

Glycated Low-Density Lipoprotein Attenuates Shear Stress–Induced Nitric Oxide Synthesis by Inhibition of Shear Stress–Activated L-Arginine Uptake in Endothelial Cells

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Little is known about the mechanism(s) of endothelial dysfunction in diabetes. In this study, the effect of nonenzymatic glycated LDL, a phenomenon induced by elevated D-glucose levels associated with diabetes, on porcine aortic endothelial cells was investigated. Two fractions of LDL from diabetic patients were separated by affinity column chromatography and are referred to herein as fraction α (nonglycated LDL) and fraction β (glycated LDL). Incubation of endothelial cells for 24 h with total LDL isolated from diabetic subjects (dLDL) increased the release of superoxide anions ($\cdot\text{O}_2^-$) by fivefold, while no effect of LDL isolated from healthy individuals (nLDL) was found. Fraction β , but not fraction α , evoked the $\cdot\text{O}_2^-$ release. In vitro–glycated LDL mimicked the effect of dLDL/fraction β on $\cdot\text{O}_2^-$ release that correlated with its degree of glycation ($R^2 = 0.96$). Moreover, nitric oxide (NO) stability (measured with a porphyrinic-based electrode) and NO bioactivity (measured by its ability to elevate cellular cGMP levels) were reduced in cells treated with dLDL by 46 and 41%, respectively. dLDL (but not nLDL or fraction α) abolished shear stress–induced L-arginine uptake. The inhibitory effect of dLDL on shear stress–induced L-arginine uptake was mimicked by in vitro–glycated LDL. The efficiency of in vitro–glycated LDL to diminish shear stress–evoked L-arginine uptake correlated with the extent of glycation ($R^2 = 0.88$). Moreover, dLDL, but not nLDL or fraction α , reduced shear stress–mediated cGMP formation and NO_x production by 47 and 88%, respectively. This effect was also mimicked by in vitro–glycated LDL, correlating with its degree of glycation ($R^2 = 0.86$). Under these experimental conditions, glycated LDL reduced shear stress–induced increase in NO synthesis by inhibition of shear stress–stimulated L-arginine uptake and NO bioactivity due to increased endothelial cell $\cdot\text{O}_2^-$ release.

These properties may contribute to the reduced vasodilatory response and the vascular complications in diabetes. *Diabetes* 48:1331–1337, 1999

An attenuated endothelium-dependent relaxation has been reported in aortas from various diabetic animals, including alloxan-induced diabetic rabbits (1) and genetically (2) and streptozotocin-induced diabetic rats (3). The exact mechanisms underlying this decreased vasodilatory response of the endothelium is unclear. Current hypotheses include enhanced release of constricting prostanoids (1), decreased bioactivity of nitric oxide (NO) (4) due to increased superoxide anion ($\cdot\text{O}_2^-$) release during high D-glucose exposure (5), and attenuated formation of NO (6,7). Most in vitro studies are focused on hyperglycemia as the main cause of endothelial dysfunction, while the effect of glycated LDL on endothelial $\cdot\text{O}_2^-$ release and shear stress response has not been investigated so far. Based on its high susceptibility for oxidation (8) and its altered cell binding (9), metabolism, and half-life (10), glycated LDL has been suggested to contribute to atherogenesis (11,12). However, in vitro–glycated LDL was used in most studies, and no direct comparison of the biological activities of in vivo– versus in vitro–glycated LDL on endothelial cells has been reported.

In addition to the mechanisms suggested to be involved in vascular dysfunction during diabetes mentioned above, reduced L-arginine utilization/uptake for NO production in diabetes has been suggested in endothelial cells (13). Interestingly, supplementation of L-arginine restored endothelial vascular function (7,13). Little is known about the mechanisms and the mediators resulting in the reduction of L-arginine to serve as substrate for NO production in diabetes. However, oxidized LDL, which is another form of modified LDL and which shares many characteristics with glycated LDL (11), has been shown to inhibit L-arginine uptake in platelets (14). Although the hypothesis that a reduced L-arginine uptake is responsible for the diminished NO production in diabetes is attractive, it is in contrast with data that suggest that endothelial cells do not depend on extracellular L-arginine for autacoid-induced NO production (15). Recently, we have shown that shear stress–activated L-arginine uptake (γ^+ system) is essential for NO production in response to shear

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AGE, advanced glycation end product; dLDL, LDL isolated from diabetic subjects; DMEM, Dulbecco's modified Eagle's medium; HBS, HEPES-buffered solution; nLDL, LDL isolated from healthy subjects; mab, monoclonal antibody; PBS, phosphate-buffered solution; SCBH, sodium cyanoborohydride; SOD, superoxide dismutase.

stress (16). The consequences of an exposure to glycated LDL on shear stress-mediated endothelial response have not been investigated so far.

Therefore, this study was designed to investigate the effect of in vivo-glycated LDL on endothelial $\cdot\text{O}_2^-$ production, NO stability and bioactivity, and shear stress response. In addition, the fraction responsible for the reported effects of diabetic LDL on endothelial function was characterized. Finally, these data were compared with those obtained from in vitro-glycated LDL to test whether artificial LDL glycation provides a suitable model for studying the consequences of LDL glycation on the biologic activity of in vivo glycated LDL.

RESEARCH DESIGN AND METHODS

Materials. Cell culture chemicals were obtained from Life Technologies (Vienna). Fetal calf serum was purchased from PAA (Linz, Austria). All other chemicals were from Sigma (Vienna). Petri dishes were from Iwaki (Vienna); six-well plates were obtained from Falcone (Linz, Austria). L -[^3H]arginine HCL was from Amersham (Vienna).

Preparation of LDL. LDL was prepared from the fasting serum of healthy male volunteers aged between 25 and 40 years (control LDL). In addition, LDL was isolated from the fasting serum of diabetic patients with normal levels of triglycerides (<150 mg/dl) and LDL (<165 mg/dl calculated as LDL cholesterol). The mean blood D-glucose concentration in the diabetic group was 177.9 ± 43.8 mg/dl ($n = 30$); healthy control subjects: 93.9 ± 12.9 mg/dl, $n = 33$; $P < 0.05$ vs. diabetic group). The HbA_{1c} of the diabetic group was significantly higher than that of control subjects (8.28 ± 1.31 vs. $5.36 \pm 0.43\%$; $P < 0.05$). BMIs from control and diabetic donors were not different (control subjects: 24.8 ± 4.5 kg/m 2 ; diabetic subjects: 28.6 ± 6.4 kg/m 2 ; NS). Isolation of LDL was performed as described previously (17). Floating LDL was recentrifuged in a linear density gradient from 1.200 to 1.020 g/ml. To prevent oxidation, all procedures were carried out under nitrogen and in the presence of 1 mg/ml of EDTA. After LDL isolation, 3 ml of the LDL-containing solution was applied to a Sephadex G-25M (10 ml; Pharmacia Biotech, Vienna) column and eluted by ammonium acetate buffer (in millimoles per liter: 250 ammonium acetate, 500 NaCl, 50 MgCl $_2$, and 0.05% NaN $_3$, pH 8.5) to remove buffer salts. The final concentration of LDL in the eluate was 2–3 mg/ml total cholesterol.

In vitro glycation of LDL. In vitro glycation of LDL was performed according to the method of Duell et al. (18). Freshly isolated LDL was diluted to a concentration of 1 mg/ml total cholesterol with phosphate-buffered solution (PBS) (in millimoles per liter: 137 NaCl, 2.7 KCl, 8 Na $_2$ HPO $_4$, and 1.5 KH $_2$ PO $_4$, pH 7.4) containing 1 mg/ml EDTA, 25 $\mu\text{mol/l}$ butylhydroxytoluene, and 50 $\mu\text{mol/l}$ diethylenetriaminepentaacetic acid. Various concentrations of D-glucose (0.5–44 mmol/l) and sodium cyanoborohydride (SCBH) (12 mg/ml) were added. This solution was sterile filtered, covered with nitrogen, sealed, and stored for 5 days at 37°C in the dark. After glycation, the solutions were dialyzed under nitrogen against PBS containing 0.01% EDTA for 2 h at 20°C (20 ml against 4 liters; dilution 1:200), followed by 40 h at 4°C (20 ml against 4 liters; dilution 1:200).

Separation of diabetic LDL in glycated and nonglycated LDL fractions. To separate the glycated LDL from the nonglycated LDL, 5 ml of freshly isolated LDL solution was applied to a 2-ml Glyko-Gel II B column (m-aminophenyl-boronate equilibrated in 0.5% acetic acid; Pierce, Vienna). The nonglycated LDL (fraction α) was eluted with 12 ml of ammonium acetate buffer, followed by a 20-ml wash-out. The glycated LDL (fraction β) was eluted with 10 ml sorbitol buffer containing (in millimoles per liter) 200 sorbitol, 500 NaCl, 100 Tris, 25 EDTA, and 0.05% NaN $_3$ (pH 8.5). Both fractions were dialyzed under nitrogen against PBS containing 0.01% EDTA for 2 h at room temperature (20 ml against 4 liters; dilution 1:200), followed by 40 h at 4°C (20 ml against 4 liters; dilution 1:200).

Measurement of LDL glycation. The degree of glycation was determined according to the method of Duell et al. (18). After glycation, 200 μl of LDL solution were mixed with 200 μl NaHCO $_3$ (4%) and 200 μl of trinitrobenzoic acid solution (0.1%) (final pH 7.1–7.2), covered with nitrogen, sealed, and incubated at 37°C in the dark. After 2 h, the degree of glycation was photometrically measured at 340 nm against a mixture of LDL and NaHCO $_3$ in PBS (in millimoles per liter: 137 NaCl, 2.7 KCl, 8 Na $_2$ HPO $_4$, 1.5 KH $_2$ PO $_4$, and 0.1 EGTA, pH adjusted at 7.4). Glycation of LDL is given as relative reduction of the detected free ϵ -amino groups of L-lysine compared with LDL treated identical to the samples but in the absence of D-glucose.

Control of LDL oxidation. Any possible oxidative modification of LDL was tested by means of its reaction with a monoclonal antibody (mab OB/04), raised against copper-oxidized LDL (19), in a sandwich fluorescence immunoassay as described recently (20) and by estimation of the lipid hydroperoxides with a method developed in this institute. Affinity of mab OB/04 did not differ between the LDL isolated from diabetic patients (dLDL) and that of control subjects (nLDL) (Fig. 2B). Hence, the in vitro glycation of LDL did not increase the affini-

ty of the mab OB/04 to the LDL samples (Fig. 2B). Furthermore, the concentrations of lipid hydroperoxides in LDL in the absence of D-glucose (<1 nmol/mg protein) did not differ from those measured in LDL treated in the presence of 22 and 44 mmol/l D-glucose, while Cu $^{2+}$ increased lipid hydroperoxides in LDL to 130.0 ± 3.6 nmol/mg protein after 4 h (data not shown).

Cell isolation and cell culture. Endothelial cells from porcine aortas were isolated as described previously (21). Briefly, aortas were prepared in the slaughterhouse, put in a sterile bag and immediately transported to the laboratory. In the laboratory, vessels were fixed in an incubation chamber, washed twice, and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 294 U/ml collagenase plus (in milligrams per milliliter): 2 bovine serum albumin, 1 trypsin inhibitor (soybean), and 0.4 DNAase I. After 30–40 min at 37°C, the cell suspension was centrifuged and resuspended in Opti/MEM containing 3% fetal calf serum for culture. After 5 days, the primary culture was split 1:4 for final culture. All experiments were performed with cells from primary culture and first passage within 10 days after isolation. Cell culture was $<99\%$ pure, tested by the typical cobblestone morphology and the lack of immunofluorescence detection of smooth muscle cells (α -actin).

Pretreatment with LDL or glycated LDL. Confluent cells were washed twice and were incubated 24 h before the experiment with serum-free DMEM containing the LDL as indicated. During this exposure time, no LDL-mediated cell cytotoxicity was found by measuring cell size, lactate dehydrogenase release, or trypan blue resistance.

Shear stress activation. Shear stress activation was performed as previously described (16). For shear stress experiments, HEPES-buffered solution (HBS) (in millimoles per liter: 145 NaCl, 5 KCl, 10 HEPES acid, and 2.5 CaCl $_2$, pH 7.4), containing 10 mg/ml bovine serum albumin to enhance viscosity, was used. Shear stress was performed on an orbital shaker for 45 min at 37°C (22). This technique induces similar changes in the alignment and the shape of the cells and NO production as that described with the cone-plate viscometer (22). The mean shear stress (F) to which the cells were exposed was calculated according to $F = \eta S 2\pi r f / \Delta h$, where η refers to the dynamic viscosity, S to the area of the well, f to the frequency, r to the radius of each dish, and h to the height of liquid. Because of limitations in the technique used, shear stress cannot be calculated properly for the entire dish. Thus, shear stress was calculated for cells at the outer edge of the dish, and was ~ 33 dyn/cm 2 .

L-Arginine uptake. Cells were grown in six-well plastic dishes. After reaching confluence, cells were preincubated with various LDL fractions described above. Before the experiment, cells were washed three times with HBS. After an exposure of the cell to shear stress (33 dym/cm 2) in HBS containing 2.5 mmol/l CaCl $_2$ for 45 min at 37°C, incubation medium was aspirated and HBS containing 2.5 mmol/l CaCl $_2$ and a mixture of L-arginine/L-[^3H]arginine was added. After 10 min, experiments were terminated by removing the incubation buffer and adding 1 ml 0.01 mol/l HCl. Cells were placed in the refrigerator for 2 h, and radioactivity of the supernatant was measured by liquid scintillation counting. Net L-arginine uptake was calculated by a subtraction of nonspecific L-[^3H]arginine binding at time-point zero from the measured L-arginine concentration in the cell supernatant.

Measurement of $\cdot\text{O}_2^-$ release. Release of $\cdot\text{O}_2^-$ was determined as the reduction of ferricytochrome c (23), as described previously (5,24). Briefly, cultured endothelial cells grown on petri dishes (diameter 10 cm) were washed twice and incubated for 24 h in DMEM containing the LDL indicated. Before the experiments, cells were washed and incubated in PBS (in millimoles per liter) 137 NaCl, 2.7 KCl, 8 Na $_2$ HPO $_4$, 1.5 KH $_2$ PO $_4$, and 0.1 EGTA, pH adjusted at 7.4) containing 10 $\mu\text{mol/l}$ ferricytochrome c in the absence or presence of 500 U/ml superoxide

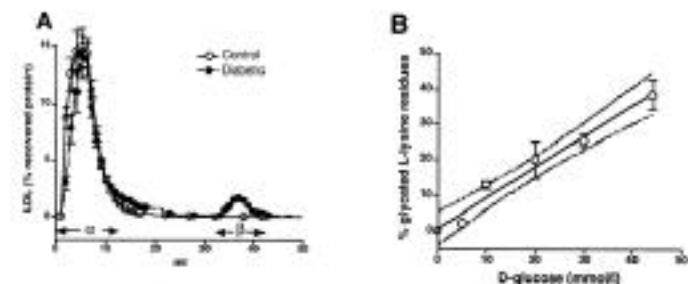


FIG. 1. Separation of LDL fractions using affinity column chromatography (A) and in vitro glycation of LDL (B). A: LDL freshly isolated from control and diabetic subjects was separated using a Glyko-Gel II B column as described in METHODS. In regard to the retention time, three fractions of LDL were found. B: Glycation of LDL (1 mg/ml total cholesterol) in the presence of SCBH after 6 days in buffer containing the concentration of D-glucose indicated ($n = 16$ –27).

dismutase (SOD). After 30 min, the reduction of ferricytochrome c was measured photometrically at 550 nm. The difference in absorption between samples in the absence and the presence of SOD was used to calculate the SOD-sensitive reduction of ferricytochrome c. Concentrations of $\cdot\text{O}_2^-$ were calculated using the molar extinction coefficient, ϵ , of the reduced form of ferricytochrome c ($\epsilon = 21,000$) (25). Neither dLDL nor in vitro-glycated LDL affected the detection of $\cdot\text{O}_2^-$ derived from the xanthine oxidase/hypoxanthine reaction in cell free experiments.

Measurement of NO synthesis. Formation of NO was monitored by measuring the synthesis of endothelial cGMP, which serves as a biological marker for NO production (26). In HBS containing 2.5 mmol/l CaCl_2 , 0.3 mmol/l L-arginine, and 1 mmol/l 3-isobutyl-1-methylxanthine (to avoid phosphodiesterase activity), cells were stimulated by shear stress. After 45 min of shear stress exposure, experimental buffer was aspirated and 1 ml 0.01 mmol/l HCl was added for cell lysis. Cellular cGMP content was measured using a radioimmunoassay (5).

Determination of NO bioactivity. Bioactivity of NO was measured by increases in endothelial cGMP formation in response to the addition of extracellularly applied NO. Cultured cells were treated as indicated and washed twice. Experiments were performed in 2.5 mmol/l CaCl_2 -containing HBS, 1 mmol/l 3-isobutyl-1-methylxanthine, and 100 $\mu\text{mol/l}$ N^ω -nitro-L-arginine (to prevent cellular NO production). To each dish, 1 $\mu\text{mol/ml}$ NO was added during constant shaking. After an incubation time of 4 min, experimental buffer was aspirated, and 1 ml 0.01 mol/l HCl was added for cell lysis. Endothelial cGMP content in response to NO application was measured as described above. 3-isobutyl-1-methylxanthine had no effect on glycated LDL-induced changes assessed by every other technique used.

NO degradation assay. Degradation of NO was determined using a porphyrinic-based NO-sensitive electrode (ISO-NO; WPI, Berlin) as previously described (4). In brief, after an incubation period of 24 h in serum-free DMEM containing the LDL type as indicated, cells were harvested by 1-min incubation with trypsin (0.02% in PBS containing 1 mmol/l EGTA), centrifuged, resuspended in 2 ml HBS (5×10^6 cells/ml), and transferred into a thermostatically controlled mixed cuvette (37°C). After an equilibration time of 3 min, 2 μmol NO were added from a saturated NO solution, and degradation of NO was monitored using a porphyrinic-based NO-sensitive electrode as previously described (5). Data represent NO detectable 20 s after NO application in percent of that found in cells without preincubation with a lipoprotein.

NO_x determination. Experiments were performed in DMEM-like solution containing the L-arginine concentration indicated (0.3 or 10.0 mmol/l). Briefly, the cells were exposed for 3 h at 0 or 33 dyn/cm^2 . The 1-ml samples were lyophilized for concentration and dissolved in 250 μl water. The stable oxidation products of NO nitrite/nitrate were measured after nitrate reductase treatment with the Griess reagent described by Kuchan and Frangos (27). NO_x was quantified by comparing absorbance of samples at 540 nm with standards.

Statistics. Group data represent mean values \pm SE. Statistical significance was evaluated with a one- or two-way analysis of variance including Scheffe's post hoc analysis. Level of significance was defined as $P < 0.05$ in all experiments.

RESULTS

Glycation of LDL

In vivo-glycated LDL. LDL isolated from control (native LDL [nLDL]) and diabetic subjects (diabetic LDL [dLDL]) were fractionated by glucose-affinity column chromatography (Glyko-Gel II B). As shown in Fig. 1A, two major fractions of

the LDL were separated in regard to the retention time. In diabetic subjects, there was a significant increase in protein found in fraction β (control subjects: $0.3 \pm 0.1\%$ of the recovered apolipoprotein B protein; diabetic subjects: $9.8 \pm 0.2\%$ of the recovered apolipoprotein B protein, $n = 18$, $P < 0.05$ vs. control group).

In vitro-glycated LDL. Pretreatment of freshly isolated LDL with various D-glucose concentrations for 6 days in the presence of SCBH achieved glycation of the LDL (Fig. 1B), while no oxidation of the LDL particle was observed (Fig. 2). The correlation of glycation with the D-glucose concentration present during the procedure followed a linear correlation (% glycation = $0.86 \times \text{D-glucose [in millimoles per liter]} + 0.76$; $R^2 = 0.97$). In vitro-glycated LDL used for this study is defined by the concentration of D-glucose present during the glycation procedure.

Release of superoxide anion. Incubation of endothelial cells with nLDL for 24 h slightly increased $\cdot\text{O}_2^-$ production (Fig. 3A). In contrast, in cells preincubated with dLDL, the release of $\cdot\text{O}_2^-$ was increased by 397% (Fig. 3A). In addition, endothelial cells were incubated for 24 h with fraction α or fraction β of the dLDL. As shown in Fig. 3A, treatment of endothelial cells with fraction α did not affect $\cdot\text{O}_2^-$ release, while a preincubation with fraction β augmented endothelial $\cdot\text{O}_2^-$ release by 361%.

In agreement with these results obtained with glycated dLDL, an incubation of endothelial cells with in vitro-glycated LDL for 24 h increased $\cdot\text{O}_2^-$ production (Fig. 3B). The effect of the in vitro-glycated LDL to increase $\cdot\text{O}_2^-$ release correlated with its glycation ($\cdot\text{O}_2^-$ [in nanomoles per 30 min $\times 10^6$ cells] = $4.39 + 2.19 \times [\text{percent glycation of LDL}] - 0.03 \times [\text{percent glycation of LDL}]^2$; $R^2 = 0.96$).

Cell-mediated degradation of NO and NO bioactivity. To test whether the increased $\cdot\text{O}_2^-$ release from cells treated with glycated LDL might affect stability of extracellular NO, the degradation of extracellularly applied NO was measured after 24-h incubation using a NO-selective electrode. Preincubation of cultured endothelial cells with nLDL or the nonglycated fraction of LDL from diabetic patients (fraction α) slightly reduced detection of extracellularly applied NO by 10.4 and 11.7%, respectively ($n = 6$; NS vs. control) (Fig. 4A). In contrast, in front of cells treated with dLDL, the detection

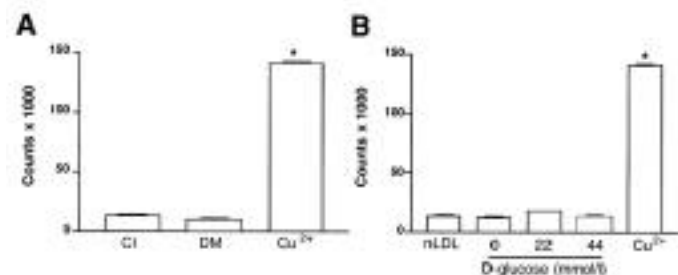


FIG. 2. Measurement of the degree of oxidation of LDL from control individuals (CI) and diabetic patients (DM) (A) and after the in vitro glycation procedure in the absence (0) or presence of 22 and 44 mmol/l D-glucose (B), using a monoclonal antibody against oxidized LDL (OB/04) (19). For comparison, affinity of mab OB/04 against LDL without any treatment (nLDL) and after 180-min oxidation with 30 $\mu\text{mol/l}$ Cu^{2+} is shown. Columns represent the means \pm SE. A: $n = 3$; B: $n = 4$. * $P < 0.05$ vs. LDL from CI and DM (A) and nontreated LDL (B).

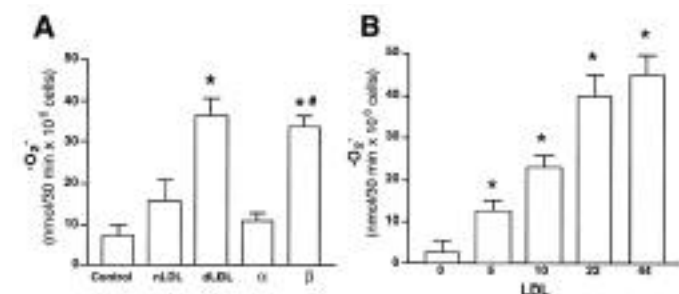


FIG. 3. Release of superoxide anions from cultured endothelial cells incubated with in vivo- (A) and in vitro- (B) glycated LDL. A: Cultured endothelial cells were incubated for 24 h in DMEM (control) or DMEM containing either 100 $\mu\text{g/ml}$ total cholesterol nLDL, dLDL, or nonglycated LDL (fraction α) or 20 $\mu\text{g/ml}$ total cholesterol glycated LDL (fraction β). * $P < 0.05$ vs. control and nLDL-treated cells, # $P < 0.05$ vs. cells treated with fraction α ($n = 8-12$). B: Endothelial cells were incubated for 24 h in DMEM containing 100 $\mu\text{g/ml}$ (total cholesterol) LDL after in vitro glycation procedure in the presence of the D-glucose concentration indicated. * $P < 0.05$ vs. cells incubated with LDL after in vitro glycation procedure in the absence of D-glucose ($n = 6$).

of applied NO was reduced by 46.4% ($n = 6$; $P < 0.05$ vs. control, nLDL, and fraction α) (Fig. 4A). In the presence of 300 U/ml SOD, no further enhanced degradation of extracellularly applied NO by dLDL-treated cells was found ($142 \pm 14.3\%$ in control; 139 ± 10.6 in nLDL-treated cells, and $128 \pm 11.7\%$ compared with the control in the absence of SOD; $n = 3$, NS).

In agreement with these findings, a treatment with dLDL reduced the bioactivity of NO, monitored by its potency to elevate endothelial cGMP concentration when applied extracellularly, by 40.6% ($n = 8$; $P < 0.05$ vs. control, nLDL, and fraction α) (Fig. 4B). In contrast, treatment of endothelial cells with nLDL or fraction α did not affect the bioactivity of NO compared with cells without treatment with LDL. An incubation of endothelial cells for 24 h with fraction β reduced the bioactivity of NO by 68.3% ($n = 4$; $P < 0.05$ vs. control, nLDL, and fraction α) (Fig. 4B). In the presence of 300 U/ml SOD, endothelial cGMP formation in response to extracellular NO did not differ between control, nLDL-, and dLDL-treated cells (control: 21.6 ± 3.1 pmol cGMP/ 10^6 cells, nLDL: 16.7 ± 4.3 pmol cGMP/ 10^6 cells, and dLDL: 18.6 ± 2.1 pmol cGMP/ 10^6 cells; $n = 3$; NS vs. control).

Shear stress-induced L-arginine uptake

Basal L-arginine uptake. Uptake of L-arginine in resting endothelial cells remained unchanged after treatment with nLDL and fraction α from dLDL (Fig. 5A). Hence, an incubation of endothelial cells with dLDL (Fig. 5A) or with in vitro-glycated LDL of various amounts of glycation (Fig. 5B) also failed to affect L-arginine in resting cells.

Shear stress-induced L-arginine uptake. We have previously shown that shear stress selectively activates uptake of L-arginine via the y^+ system (21). Interestingly, dLDL diminished shear stress-induced L-arginine uptake by 92.4%, while neither nLDL nor fraction α from diabetic patients affected shear stress-induced L-arginine uptake (Fig. 6A). In agreement with the finding that dLDL abolished shear stress-mediated L-arginine uptake, in vitro-glycated LDL also diminished shear stress-induced L-arginine uptake (Fig. 6B). This effect correlated with the degree of glycation of the LDL (L-arginine uptake [in nanomoles per 10^6 cells every 10 min] = $0.23 -$

$0.012 \times [\text{percent glycation of LDL}] + 0.0001 \times [\text{percent glycation of LDL}]^2$; $R^2 = 0.88$). In contrast to a preincubation with dLDL, a exposure of endothelial cells to the $\cdot\text{O}_2^-$ -generating mixture xanthine oxidase (300 $\mu\text{U/ml}$) and hypoxanthine (1 mmol/l) for 4 h did not affect shear stress-induced uptake of L-arginine (data not shown). In addition, 300 U/ml SOD did not prevent dLDL/glycated LDL-induced reduction of L-arginine uptake (data not shown).

Shear stress-mediated NO synthesis. Shear stress-induced uptake of L-arginine was recently shown to be essential for shear stress-mediated NO production (16). Therefore the effect of glycated LDL on shear stress-induced NO formation was assessed. Incubation of cultured endothelial cells for 24 h with nLDL did not affect shear stress-induced NO production in the presence of 0.3 mmol/l L-arginine (Fig. 7A). In contrast, dLDL reduced shear stress-induced NO production by 46.8% ($n = 12$; $P < 0.05$ vs. control, nLDL), while fraction α isolated from diabetic patients had no effect (Fig. 7A). The attenuated NO formation in cells preincubated with dLDL was normalized when 10 mmol/l L-arginine was added during shear stress activation (Fig. 7A) (dLDL + arginine, $n = 6$; NS vs. control, nLDL, and fraction α , $P < 0.05$ vs. dLDL). In contrast to 10 mmol/l L-arginine, SOD and the intracellular $\cdot\text{O}_2^-$ scavenger tiron failed to ameliorate reduced cGMP formation in dLDL-treated cells. In the presence of 300 U/ml SOD or 10 mmol/l tiron, shear stress-mediated cGMP increase in cells pretreated with dLDL (100 $\mu\text{g/ml}$ total cholesterol) was reduced compared with that found in cells treated with nLDL (100 $\mu\text{g/ml}$ total cholesterol) by 43.1 ± 5.2 and $46.9 \pm 8.4\%$, respectively. In agreement with our data on cGMP, a preincubation of endothelial cells with dLDL (100 $\mu\text{g/ml}$ total cholesterol) for 24 h reduced flow-mediated (33 dyn/cm² for 2 h) NO_x production in the presence of 0.3 mmol/l L-arginine from 43.9 ± 17.7 nmol NO_x/mg protein (nLDL-treated cells) to 5.3 ± 11.9 ($n = 8$; $P < 0.05$ vs. nLDL-treated cells), while the dLDL-mediated reduction of flow-induced NO_x production was ameliorated in the presence of 10 mmol/l L-arginine (33.1 ± 19.4 ; $n = 6$; NS vs. nLDL-treated cells).

The effect of dLDL on shear stress-induced NO/cGMP formation in the presence of L-arginine was mimicked by in vitro-glycated LDL (Fig. 7B) and similarly correlated with the amount of glycation of the LDL as observed for shear stress-mediated L-arginine uptake.

In contrast to its effect on shear stress-mediated cGMP formation, dLDL failed to affect cGMP formation in response to

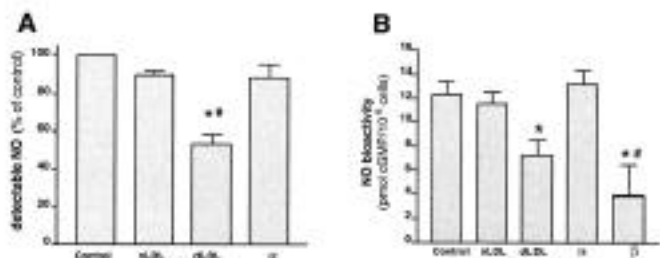


FIG. 4. Effect of a treatment of endothelial cells with various LDL fractions on NO stability (A) and bioactivity (B). Endothelial cells were treated for 24 h in DMEM (control) or DMEM containing 100 $\mu\text{g/ml}$ of total cholesterol nLDL, dLDL, or the nonglycated fraction separated from dLDL (fraction α) or 20 $\mu\text{g/ml}$ total cholesterol isolated glycated LDL (fraction β ; B only). A: Columns show detectable NO in HBS after extracellular application of 1 $\mu\text{mol/ml}$ NO from a saturated solution given in percent of that found in cells not treated with the lipoproteins. * $P < 0.05$ vs. control and nLDL, ** $P < 0.05$ vs. fraction α ($n = 6$). B: Bioactivity of extracellularly applied NO (1 $\mu\text{mol/ml}$) was measured by monitoring increases in endothelial cGMP levels in response to NO application in the presence of 100 $\mu\text{mol/l}$ N^o-nitro-L-arginine. Each column represents the mean \pm SE. * $P < 0.05$ vs. control and nLDL, ** $P < 0.05$ vs. fraction α ($n = 8$).

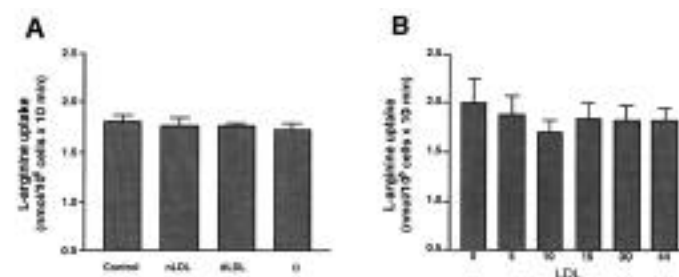


FIG. 5. Effect of the incubation of endothelial cells with various LDL fractions on basal L-arginine uptake in resting cells. A: Endothelial cells were treated for 24 h in DMEM (control) or DMEM containing 100 $\mu\text{g/ml}$ of total cholesterol nLDL, dLDL, or the nonglycated fraction separated from dLDL (fraction α). B: Endothelial cells were incubated for 24 h in DMEM containing 100 $\mu\text{g/ml}$ (total cholesterol) LDL after in vitro glycation procedure in the presence of the D-glucose concentration indicated. Each column represents the mean \pm SE ($n = 9$).

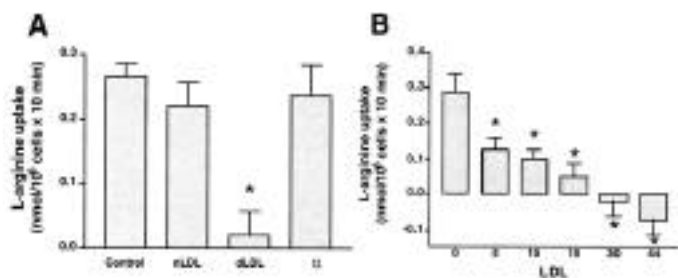


FIG. 6. Effect of the incubation of endothelial cells with various LDL fractions on shear stress-induced L-arginine uptake. After washing twice, cells were stimulated by shear stress (33 dyn/cm² for 45 min at 37°C). **A:** Endothelial cells were treated for 24 h in DMEM (control) or DMEM containing 100 µg/ml of total cholesterol nLDL, dLDL, or the nonglycated fraction separated from dLDL (fraction α). **P* < 0.05 vs. control, nLDL, and fraction α (*n* = 8). **B:** Endothelial cells were incubated for 24 h in DMEM containing 100 µg/ml (total cholesterol) LDL after in vitro glycation in the presence of the D-glucose concentration indicated. **P* < 0.05 vs. uptake of L-arginine in cells incubated with LDL after in vitro glycation in the absence of D-glucose (*n* = 6). Each column represents the mean ± SE.

A23187. In cells treated with nLDL or dLDL, 1 µmol/l A23187 increased endothelial cGMP levels from 3.39 ± 0.02 to 20.91 ± 1.06 pmol cGMP/10⁶ cells and from 2.54 ± 0.17 to 20.08 ± 2.64 pmol cGMP/10⁶ cells (*n* = 6; NS vs. nLDL-treated cells).

DISCUSSION

Numerous reports have been published in recent years demonstrating the effect of hyperglycemia on endothelial-dependent relaxation and/or NO formation (7,21,28–30). In addition, the interaction of advanced glycation end products (AGEs) with endothelial cells has been described in terms of effects on cell proliferation (31), expression of NO synthase (32), and cellular oxidant stress (33). High glycated hemoglobin and other AGEs have been reported to mediate defective autacoid-induced endothelium-dependent relaxation (34,35). In contrast, to the best of our knowledge, an effect of early glycation products of LDL on endothelial cell function has not been reported so far. It is known that the nonenzymatic glycation of LDL alters its bioactivity, metabolism (10), binding (9), and susceptibility for oxidation (8). Increased plasma levels of glycated LDL have been reported not only in diabetes (36), but also in hypercholesterolemia (37). Based on these findings, glycated LDL has been hypothesized to constitute a risk for atherosclerosis in both diabetes and hypercholesterolemia (11,12).

In this study, we have separated two fractions of LDL from diabetic subjects. In agreement with the reports of Klein et al. (38), there was significantly more glycated LDL (fraction β) in LDL from diabetic patients than in that from healthy subjects. For this study, we have compared the effects of LDL isolated from healthy (nLDL) and diabetic (dLDL) subjects as well as that of fraction α and fraction β. In addition, to assess the effect of glycation of LDL of identical lipid composition independent of the diabetic milieu, we have glycated nLDL in vitro at various D-glucose concentrations.

The increase in $\cdot\text{O}_2^-$ release of endothelial cells exposed to dLDL is consistent with observations in macrophages (39). While fraction α of dLDL had no effect on endothelial $\cdot\text{O}_2^-$ release, fraction β mimicked the effect of unfractionated dLDL on $\cdot\text{O}_2^-$ release. These findings suggest that glycation of

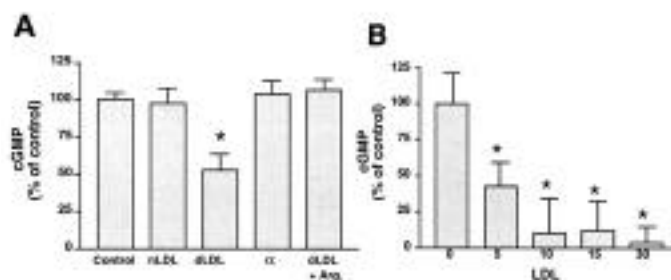


FIG. 7. Effect of the incubation of endothelial cells with various LDL fractions on shear stress-mediated NO production, measured by increases in endothelial cGMP content. After two washings, cells were stimulated in the presence of 0.3 or 10 mmol/l L-arginine (dLDL + arginine [A]) by shear stress (33 dyn/cm² for 45 min at 37°C). **A:** Endothelial cells were treated for 24 h in DMEM (control) or DMEM containing 100 µg/ml of total cholesterol nLDL, dLDL (dLDL + arginine), or the nonglycated fraction separated from dLDL (fraction α). **P* < 0.05 vs. control, nLDL, and fraction α (*n* = 4–8). **B:** Endothelial cells were incubated for 24 h in DMEM containing 100 µg/ml (total cholesterol) LDL after in vitro glycation in the presence of the D-glucose concentration indicated. **P* < 0.05 vs. cGMP increase in cells incubated with LDL after in vitro glycation in the absence of D-glucose (*n* = 9). Each column represents the percent of cGMP increases to shear stress measured in the control subjects ± SE.

LDL and/or differences in composition of fraction α and β (38) initiate $\cdot\text{O}_2^-$ release from the endothelium. The observations that in vitro-glycated LDL mimicked the effect of dLDL/fraction β on $\cdot\text{O}_2^-$ release and that the amount of $\cdot\text{O}_2^-$ produced correlated with the degree of LDL glycation indicate that LDL glycation per se alters the bioactivity of the LDL.

In agreement with these findings, the stability and bioactivity of exogenous NO was reduced by adding dLDL/fraction β (but not nLDL/fraction α) to endothelial cells. Since NO reacts very rapidly with $\cdot\text{O}_2^-$, one can speculate that the reduction of exogenous NO stability/bioactivity reported herein is due to enhanced $\cdot\text{O}_2^-$ release by dLDL/fraction β-treated cells. This interpretation is consistent with the view that an imbalance of NO and $\cdot\text{O}_2^-$ production contributes to vascular dysfunction in diabetes (40). In view of the absence of evidence of oxidative modification of the glycated LDL in these studies, these observations indicate that glycation of LDL per se independent of oxidative modification (39) can induce $\cdot\text{O}_2^-$ production by endothelial cells.

L-arginine supplementation has been reported to ameliorate impaired endothelial-dependent relaxation in diabetes (7,13). In contrast to this normalizing effect of L-arginine in patients with pathological decreased endothelial-dependent relaxation or in vessels derived from animals with diabetes, NO formation in healthy individuals cannot be further improved by added L-arginine (7,13). In agreement with these findings, Mitchell et al. (41) reported that under control conditions, endothelial NO production does not depend on extracellular L-arginine. This might be due to recycling of L-citrulline to L-arginine (15), while during diabetes, L-arginine recycling might be attenuated (7,13). Although there is evidence that endothelial cells can be depleted of L-arginine, which results in decreased endothelium-dependent relaxation (42), the mechanism(s) of this L-arginine depletion is unknown. Glycated LDL had no effect on basal L-arginine uptake in resting cells in the present studies. This is in contrast to the reported inhibition of L-arginine uptake by oxidized LDL in platelets (14). However, shear stress-induced L-arginine uptake was abolished by dLDL. This effect was mimicked by fraction β

and by in vitro-glycated LDL in a glycation-dependent manner. These data indicate that glycation of the LDL particle is responsible for its inhibitory effect on shear stress-mediated L-arginine uptake.

The inhibitory property of fraction β on shear stress-induced L-arginine uptake is in contrast to the effect of hyperglycemia, which increased uptake of L-arginine in endothelial cells (43). This difference might be of particular importance in regard to the hypothesis that impaired L-arginine utilization might contribute to decreased endothelium-dependent relaxation in diabetes (44) and hypercholesterolemia (45). Our findings that fraction β abolished shear stress-mediated L-arginine uptake, together with reports of reduced plasma L-arginine levels in diabetes (13), might explain endothelial L-arginine depletion in diabetes.

We have previously shown that, in contrast to agonist-induced NO formation, shear stress-evoked NO biosynthesis critically depends on L-arginine uptake (16). In agreement with these findings, dLDL but not nLDL/fraction α diminished shear stress-mediated NO production. This effect was mimicked by in vitro-glycated LDL in a glycation-dependent manner like that observed for L-arginine uptake. Since endothelial NO production in dLDL-treated cells was normalized in the presence of a very high L-arginine concentration (i.e., 10 mmol/l), these data indicate that the inhibition of shear stress-evoked L-arginine uptake by glycated LDL is responsible for the reduced NO production in dLDL-treated cells. These findings are consistent with reports that L-arginine ameliorates impaired endothelium-dependent relaxation in diabetic animals (7,13).

The present findings suggest that besides hyperglycemia and AGEs, glycation of LDL might contribute to endothelial dysfunction in diabetes. Because of its property to reduce shear stress-induced L-arginine uptake and to elevate endothelial $\cdot\text{O}_2^-$ release, glycated LDL interferes with the synthesis and the activity of NO as the most prominent vasorelaxing factor. Thus, glycated LDL might constitute an important risk factor for endothelial dysfunction and atherosclerosis in diabetes.

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