Modifications Induced by LDL From Type 1 Diabetic Patients on Endothelial Cells Obtained From Human Umbilical Vein

Rosa Anna Rabini, Nelvio Cester, Roberto Staffolani, Eleonora Salvolini, Natalia Moretti, Arianna Vignini, Daniele Fumelli, and Laura Mazzanti

The aim of the present work was to analyze the effect of LDL obtained from type 1 diabetic patients in good metabolic control on human umbilical vein endothelial cells (HUVECs) after a short incubation period to detect possible atherogenic modifications of endothelial properties. Cultured HUVECs were incubated for 3 h with culture medium alone (control HUVEC), with native LDL from 12 healthy men (control LDL), or with native LDL from 12 type 1 diabetic men (type 1 LDL) (100 µg/ml). After the incubation, the following parameters were evaluated: nitric oxide synthase (NOS) activity, cytoplasmic Ca^{2+} levels, Na^+-K^+-ATPase activity, plasma membrane fluidity determined by means of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), and plasma membrane conjugated diene (CD) content. The same experiments were repeated after bradykinin stimulation or in the presence of the antioxidant butylated hydroxytoluene (BHT), and nitric oxide (NO) production in intact HUVECs was also evaluated. HUVECs incubated with control LDL in comparison with control HUVECs showed a decreased fluidity of the membrane surface evaluated by TMA-DPH and a higher CD content. These alterations were prevented by the presence of BHT. HUVECs incubated with type 1 LDL in comparison with both control HUVECs and cells incubated with control LDL showed *1*) increased NOS and Na^+-K^+-ATPase activity, cytoplasmic Ca^{2+} levels, and CD content, and *2*) decreased fluidity of the membrane surface evaluated by TMA-DPH. These modifications were blunted—but not abolished—by the presence of BHT. After bradykinin stimulation either in the absence or in the presence of BHT, both cytoplasmic Ca^{2+} levels and NO production were increased in control HUVECs and in HUVECs incubated with control LDL, while a reduced response was observed in HUVECs incubated with type 1 LDL. The alterations observed in the endothelial function after the cell-LDL interaction might play a central role in the atherogenic process in diabetes. *Diabetes* 48:2221–2228, 1999

From the Department of Diabetology (R.A.R., R.S., D.F.), INRCA Hospital, Ancona; the Department of Obstetrics and Gynecology (N.C.) and the Institute of Biochemistry (E.S., N.M., A.V., L.M.), University of Ancona School of Medicine, Ancona, Italy.

Address correspondence and reprint requests to Prof. Laura Mazzanti, Institute of Biochemistry, School of Medicine, University of Ancona, via P. Ranieri 65, Ancona, Italy. E-mail: mazzanti@popcsi.unian.it.

Received for publication 15 October 1998 and accepted in revised form 5 August 1999.

BHT, butylated hydroxytoluene; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CD, conjugated diene; DPH, 1,6-diphenyl-1,3,5-hexatriene; ECBGM, endothelial cell basal growth medium; EDRF, endothelium-derived relaxing factor; eNOS, endothelial constitutive nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide; NOS, nitric oxide synthase; TMA-DPH, 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene.

Endothelial dysfunction plays a pivotal role in the initiation and progression of atherosclerosis. In fact, the vascular endothelium modulates the vessel tone by releasing both relaxing and contractile factors (1), regulates the adherence of mononuclear cells to its surface and the vascular permeability (2,3), and produces substances involved in the regulation of hemostasis and tissue proliferation (4). All of these processes have been demonstrated to be altered in human diabetes, which is characterized by an imbalance between endothelium-derived vasorelaxing and vasoconstrictor factors, a modified adherence of mononuclear cells to the endothelium, an altered vascular permeability, and a hypercoagulable state with platelet activation (5). These observations have recently led to the hypothesis that a modified interaction between circulating molecules and/or cells and the vessel wall might be at the basis of the endothelial dysfunction in diabetes (6).

LDL is able to modify in vitro endothelial cell functions. High concentrations of native LDL alter endothelial eicosanoid metabolism (7) and mononuclear cell attachment by changing the cell membrane lipid composition and dynamics (8). Oxidized LDLs have also been demonstrated to induce potentially atherogenic changes in membrane physicochemical properties and in cytosolic calcium concentrations in bovine aortic endothelial cells (9). Moreover, recent reports demonstrated that native LDL binding to the LDL receptor induces the endothelial expression of adhesion molecules through a rise in intracellular calcium (10).

The aim of the present work was to analyze the effect of LDL obtained from normolipidemic type 1 diabetic patients in good metabolic control on human umbilical vein endothelial cells (HUVECs) after a short incubation period to detect possible atherogenic modifications of endothelial properties. It might be hypothesized that LDL from type 1 diabetic patients exerts an effect similar to oxidized LDL (9) or high concentrations of native LDL (8) on the endothelial cell membrane physicochemical properties, evaluated by means of the measurement of fluidity both in the more superficial and in the deeper part of the membrane. Such an action might be mediated by a peroxidative stress exerted by LDL from diabetic patients that we evaluated as conjugated diene (CD) for-

mation. Secondary effects of the endothelial membrane modifications might be changes in cytosolic Ca^{2+} concentration caused by altered transmembrane calcium fluxes and modifications in nitric oxide synthase (NOS) activity, which regulates the vessel tone by modifications in nitric oxide (NO) production and is known to be modulated by cytosolic Ca^{2+} levels (10). In the present work, we also studied the action of LDL on the activity of Na^+-K^+-ATPase, which is an integral membrane protein highly sensitive to modifications in membrane fluidity and which is also dependent on NO synthesis through a not yet elucidated mechanism (11).

RESEARCH DESIGN AND METHODS

HUVECs were purchased from Mascia Brunelli (Milan, Italy) and cultured in endothelial cell basal growth medium (ECBGM) with addition of low serum growth supplement. At 80% confluence (about 800,000 cells/ml), cultured HUVECs were used for three different incubation experiments.

Experiment 1. In experiment 1, incubation was for 3 h at 37°C with ECGBM alone (control HUVEC), with native LDL from healthy subjects (control LDL), or with native LDL from type 1 diabetic patients (type 1 LDL) (100 µg/ml in ECGBM). After the incubation, the monolayers were removed by scraping and resuspended in fresh culture medium or in Tris-HCl (50 mmol/l, pH 7.4) for the fluorescence experiments, and the following parameters were evaluated: NOS activity, cytoplasmic Ca^{2+} levels, Na^+-K^+-ATPase activity, plasma membrane fluidity determined by means of both 1,6-diphenyl-1,3,5-hexatriene (DPH) and its derivative 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), and plasma membrane CD content. These incubation experiments were performed with fresh LDL obtained from 12 healthy men and 12 age-matched men affected by type 1 diabetes but in good metabolic control. Clinical characteristics of the study subjects are shown in Table 1. The two groups differed only in terms of blood glucose concentrations and HbA_{1c} levels. Clinical laboratory parameters were measured by routine laboratory methods. All the study subjects were normotensive and were not smokers. No subject was taking any medication, except for insulin in the type 1 diabetic patients. None of the patients was affected by nephropathy or neuropathy; three patients showed background retinopathy.

Experiment 2. At a later time, LDLs were again obtained from eight healthy subjects and eight type 1 diabetic patients from the two groups previously described and immediately used for further incubation experiments with HUVECs under the conditions described above. At the end of the incubation period, an aliquot of HUVECs was immediately used for the determination of NO directly produced by cells, cytoplasmic Ca^{2+} levels, Na^+-K^+-ATPase activity, plasma membrane fluidity determined by both DPH and TMA-DPH, and plasma membrane CD content. The other aliquot was stimulated with 200 nmol/l bradykinin, and the same parameters were evaluated after stimulation.

Experiment 3. LDLs obtained from the patients of experiment 2 were also incubated with HUVECs for 3 h at 37°C in the presence of 20 µmol/l of the antioxidant butylated hydroxytoluene (BHT) (12). NO directly produced by cells, cytoplasmic Ca^{2+} levels, Na^+-K^+-ATPase activity, plasma membrane fluidity determined by both DPH and TMA-DPH, and plasma membrane CD content were then measured. After the incubation, an aliquot was stimulated with 200 nmol/l bradykinin, and NO production and cytoplasmic Ca^{2+} levels were determined.

Study protocol. LDLs from type 1 diabetic patients and respective control subjects were run in the same experiment. The study was performed in accordance with the principles of the Declaration of Helsinki as revised in 1996, and informed consent was obtained from the patients.

Statistical analysis. Results are expressed as means ± SD. Statistical analyses were performed using the Student's t test for paired data. Differences were considered significant with $P < 0.05$.

Preparation and characterization of LDLs. Plasma was separated from freshly drawn human blood, and native lipoproteins (LDL: density between 1.025 and 1.063 g/ml) were isolated by discontinuous density gradient ultracentrifugation as described by Chen et al. (13). The concentrations of triglycerides, phospholipids, and cholesterol were determined in LDL as previously described (14). LDL lipid hydroperoxide concentrations were measured by the ferrous oxidation–xylenol orange assay according to Jiang et al. (15).

NOS activity. NOS activity was measured spectrophotometrically by following the oxidation of oxyhemoglobin (λ max 426 nm) to methemoglobin (λ max 405 nm) by NO at 37°C, as previously described (16). Oxyhemoglobin was prepared by reduction. Briefly, human hemoglobin (60 mg; Sigma, St. Louis, MO) and sodium dithionite (120 mg) were dissolved in distilled water (2 ml) and gently agitated in a flat dish for 15 min. The hemoglobin mixture was then chromatographed on a column of Sephadex G-25 (1.5 × 30 cm). The reduced fraction, with its characteristic bright red color, was isolated and stored frozen (–20°C). The NOS assay mixture consisted of 200 µmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 100 µmol/l L-Arg, 100 µmol/l NADPH, 1.6 µmol/l oxyhemoglobin, and 12 µmol/l (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride. The reaction was initiated with 50 µl of HUVEC homogenate, which was mixed with the reaction medium with the aid of a glass plumper. Cells were counted in each sample before homogenizing platelets to ensure that the same amount of cells was used. NO production was calculated from the decrease in absorbance at 426 nm (with an estimated extinction coefficient of 98,000 $l \cdot mol^{-1} \cdot cm^{-1}$). NOS activity was expressed in picomoles NO produced per minute per milligram of protein.

To ensure the NOS specificity of the oxidation of oxyhemoglobin, the reaction was also performed after a 20-min preincubation of HUVEC homogenate with 100 mmol/l nitro-L-arginine methyl ester or N^G amino arginine, which completely inhibited the decrease in absorbance at 426 nm. Both substances are L-arginine analogs affecting NOS activity by competitive inhibition (17).

Intracellular Ca^{2+} concentrations. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in intact HUVECs using the fluorescent probe Fura 2-AM as previously described (18). Determinations were performed in a Perkin-Elmer/Cetus (Norwalk, CT) MPF-66 spectrofluorometer at 37°C according to the method of Rao (19). Fluorescence intensity was read at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). Calibration was carried out as described by Grynkiewicz et al. (20) with

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where K_d is the dissociation constant of the Ca^{2+}–Fura 2 interaction in the cytosolic environment; R is the ratio of the fluorescence intensities at excitation wavelengths 340 and 380 nm; R_{min} and R_{max} are the ratios of the fluorescence intensities without Ca^{2+} and with saturating levels of Ca^{2+}, respectively; and S_{f2} and S_{b2} are fluorescence intensities at 380 nm without Ca^{2+} and with saturating levels of Ca^{2+}, respectively. R_{min} and S_{f2} were measured after cellular lysis with 25% Triton and addition of 10 mmol/l EGTA, pH 8.3. R_{max} and S_{b2} were determined after lysis and addition of 10 mmol/l $CaCl_2$. Autofluorescence was subtracted before performing the Ca^{2+} calibration procedure.

Na^+-K^+-ATPase assay. The Na^+-K^+-activated Mg^{2+}-dependent ATPase activity was determined on HUVECs by the Kitao and Hattori method (21). ATPase activity was assayed by incubating 1 ml of HUVECs (2×10^6 cells/ml) after sonication at 37°C in the reaction medium containing $MgCl_2$ (5 mmol/l), NaCl (140 mmol/l), and KCl (14 mmol/l) in 40 mmol/l Tris-HCl, pH 7.7. The ATPase reaction was started by the addition of 3 mmol/l Na_2ATP and stopped 20 min later by the addition of 1 ml of 15% trichloroacetic acid. Tubes were centrifuged at 1,100 g for 10 min, and the inorganic phosphate hydrolyzed from the reaction was measured in the supernatant by a colorimetric assay using KH_2PO_4 as standard (22). The ATPase activity assayed in the presence of 10 mmol/l ouabain was subtracted from the total Mg^{2+}-dependent ATPase activity to calculate the activity of Na^+-K^+-ATPase. Protein concentration was determined as described by Lowry et al. (23) using serum albumin as a standard.

Membrane fluidity. The HUVEC plasma membrane fluidity was studied by determining the fluorescence anisotropies (reciprocal of fluidity) of the probes DPH and TMA-DPH, which are incorporated, respectively, in the deeper hydrophobic part of the membrane and at the lipid-water interface of the membrane bilayer (24). Cell incubations with DPH and with TMA-DPH were performed as described by Mazzanti et al. (25) and by Sheridan and Block (26),

TABLE 1
Clinical characteristics of subjects from whom LDLs were obtained

	Healthy men	Type 1 diabetic men
n	12	12
Age (years)	37 ± 8	33 ± 8
BMI (kg/m^2)	24 ± 3	23 ± 3
Diabetes duration (years)	—	8 ± 5
Fasting plasma glucose (mmol/l)	5.2 ± 0.3	7.4 ± 0.9*
HbA_{1c} (%)	5.3 ± 0.3	7.2 ± 0.8*
Plasma LDL cholesterol (mmol/l)	2.61 ± 0.23	2.53 ± 0.18
Plasma HDL cholesterol (mmol/l)	1.23 ± 0.09	1.18 ± 0.10
Plasma triglycerides (mmol/l)	1.41 ± 0.22	1.38 ± 0.28

Data are means ± SD or n. *$P < 0.05$.

TABLE 2
Composition of LDL from healthy subjects and patients affected by type 1 diabetes

	Control LDL	Type 1 LDL
n	12	12
Proteins (%)	30.04 ± 5.96	29.84 ± 6.34
Triglycerides (%)	2.27 ± 1.02	2.98 ± 0.96
Phospholipids (%)	27.12 ± 5.13	26.51 ± 6.84
Cholesterol (%)	40.57 ± 8.09	40.67 ± 9.12
Lipid hydroperoxides (μmol/mg phospholipids)	163.51 ± 45.23	171.12 ± 41.29

Data are means ± SD or *n*.

respectively. Briefly, 3 μl of DPH or TMA-DPH (10^{-3} mol/l) were incubated for 10 or 5 min, respectively, at room temperature (23°C) with 2 ml of endothelial cells (2×10^6 cells/ml) in 50 mmol/l Tris-HCl buffer solution, pH 7.4. The incubation times were chosen in order to avoid probe diffusion within the cells, which was previously checked by the 2,4,6-trinitrobenzenesulfonic acid method (27).

Fluorescence intensities (100 readings each) of the vertical and horizontal components of the emitted light were measured on a Perkin-Elmer/Cetus MPF66 spectrofluorometer equipped with two glass prism polarizers (excitation wavelength 365 nm, emission wavelength 430 nm). Steady-state fluorescence anisotropy of DPH and TMA-DPH was calculated using

$$r = \frac{I_V G - I_h}{I_V + 2I_h}$$

where G is the instrumental factor that corrects the r value for an unequal detection of vertically (I_V) and horizontally (I_h) polarized light.

Plasma membrane CD content. Endothelial plasma membranes were prepared as described by Thorin et al. (9). Briefly, HUVECs were detached by scraping, and the suspension was centrifuged for 5 min at 400 *g*. The pellet was suspended in hypotonic buffer containing Na_2HPO_4 (0.61 mmol/l), KH_2PO_4 (0.38 mmol/l), $MgSO_4$ (0.2 mmol/l), and dithiothreitol (1 mmol/l), pH 7.4, dispersed in a Dounce homogenizer, and then centrifuged for 15 min at 20,000 *g* at 4°C. Lipids were extracted from the resulting pellet with chloroform-methanol 2:1 according to the method of Folch (28). All aqueous phase procedures were conducted in the presence of EDTA to avoid possible lipid peroxidation. CDs were measured on the extracted chloroform-free lipid under N_2 flux in 3 ml cyclohexane as described by Esterbauer et al. (29), measuring the absorbance at 234 nm against a cyclohexane blank. The amount of CDs was calculated using the molar absorbance of CD (E_{234nm} = 27,000 l · mol^{-1} · cm^{-1}). Results are expressed as nanomoles CD per milligram of lipid.

NO production. NO released by endothelial cells was directly measured in the intact HUVEC suspension using an isolated NO meter and its associated probe (IsoNO Mk-II; World Precision Instruments, Sarasota, FL) equipped with the Duo. 18 Data Acquisition System, as recently described by Chakravarthy et al. (30). NO gas diffuses through to the probe tip and is oxidized at the working electrode, resulting in an electrical current proportional to its concentration. The NO probe was calibrated according to the chemical calibration method of Tsukahara (31). NO production was determined directly in the HUVEC suspension medium after addition of 100 μmol/l L-Arg, which induced a rapid increase in NO release measurable 30 s after stimulation.

RESULTS

No significant difference was observed in triglycerides, phospholipids, cholesterol concentrations, or in lipid hydroperoxide levels in LDL obtained from type 1 diabetic patients compared with LDL isolated from healthy subjects (Table 2).

Incubation experiments with HUVECs in the basal state. The incubation of HUVECs for 3 h with ECGBM alone did not cause significant changes in any of the parameters studied with respect to the basal levels.

NOS activity was not significantly changed after the incubation of HUVECs with control LDL, while it was greatly enhanced after the incubation with type 1 LDL (Fig. 1: $P < 0.01$ type 1 LDL vs. control HUVEC, $P < 0.01$ type 1 LDL vs. control LDL). Similarly, the $[Ca^{2+}]_i$ was not changed in HUVECs incubated with control LDL, while it was significantly increased after incubation with type 1 LDL (Fig. 2: $P < 0.01$ type 1 LDL vs. control HUVEC, $P < 0.01$ type 1 LDL vs. control LDL). LDL from healthy subjects did not affect HUVEC Na^+-K^+-ATPase activity, which was, on the contrary, significantly increased after the incubation of endothelial cells with LDL from type 1 diabetic patients (Fig. 3: $P < 0.01$ type 1 LDL vs. control HUVEC, $P < 0.01$ type 1 LDL vs. control LDL).

The effect of the incubation with LDL on HUVEC plasma membrane fluidity is shown in Table 3. The incubation with both control and type 1 LDL did not cause significant changes in the r values of DPH. On the contrary, higher TMA-DPH r val-

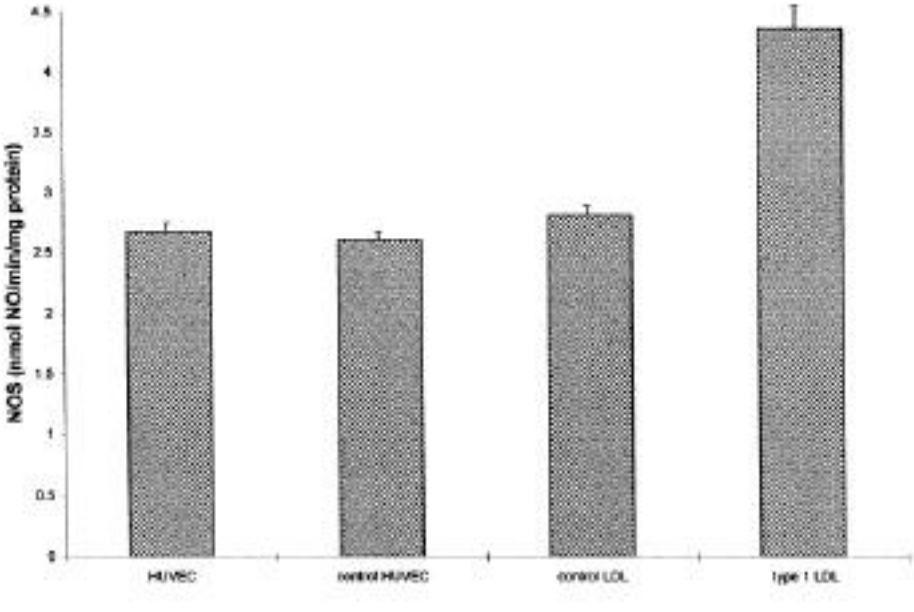

FIG. 1. NOS activity in HUVECs in the basal state (HUVEC) and after incubation with culture medium alone (control HUVEC), with LDL from healthy subjects (control LDL), and with LDL from patients affected by type 1 diabetes (type 1 LDL). Means ± SD are shown. $P < 0.01$ type 1 LDL vs. HUVEC, control HUVEC, and control LDL.

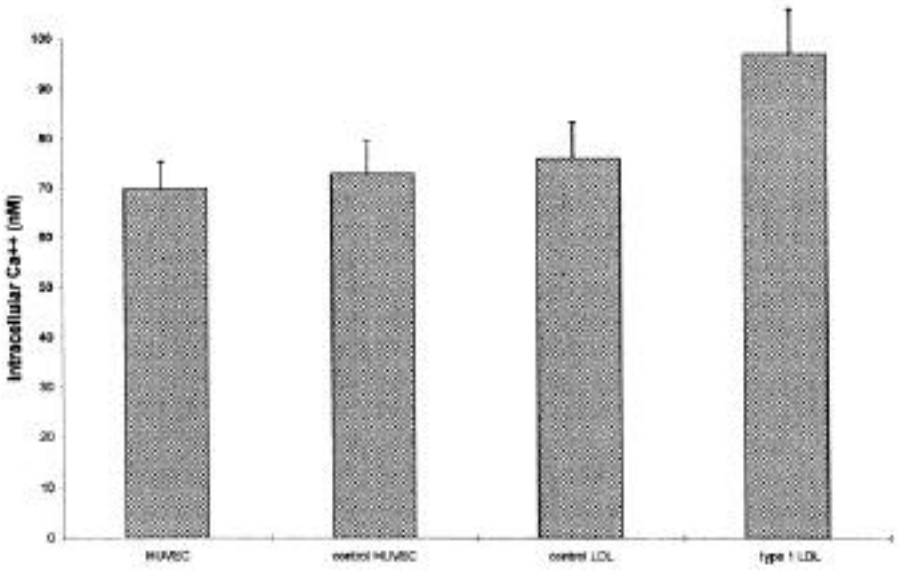

FIG. 2. $[Ca^{2+}]_i$ in HUVECs in the basal state (HUVEC) and after incubation with culture medium alone (control HUVEC), with LDL from healthy subjects (control LDL) and with LDL from patients affected by type 1 diabetes (type 1 LDL). Means ± SD are shown. $P < 0.01$ type 1 LDL vs. HUVEC, control HUVEC, and control LDL.

ues (i.e., decreased fluidity of the superficial part of the membrane) were observed after the incubation with control LDL compared with HUVECs in the basal state and with HUVECs incubated with the culture medium alone ($P < 0.05$). Type 1 LDL caused a further increase in the TMA-DPH anisotropy values in comparison not only with basal levels ($P < 0.01$) but also with cells incubated with control LDL ($P < 0.05$). Table 2 shows the action of the incubation with LDL on the CD formation in HUVECs. CDs were significantly increased after the exposure of endothelial cells to control LDL compared with either basal levels or cells incubated with culture medium ($P < 0.05$). The incubation with type 1 LDL formed significantly more CDs than did control LDL ($P < 0.01$).

Incubation experiments with bradykinin activation. The additional incubation experiments performed as described in RESEARCHDESIGNANDMETHODS (experiment 2) confirmed the results described above with HUVECs in the basal state (data not shown). In fact, control LDL induced no significant change in HUVEC $[Ca^{2+}]_i$, Na^+-K^+-ATPase activity, or DPH anisotropy values, while significant increases were observed in TMA-DPH anisotropy levels and CDs with respect to HUVECs incubated with culture medium alone ($P < 0.01$). LDLs obtained from type 1 diabetic patients induced significant elevations in $[Ca^{2+}]_i$, Na^+-K^+-ATPase activity, TMA-DPH anisotropy levels, and CDs with respect to both HUVECs incubated with culture medium alone ($P <$

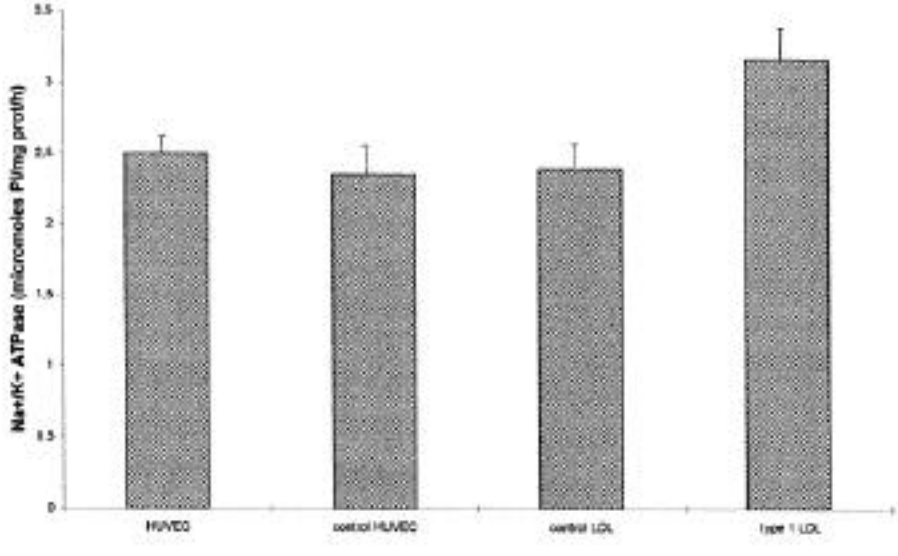

FIG. 3. Na^+-K^+-ATPase activity in HUVECs in the basal state (HUVEC) and after incubation with culture medium alone (control HUVEC), with LDL from healthy subjects (control LDL) and with LDL from patients affected by type 1 diabetes (type 1 LDL). Means ± SD are shown. $P <$ 0.01 type 1 LDL vs. HUVEC, control HUVEC, and control LDL.

TABLE 3
Plasma membrane fluorescence anisotropy of DPH and TMA-DPH and CD content

	HUVEC	Control HUVEC	HUVEC + control LDL	HUVEC + type 1 LDL	HUVEC + control LDL + BHT	HUVEC + type 1 LDL + BHT
n	12	12	12	12	8	8
Fluorescence anisotropy of DPH	0.285 ± 0.024	0.281 ± 0.029	0.298 ± 0.036	0.312 ± 0.043	0.301 ± 0.026	0.307 ± 0.024
Fluorescence anisotropy of TMA-DPH	0.344 ± 0.011	0.342 ± 0.013	0.379 ± 0.011*	0.402 ± 0.015†‡	0.345 ± 0.016	0.380 ± 0.012*‖
CD content (nmol/mg lipid)	146 ± 12	151 ± 15	188 ± 14*	306 ± 39‡§	162 ± 18	190 ± 16*‖

Data are means ± SD or *n*. Fluorescence anisotropy and CD content were determined in HUVECs in the basal state (HUVEC) and after incubation with culture medium alone (control HUVEC), with LDL from healthy subjects (HUVEC + control LDL), and with LDL from patients affected by type 1 diabetes (HUVEC + type 1 LDL) in the absence or in the presence of 20 µmol/l of the antioxidant BHT. *$P < 0.05$ vs. HUVEC and control HUVEC; †$P < 0.05$ vs. HUVEC + control LDL; ‡$P < 0.01$ vs. HUVEC and control HUVEC; §$P < 0.01$ vs. HUVEC + control LDL; ‖$P < 0.01$ vs. HUVEC + type 1 LDL.

0.01) and HUVECs incubated with control LDL ($P < 0.01$), while they did not affect DPH anisotropy values.

NO production directly measured in the intact HUVEC suspension using the NO meter was not changed after incubation with LDL from control subjects in comparison with cells incubated in ECGBM alone, while a significant increase was observed after incubation with LDL from type 1 diabetic patients (Fig. 4: $P < 0.01$ type 1 LDL vs. control HUVEC, $P < 0.01$ type 1 LDL vs. control LDL).

After bradykinin stimulation, NO production significantly increased in HUVECs incubated with culture medium alone or with control LDL ($P < 0.01$), while no significant change was observed in HUVECs previously incubated with type 1 LDL (Fig. 4). In a similar way, bradykinin caused a significant increase in $[Ca^{2+}]_i$ in HUVECs incubated with ECGBM (125 ± 21 vs. 73 ± 7 nmol/l in the basal state, $P < 0.01$) and in HUVECs incubated with control LDL (121 ± 20 vs. 75 ± 8 nmol/l in the basal state, $P < 0.01$), while HUVECs previously incubated with type 1 LDL showed a blunted response of $[Ca^{2+}]_i$ to bradykinin addition (123 ± 22 vs. 95 ± 9 nmol/l in the basal state, $P < 0.05$). Bradykinin did not affect Na^+-K^+-ATPase activity, DPH and TMA-DPH anisotropy values, or CD content.

Incubation experiments in the presence of antioxidant. When the incubation experiments were performed in the presence of BHT, control LDL did not cause any significant change in HUVEC CD content or in TMA-DPH anisotropy values (Table 3). Moreover, in the presence of BHT, type 1 LDL caused a less relevant increase in HUVEC CD content or in TMA-DPH anisotropy values, although it was always statistically significant in comparison with HUVECs incubated with culture medium alone ($P < 0.05$, Table 3). In a similar way, in the presence of BHT, type 1 LDL caused a less relevant increase in both HUVEC NO production and $[Ca^{2+}]_i$ in comparison with HUVECs incubated with culture medium alone (NO production = 141 ± 23 nmol · l^{-1} · l^{-1} NO released × 10^6 cells [$P < 0.05$] vs. control HUVEC; $[Ca^{2+}]_i$ = 83 ± 7 nmol/l [$P < 0.05$] vs. control HUVEC). Na^+-K^+-

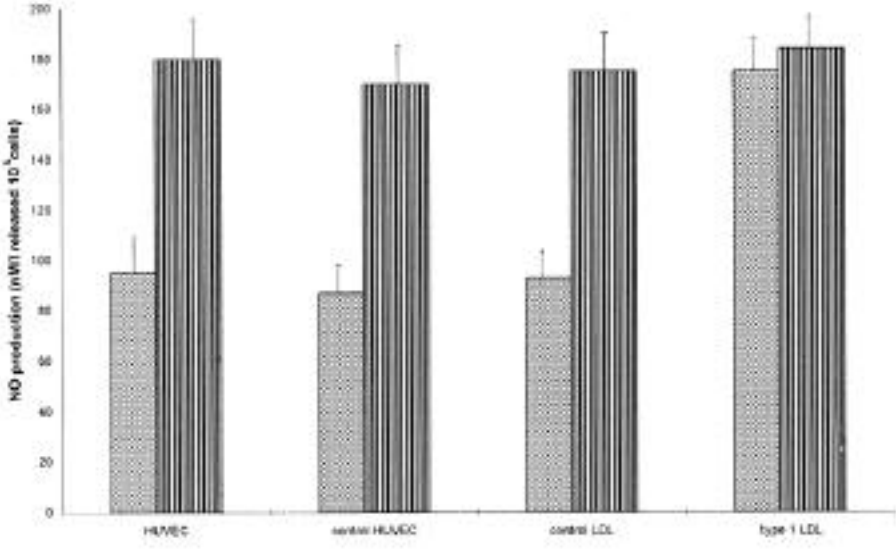

FIG. 4. NO produced by HUVEC before (HUVEC) and after incubation with culture medium alone (control HUVEC), with LDL from healthy subjects (control LDL), and with LDL from patients affected by type 1 diabetes (type 1 LDL) in the basal state (▨) and after bradykinin stimulation (▥). Means ± SD of eight experiments are shown. $P < 0.01$ type 1 LDL vs. HUVEC, control HUVEC, and control LDL in the basal state.

ATPase activity was not affected by type 1 LDL incubation in the presence of BHT.

The addition of BHT to the medium did not affect the altered Ca^{2+} or NO responses to bradykinin in HUVECs incubated with LDL from patients with type 1 diabetes.

DISCUSSION

The incubation of HUVECs with LDL was associated with a significant decrease in the fluidity of the more superficial part of the plasma membrane, as demonstrated by the higher TMA-DPH r values, while the deeper and more hydrophobic region of the membrane probed by the DPH fluorescence did not seem to be modified by either LDL from healthy subjects or LDL from type 1 diabetic patients. These data are in agreement with the previous observations of Pritchard et al. (8), who observed a reduced fluidity of plasma membranes of HUVECs after a prolonged (4 days) incubation with atherogenic LDL concentrations and hypothesized a relation between this change and an enhanced endothelial cell binding of monocytes. In addition, the present study indicates that this modification may occur after a short-term incubation with control LDL and is deeper when the incubation is performed with LDL from type 1 diabetic patients. It might therefore be hypothesized that short-term interactions between LDL and endothelial cells affect the plasma membrane surface in a possible atherogenic way, since modulations in fluidity influence not only mononuclear cell attachment (8) but also endothelial cell plasma membrane transport and receptor function (32).

The decreased fluidity of the plasma membrane surface might be partly due to the peroxidative damage caused by the incubation of HUVECs with LDL, as demonstrated by the increased CD content observed in both endothelial cells incubated with LDL from healthy subjects and, to a greater extent, in cells incubated with LDL from type 1 diabetic patients. This hypothesis is confirmed by the experiments performed in the presence of the antioxidant BHT, which completely prevented the modifications in the CD content and in the surface fluidity of HUVEC membrane caused by incubation with LDL from healthy subjects. Moreover, the antioxidant was also able to significantly reduce—but not abolish—the modifications induced in HUVECs by LDL from type 1 diabetic patients, suggesting that these lipoproteins exert their action on endothelial cells at least partially by peroxidative damage.

The occurrence of membrane lipid peroxidation might play a central role in endothelial dysfunction in diabetes, since the generation of oxygen-derived free radicals has been reported to mediate the abnormal endothelium-dependent relaxation of aorta exposed to elevated glucose in vitro (33). The present data suggest that a further mechanism causing an oxidative stress in endothelial cells during diabetes might be the interaction between circulating LDL and the plasma membrane.

It was recently observed that the generation of O_2^- in endothelial cells results in elevation of intracellular Ca^{2+} release, Ca^{2+} entry, and endothelium-derived relaxing factor (EDRF) formation to bradykinin (34). In the present work we found that the relevant increase in the CD content of HUVECs exposed to LDL from diabetic patients was accompanied by a higher cytosolic Ca^{2+} concentration and an enhanced NOS activity in comparison with both control cells and cells incubated with LDL from healthy subjects. It might be hypothesized that the changes induced by LDL from diabetic patients in endothelial cell Ca^{2+} concentration and NOS activity are mediated, at least partially, by peroxidative damage, as previously demonstrated for the similar alterations caused by high D-glucose in endothelial Ca^{2+}/EDRF signaling (34). This hypothesis is also supported by the confirmatory experiments performed in the presence of antioxidant, which caused a contemporaneous reduction in both the peroxidative damage of the endothelial membrane evaluated as CD content and in the cellular Ca^{2+} concentration and NO production.

Graier et al. (34) suggested that the interaction of O_2^- with outer parts of the cell membranes might be responsible for the altered endothelial Ca^{2+}/EDRF signaling caused by glucose. In a similar way, our finding of a reduced fluidity of the more superficial part of the plasma membrane in HUVECs exposed to LDL from diabetic patients in comparison with control cells and with cells incubated with LDL from healthy subjects seems to suggest a central role of the cellular surface in these modifications.

The increased HUVEC cytosolic Ca^{2+} concentrations caused by the incubation with LDL from diabetic patients might be the direct determinant of the enhanced NOS activity observed under the same conditions, since endothelial constitutive NOS (eNOS) is Ca^{2+}-dependent (35). Moreover, this isoenzyme is partly membrane-associated because of binding of the NH_2-terminal myristate to plasma membrane (35). Therefore, a modification of the plasma membrane might also cause an altered activity of this enzyme through an altered membrane binding, as one can hypothesize concerning Na^+-K^+-ATPase, an integral plasma membrane protein that shows an increased activity after incubation of HUVECs with LDL from type 1 diabetic patients. Na^+-K^+-ATPase activity is also dependent on NO synthesis, since sodium nitroprusside, which directly releases NO, reverses the inhibition of rabbit aorta Na^+-K^+-ATPase caused by either hyperglycemia or removal of the endothelium (11).

The enhanced endothelial NOS activity caused by a short-term incubation with LDL from diabetic patients is consistent with the enhanced vessel relaxation taking place in the early stages of diabetes (36). Moreover, the increased NO release might exert cytotoxic effects in the presence of high concentrations of oxygen-reactive species, as occurs during hyperglycemia. In fact, NO can rapidly react with superoxide anion to produce the strong oxidant peroxynitrite (37).

Data obtained on NO production directly measured in intact endothelial cells give further support to the observations concerning NOS activity in cell homogenates, confirming that eNOS activity in intact cells is also altered by the action of LDL from type 1 diabetic patients. Experiments performed after bradykinin stimulation suggest a further possible mechanism of damage on intact HUVECs caused by LDL from diabetic patients, i.e., the blunted response of intracellular Ca^{2+} and NO production to bradykinin addition. This observation indicates that diabetic LDLs impair the HUVEC receptor-mediated cytosolic Ca^{2+} increase and the subsequent eNOS stimulation caused by bradykinin, as described recently in endothelial cells exposed to elevated glucose concentrations (38). This action might be physiologically significant, suggesting that exposure to diabetic LDLs might make endothelial cells less responsive to stimuli inducing in vivo endothelial-dependent relaxation. Interestingly, this effect

was not inhibited by the presence of an antioxidant, suggesting that the effect of diabetic LDLs on HUVECs is not explained only by peroxidative damage of the membrane. The decrease in the fluidity of the more superficial part of the membrane caused by diabetic LDLs also in the presence of the antioxidant BHT might be at the basis of the reduced bradykinin-initiated Ca^{2+} signaling. In fact, a recent work performed on porcine aortic endothelial cells reported a negative correlation between membrane viscosity (i.e., the inverse of fluidity) and agonist-stimulated intracellular Ca^{2+} release, suggesting that this relation might also be mediated by a modulation of phospholipase C activity or a modification of membrane polarization (39).

It might be argued that the experiments performed in the present work are not relevant for the in vivo situation, since plasma contains antioxidants, and therefore, incubation of endothelial cells with LDL alone creates an environment favoring LDL oxidation that does not exist in vivo. However, in a recent work by our group, we described similar alterations in HUVECs caused by plasma from type 1 diabetic pregnant women (40).

The mechanisms by which native LDL from healthy and diabetic patients causes the observed alterations in HUVECs in vitro deserve further investigation. LDL from type 1 diabetic patients in good metabolic control did not show any relevant change in the main lipid classes or in levels of lipid hydroperoxides in comparison with LDL from healthy subjects, so that a subtler difference in the lipid composition must be hypothesized to explain the different and/or the deeper effects exerted on HUVECs by diabetic LDL (for instance, a modification in the concentrations of distinct fatty acids). A role of glycation of LDL must also be taken into consideration, since glycated LDL have been reported to exert an increased atherogenic action (41).

Further studies are also needed to elucidate the role of the LDL receptor in these modifications caused by short-term interactions of LDL with HUVECs. Based on the present data, it can be suggested that this short-term interaction modifies the physicochemical properties of the lipid moiety of the more superficial part of the plasma membrane. It can be hypothesized that the observed alterations in the activity of integral membrane-bound enzymes, such as Na^+-K^+-ATPase, and/or in transmembrane Ca^{2+} fluxes, with secondary enhancement of NOS activity, might be dependent on changes of the protein arrangement within the lipid moiety. Moreover, as mentioned above, a key regulatory role of membrane fluidity on the endothelial cell Ca^{2+} signaling has been recently suggested based on the negative correlation observed between bradykinin-initiated Ca^{2+} release and membrane viscosity (39).

LDLs have been implicated as one of the major factors that could modulate the development of atherosclerosis. Both native and oxidized lipoproteins can interact with the endothelial cells lining the vessel wall and might exert a role in the endothelial dysfunction observed in both atherosclerosis and diabetes (5,6). The present work demonstrated that the short-term incubation of endothelial cells with LDL obtained from type 1 diabetic patients causes deep modifications in both the plasma membrane and the cytosolic compartment, both of which are not present or are present to a smaller extent after incubation with LDL from healthy subjects. The alterations observed in endothelial function after the cell-lipoprotein interaction might play a central role in the atherogenic process.

ACKNOWLEDGMENTS

The present work was supported by a grant (60%) from the University of Ancona to L.M.

REFERENCES

1. Vane JR, Anggard EE, Botting RM: Regulatory functions of the vascular endothelium. *N Engl J Med* 323:27–36, 1990
2. Bevilacqua MP: Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol* 11:767–804, 1993
3. Pedrinelli R, Giampietro O, Cramassi F, Melillo E, Dell'Omo G, Catapano G, Matteucci E, Talarico L, Morale M, De Negri F, Di Bello V: Microalbuminuria and endothelial dysfunction in essential hypertension. *Lancet* 344:14–18, 1994
4. Bloom AL: The role of the endothelium in the regulation of haemostasis. In *Endothelial Cell Function in Diabetic Microangiopathy: Problems in Methodology and Clinical Aspects*. Vol. 9. Molinatti GM, Bar RS, Belfiore F, Porta M, Eds. Basel, Karger, 1990, p. 139–155
5. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Cayatte AJ, Rozek MM: Pathogenesis of the atherosclerotic lesion: implications for diabetes mellitus. *Diabetes Care* 15:1156–1167, 1992
6. Schror K: Blood vessel wall interactions in diabetes. *Diabetes* 46:S115–S118, 1997
7. Pritchard KA Jr, Wong PY-K, Stemerman MB: Atherogenic concentrations of low-density lipoprotein enhance endothelial cell generation of epoxyeicosatrienoic acid products. *Am J Pathol* 136:1381–1391, 1990
8. Pritchard KA Jr, Schwarz SM, Medow MS, Stemerman MB: Effect of low-density lipoprotein on endothelial cell membrane fluidity and mononuclear cell attachment. *Am J Physiol* 260:C43–C49, 1991
9. Thorin E, Hamilton C, Dominiczak AF, Dominiczak MH, Reid JL: Oxidized LDL-induced changes in membrane physicochemical properties and $[Ca^{2+}]_i$ of bovine aortic endothelial cells: influence of vitamin E. *Atherosclerosis* 114:185–195, 1995
10. Allen S, Khan S, Al-Mohanna F, Batten P, Yacoub M: Native low-density lipoprotein–induced calcium transients trigger VCAM-1 and E-selectin expression in cultured human vascular endothelial cells. *J Clin Invest* 101:1064–1075, 1998
11. Gupta S, Sussman I, McArthur CS, Tornheim K, Cohen RA, Ruderman NB: Endothelium-dependent inhibition of Na^+-K^+-ATPase activity in rabbit aorta by hyperglycemia: possible role of endothelium-derived nitric oxide. *J Clin Invest* 90:727–732, 1992
12. Edelstein C, Scanu AM: Precautionary measures for collecting blood destinated for lipoprotein isolation. In *Methods in Enzymology*. Segrest JP, Albers JJ, Eds. New York, Academic, 1986, p. 151–155
13. Chen LY, Mehta P, Mehta JL: Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidized LDL on platelet function. *Circulation* 93:1740–1746, 1996
14. Rabini RA, Ferretti G, Galassi R, Taus M, Curatola G, Tangorra A, Fumelli P, Mazzanti L: Modified fluidity and lipid composition in lipoproteins and platelet membranes from diabetic patients. *Clin Biochem* 27:381–385, 1994
15. Jiang Z-Y, Hunt JV, Wolff SP: Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density lipoprotein. *Anal Biochem* 202:384–389, 1992
16. Muragandam A, Mutus B: Isolation of nitric oxide synthase from human platelets. *Biochim Biophys Acta* 1200:1–6, 1994
17. Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB: Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* 47:945–952, 1998
18. Mazzanti L, Rabini RA, Faloia E, Fumelli P, Bertoli E, DePirro R: Altered cellular calcium and sodium transport in diabetes mellitus. *Diabetes* 39:850–854, 1990
19. Rao GHR: Measurement of ionized calcium in normal human blood platelets. *Anal Biochem* 169:400–404, 1988
20. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 6:3440–3450, 1985
21. Kitao T, Hattori K: Inhibition of erythrocyte ATPase activity by aclacinomycin and reverse effect of ascorbate on ATPase activity. *Experientia* 39:1362–1364, 1983
22. Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. *J Biol Chem* 66:375–400, 1925
23. Lowry OH, Rosenbrough NJ, Farr AL, Randall RT: Protein measurement with the Folin phenol reagent. *J Biol Chem* 192:265–267, 1951

24. Duportail G, Weinreb A: Photochemical changes of fluorescent probes in the membranes and their effect on the observed fluorescent anisotropy values. *Biochim Biophys Acta* 736:171–177, 1983
25. Mazzanti L, Staffolani R, Rabini RA, Cugini AM, Cester N, Romanini C, Faloia E, De Pirro R: Modifications in platelet membrane transport functions in insulin-dependent diabetes mellitus and in gestational diabetes. *Biochim Biophys Acta* 1139:65–69, 1992
26. Sheridan NP, Block ER: Plasma membrane fluidity measurements in intact endothelial cells: effect of hyperoxia on fluorescence anisotropies of 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene. *J Cell Physiol* 134: 117–123, 1988
27. Grunberger D, Haimovitz R, Shinitzky M: Resolution of plasma membrane lipid fluidity in intact cells labeled with diphenylhexatriene. *Biochim Biophys Acta* 688:764–774, 1982
28. Folch J, Lees M, Sloane-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:466–468, 1957
29. Esterbauer H, Striegl H, Puhl H, Rotheneder M: Continuous monitoring of in vitro oxidation of human low-density lipoprotein. *Free Radic Res Commun* 6:67–75, 1989
30. Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB: Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* 47:945–952, 1998
31. Tsukahara H: Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 193:722–729, 1993
32. Block ER, Edwards D: Effect of plasma membrane fluidity on serotonin transport by endothelial cells. *Am J Physiol* 253:C672–C678, 1987
33. Tesfamariam B, Cohen RA: Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* 263:H321–H326, 1992
34. Graier WF, Simecek S, Kukovetz WR, Kostner GM: High D-glucose–induced changes in endothelial Ca^{2+}/EDRF signaling are due to generation of superoxide anions. *Diabetes* 45:1386–1395, 1996
35. Knowles RG, Moncada S: Nitric oxide synthases in mammals. *Biochem J* 298:249–258, 1994
36. Mogensen CE: Glomerular filtration rate and renal plasma flow in short-term and long-term juvenile diabetes mellitus. *Scand J Clin Lab Invest* 28:91–100, 1971
37. Van der Vliet A, Eiserich JP, O'Neill CA, Halliwell B, Cross CE: Tyrosine modification by reactive nitrogen species: a closer look. *Arch Biochem Biophys* 319:341–349, 1995
38. Pieper GM, Dondlinger LA: Antioxidant pyrrolidine dithiocarbamate prevents defective bradykinin-stimulated calcium accumulation and nitric oxide activity following exposure of endothelial cells to elevated glucose concentration. *Diabetologia* 41:806–812, 1998
39. Graier WF, Hoebel BG, Paltauf-Doburzynska J, Kostner GM: Effects of superoxide anions on endothelial Ca^{2+} signaling pathways. *Arterioscler Thromb Vasc Biol* 18:1470–1479, 1998
40. Salvolini E, Rabini RA, Martarelli D, Moretti N, Cester N, Mazzanti L: A study on human umbilical cord endothelial cells: functional modifications induced by plasma from insulin-dependent diabetes mellitus (IDDM) patients. *Metab - olism* 48:554–557, 1999
41. Lopes-Virella MF, Klein RL, Lyons TJ, Stevenson HC, Witztum JL: Glycosylation of low-density lipoprotein enhances cholesteryl ester synthesis in human monocyte-derived macrophages. *Diabetes* 37:550–554, 1988