinction over 15 s was measured spectrophotometrically. Superoxide dismutase activity in the homogenate was determined by an enzymatic assay method with a commercial kit (Wako Pure Chemical Industry) according to the manufacturer's instructions.

Determination of NO Production—NO production was measured by means of the chemiluminescent assay described previously (*21*). Briefly, 100 μl of the conditioned medium was brought to 1 0 ml with ice cold 20 mM Tris-HCl buffer, pH 7.4, and then the solution was heated at 90°C for 30 min to denature proteins. After cooling in ice, the sample solution was sonicated for 10 s and then centrifuged at 14,000 ×*g* for 20 min. The supernatant was ultrafiltrated at 4,000 ×*g* for 30 min through a prewashed Ultrafree microcentrifuge filter unit with a molecular weight cutoff of 30 kDa. The eluate (200 μl) was added to the reducing solution (80 μl) consisting of 500 μM NADPH and 0.85 U/ml nitrate reductase. After incubation at 25°C for 1 h, 40 μl of 2.4 N NaOH was added to the reaction mixture and the solution was kept in ice until analysis. An aliquot (20 μl) of the solution was injected into the gas-purge vessel with 5 ml of a 1% sodium iodide solution in glacial acetic acid for the chemical reduction of nitrite to NO. The NO gas generated was then carried by a N_2 stream into the chemiluminescence detector (Sievers NO analyzer Model 280; Taiyo Toyo Sanso, Osaka), where it could be detected through a reaction with ozone (*22*). Signals were recorded with a digital integrator (Hewlett-Packard Model 3396).

Measurement of ESR Spectra—Electron spin resonance (ESR) spectra were measured at room temperature according to the method described previously (*23*). After treatment with the lipoprotein, HAECs were mildly washed twice with 15 mM Hepes-saline buffer, pH 7.4, and then treated an additional three times at 37°C for 1 min with the same buffer containing 1 μM phorbol myristate acetate (PMA) to activate endothelial cells After washing the cells, 15 mM Hepes-saline buffer, pH 7.4, containing 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (ICN Biomedical, Aurora, OH) was freshly added to the culture dish and the cells were incubated at 37°C for 30 min. An ESR spectrum of the buffer collected by centrifugation was obtained with an ESR spectrometer system, model FE-1-X (JEOL, Tokyo). The magnetic field was set at 334.0 ± 5 mT, with a microwave power of 8 mW, modulation amplitude of 0.1 mT, time constant of 0.03 s, scan time of 20 s, and 50 accumulation.

Statistical Analysis—The results are expressed as means ± SE. Statistical evaluation of the data was performed using the unpaired Student's *t*-test and ANOVA, followed by Fisher's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Detection of Cross-Linking of Apolipoproteins in HDL Treated with Peroxynitrite—The major protein bands stained with Coomassie brilliant blue for the native HDL and prepared NO_2-HDL each correspond to the molecular mass, 27 kDa, estimated for an apolipoprotein AI (apoAI) (Fig. 1A). Other faintly stained bands corresponding to molecular masses of 72, 54, and 41 kDa were just visible for NO_2-HDL, and peroxynitrite induced the enhancement of these proteins in a dose-dependent manner. The apolipoprotein content of each of the bands was probed in immunoblots with anti-apoAI and anti-apoAII monoclonal antibodies (Fig. 1, B and C). In addition to bands stained for apoAI or apoAII alone, 41 kDa bands consistent with

apoAI-apoAII heterodimers stained with both anti-apoAI and anti-apoAII were identified. A series of higher molecular mass bands (>50 kDa) apparently stained for apoAI alone were assumed to be apoAI polymers. An immunoblot with anti-nitrotyrosine antibodies revealed nitrotyrosine in all bands stained with Coomassie Brilliant Blue (data not shown). In addition, to determine whether or not nitration of the lipoprotein as well as oxidation were caused by peroxynitrite, we attempted to detect lipid peroxidation in the prepared NO-lipoproteins by means of a thiobarbituric acid reactive substance (TBARS) assay according to the method reported by Yagi *et al.* (*24*). The TBARS values for the prepared NO_2-HDL were much lower than those for oxidized HDL obtained after incubation of native HDL with 10 μM Cu^{2+} for 16 h, and did not change significantly compared to in the case of the native HDL (data not shown). This finding demonstrated that treatment with peroxynitrite caused little oxidation of lipids in HDL under the conditions used in this study.

Detection of Nitrotyrosine in NO_2-Lipoprotein and Susceptibility of Lipoproteins to Peroxynitrite-Induced Nitration—Peroxynitrite can oxidize lipoproteins and generate nitrotyrosine from either free or protein-bound tyrosine. To determine the content of nitrotyrosine in nitrated lipoprotein, we analysed the 3-nitrotyrosine in the NO_2-HDL hydrolysate by HPLC (Fig. 2A). Figure 2A (b) shows a representative HPLC chromatogram for HDL not treated with peroxynitrite. In Fig. 2A (c) and (d), a well-defined peak at the retention time (11.8 min) of 3-nitrotyrosine can be seen for lipoproteins treated with 1 mM peroxynitrite. This peak was confirmed to be 3-nitrotyrosine by the fact that authentic 3-nitrotyrosine added to the samples was co-eluted with the peak [Fig. 2A (e)]. The increase in the peak area of 3-nitrotyrosine detected with this system was dose-dependent on peroxynitrite treatment (Fig. 2B). Treatment of lipoprotein with 1 mM peroxynitrite increased the 3-nitrotyrosine level in NO_2-HDL to 28.5 mmol/mol and the tyrosine residue level in NO_2-LDL to 15.0 mmol/mol. HDL

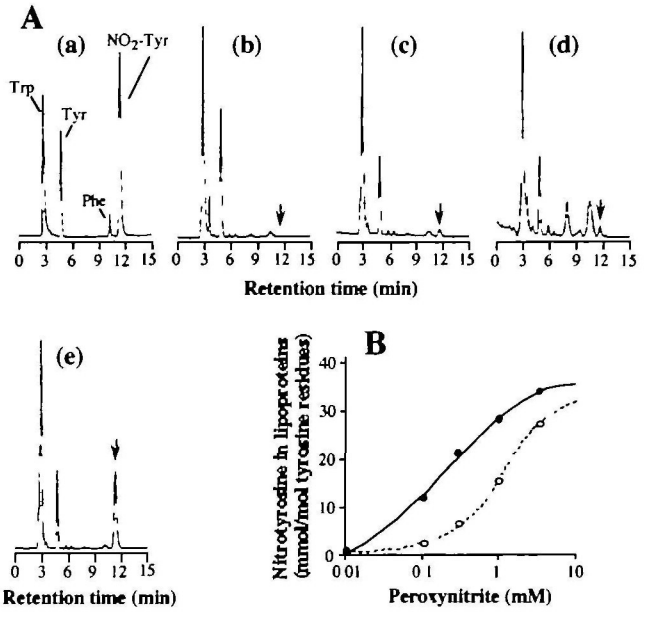

Fig 2 **Determination of nitrotyrosine residues in NO_2-lipoprotein and susceptibility of lipoprotein to nitration by peroxynitrite.** (A) HPLC chromatograms of NO_2-lipoproteins. A standard mixture (a) of Trp (800 μM), Phe (100 μM), Tyr (100 μM), and 3-nitrotyrosine (100 μM), acid hydrolysates of HDL treated without (b) or with (c) 1 mM peroxynitrite, an acid hydrolysate of LDL (d) treated with 1 mM peroxynitrite, and a mixture (e) containing acid hydrolysates of HDL treated with peroxynitrite and 50 μM 3-nitrotyrosine were subjected to HPLC analysis, respectively. Lyophilized NO_2-lipoprotein preparations were hydrolyzed with 5 7 M HCl containing 0.5% 2-mercaptoethanol and 0 01% phenol in evacuated and sealed tubes at 100°C for 24 h. Hydrolyzed samples were lyophilized and then resolved in 2 ml H_2O before analysis The expected position of the nitrotyrosine peak is indicated by an arrow (B) Nitrotyrosine levels in lipoprotein treated with various concentrations of peroxynitrite Quantitative data as to the nitrotyrosine contents in NO_2-HDL (closed circles) and NO_2-LDL (open circles), and estimated from the peak area on HPLC analysis are shown in the figure Each point is the mean of four determinations in two independent experiments

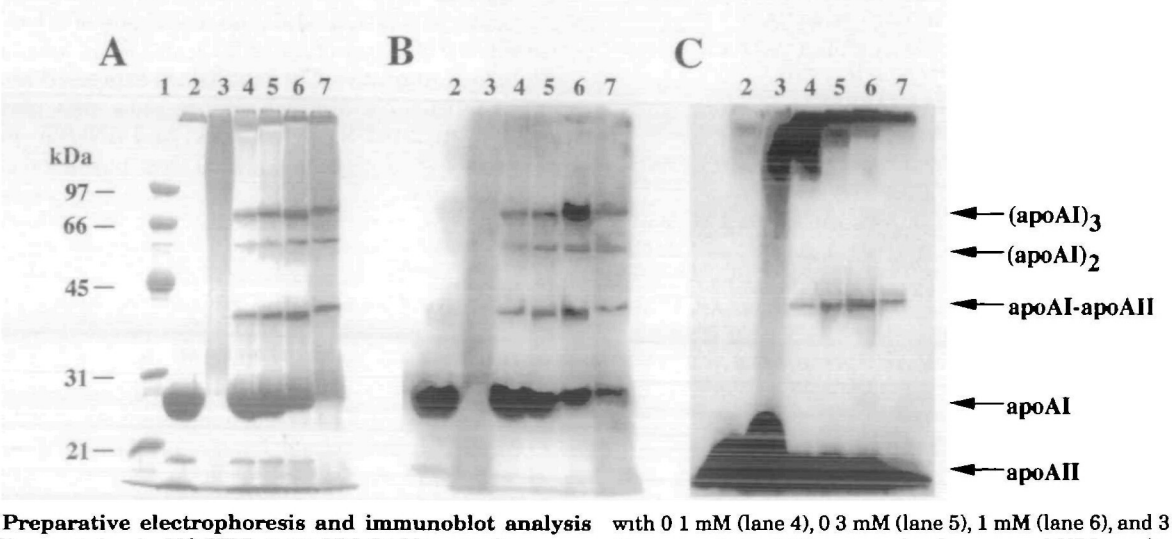

Fig 1. **Preparative electrophoresis and immunoblot analysis of apolipoproteins in NO-HDL.** 7.5% SDS-PAGE, with Coomassie Brilliant Blue staining (A), anti-apoAI immunoblotting (B), and anti-apoAII immunoblotting (C) of native HDL (lane 2), Cu^{2+}-oxidized HDL (lane 3), and NO_2-HDL (lanes 4–7), was performed under reducing conditions. NO_2-HDLs were prepared by treatment of native HDL with 0 1 mM (lane 4), 0 3 mM (lane 5), 1 mM (lane 6), and 3 mM (lane 7) peroxynitrite. A 20 μg sample of a prepared HDL per lane was applied to the gel. Molecular weight markers are shown in lane 1, and the expected forms of apolipoproteins are indicated on the right The results shown are for a single representative experiment of three independent ones.

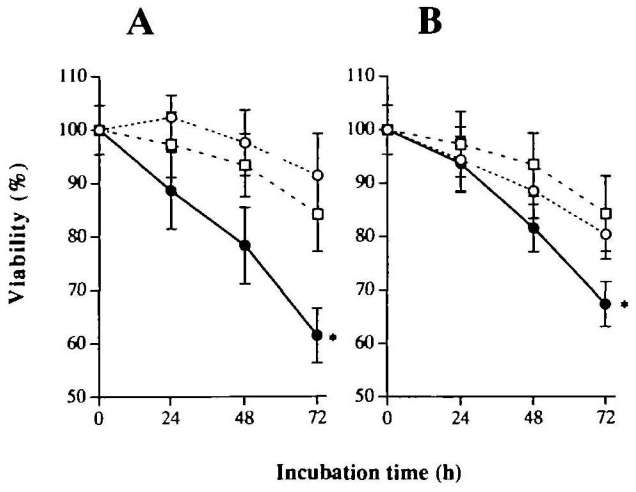

Fig 3 **Effect of NO_2-lipoprotein on the viability of HAECs.** 100 μg/ml NO_2-HDL (A) or NO_2-LDL (B) (nitrated lipoprotein prepared by the addition of 100 μM peroxynitrite to the lipoprotein particles) was added to the medium of cultured HAECs and the media were incubated for the times indicated Cell viability was quantified using WST-1 as described under "MATERIALS AND METHODS" The numbers of vital HAECs before lipoprotein treatment are expressed as a percentage of the maximum level (defined as 100%) Values are the means of $n = 3 \pm$ SE and are representative of three separate experiments The symbols are as follows ○, native lipoproteins, ●, NO_2-lipoproteins, □, untreated control *$p < 0.05$ *vs.* untreated control.

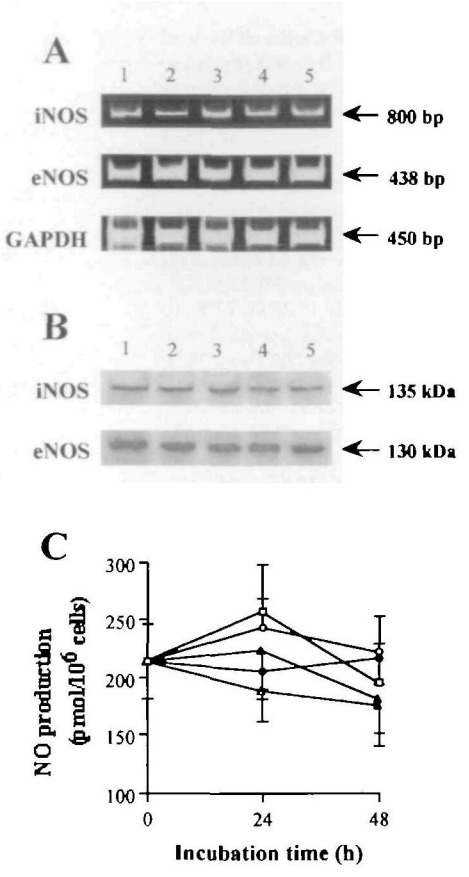

Fig 4 **NOS expression and NO production in HAECs treated with NO_2-lipoprotein.** (A) RT-PCR analysis of total RNA (0 6 μg) from HAECs stimulated with and without 100 μg/ml of lipoprotein was performed according to the method described under "MATERIALS AND METHODS." A 10 μl aliquot of each RT-PCR product was electrophoresed on a 5% polyacrylamide gel in TBE buffer and stained with ethidium bromide. The expected sizes of the cDNA products are indicated on the right (B) Western blotting of total protein (20 μg) from the HAEC homogenate was performed according to the method described under "MATERIALS AND METHODS" These two typical photographs were taken in a single representative experiment of three independent ones. The lanes are as follows: (1) untreated control; (2) native HDL, (3) native LDL, (4) NO_2-HDL, (5) NO_2-LDL (C) Total NO-metabolites in the conditioned medium of HAECs were measured according to the method described under "MATERIALS AND METHODS" Values are the means of $n = 3 \pm$ SE and are representative of three separate experiments. The symbols are as follows ○, native HDL, ●, NO_2-HDL, △, native LDL; ▲, NO_2-LDL, □, untreated control.

nitration by peroxynitrite increased dose-dependently and reached a maximum as a result of treatment with 2 mM peroxynitrite for 5 min, and LDL required more than HDL to reach a maximum. These findings indicated that HDL is more susceptible to peroxynitrite-induced nitration than LDL

Effect of NO_2-Lipoprotein on HAEC Viability—Both NO_2-HDL and NO_2-LDL (prepared by the addition of 100 μM peroxynitrite) were cytotoxic toward HAECs in a time-dependent manner (Fig. 3). The viability of the cells decreased to 60 or 70% at as early as 72 h incubation with 100 μg of protein/ml of NO_2-HDL or NO_2-LDL. After 96 h, the HAECs were injured regardless of the presence or absence of NO_2-lipoprotein, probably because of serum starvation, and the effect of NO_2-lipoprotein became less prominent (data not shown). Incubation with native HDL or LDL did not induce any decrease in cell viability beyond the control level (about 8 to 18% of total cells). This decrease in the control group was thought to be due to both serum deprivation and the lack of growth factors in the medium. Incubation with oxidized HDL or oxidized LDL, which was obtained on incubation of native HDL with 10 μM Cu^{2+} for 16 h, decreased the HAEC viability to 56 or 62%, respectively (data not shown).

Cell viability was quantified by trypan blue exclusion after collection by trypsinization. In the group with incubation with either NO_2-HDL or NO_2-LDL, the number of viable HAECs significantly decreased compared with the maximal level of HAECs before lipoprotein treatment. Light-microscopical observation demonstrated great increases in floating cells and expanded cells for NO_2-lipoprotein-treated HAECs, but not for an untreated control.

Effects of NO_2-Lipoprotein on NOS Expression and NO Production in HAECs—To determine the causes of the endothelial dysfunction induced by NO_2-lipoprotein, we investigated the effect of NO_2-lipoprotein on NOS expression in HAECs. Exposure to NO_2-lipoprotein for 48 h does not affect the mRNA expression of either inducible NOS (iNOS) or endothelium NOS (eNOS), as detected on RT-PCR analysis (Fig. 4A). Western blotting of both NOS isozymes also failed to reveal changes in iNOS and eNOS expression in HAEC homogenates stimulated with NO_2-HDL or NO_2-LDL (Fig. 4B). Additionally, significant NO production was not detected in the culture medium of HAECs treated with NO_2-HDL or NO_2-LDL, in contrast to the level in untreated or native lipoprotein-treated HAECs (Fig. 4C). These findings suggested that NO production

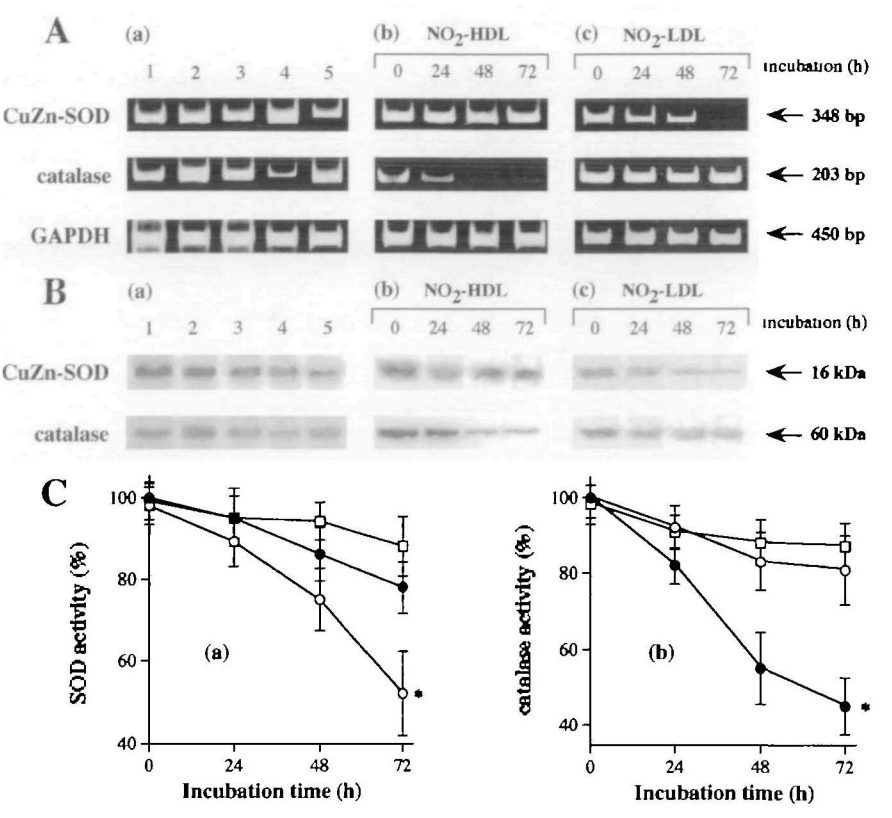

Fig 5 **Regulation of CuZn-SOD and catalase in HAECs by NO_2-lipoprotein treatment.** (A) The mRNA expression of CuZn-SOD and catalase in NO_2-lipoprotein-treated HAECs. (a) RT-PCR analysis of total RNA (0 6 μg) from HAECs stimulated with and without 100 μg/ml of lipoprotein was performed as described under "MATERIALS AND METHODS" The lanes are as follows: (1) untreated control, (2) native HDL; (3) native LDL, (4) NO_2-HDL, (5) NO_2-LDL (b and c) Total RNA of HAECs treated with NO_2-HDL or NO_2-LDL for the times indicated was subjected to RT-PCR analysis The expected sizes of the cDNA products are indicated on the right (B) The expression of CuZn-SOD and catalase protein in NO_2-lipoprotein-treated HAECs. (a) Western blots of total protein (20 μg) from homogenates of HAECs stimulated with and without 100 μg/ml of lipoprotein were obtained as described under "MATERIALS AND METHODS" The lanes are as follows (1) untreated control; (2) native HDL, (3) native LDL; (4) NO_2-HDL, (5) NO_2-LDL (b and c) Homogenates of HAECs treated with NO_2-HDL or NO_2-LDL for the times indicated were subjected to Western blot analysis. The sizes of the bands stained are indicated on the right Both typical photographs shown are for a single representative experiment of three independent ones (C) SOD and catalase activities in NO_2-lipoprotein-treated HAECs. The activities of SOD (a) and catalase (b) in HAECs stimulated without (open squares) and with a NO_2-lipoprotein [NO_2-HDL (closed circles), NO_2-LDL (open circles)] were determined according to the method described under "MATERIALS AND METHODS" Each of the activities in HAECs before lipoprotein treatment is defined as 100%. Values are the means of $n = 3 \pm$ SE and are representative of three separate experiments $^*p < 0.05$ *vs.* untreated control

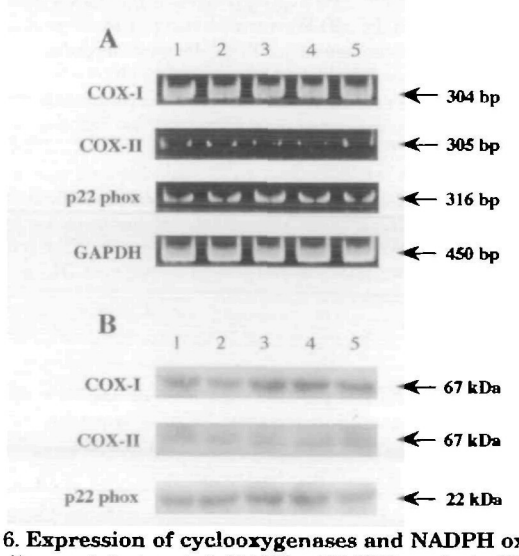

Fig 6. **Expression of cyclooxygenases and NADPH oxidase in NO_2-lipoprotein-treated HAECs.** RT-PCR analysis (A) of total RNA (0.6 μg) and Western blot analysis (B) of cell homogenates (20 μg) of HAECs stimulated with and without 100 μg/ml of lipoprotein for COX-I, COX-II, and p22 phox were performed according to the methods described under "MATERIALS AND METHODS." The mean values for three experiments, triplicate determinations, are shown In A, the expected sizes of the cDNA products are indicated on the right The lanes are as follows (1) untreated control, (2) native HDL; (3) native LDL; (4) NO_2-HDL, (5) NO_2-LDL.

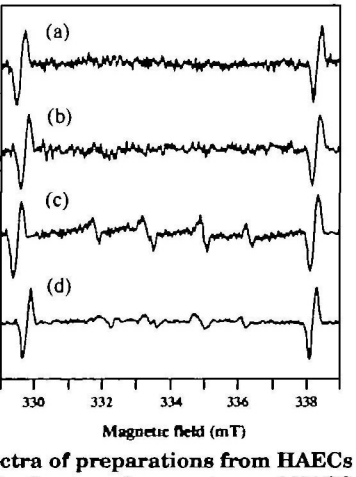

Fig. 7. **ESR spectra of preparations from HAECs treated with NO_2-lipoprotein.** Spectra of preparations of HAECs treated with 100 μg/ml of native HDL (a), native LDL (b), NO_2-HDL (c), and NO_2-LDL (d) The experimental conditions are given under "MATERIALS AND METHODS." The illustrated spectra are for one of four separate experiments

does not contribute to the mechanism of the endothelial dysfunction induced by NO_2-lipoprotein.

Expression of Reactive Oxygen Species-Related Enzymes in NO_2-Lipoprotein-Treated HAECs—We next turned our attention to reactive oxygen species as a potential factor

related to NO_2-lipoprotein-induced endothelial cell injury, and assayed the expression of CuZn-SOD and catalase in NO_2-lipoprotein-treated HAECs. Stimulation of HAECs with NO_2-HDL resulted in time-dependent decreases in the mRNA level and protein expression of catalase, although there was little effect on the reduced expression of CuZn-SOD (Fig. 5, A and B). Densitometric analysis of the catalase protein in HAECs treated with NO_2-HDL for 48 h showed a 42% decrease compared to in cells exposed to native HDL. In the group stimulated with NO_2-LDL for 48 h, both the CuZn-SOD mRNA and protein levels, but not that of catalase, markedly decreased in HAECs compared with the levels in the corresponding native LDL group. The catalase activity in NO_2-HDL-treated HAECs was significantly lower than in cells induced by native HDL, which paralleled the reduction of the band detected on Western blotting. The same was true of the SOD activity in NO_2-LDL-treated cells (Fig. 5C). Incubation of HAECs with NO_2-lipoprotein failed to alter the expression of cyclooxygenases (COX-I and COX-II) or p22 phox, as detected on RT-PCR analysis and Western blotting (Fig. 6).

Radical Generation from HAECs Stimulated with NO_2-Lipoprotein—We examined radical generation from cultured HAECs by ESR spectroscopy in the presence of the spin trap DMPO. Although no signals were observed for native-lipoprotein-treated HAECs, ESR signals, including a prominent 1 2 2 1 quartet signal indicative of DMPO-OH, were observed for HAECs treated with NO_2-lipoprotein (Fig. 7).

DISCUSSION

The present study provided direct evidence that reactive oxygen species are associated with the injury to human aortic endothelial cells exposed to peroxynitrite-treated lipoprotein. The results also shed new light on investigations of the mechanism of the onset and development of atherosclerosis. Several researchers have pointed out the association between the generation of peroxynitrite and the development of atherosclerosis (*3, 4, 25*). Since apolipoproteins, which are localized on the surface of lipoprotein particles, are known to play an important role in binding to cells, we first investigated intermolecule modification of the surface of HDL particles treated with peroxynitrite. Interestingly, treatment with peroxynitrite caused the cross-linking of surface apolipoproteins, and the formation of polymeric molecules of apolipoproteins such as apoAI-apoAII heterodimers and apoAI polymers. Since the formation of similar molecules was reported to occur during incubation of tyrosyl radicals with HDL (*26*), their formation may be due to the initial cross-linking of apoAI and apoAII by peroxynitrite to yield an active heterodimer. We also showed that the apolipoproteins simultaneously underwent nitration on peroxynitrite treatment, and that nitrotyrosines in the molecules could be sufficiently detected by HPLC. The data presented in Fig. 2B indicated that HDL exhibits greater susceptibility to nitration by peroxynitrite than LDL under *in vitro* conditions. The difference in susceptibility between HDL and LDL may be due to the composition and localization of apolipoproteins in the particles. The nitrotyrosine concentration in LDL isolated from the serum of atherosclerotic patients was about 0.8 mmol/mol of tyrosine residues, as indicated by Heinecke *et al.*, and its concentration was above the detectable limit (0.2 mmol/mol of tyrosine) with the HPLC method, suggesting that detection by HPLC may be useful for nitrotyrosine in proteins in atherosclerotic patients.

Oxidized LDL (oxLDL), which is thought to play a central role in atherogenesis, exhibits a potential cytotoxic effect on cultured vascular cells (*12*). In addition, Heinecke *et al.* reported that nitrated LDL is localized in the serum of patients with atherosclerosis, and that its degree of LDL nitration in the patients' serum is as much as that of LDL nitrated by about 20 to 30 μM peroxynitrite (*10*). We therefore hypothesized that NO_2-HDL, whose peroxynitrite-induced nitration proceeded at a rather high level, is present in the serum or tissue at sites of arteriosclerosis. We next investigated whether or not peroxynitrite-induced nitration of HDL and LDL would have a toxic effect on endothelial cells. As shown in Fig. 3, 72 h incubation with either NO_2-HDL or NO_2-LDL significantly decreased the viability of cultured HAECs, and the cytotoxicity was more dramatic in the group treated with NO_2-HDL than in the NO_2-LDL group. This phenomenon may be explained by the results showing that HDL is more susceptible to nitration than LDL on the addition of peroxynitrite. Moreover, counts of NO_2-lipoprotein-treated HAECs by means of trypan blue exclusion paralleled the data obtained using a WST-1, and some cells were observed to be expanded and detached during incubation with NO_2-lipoprotein on microscopic investigation. These data indicated that NO_2-lipoprotein somehow harms cultured human aortic endothelial cells. We found it very interesting that HDL, which is well known as an antiatherogenic factor, was converted on treatment with peroxynitrite to a toxic factor that caused endothelial cell disturbance. Moreover, we obtained data showing that NO_2-lipoprotein was as harmful to endothelial function as Cu^{2+}-oxidized lipoprotein. Since NO_2-HDL exhibits low affinity for Cu^{2+}-oxidized HDL binding protein on the surface of cultured HAECs (unpublished data), NO_2-HDL may induce cytotoxicity toward aortic endothelial cells *via* a mechanism similar to that in the case of oxidized HDL.

Previous reports have indicated that atherogenic oxLDL can cause endothelial-dependent vasomotor abnormalities through inhibition of endothelial-derived NO (*27*). The RT-PCR and Western blot data in Fig. 4 indicated that neither NO_2-HDL nor NO_2-LDL can elicit expression of NOS isozymes (iNOS and eNOS) in cultured HAECs, suggesting that NO production may not participate in the mechanism of NO_2-lipoprotein-induced endothelium dysfunction. A cell must maintain a balance between normal interactions between oxidants and antioxidant defense systems. The removal of toxic oxygen metabolites is the putative function of antioxidant enzymes such as CuZn-SOD and catalase. We therefore hypothesized that the accumulation of oxygen-free radicals induced by reduced antioxidative enzymes may be related to dysfunctional states exhibited by the endothelium after exposure to NO_2-lipoprotein. In order to test this hypothesis, we investigated whether or not NO_2-lipoprotein regulates the expression and activity of the antioxidant enzymes in HAECs. As shown in Fig. 5, incubation with NO_2-HDL significantly decreased the expression of both the mRNA and protein of catalase, and similar data were obtained with regard to enzyme activity. Interestingly, in HAECs stimulated with NO_2-LDL, reduced expression of CuZn-SOD, but not of catalase, was observed. Because the

data obtained in our preliminary study showed that NO_2-HDL failed to bind to a scavenger receptor that was reported to be the specific receptor of NO_2-LDL (*28*) on the surface of endothelial cells (unpublished data), the decreased expression of different enzymes caused by these NO_2-lipoproteins may be due to the different receptors for NO_2-HDL and NO_2-LDL on endothelial cells.

Cyclooxygenase is also capable of generating superoxide anions through a mechanism that involves conversion of nicotinamide adenine dinucleotide phosphate (NADPH) to an intermediate radical form of the enzyme such as prostaglandin G_2, which is converted to prostaglandin H_2 (*29*), although NADPH oxidase is well known to progressively catalyze the production of superoxide anions. However, NO_2-lipoprotein had little effect on the expression of these enzymes in endothelial cells (Fig. 6). Although the superoxide anion is continuously produced at a low level from endothelial cells, even nonstimulated cells, NO_2-HDL may cause relative accumulation of the superoxide anion through decreased expression of catalase, an antioxidant enzyme, in endothelial cells

The additional data shown in Fig. 7 enabled us to confirm the hypothesis presented above, since we obtained evidence that radical generation in HAECs treated with NO_2-lipoprotein was enhanced. On ESR analysis, it was observed that NO_2-lipoprotein-treated HAECs gave rise to a DMPO-OH signal in ESR spectra. DMPO reacts with superoxide to form DMPO-OOH, which rapidly breaks down into DMPO-OH, which is the same DMPO-adduct as that formed through direct trapping of OH radicals (*30*). These results demonstrated that treatment with NO_2-lipoprotein might produce either a superoxide anion or its secondary product, a more reactive hydroxyl radical, in cells.

In conclusion, the data obtained in the present study indicate that human vascular endothelial cells exposed to nitrated lipoprotein generate free oxygen radicals, especially superoxide anions and OH radicals, as a result of the reduced expression of antioxidant enzymes, but that there is little association with the enzymes that produce NO and reactive oxygen species, and these free oxygen radicals participate in the pathobiological mechanism of nitrated lipoprotein-induced endothelial dysfunction.

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