Glycated High-Density Lipoprotein Regulates Reactive Oxygen Species and Reactive Nitrogen Species in Endothelial Cells

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Nonenzymatic glycosylation of plasma proteins may contribute to the excess risk of developing atherosclerosis in patients with diabetes mellitus. Although it is believed that high-density lipoprotein (HDL) is glycosylated at an increased level in diabetic individuals, little is known about a possible linkage between glycated HDL and endothelial dysfunction in diabetes. To clarify whether glucose-modified HDL affects the function of endothelial cells, we first examined herein the level of H_2O_2 generation from cultured human aortic endothelial cells (HAECs) exposed to a glycated oxidized HDL (gly-ox-HDL) prepared in vitro. Incubation for 48 hours with 100 μg/mL of gly-ox-HDL induced significant release of H_2O_2 from cells and gly-ox-HDL–induced H_2O_2 formation was inhibited in the presence of diphenyleneiodonium, an inhibitor of NADPH oxidase. In addition, stimulation of HAECs with gly-ox-HDL for 48 hours elicited a marked downregulation of catalase and Cu^{2+}, Zn^{2+}-superoxide dismutase (CuZn-SOD), suggesting H_2O_2 formation by gly-ox-HDL to be due to a disturbance involving oxidant and antioxidant enzymes in the cells. Treatment of HAECs with gly-ox-HDL attenuated the expression of endothelial nitric oxide synthase (eNOS), but not inducible nitric oxide synthase (iNOS), and this was followed by decreased production of nitric oxide (NO) by the cells. Furthermore, in vitro experiments with glycated HDL (gly-HDL) in the presence of 2 mmol/L EDTA and Cu^{2+}-oxidized HDL suggested the effect of gly-HDL on endothelial function to be possibly potentiated by additional oxidative modification. Taking all of the above findings together, gly-ox-HDL may lead to the deterioration of vascular function through altered production of reactive oxygen species and reactive nitrogen species in endothelial cells.

ACCELERATED ATHEROSCLEROSIS is a major complication of diabetes mellitus, but the role of associated risk factors, such as dyslipidemia, hypertension, obesity, and hyperglycemia, per se, has not been fully elucidated. Epidemiologic studies have identified an inverse relationship between the plasma level of high-density lipoprotein (HDL) and the occurrence of atherosclerosis.[1] Although individuals with type 2 diabetes often have low HDL levels, type 1 diabetics develop equally severe atherosclerosis despite normal or even elevated HDL levels.[2] Gowri et al suggested that compositional alterations in HDL_2 in poorly controlled type 2 diabetic subjects may reduce its antiatherogenic properties,[3] and the HDL apoproteins in the sera of hyperglycemic individuals are more highly glycated than those in normoglycemic individuals.[4] Thus, functional alterations of HDL as a result of hyperglycemia may contribute to accelerated atherosclerosis.

Oxidative modification of low-density lipoprotein (LDL) in vivo and its accelerated uptake by arterial wall macrophages have been implicated in the development of atherosclerosis,[5] and there is evidence that glycation of LDL increases its susceptibility to oxidation.[6] Several observations suggest that HDL is also oxidized in vivo. Bowry et al[7] reported that most of the measurable lipid peroxides in plasma are found in the HDL fraction, and there are particles in HDL that contain no lipophilic antioxidants. Suzukawa et al[8] found that HDL contains polyunsaturated fatty acids that are susceptible to oxidation in vitro and that each HDL particle carries fewer molecules of the antioxidant vitamin E than LDL.

In the process of nonenzymatic glycosylation, glucose reacts with amino acids or nucleic acids to form Schiff bases, which then rearrange chemically into early glycosylation products; these in turn restructure into advanced glycation end-products (AGEs).[9] Endothelial cells express receptors for AGEs (RAGEs),[10] which facilitate the internalization of these products into endothelial cells and their transfer into the subendothelial space. AGEs could conceivably impair endothelium-dependent relaxation by several mechanisms[11,12] that involve the glycosylation and oxidative modification of LDL, which in turn disrupts the formation and action of nitric oxide (NO),[13] as well as the direct quenching of NO by AGEs.[14] Furthermore, glycated LDL may be atherogenic, since it is cleared from serum more slowly than LDL, has less affinity for the LDL receptor, and is more avidly internalized by human monocyte-derived macrophages.[6]

Reactive oxygen species are thought to play an important role in the pathophysiology of diabetes mellitus.[15,16] The exact mechanism underlying hyperglycemia-induced endothelial dysfunction through these free radicals is unclear. Current hypotheses include enhanced release of constricting prostanoids,[17] decreased bioactivity of NO[18] due to increased superoxide anion ($\cdot O_2^-$) release during high D-glucose exposure,[19] and attenuated formation of NO.[20,21] Recently, Galle et al reported that glycated LDL enhances inactivation of NO via increased production in endothelial cells.[22] Although glycation of HDL is also known to occur under hyperglycemic conditions in a time- and glucose concentration–dependent manner, the effects of glycated HDL on endothelial function have seldom been investigated. Since there is evidence of a linkage between endo-

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thelial dysfunction in diabetes mellitus and oxidative stress, we focused on the production of reactive oxygen species and reactive nitrogen species in cells as a pivotal factor in endothelial dysfunction and measured the levels of free radicals of oxygen and nitrogen generated from endothelial cells stimulated by glycated HDL. Moreover, we investigated the mechanism of these free radical productions by the stimulated cells from both the aspect of denaturing of HDL components by glycation and that of alterations of oxidant and antioxidant enzymes in the cells.

MATERIALS AND METHODS

Preparation of Modified Lipoproteins

Native HDL was isolated from fresh normolipidemic human serum by sequential ultracentrifugation as described previously.[23] The HDL fraction ($1.063 < d < 1.210$) was pooled and dialyzed extensively against 50 mmol/L phosphate-buffered saline (PBS), pH 7.5, containing 1 mmol/L EDTA. To prepare glycated lipoprotein, lipoproteins at a final concentration of 0.5 mg/mL were incubated at 37°C for 7 days in PBS, pH 7.4, supplemented with 0 to 200 mmol/L D-glucose (containing 0.0005% iron, 0.0005% lead, and 0.0001% arsenic) (Wako Pure Chemical Industry, Osaka, Japan). To prepare oxidized lipoprotein, lipoproteins at a final concentration of 0.5 mg/mL were incubated at 37°C for 16 hours in the presence of 10 μmol/L $CuSO_4$. In some experiments, 2 mmol/L EDTA was added to prevent oxidation brought about by the presence of trace metal ions. Control samples were incubated in the same buffer without D-glucose and EDTA. These products were then dialyzed extensively against PBS, pH 7.4, containing 1 mmol/L EDTA to remove glucose or copper ions, and each lipoprotein was stored in a dark environment at 4°C until required for use.

Cell Culture

HAECs (Sanko Junyaku, Tokyo, Japan) were cultured as previously described.[24] The cells were suspended in an endothelial cell growth medium (EBM-2) (Sanko Junyaku) containing 2% heat-inactivated fetal bovine serum, antibiotics, and several growth factors, and they were seeded at a density of 5×10^5 cells per dish on type I collagen-coated 100-mm dishes. After incubation for 3 days at 37°C, 5% CO_2 and 95% air environment, 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 hours. After treatment with the lipoproteins, adherent cells were gently washed 3 times with 4 mL of PBS, and the cells were harvested from the dish with a rubber policeman. They were then centrifuged at $400 \times g$ for 5 minutes and washed twice with ice-cold PBS, and the cells were stored at −80°C until required for analysis. In all experiments, cells were used at passages 4 and 5, and endothelial cobblestone morphology was confirmed microscopically before their use.

Determination of H_2O_2

H_2O_2 content in the medium was fluorometrically measured according to the methods reported by Zaitsu and Ohkura.[25] A fluorogenic substrate solution containing 5 mmol/L 3-(*p*-hydroxyphenyl) propionic acid was added to the conditioned medium. After incubation for 30 minutes, 1 U of horseradish peroxidase was added to the reaction mixture. After standing for 5 minutes, fluorescence intensities of the solutions were measured on a spectofluorometer with an excitation wavelength of 355 nm and an emission wavelength of 405 nm.

Determination of the Activities of Catalase and Cu^{2+}, Zn^{2+}-Superoxide Dismutase

Catalase activity was measured by the decrease in extinction at 240 nm based on H_2O_2 decomposition.[26] A 50-μg sample of HAEC homogenate was added to 30 mmol/L H_2O_2 solution at 20°C, and the decrease in extinction during a 15-second interval was measured spectrophotometrically. Superoxide dismutase activity in the homogenate was determined by an enzymatic assay method using a commercial kit (Wako Pure Chemical Industries) according to the manufacturer's instructions.

Western Blot Analysis

Western blotting of the HAEC homogenate was performed as previously described.[27] A 20-μg sample ($\simeq 2 \times 10^6$ cells) of the protein was lysed in 0.1% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (7.5% and 12% acrylamide, 1.5-mm thick slab gel) under reducing conditions. The proteins were then transferred to a polyvinylidene difluoride (PVDF) (Millipore, Tokyo, Japan) membrane by electroblotting, and the membrane was blocked for 12 hours at 4°C in Block Ace (Dai-Nippon-Pharmaceutical, Osaka, Japan) and incubated with 1 μg/mL of anti-human Cu^{2+}, Zn^{2+}-superoxide dismutase (CuZn-SOD) antibody (The Binding Site, Birmingham, England), anti-human catalase antibody (The Binding Site), anti-inducible NOS (anti-iNOS) (Transduction Laboratories, Lexington, KY), and anti-endothelial NOS (anti-eNOS) antibody (Transduction Laboratories). After washing, the membrane was incubated with 1 μg/mL of anti-immunoglobulin G antibody conjugated to horseradish peroxidase. Peroxidase activity on the membrane was visualized using an enhanced chemiluminescence substrate system (Amersham, Arlington Heights, IL).

Reverse-Transcription Polymerase Chain Reaction Analysis

Reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine the mRNA levels of eNOS, iNOS, catalase, and CuZn-SOD in the lipoprotein-treated HAECs as previously described.[27] Single-stranded cDNA was prepared from 0.6 μg ($\simeq 5 \times 10^5$ cells) of total RNA by avian myeloblastosis virus (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 (Boehringer Mannheim, Germany) using a thermal cycler. The RT reaction was performed at 50°C for 30 minutes to maximize cDNA synthesis. PCR conditions were as follows: 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 4 minutes, followed by cooling to 4°C. PCR products were analyzed on 5% acrylamide gel stained with ethidium bromide using the DNA molecular weight marker XIII.

Determination of NO_2^-/NO_3^-

NO_2^-/NO_3^- content of the culture medium was measured by chemiluminescent assay, as described previously.[28] Briefly, conditioned medium was heated at 90°C for 30 minutes to denature the proteins in samples. After cooling the solution in ice, the sample solution was sonicated and centrifuged at $14{,}000 \times g$ for 20 minutes. The supernatant was ultrafiltered through a prewashed Ultrafree microcentrifuge (Millipore) filter unit with a molecular weight cut-off of 30 kd. The filtrate was added to the reducing solution consisting of 500 μmol/L NADPH and 0.85 U/mL nitrate reductase. After incubation at 25°C for 1 hour, 2.4N NaOH was added to the reaction mixture and the solution was kept in ice until the analysis. An aliquot of the solution was injected into the gas-purge vessel with a 1% solution of sodium iodide in glacial acetic acid for the chemical reduction of nitrite to NO, and the NO gas generated was then carried by N_2 stream into the chemiluminescence detector (Sievers NO analyzer Model 280, Taiyo Toyo Sanso, Osaka, Japan), in which it could be detected by reaction with ozone.

Signals were recorded on a digital integrator (Hewlett-Packard Model 3396, Palo Alto, CA).

Level of HDL Glycation

The relative degree of HDL glycation was detected by affinity chromatography with m-aminophenylboronic acid gel.[29] Columns packed with the gel were equilibrated with 0.25 mmol/L ammonium acetate buffer, pH 8.0, containing 50 mmol/L $MgCl_2$ at 20°C. After equilibration, 2 mg of each HDL was added and allowed to soak into the gel, followed by perfusion of the column with the equilibration buffer. HDL that was tightly retained by the column was eluted by perfusion with 0.1 mol/L Tris-HCl buffer, pH 8.5, containing 200 mmol/L sorbitol. The release of HDL from the column was detected by an increase in absorption at 280 nm. The amount of glycated products bound to the column is indicated as the ratio of the glycated products to the total preparation.

AGEs produced in modified HDL solution were measured fluorometrically at 440 nm with excitation at 370 nm.[30]

Determination of HDL Oxidation

The amount of lyso-phosphatidylcholine (LPC) produced was measured by enzyme-linked immunosorbent assay (ELISA) with the anti-oxidized HDL antibody 9F5-3a according to a method described previously.[23] Lipid peroxide formation in the modified HDL prepared was monitored by the thiobarbituric acid reaction, according to Yagi's method.[31]

Statistical Analysis

Statistical evaluation of the data was performed using the unpaired Student's *t* test and analysis of variance (ANOVA) followed by Fisher's test. A *P* value less than .05 was considered statistically significant.

RESULTS

Incubation with 100 μg/mL of glycated oxidized HDL (gly-ox-HDL) (prepared by incubation for 7 days with 100 mmol/L glucose) enhanced the H_2O_2 content in the conditioned medium of HAECs in a manner dependent on the dose of glucose used for preparation, as shown in Fig 1A. When H_2O_2 formations in the conditioned medium of HAECs treated with modified HDLs were compared, oxidized HDL (ox-HDL) (prepared by incubation for 16 hours with 10 μmol/L $CuSO_4$) and gly-ox-HDL were found to induce H_2O_2 at a similar level, whereas glycated nonoxidized HDL (gly-HDL) (prepared by incubation for 7 days with 100 mmol/L glucose and 2 mmol/L EDTA) showed no H_2O_2 formation. To clarify the source of reactive oxygen species generated from HAECs, effects of preincubation with oxidant inhibitors on gly-ox-HDL-induced formation of H_2O_2 from the cells were assayed (Fig 1B). Preincubation with vitamin C (Wako Pure Chemical Industry), known to work as a free radical scavenger, significantly reduced gly-ox-HDL–induced H_2O_2 production from HAECs (30% ± 6% of that in the group treated with gly-ox-HDL alone). Allopurinol (Wako Pure Chemical Industry) (85% ± 6% of gly-ox-HDL alone) and aspirin (Sigma, St Louis, MO) (90% ± 6% of gly-ox-HDL alone) slightly but insignificantly reduced H_2O_2 whereas N^G-nitro-L-arginine methyl ester (L-NAME) (Research Biochemicals International, Natrick, MA) did not interfere the level of H_2O_2 in the conditioned medium. In contrast, pretreatment with diphenylene iodonium (DPI) (Sigma) markedly inhibited gly-ox-HDL–induced formation of H_2O_2 (48% ± 5% of gly-ox-HDL alone), and the significant inhibition by DPI was also

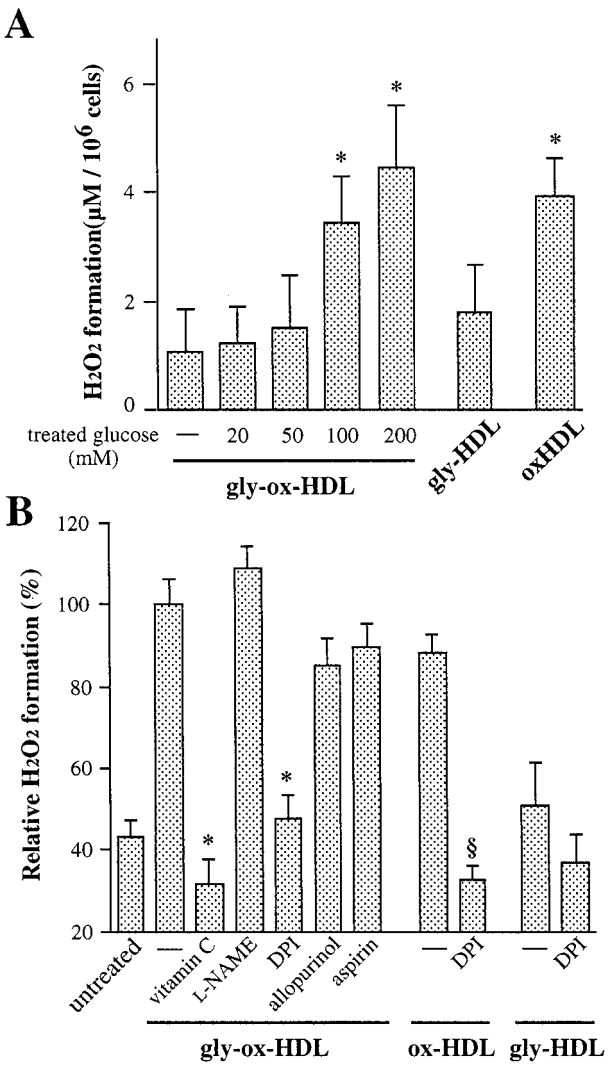

Fig 1. H_2O_2 generation from glycated HDL-treated HAECs. (A) H_2O_2 amounts generated from HAECs treated with modified HDL. Values are the means ± SD of 3 determinations and are representative of 3 separate experiments. *Significant difference from the control, *P* < .05. (B) Effect of inhibitors of oxidant enzymes on H_2O_2 generation from HAECs treated with modified HDL. HAECs were preincubated with either 5 μmol/L vitamin C, 200 μmol/L L-NAME, 500 μmol/L allopurinol, 100 μmol/L aspirin, or 5 μmol/L DPI for 30 minutes at 37°C in the presence of 5 mmol/L L-arginine and 3 μmol/l TB_4. HAECs were then incubated for 12 hours with 100 μg/mL modified HDL. Values are the means ± SD of 3 determinations and are representative of 3 separate experiments. *Significant difference from gly-ox-HDL alone, *P* < .05. §Significant difference from ox-HDL alone, *P* < .05.

detected in the group of HAECs treated with ox-HDL, but not the gly-HDL group. Although incubation of HAECs with gly-ox-HDL failed to alter the expression of xanthine oxidase and cyclooxygenases, the modified HDL induced a slight increase in expression of the mRNA and protein of p22 phox, a subunit of NADPH oxidase, in cultured HAECs (data not shown). This provides evidence that superoxide anion production by NADPH oxidase may contribute to H_2O_2 generation from HAECs treated with either gly-ox-HDL or ox-HDL.

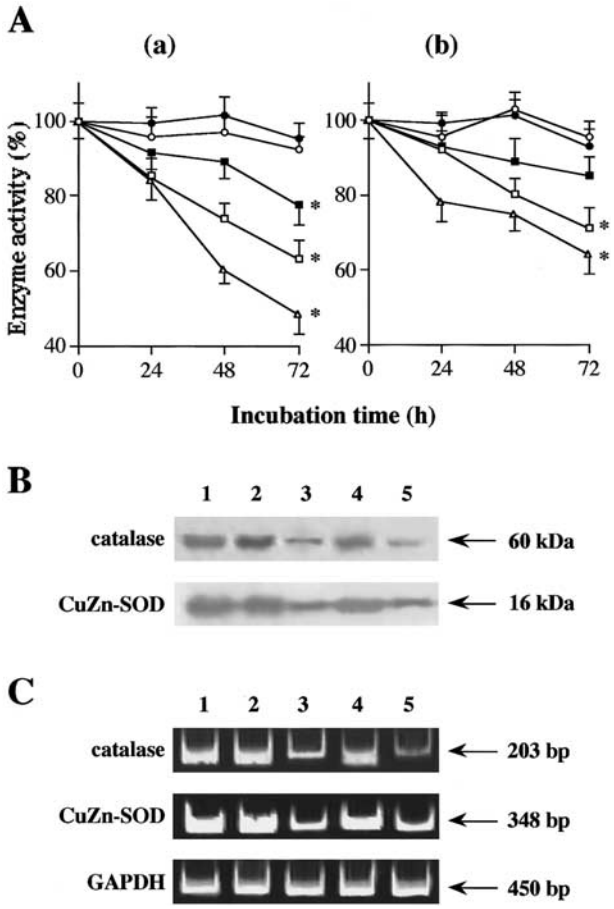

Fig 2. Regulation of catalase and CuZn-SOD in glycated HDL-treated HAECs. (A) The activities of catalase and SOD in HAECs treated with modified HDL. Each activity of catalase (a) and SOD (b) and in HAECs stimulated without (○) and with native HDL (●), ox-HDL (□), gly-HDL (■), or gly-ox-HDL (△) was determined. The activity in HAECs before lipoprotein treatment was assumed to be 100%. Values are the means ± SD of 3 determinations and are representative of 3 separate experiments. *$P < .05$ *v* untreated control. (B) Expression of catalase and CuZn-SOD protein in HAECs treated with modified HDL. Western blotting of total protein (20 μg) from the homogenate of HAECs stimulated with modified HDL was performed. The lane labels are (1) untreated control; (2) native HDL; (3) ox-HDL; (4) gly-HDL; (5) gly-ox-HDL. The size of the bands stained is indicated on the right. Both typical photographs shown are from a single representative experiment. (C) RT-PCR analysis of catalase and CuZn-SOD in HAECs treated with modified HDL. Total RNA (0.6 μg) from HAECs stimulated with modified HDL was used for determination. The lane labels are (1) untreated control; (2) native HDL; (3) ox-HDL; (4) gly-HDL; (5) gly-ox-HDL. The expected sizes of the cDNA products are indicated on the right.

We also determined the enzyme activities of both CuZn-SOD and catalase in glycated lipoprotein-treated HAECs. As shown in Fig 2A, the activity of catalase in gly-ox-HDL-treated HAECs was significantly lower than that in cells stimulated with native HDL. Stimulation of HAECs with 100 μg/mL of either gly-ox-HDL or ox-HDL for 48 hours significantly decreased the protein expressions of catalase (39% ± 7% and 53% ± 4% of untreated control, respectively) and CuZn-SOD (46% ± 5% and 53% ± 8% of the control, respectively), paralleling the reductions in activity. Although there was a slight effect on the reduced expression of catalase (83% ± 4% of untreated control) in the cells treated with gly-HDL (Fig 2B and C), densitometric analysis showed no alteration of CuZn-SOD protein in the HAECs treated for 48 hours with gly-HDL when compared to the cells exposed to native HDL.

Next, the effect of glycated lipoprotein on NO production and NOS expression in HAECs was investigated. Exposure to 100 μg/mL of modified HDL for 72 hours significantly attenuated NO_2^-/NO_3^- production by the cells, as detected with a NO analyzer (Fig 3A). Western blotting failed to detect iNOS in HAEC homogenates stimulated by the modified HDL, but a significant decrease in eNOS protein in the cells stimulated with ox-HDL (32% ± 6% of untreated control), gly-HDL (53% ± 8% of the control), and gly-ox-HDL (31% ± 9% of the control), was induced as compared with that of the control group treated with HDL (Fig 3B). Significantly decreased expression of eNOS mRNA was also detected in the HAECs treated with these modified HDLs compared to the level in untreated and native HDL-treated HAECs (Fig 3C).

To clarify the mechanism of gly-HDL–induced alterations of free radicals, we measured the levels of glycation and oxidation in gly-HDL particles. When glycation was determined by affinity chromatography with m-aminophenylboronic acid gel, treatment of HDL with glucose increased the amount of glucose-binding HDL particles in a dose-dependent and time-dependent manner (Fig 4A). Slightly fewer proteins bound to the column in the gly-HDL preparation than in the gly-ox-HDL preparation. Thus, in vitro glycation of HDL enhances glucose binding to apolipoproteins and other proteins in the particles. In fluorescent determination of the amount of AGEs in the gly-HDL particles, we found that incubation of HDL with glucose increased the level of the produced AGEs in a dose-dependent manner and almost all of the low-molecular derivatives generated were removed by sufficient dialyses with membranes with a molecular weight cut-off of 10 kd (Fig 4B). The level of the high-molecular fraction of AGE derivatives significantly increased in the gly-ox-HDL preparation after dialysis, while a very small amount of high-molecular AGEs was detected in the gly-HDL preparations.

When lipid peroxide formation was measured in gly-HDL particles, greater formation of thiobarbituric acid reactive substance (TBARS) was induced in the gly-ox-HDL than in the untreated control (Fig 5A). In contrast, the thiobarbituric acid (TBA) value in the gly-HDL preparation was unchanged. Determination of LPC amount in native HDL, ox-HDL, and gly-HDL revealed the LPC formation in the gly-ox-HDL particles to be increased in a glucose concentration-dependent manner during glycation of HDL, while untreated HDL contained only minimal amounts of LPC (Fig 5B). The amount of LPC in the gly-HDL was the same as that in the untreated control. In contrast, oxidative modification of HDL with 10 μmol/L Cu^{2+} for 16 hours induced a marked increase in the amount of LPC in the particles.

DISCUSSION

Hyperglycemia is generally accepted as the major cause of vascular complications in diabetics and a glucose-induced functional change in vascular endothelial cells may be a key

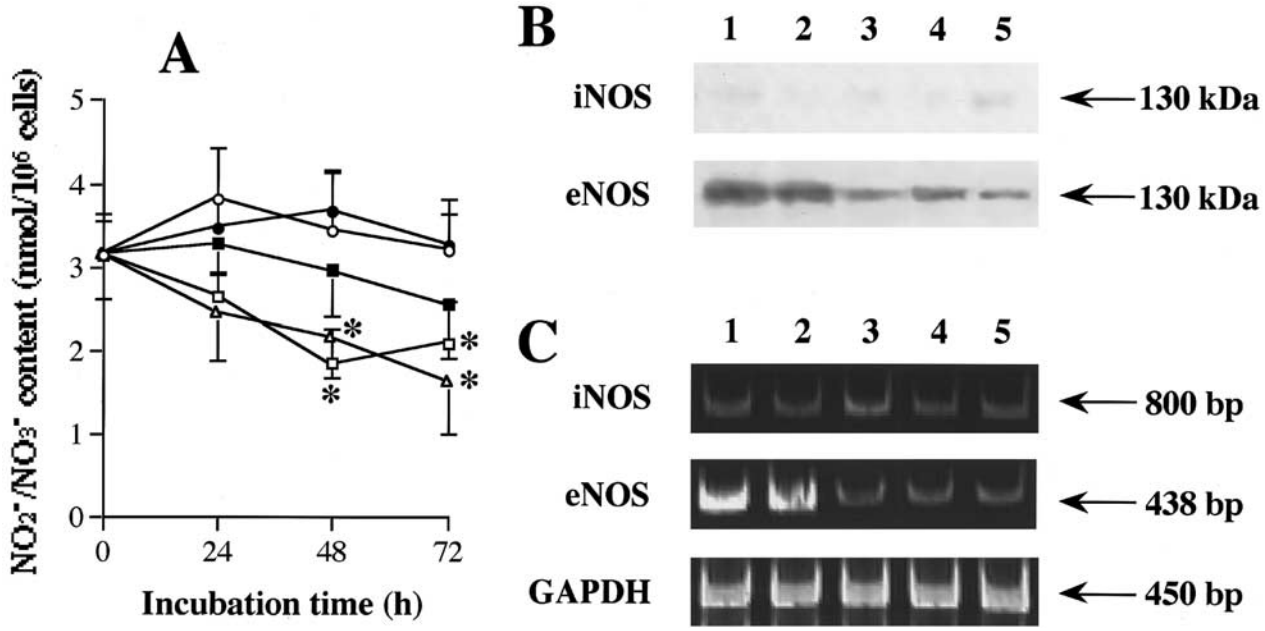

Fig 3. NO production and NOS expression in glycated HDL-treated HAECs. (A) NO_2^-/NO_3^- content in the conditioned medium of HAEC treated with modified HDL. Values are the means ± SD of 3 determinations and are representative of 3 separate experiments. ○, Untreated control; ●, native HDL; □, ox-HDL; ■, gly-HDL; △, gly-ox-HDL. *$P < .05$ *v* untreated control. (B) Expression of NOS protein in HAECs treated with modofied HDL. Western blotting of total protein (20 μg) from the homogenate of HAECs stimulated with modified HDL was performed. These 2 typical photographs are from a single representative experiment, performed independently in triplicate. The lane labels are (1) untreated control; (2) native HDL; (3) ox-HDL; (4) gly-HDL; (5) gly-ox-LDL. (C) RT-PCR analysis of NOS in HAECs treated with modified HDL. Total RNA (0.6 μg) from HAECs stimulated with modified HDL was used for determination. The expected sizes of the cDNA products are indicated on the right. The lane labels are (1) untreated control; (2) native HDL; (3) ox-HDL; (4) gly-HDL; (5) gly-ox-LDL.

event in the development of diabetic vascular complications.[32] Moreover, Lyons has suggested that increased glycation of LDL may play a role in accelerated development of atherosclerosis in diabetics.[33] The purpose of the present study was to determine whether gly-HDL alters the formation of free radicals observed in diabetics. The present study provides direct evidence that a marked upregulation of reactive oxygen species and a downregulation of reactive nitrogen species formed from HAECs are induced during exposure to glucose-treated HDL. Since lipoproteins are exposed for a long time to high glucose concentrations in the serum of hyperglycemic individuals, elevated levels of gly-HDL were suggested to be maintained in the serum for several days. In these experiments, HDLs treated with high glucose concentrations were used to observe the effects of short-term exposure and to clarify the role on endothelial functions. The findings obtained in our study indicate that HDL modified by glucose induced H_2O_2 formation from HAECs in a manner dependent on the glucose dose used for preparation. To identify a probable causal factor for the formation of reactive oxygen species in the cells, the alterations of oxidant and antioxidant enzymes in HAECs treated with gly-ox-HDL were first investigated, since the generation of free radicals is maintaind by a balance between oxidants and antioxidant defense systems. As for the potential sources of reactive oxygen species, xanthine oxidase, cyclooxygenases, eNOS,[34] and NADPH oxidase[35] are well known to be responsible for progressive production of endothelial superoxide anion. As shown in Fig 1B, H_2O_2 generation induced by gly-ox-HDL was significantly inhibited by preincubation with DPI, a selective inhibitor of NADPH oxidase[36] and eNOS.[37] However, L-NAME in excess of L-arginine and (6R)-5,6,7,8-tetrahydrobiopterin (TB_4) did not reduce H_2O_2 formation by gly-ox-HDL, suggesting that eNOS may not be included in H_2O_2 generation from HAECs treated with gly-ox-HDL. As the levels of allopurinol and aspirin used in the present study had been already shown to completely block xanthine oxidase activity[38] and arachidonic acid metabolism by cyclooxygenase,[9] the results of the present study indicate the generation of H_2O_2 induced by gly-ox-HDL not to be related to the activity of these enzymes. Our analytical Western blot and RT-PCR data showed that a slight upregulation of the protein and mRNA of p22 phox, a subunit of NADPH oxidase, in HAECs is induced by treatment with gly-ox-HDL. In contrast, gly-ox-HDL did not interfere with the expressions of xanthine oxidase and cyclooxygenases in the cells (unpublished data). These observations suggest that H_2O_2 formation by glucose-treated HDL may be mainly mediated by the activity of NADPH oxidase in HAECs. The removal of toxic oxygen metabolites is generally regarded as a putative function of antioxidant enzymes such as CuZn-SOD and catalase. We hypothesized that accumulation of oxygen free radicals due to reduced antioxidative enzymes is related to dysfunctional states exhibited by the endothelium after exposure to gly-HDL. In order to test this hypothesis, we investigated whether gly-HDL regulates the expression and activity of antioxidant enzymes in HAECs. Incubation with gly-ox-HDL significantly diminished the expression of both the mRNA and the protein of catalase, and similar data were also obtained in regard to the activity of the enzyme in Fig 2. The data we

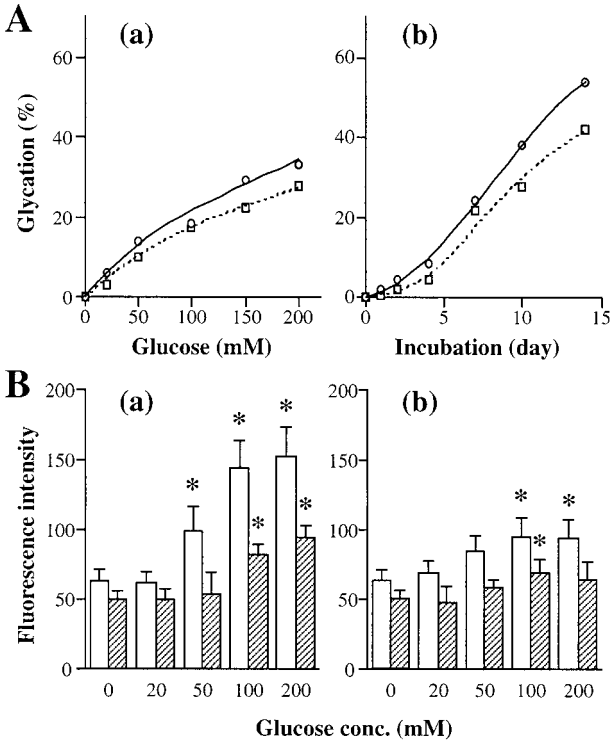

Fig 4. Determination of glycated products in gly-ox-HDL particles. (A) Dose response (a) and time course (b) curves of glucose binding to HDL. The figure shows the content of proteins bound tightly to m-aminophenylboronic acid gel in HDL glycated with 200 mmol/L glucose in the absence (○) or presence (□) of 2 mmol/L EDTA by estimates of the peak area. Each point is the mean of three determinations in 2 independent experiments. (B) AGEs in glycated HDL preparations. AGEs in HDL glycated with 200 mmol/L glucose in the absence (a) and presence (b) of 2 mmol/L EDTA were quantitated by measuring the fluorescence intensity of solutions at 440 nm with excitation at 370 nm. A 1-mg sample of modified HDL preparations was assayed before (□) or after (▨) sufficient dialysis against PBS. Each bar is the mean ± SD of 4 determinations in 2 independent experiments. *Significant difference from the untreated control, $P < .05$.

obtained demonstrate that treatment with gly-HDL may produce either a superoxide anion, or its secondary product, H_2O_2, which then disrupts the balance between oxidant and antioxidant enzymes, such as NADPH oxidase, catalase, and CuZn-SOD. As it was previously reported that glycated LDL exhibits a cytotoxic effect on cultured vascular cells through free radical release,[33] and gly-HDL induces cellular apoptosis,[40] the formation of reactive oxygen species based on the disruption of these oxidant-related enzymes may participate in the mechanism underlying gly-HDL–induced apoptosis of HAECs.

Glycated LDL reportedly enhances NO inactivation in endothelial cells.[22] Moreover, previous reports have indicated that atherogenic ox-LDL can produce endothelial-dependent vasomotor abnormalities via inhibition of endothelial-derived NO.[41] In the present study, we found that gly-ox-HDL attenuates expression of mRNA and the protein of eNOS, but not iNOS, as shown by analysis of data from both RT-PCR and Western blot (Fig 3), and that this is followed by reduced production of NO in cultured endothelial cells, demonstrating that decreased production of NO from endothelial cells induced by gly-HDL is mediated by downregulation of eNOS, but not iNOS. Endothelial cells reportedly fail to protect themselves against apoptosis by decreasing production of eNOS-induced NO.[41,42] As demonstrated in these experiments, the eliminated release of eNOS-derived NO from HAECs stimulated with gly-HDL is apparently associated with endothelial dysfunction leading to cellular apoptosis. The differing alterations in oxygen and nitrogen radicals generated from gly-HDL–treated HAECs, as indicated

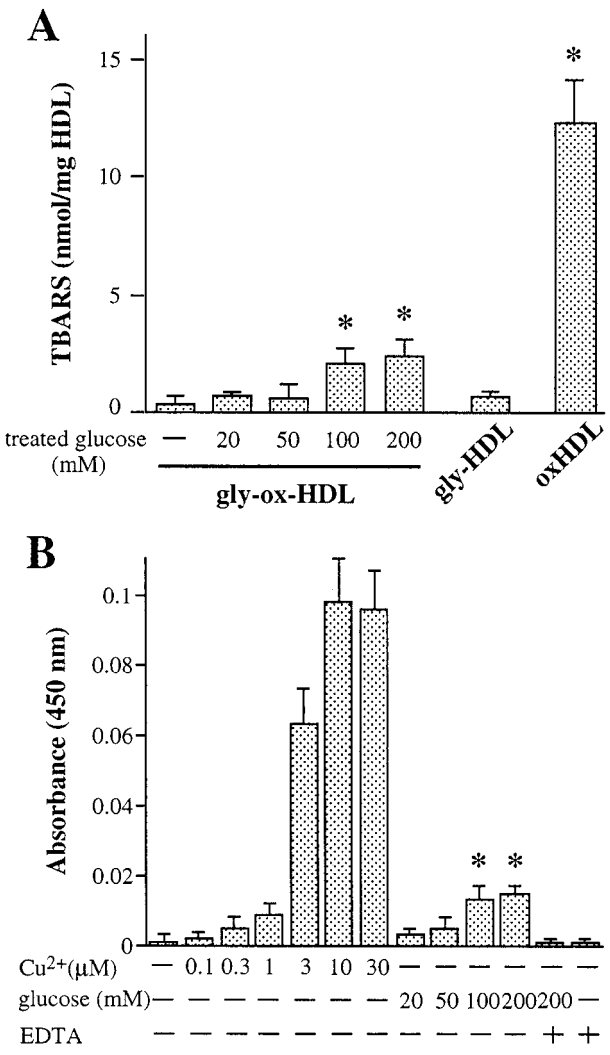

Fig 5. Determination of oxidized products in gly-ox-HDL particles. (A) TBARS levels in HDL preparations treated with glucose. HDL glycated with the concentration of glucose indicated in the absence or presence of 2 mmol/L EDTA for 7 days was assayed. Each bar represents the mean ± SD of 3 determinations, and is representative of 3 separate experiments. (B) LPC levels in HDL preparations treated with glucose. HDL glycated with the concentration of glucose indicated in the absence or presence of 2 mmol/L EDTA for 7 days was assayed. HDL incubated for 16 hours with the concentration of $CuSO_4$ indicated was measured as a positive control. Each bar displays the mean ± SD of 3 determinations in 3 separate experiments. Formation of TBARS and LPC in the particles of the modified HDL was measured by standardization to protein content. *Significant difference from the control, $P < .05$.

by the gly-ox-HDL data, are thought to be a problem requiring prompt resolution.

To identify the causal factor in the gly-HDL particles, levels of both glycated and oxidized products formed by treatment of HDL particles with glucose were measured. It is widely known that oxidative cleavage and the following AGE generation of glucose-bound protein are suppressed by both the chelation of trace metal ions by EDTA and the presence of an oxidation inhibitor, such as 2,6-di-t-butyl-4-methylphenol or t-butyl hydroxyanisole. Therefore, EDTA, which has little effect on the stereochemical structures of lipoproteins, was used for preparation of glycated non-oxidized HDL. As shown in Fig 4, the amounts of glycated products in the gly-ox-HDL and gly-HDL particles increased in a manner dependent on the dose and timing of glucose treatment, demonstrating that HDL containing glucose-bound proteins is produced by incubation with glucose despite the presence of EDTA. The nonenzymatic glycation of amino groups by reducing sugars through the Maillard reaction culminates in the formation of AGEs. In vivo, AGEs accumulate with aging, and at an accelerated rate in diabetes.[43] Furthermore, AGEs have been widely implicated as triggers in structural and functional aspects of the vascular complications of diabetes.[9,44] We therefore attempted to fluorometrically measure the amounts of AGE produced in gly-HDL preparations. When HDL solution was incubated in the presence of glucose, the levels of AGE derivatives in the HDL particles increased in a dose-dependent manner of glucose. In the gly-HDL group, lower production of AGEs was induced as compared to gly-ox-HDL, and this may be due to inhibition of the Maillard reaction by a chelating trace metal ion in glucose solution with EDTA. Since previous report showed that low-molecular AGEs induce the generation of reactive oxygen species from endothelial cells via its binding to RAGE,[45] we investigated whether high-molecular fraction removed the low-molecular AGEs with sufficient dialysis alters the production of free radicals from HAECs. The gly-HDL products, which possess a few of the high-molecular AGEs within the particles after dialysis, reduced the expressions of catalase and NOSs in HAECs, but had little effect on either CuZn-SOD or NADPH oxidase (Figs 2 and 3). The data suggest that both glucose binding and the additional formation of AGEs may be at least partially attributed to altered generations of oxygen free radicals. Recent report showed that oxygen radicals such as H_2O_2 can stimulate glycation of proteins.[46] This fact demonstrates that H_2O_2 secreted from HAEC during the exposure with gly-HDL, as shown in Fig 1, may induce an additional glycation of the HDL particle and the consequent deteriorated HDL may cause dysfunction of HAEC via increased generation of oxygen radicals.

It was previously reported that glycation of HDL increases its susceptibility to oxidation through reduced paraoxonase activity by glucose exposure,[47] and LPC, which is formed during oxidative modification of lipoproteins, stimulates superoxide anion production in endothelial cells through a NADH/NADPH oxidase-dependent mechanism.[48] We therefore measured the formation of both lipid peroxides and LPC after preparation of gly-HDL. The formations of both TBARS and LPC were enhanced in the gly-ox-HDL group prepared by treatment with 200 mmol/L glucose for 7 days, as shown in Fig 5. Although superoxide anion formation is induced during conversion of Amadori compounds to AGEs in the Maillard reaction, this result indicated long-term glucose exposure to result in the formation of oxidative products, such as LPC, in HDL particles. To clarify the role of oxidation in gly-ox-HDL-induced alterations of endothelial function, HDL treated with 10 μmol/L $CuSO_4$ for 16 h (ox-HDL) was used in all experiments. The result showed that changes detected in the incubation of HAECs with ox-HDL were similar to those with gly-ox-HDL in the experiments shown in Figs 1 through 3. This raises the possibility that enhanced productions of lipid peroxide and LPC may play a key role in the altered formation of free radicals from endothelial cells by gly-HDL. Although a slight reduction of catalase was seen in the group of HAECs treated with gly-HDL, in disagreement with those exposed to ox-HDL and gly-ox-HDL, the altered expression of catalase caused by each of these HDLs may be explained by the binding of gly-HDL and ox-HDL to endothelial cells via different receptors. Thus, these findings indicate that the generations of free radicals from endothelial cells by gly-HDL stimulation may be potentiated by additional oxidative modification, and the effect of gly-ox-HDL may be due to synergism between the glycation and oxidation in HDL particles.

In conclusion, the data obtained in the present study indicate that exposure of human vascular endothelial cells to gly-HDL downregulates NO production and upregulates free-oxygen radical production, especially the production of superoxide anions and H_2O_2, through the reduced expression of antioxidant enzymes, and that alterations of these free radicals may participate in the pathobiological mechanism underlying glycated lipoprotein-induced endothelial dysfunction. These findings suggest that HDL treated with glucose modifies both glucose binding to the particle and additional oxidation, and that oxidized particles including some LPC may be associated with gly-HDL–induced alterations of free radicals from HAECs.

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