Glycoxidized Low-Density Lipoprotein Enhances Monocyte Chemoattractant Protein-1 mRNA Expression in Human Umbilical Vein Endothelial Cells: Relation to Lysophosphatidylcholine Contents and Inhibition by Nitric Oxide Donor

Kazuo Sonoki, Mototaka Yoshinari, Masanori Iwase, Kenzo Iino, Kojiro Ichikawa, Shigehiro Ohdo, Shun Higuchi, and Mitsuo Iida

Low-density lipoprotein (LDL) may undergo more glycation or oxidation in patients with diabetes mellitus than in nondiabetic subjects. We investigated whether glycoxidized LDL (goLDL) induces monocyte chemoattractant protein-1 (MCP-1) mRNA expression through activation of nuclear factor-kappaB (NFκB), and determined the effect of nitric oxide (NO) on MCP-1 mRNA expression in human umbilical vein endothelial cells (HUVEC). Oxidized (oxLDL) or goLDL enhanced MCP-1 mRNA expression in HUVEC, and preincubation with NOR3, a NO donor, abrogated such stimulation. goLDL increased NFκB-DNA binding activity in HUVEC and this effect was also suppressed by NOR3. We measured lysophosphatidylcholine (lyso-PC) contents in modified LDL using electrospray ionization liquid chromatography/mass spectrometry (LC/MS) to identify its molecular species. MCP-1 mRNA expression and NFκB activation correlated significantly with palmitoyl- and stearoyl-lyso-PC contents in LDL. Our results suggest that LDL modified by glycation and oxidation may contribute to the development of accelerated atherosclerosis in the presence of diabetes, a process that may be prevented by increased vascular NO availability.

IT IS WELL ESTABLISHED that oxidative modification of low-density lipoprotein (LDL) plays a causal role in human atherogenesis. The development of atherosclerosis is accelerated in patients with diabetes mellitus, and LDL in a diabetic state is susceptible to glycation[1,2] and oxidation.[3,4] Recent immunohistochemical studies of human atherosclerotic lesions demonstrated that advanced glycation end products (AGE) and oxidized LDL (oxLDL) are colocalized in the cytoplasm of macrophage-derived foam cells.[5] Therefore, it is conceivable that glycoxidized LDL (goLDL) may contribute to the development and progression of atherosclerosis in diabetes mellitus.

LDL oxidized by enzymes or copper ion induces the expression of chemokines, including monocyte chemoattractant protein-1 (MCP-1), in various cells.[6-8] MCP-1 has been implicated as a pathogenetic factor in the infiltration of monocytes into the subendothelial space,[9,10] which is a crucial initial step in atherogenesis. Monocyte penetration through the vascular endothelium is thought to occur in response to a gradient of MCP-1 released from activated endothelial cells.[11] Although oxLDL upregulates the gene expression of MCP-1 by activating the transcription factor nuclear factor-kappaB (NFκB) in endothelial cells,[12] whether goLDL also activates MCP-1 expression similar to oxLDL has not yet been determined.

Nitric oxide (NO), a potent vasodilator released by endothelial cells, is thought to play a key role in preventing the development of atherosclerosis by inhibiting smooth muscle cell proliferation,[13] endothelin generation,[14] and leukocyte adhesion to the endothelium.[15] NO suppresses MCP-1 expression induced by tumor necrosis factor-alpha (TNFα) in human endothelial cells[16] and by lipopolysaccharide in rabbit smooth muscle cells[7] by inhibiting NFκB activation. The present study was designed to investigate whether goLDL induces MCP-1 mRNA expression through NFκB activation, and to determine any protective effect for NO on MCP-1 expression in human umbilical vein endothelial cells (HUVEC). In addition, we measured 2 molecular species of lysophosphatidylcholine (lyso-PC) contents (16:0, 18:0), the main constituent of oxidation, in modified LDL and investigated the relationship between lyso-PC contents and MCP-1 mRNA expression or NFκB activation in HUVEC.

MATERIALS AND METHODS

Cell Culture

HUVEC were isolated from umbilical cord veins using 0.25% trypsin (Difco Laboratories, Detroit, MI) according to the method of Jaffe et al[17] with minor modifications. Cells were grown in M199 medium supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Life Technologies, Rockville, MD), 100 μg/mL heparin (Sigma Chemical Co, St Louis, MO), 20 μg/mL endothelial cell growth supplement (Upstate Biotechnology, New York, NY), 0.33 mg/mL piperacillin sodium (Sankyo Co, Tokyo, Japan) in a humidified atmosphere containing 5% CO_2. The cells were used prior to the 6th passage.

Isolation and Modification of LDL

LDL was isolated from human plasma using density-gradient ultracentrifugation.[18] In brief, venous blood samples were collected into tubes containing EDTA from consented healthy volunteers after an overnight fast. After centrifugation, the plasma was adjusted to a density of 1.21 g/mL by adding KBr. In centrifuge tubes, a discontinuous density gradient was created by overlaying 2.8 mL plasma solution with 6.6 ml HEPES buffer solution (0.02 mol/L HEPES, 0.4 mol/L NaCl, and 0.4 mol/L NaOH, pH 7.4, $d = 1.007$ g/mL). The tubes were ultracentrifuged at 65,000 rpm for 3 hours at 15°C. The LDL fraction was aspirated and dialyzed by 0.15 mol/L NaCl, pH 7.4. Thereafter, the LDL solution was sterilized through a filter with a pore size of 0.45 μm

From the Department of Medicine and Clinical Science, Graduate School of Medical Sciences, and the Department of Clinical Pharmacokinetics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

Submitted August 27, 2001; accepted March 4, 2002.

Address reprint requests to Masanori Iwase, MD, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan.

0026-0495/02/5109-0009$35.00/0
doi:10.1053/meta.2002.34703

(Millipore, Bedford, MA). EDTA at a final concentration of 0.26 mmol/L was added to an aliquot of LDL solution, which was stored at 4°C under N_2 gas, as a native LDL (nLDL) preparation until final dialysis.

Glycated LDL (gLDL) was obtained by incubating an aliquot of the LDL solution with 200 mmol/L glucose at 37°C for 3 days. oxLDL was prepared by incubation with 5 μmol/L $CuSO_4$ at 37°C for 12 hours after dialysis with 0.15 mol/L NaCl, pH 7.4. goLDL was prepared by incubating gLDL with 5 μmol/L $CuSO_4$ at 37°C for 12 hours. Thereafter, all LDL preparations were dialyzed with 0.15 mol/L NaCl and 0.26 mmol/L EDTA, pH 7.4, and then stored at 4°C under N_2 gas. The protein concentration of each LDL preparation was determined by the Coomassie brilliant blue method using bovine serum albumin as a standard (Nacalai Tesque, Kyoto, Japan).

Electrophoresis of Lipoprotein and Measurement of Lipid Peroxidation

Electrophoresis of lipoprotein was carried out to ascertain the purity of the LDL fraction and to measure the electrophoretic mobility using a commercial kit (Titan Gel Lipoproteins; Helena Laboratories, Saitama, Japan). In brief, 1 μL of nLDL and human serum were loaded on an agarose gel sheet and electrophoresed in barbital buffer at a voltage of 100 V for 25 minutes. The gel sheet was dried and stained with 0.04% Fat Red 7B in methanol.

Lipid peroxidation was estimated by thiobarbituric acid-reactive substances (TBARS) method of Yagi[19] with minor modifications. In brief, 200 μg of protein of each modified LDL was mixed with 10% phosphotungistic acid and was centrifuged. The sediment was mixed with 0.67% thiobarbituric acid solution and then heated at 95°C for 60 minutes. After cooling, n-butanol was added and centrifuged. The absorbance of the n-butanol layer was read at 532 nm by spectrophotometer (U-1100, Hitachi, Tokyo, Japan). Serial dilutions of 1,1,3,3-tetramethoxypropane (Sigma), which yields malondialdehyde (MDA), was used to construct the standard curve. TBARS was expressed as nanomoles of MDA per milligram LDL protein.

Measurement of lyso-PC in Modified LDL by Electrospray Ionization Liquid Chromatography/Mass Spectrometry

The content of lyso-PC in modified LDL was measured by liquid chromatography/mass spectometry (LC/MS).[20] First, the modified LDL was concentrated and dialyzed with 0.05 mol/L HEPES buffer containing 1 mol/L NaCl and 1 mol/L NaOH, pH 7.4, by centrifuging in an Ultrafree-15 centrifugal filter (Millipore). After determination of the protein concentrations of each LDL, lipids were extracted from 50 μL of LDL supplemented with 500 ng of 1,2-Dimyristoyl-sn-Glycero-3-Phosphatidylcholine (DMPC; Avanti Polar-Lipids, Alabaster, AL) as an internal standard, according to the method of Bligh and Dyer.[21] Lipid extracts were evaporated under N_2 gas, and the residue was dissolved in 100 μL chloroform. Phospholipids were separated from the extracted lipids by the method of Kaluzny et al[22] using aminopropyl solid-phase extraction chromatography (BAKERBOND spe Columns; J.T. Baker Inc, Phillipsburg, NJ). The separated phospholipid fractions were evaporated under N_2 gas and resuspended in 200 μL methanol. Phospholipids were immediately introduced into the electrospray mass spectrometer (LCQ; ThermoQuest, Tokyo, Japan) via high-performance liquid chromatography (HPLC; LC-10, Shimazu, Kyoto, Japan). Reverse-phase high-performance liquid chromatography (HPLC) was performed by injecting 20 μL of isolated phospholipids in methanol into an STR-ODS analytical microcolumn (150 mm $\times$ 2.0 mm, 5 μmol/L; Shimazu) at a flow rate of 0.3 mL/min and eluting with a mobile solvent of methanol/acetonitrile/deionized water (84:14:2, vol/vol/vol). The mass spectrometer was operated in positive mode employing the "full-scan" function set from m/z 100 to 1,000. Quantitative analysis of phospholipids was performed essentially as described by Han et al,[23] using DMPC as an internal standard. Phospholipids were quantitated based on their ion intensity relative to the internal standard. LC/MS chromatogram of LDL showed the peaks of palmitoyl-lyso-PC, stearoyl-lyso-PC, and DMPC (used as an internal standard), at retention times of 2.03, 2.50, and 5.54 minutes, respectively. The electrospray ionization mass spectrum of LDL at retention time 2.03 minutes showed 4 masses for palmitoyl-lyso-PC: m/z 496.5, 497.5, 518.4, and 519.4, which resembled the masses of the standard product of palmitoyl-lyso-PC. The concentration of palmitoyl-lyso-PC was calculated by integrating the relative intensities of the 4 masses for 10 minutes after application in comparison with the standard DMPC. The concentration of stearoyl-lyso-PC in each LDL was calculated similarly by integrating the relative intensities of stearoyl-lyso-PC′s 4 masses, m/z 524.6, 525.5, 546.5, and 547.6. The coefficient of variation for electrospray LC/MS assay was 4.8% (n = 12) with 5 ng/mL DMPC as the internal control. Standard curve experiments showed that the electrospray LC/MS method was linear over a wide range (0.1 to 5.0 ng/μL of palmitoyl- or stearoyl-lyso-PC).

Measurement of MCP-1 mRNA Expression

Cells were incubated with or without 100 μg/mL modified LDL in M199 containing 2% FCS for 4 hours. MCP-1 mRNA content was then measured by Northern blot analysis. In brief, total RNA was isolated from HUVEC by using Isogen (Nippon Gene, Tokyo, Japan). Total RNA (15 μg) was separated by electrophoresis and transferred to Hybond-N+ nylon membranes (Amersham International, Bucking-

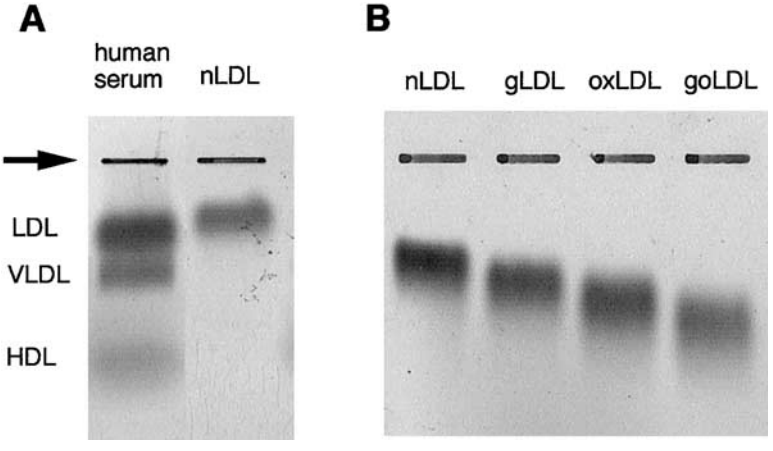

Fig 1. (A) Agarose gel electrophoresis of LDL. Human serum or nLDL was applied to agarose gel electrophoresis, and stained with Fat Red 7B. Arrow indicates the site of application. (B) Electrophoretic mobility of nLDL, gLDL, oxLDL, and goLDL.

Table 1. Parameters of Oxidation in LDL Modified In Vitro

	nLDL	gLDL	oxLDL	goLDL
Electrophoretic mobility (mm)	7.58 ± 0.45	8.56 ± 0.48	9.56 ± 0.36*	11.08 ± 0.54†§¶
TBARS (nmol MDA/mg protein)	1.26 ± 0.08	1.34 ± 0.10	7.73 ± 0.94†§	7.90 ± 0.82†§
Palmitoyl-lyso-PC (μg/mg protein)	3.32 ± 0.30	7.62 ± 0.34*	14.69 ± 1.77†‡	19.81 ± 2.46†§¶
Stearoyl-lyso-PC (μg/mg protein)	2.61 ± 0.34	5.18 ± 0.48*	9.71 ± 0.84†‡	11.85 ± 1.58†§

NOTE. Values are means ± SEM of 5 measurements for electrophoretic mobility and TBARS or 6 measurements for lyso-PC.
Abbreviations: TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; lyso-PC, lysophosphatidylcholine.
*$P < .05$, †$P < .001$ *v* nLDL; ‡$P < .01$, §$P < .001$ *v* gLDL; ¶$P < .05$ *v* oxLDL.

hamshire, England). Hybridization was performed for 1 hour at 65°C in QuikHyb hybridization solution (Stratagene, La Jolla, CA) with a human MCP-1 cDNA (R&D Systems, Minneapolis, MN) or GAPDH cDNA (Oncogene Research Products, Cambridge, MA), that had been labeled with (γ-^{32}P) adenosine triphosphate (ATP) (Amersham) by T4 polynucleotide kinase (Promega, Madison, WI). Autoradiography and quantitative analysis were performed with a Bio-Imaging Analyzer (Fuji Photo Film Co, Kanagawa, Japan).

Measurement of NFκB Activity

HUVEC were incubated with or without 100 μg/mL of modified LDL in M199 medium containing 2% FCS for 2 hours, and nuclear extracts were then prepared by a modified method of Schreiber et al.[24] In brief, after HUVEC were stimulated with modified LDL, the cells were washed with phosphate-buffered saline (PBS) and suspended in 0.4 mL buffer. After incubation on ice for 15 minutes, 25 μL of 10% Nonidet P-40 (Sigma) was added. The mixture was centrifuged and the nuclear pellet was suspended in 50 μL ice-cold buffer. The suspension was sonicated and shaken at 4°C for 15 minutes. The supernatant of nuclear extracts, spun at 13,000 × *g* at 4°C for 5 minutes, was stored at -80°C until use after determination of protein concentrations.

Electrophoretic mobility shift assay (EMSA) was performed by incubating nuclear proteins (4 μg) in 10 μL of binding buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 4% glycerol, and 0.05 mg/ml poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ) for 10 minutes at room temperature. Next, 1 μl of ^{32}P-labeled NFκB oligonucleotide probe (Promega) was added, and the reaction mixture was incubated for 20 minutes at room temperature. For competition assays, 100-fold concentrations of unlabeled oligonucleotides were added to the nuclear proteins. For supershift experiments, antibodies against p50 and p65 (Santa Cruz Biotechnologies, Santa Cruz, CA) were added to

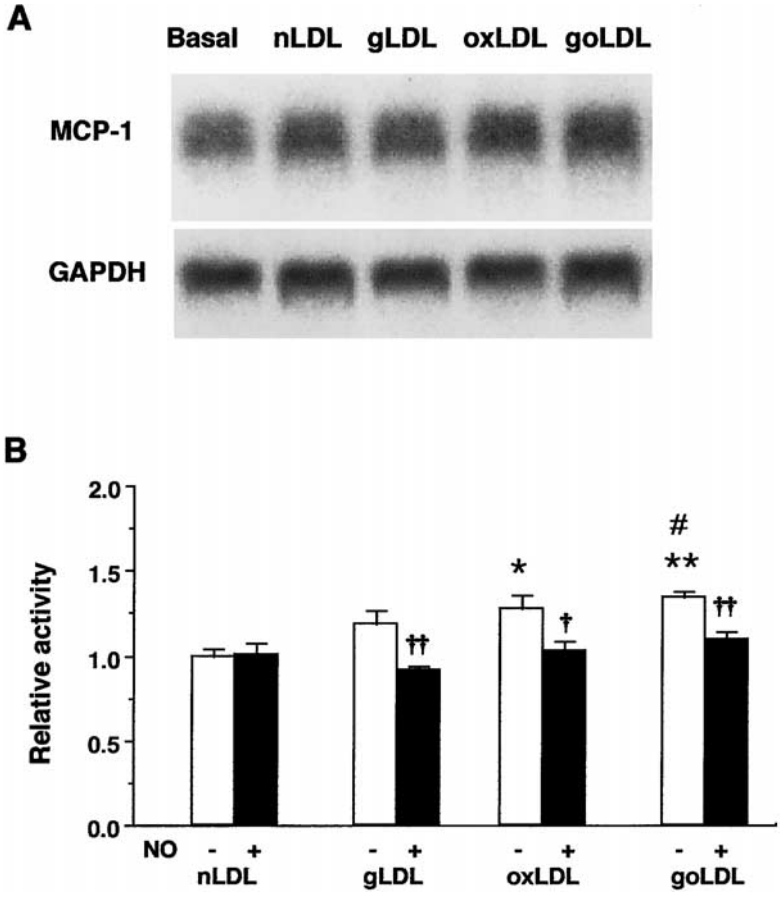

Fig 2. (A) Northern blot analysis of MCP-1 mRNA induced by modified LDL in HUVEC. Cells were incubated with or without 100 μg/mL modified LDL for 4 hours. (B) Relative intensity of MCP-1 mRNA expression corrected by GAPDH when the expression in the basal state was considered to be 1.0. (□) Expression without NOR3, a NO donor; (■) expression with NOR3 preincubation. Each bar represents the mean ± SEM of 4 experiments. *$P < .05$, **$P < .01$ *v* nLDL; #$P < .05$ *v* gLDL; †$P < .05$, ††$P < .01$ *v* without NOR3.

the nuclear proteins and then incubated for 10 minutes at room temperature. All samples were loaded onto 6% polyacrylamide gel and electrophoresed. Gel contents were dried and autoradiographed by a Bio-Imaging Analyzer.

Effect of NO Donor on MCP-1 mRNA and NFκB

HUVEC were incubated with or without 100 μmol/L of 4-ethyl-2-hydroxyimino-5-nitro-3-hexenamide (NOR3), NO donor (Dojindo Laboratories, Kumamoto, Japan) in M199 containing 2% FCS, 1 hour before incubation with the modified LDL. The expression of MCP-1 mRNA and NFκB activity were determined as described above.

Statistical Analysis

Data were presented as the mean ± SEM. Statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by Fisher's test to detect significant differences in multiple comparisons. Relationships between 2 continuous variables were examined by Pearson's correlation analysis. A P value of less than .05 was considered statistically significant.

RESULTS

Isolated nLDL showed a single band located in the same position as LDL found in human serum in agarose gel electrophoresis stained by Fat Red 7B (Fig 1A), indicating no protein contaminations in nLDL (stained by Sudan Black B; data not shown). Figure 1B shows the electrophoretic mobility of nLDL, gLDL, oxLDL, and goLDL. The distance from the applied line to the band was significantly greater in oxLDL than in nLDL (Table 1). The mobility distance was significantly greater in goLDL than in nLDL, gLDL, and oxLDL, respectively.

Lipid peroxidation measured by TBARS was significantly higher in oxLDL and goLDL compared with nLDL and gLDL, respectively (Table 1). Palmitoyl- and stearoyl-lyso-PC contents expressed as micorgrams per milligram LDL protein were significantly higher in gLDL than in nLDL, respectively, and those in oxLDL and goLDL were significantly higher than in nLDL and gLDL, respectively. Palmitoyl-lyso-PC content was significantly higher in goLDL compared to oxLDL (Table 1).

Expression of MCP-1 mRNA levels was evaluated in HUVEC incubated with nLDL or modified LDL for 4 hours using Northern blot analysis (Fig 2A). MCP-1 mRNA expression relative to GAPDH mRNA was significantly higher in oxLDL and goLDL compared with nLDL (Fig 2B). The addition of NOR3, a NO donor, significantly suppressed the expression of MCP-1 mRNA in HUVEC incubated with gLDL, oxLDL and goLDL (Fig 2B). As shown in Fig 3, MCP-1 mRNA expression correlated significantly with palmitoyl-lyso-PC (Fig 3A), as well as with stearoyl-lyso-PC contents in LDL (Fig 3B).

A weak NFκB-specific band was identified in the basal state by unlabeled NFκB oligonucleotide probe and by the supershift assay using specific antibodies against p50 and p65 (Fig 4A). We also determined NFκB-DNA binding activity in HUVEC following exposure to 100 μg/mL of native or modified LDL for 2 hours (Fig 4B). The density of the NFκB-specific band relative to the basal state was significantly higher when the cells were incubated with goLDL compared with nLDL or gLDL (Fig 4C). NO donor significantly reduced the density of the NFκB-specific band of goLDL (Fig 4C). As shown in Fig 5, NFκB activity correlated significantly with palmitoyl-lyso-PC (Fig 5A), as well as with stearoyl-lyso-PC contents in LDL (Fig 5B).

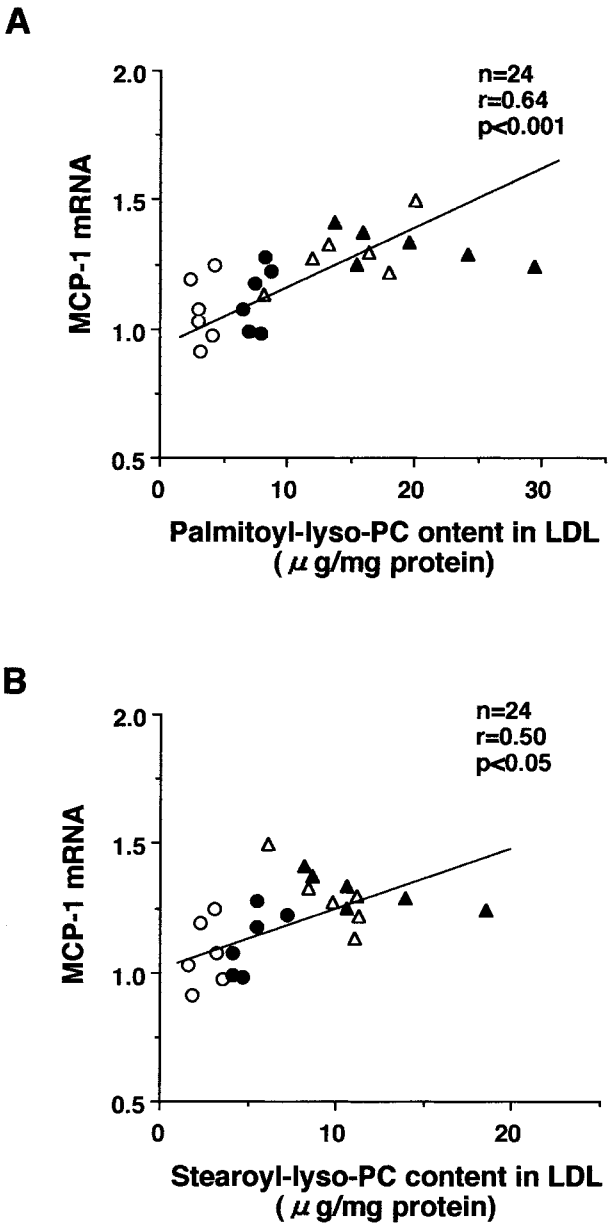

Fig 3. Correlation of MCP-1 mRNA expression with the contents of (A) palmitoyl-lyso-PC or (B) stearoyl-lyso-PC. (○) nLDL; (●) gLDL; (△) oxLDL; (▲) goLDL.

DISCUSSION

The present study demonstrated that HUVEC incubated with oxLDL or goLDL enhanced MCP-1 mRNA expression, and preincubation with NO donor reversed the stimulation of MCP-1 mRNA expression. NFκB-DNA binding activity was increased in HUVEC incubated with goLDL and was also suppressed by NO donor. We measured lyso-PC by electro-

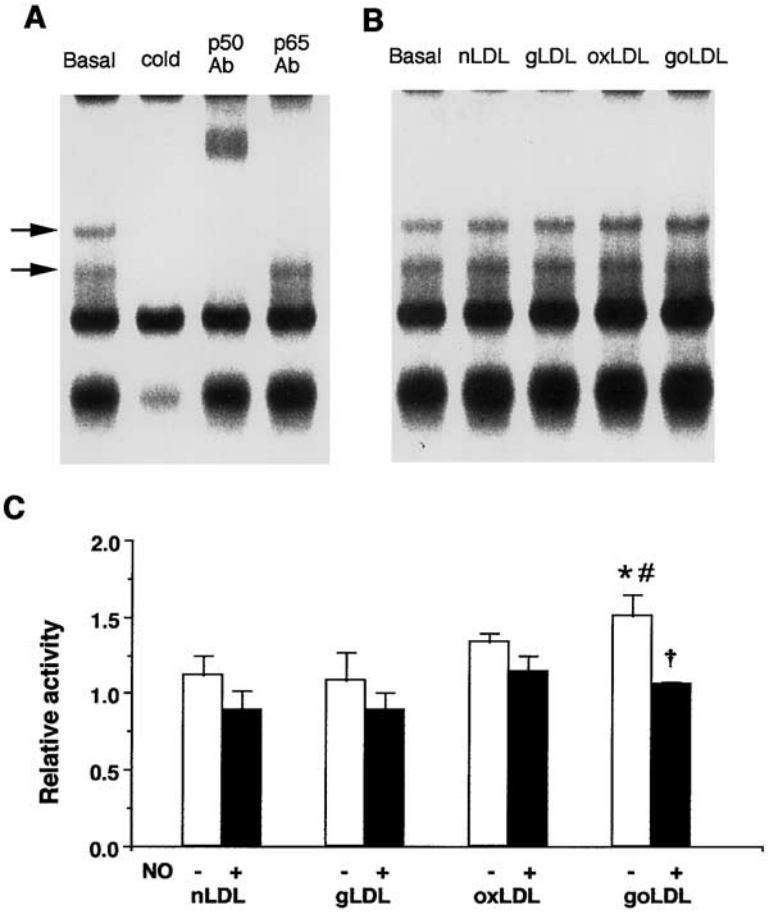

Fig 4. (A) NFκB-DNA binding activity was determined by an electrophoretic mobility shift assay. NFκB-specific bands (indicated by 2 arrows) were identified by competitive (lane 2) and supershift assays (lanes 3 and 4). (B) NFκB-DNA binding activity in HUVEC exposed to nLDL, gLDL, oxLDL, and goLDL. (C) NFκB-DNA binding activity relative to the basal state. (□) Activity without NOR3; (■) activity with NOR3 preincubation. Data are mean ± SEM of 4 experiments. *$P < .05$ *v* nLDL; #$P < .05$ *v* gLDL; †$P < .05$ *v* without NO.

spray to identify its molecular species. MCP-1 mRNA expression and NFκB activation correlated significantly with palmitoyl- and stearoyl-lyso-PC contents in LDL, respectively.

It has been reported that minimally modified LDL by oxidation induces MCP-1 mRNA expression in endothelial cells.[6,25] Minimally modified LDL has been defined as LDL that has mild lipid peroxidation and binds native LDL receptors but not scavenger receptors.[6] Recently, it was reported that highly oxidized LDL (TBARS 182 nmol/mg protein) upregulated lectin-like receptor for oxLDL (LOX-1) and activated MCP-1 mRNA.[26] Since it was reported that more oxidized goLDL than that in our study (TBARS 26.8 nmol/mg protein) was metabolized through native LDL receptors but not by scavenger receptors,[27] our goLDL seems to bind native LDL receptors and activate MCP-1 mRNA expression in HUVEC as minimally modified LDL. Since the effects of modified LDL and NO donor on HUVEC were investigated only at particular time, time studies will be required to clarify these effects in detail.

Bucala et al[28] demonstrated that incubation of LDL with 200 mmol/L glucose for 3 days resulted in the formation of readily measurable levels of AGE on both lipid and apoprotein. Other studies demonstrated that AGE induced the expression of heme oxygenase, tissue factor, and vascular cell adhesion molecule-1 (VCAM-1) genes by activating NFκB in cultured endothelial cells.[29-31] gLDL mimics oxLDL in terms of accumulation of cholesteryl ester in macrophages[32] and the expression of adhesion molecules in endothelial cells.[33] The present study showed that lyso-PC contents were significantly higher in gLDL prepared by 3-day incubation under 200 mmol/L glucose than in nLDL, indicating that oxidation occurred in the glycation process in LDL. However, gLDL did not stimulate MCP-1 mRNA expression in HUVEC. The glucose autoxidation in our gLDL might be too mild to elicit biologic responses in HUVEC. On the other hand, gLDL seems to be oxidized more readily than nLDL, because palmitoyl-lyso-PC content was significantly higher in goLDL than in oxLDL. This is in line with the report of Sakurai et al[34] who showed that gLDL incubated with Fe^{3+} caused a higher level of lipid peroxidation than nLDL. Stimulation of MCP-1 mRNA expression by goLDL was significantly enhanced compared to nLDL and gLDL. These findings suggest that the co-occurrence of glycation and oxidation in LDL strongly promotes its atherogenecity.

Lyso-PC increased the DNA-binding activity of NFκB partly through a protein kinase C (PKC)-mediated pathway[35] and stimulated MCP-1 gene expression in HUVEC.[36] Lyso-PC increased vascular superoxide anion production via PKC activation in intact vessels,[37] and stimulated O_2^- production partly through a membrane-associated NADH-dependent O_2^- production system.[38] Superoxide is considered as a second messenger

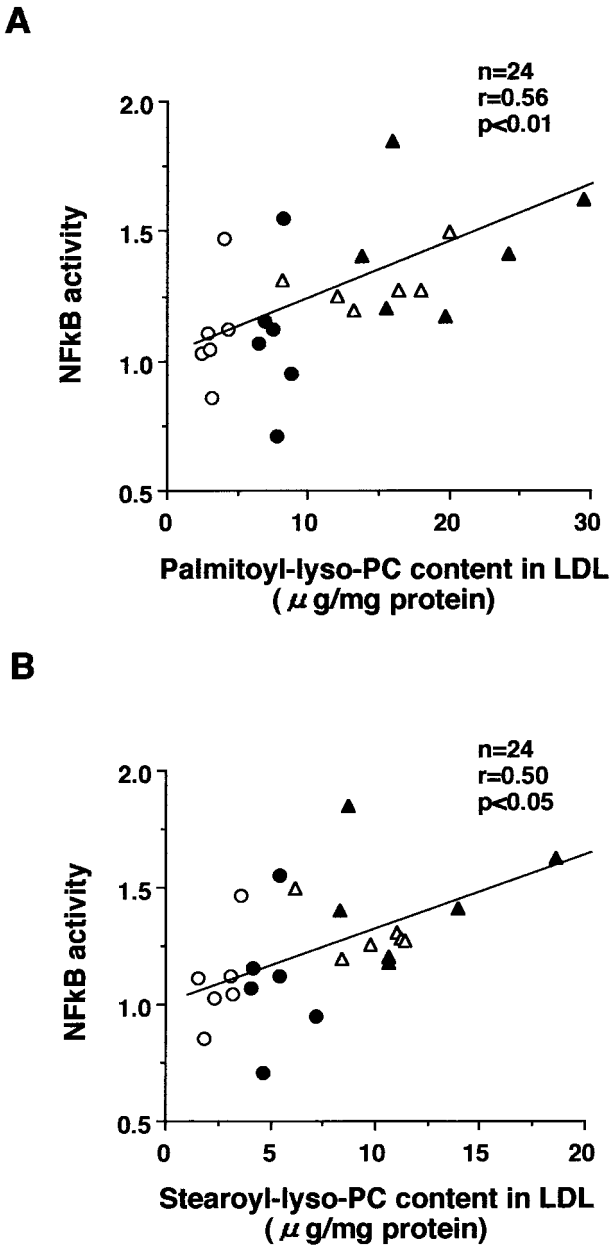

Fig 5. Correlation of NFκB-DNA binding activity with the contents of (A) palmitoyl-lyso-PC and (B) stearoyl-lyso-PC. (○) nLDL; (●) gLDL; (△) oxLDL; (▲) goLDL.

for activating the transcription factor NFκB, followed by the expression of MCP-1 mRNA in human smooth muscle cells[39] and in mouse mesangial cells.[40] In the present study, therefore, increased lyso-PC content in goLDL may cause MCP-1 gene expression in response to NFκB activation probably due to the generation of superoxide in HUVEC. We have previously measured palmitoyl- and stearoyl-lyso-PC contents in LDL using HPLC, and showed that lyso-PC contents of both species were higher in diabetic patients than in nondiabetic control.[41] In addition, diabetic patients with macroangiopathy had higher levels of palmitoyl-lyso-PC than those without macroangiopathy, whereas stearoyl-lyso-PC levels did not differ between the 2 groups. In the present study, however, palmitoyl- and stearoyl-lyso-PC contents in LDL showed similar correlation with MCP-1 mRNA expression and NFκB activation in HUVEC. Since palmitoyl- and stearoyl-lyso-PC are known to exhibit certain biologic differences,[42,43] the differences between the 2 species of lyso-PC related to the atherogenetic activities other than MCP-1 gene expression, remain to be clarified.

NO donors significantly attenuated lyso-PC-induced upregulation of p-selectin by suppressing PKC activation in cat endothelial cells.[44] In addition, MCP-1 expression and NFκB activation were reduced by NO donor in rabbit smooth muscle cells.[7] In the present study, we demonstrated that NO donor significantly suppressed MCP-1 mRNA expression and NFκB activation induced by goLDL in HUVEC. In this regard, Katsuyama et al[45] reported that NO donors inhibit the expression of inducible NO synthase (iNOS) and activation of NFκB induced by interleukin-1β in rat smooth muscle cells, by directly inhibiting the phosphorylation and subsequent degradation of IκBα, the cytoplasmic inhibitor of NFκB. In contrast, Spiecker et al[46] reported that NO donor decreased TNFα-induced VCAM-1 expression in human endothelial cells via inhibition of NFκB activation, by increasing the expression and nuclear translocation of IκBα, but not by preventing the phosphorylation and degradation of IκBα. The apparent discrepancy between these studies may be due to the different cell types, species, cytokines, and NO donors used in the experiments. Since it has been reported that vascular NO availability is reduced in diabetes mellitus,[47-49] goLDL may induce MCP-1 mRNA expression in endothelial cells under diabetic condition, leading to accelerated atherosclerosis.

In conclusion, goLDL stimulated HUVEC and upregulated MCP-1 mRNA expression at least in part due to an enhanced NFκB activity, which was completely inhibited by a NO donor. Palmitoyl- and stearoyl-lyso-PC in LDL correlated with NFκB activity and MCP-1 gene expression, respectively. The present study suggests that LDL modified by glycation and oxidation may contribute to the development of accelerated atherosclerosis under diabetic condition, a process that could be prevented by increased vascular NO availability.

REFERENCES

1. Cohen MP, Lautenslager G, Shea E: Glycated LDL concentrations in non-diabetic and diabetic subjects measured with monoclonal antibodies reactive with glycated apolipoprotein B epitopes. Eur J Clin Chem Clin Biochem 31:707-713, 1993

2. Klein RL, Laimins M, Lopes-Virella MF: Isolation, characterization, and metabolism of the glycated and nonglycated subfractions of low-density lipoproteins isolated from type I diabetic patients and nondiabetic subjects. Diabetes 44:1093-1098, 1995

3. Kobayashi K, Watanabe J, Umeda F, et al: Glycation accelerates the oxidation of low density lipoprotein by copper ions. Endocrinol J 42:461-465, 1995

4. Bowie A, Owens D, Collins P, et al: Glycosylated low density

lipoprotein is more sensitive to oxidation: Implications for the diabetic patient? Atherosclerosis 102:63-67, 1993

5. Imagawa Y, Sakata N, Takebayashi S, et al: In vivo and in vitro evidence for the glycoxidation of low density lipoprotein in human atherosclerotic plaques. Atherosclerosis 150:343-355, 2000

6. Cushing SD, Berliner JA, Valente AJ, et al: Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci USA 87:5134-5138, 1990

7. Tsao PS, Wang BY, Buitrago R, et al: Nitric oxide regulates monocyte chemotactic protein-1. Circulation 96:934-940, 1997

8. Klouche M, Gottschling S, Gerl V, et al: Atherogenic properties of enzymatically degraded LDL: Selective induction of MCP-1 and cytotoxic effects on human macrophages. Arterioscler Thromb Vasc Biol 18:1376-1385, 1998

9. Yla-Herttuala S, Lipton BA, Rosenfeld ME, et al: Expression of monocyte chemoattractant protein 1 in macrophage rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 88:5252-5256, 1991

10. Nelken NA, Coughin SR, Gordon D, et al: Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 88: 1121-1127, 1991

11. Navab M, Imes SS, Hama SY, et al: Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. J Clin Invest 88:2039-2046, 1991

12. Collins T: Endothelial nuclear factor-κB and initiation of the atherosclerotic lesion. Lab Invest 68:499-508, 1993

13. Garg UC, Hassid A: Nitric-oxide generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 83:1774-1777, 1989

14. Boulanger C, Luscher T: Release of endothelin from the porcine aorta: Inhibition by endothelium-derived nitric oxide. J Clin Invest 85:587-590, 1990

15. Kubes P, Suzuki M, Granger DN: Nitric oxide: An endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci USA 88:4651-4655, 1991

16. Zeiher AM, Fisslthaler B, Schray-Utz B, et al: Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. Circ Res 76:980-986, 1995

17. Jaffe EA, Nachman RL, Becker CG, et al: Culture of human endothelial cells derived from umbilical veins. J Clin Invest 52:2745-2756,1973

18. Vieira OV, Laranjinha JAN, Madeira VMC, et al: Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants. J Lipid Res 37:2715-2721, 1996

19. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med 15:212-216, 1976

20. Watson AD, Leitinger N, Navab M, et al: Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J Biol Chem 272: 13597-13607, 1997

21. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917, 1959

22. Kaluzny MA, Duncan LA, Merritt MV, et al: Rapid separation of lipid classes in high yield and purity using bonded phase columns. J Lipid Res 26:135-140, 1985

23. Han X, Gubitosi-Klug RA, Collins BJ, et al: Alterations in individual molecular species of human platelet phospholipids during thrombin stimulation: Electrospray ionization mass spectrometry-facilitated identification of the boundary conditions for the magnitude and selectivity of thrombin-induced platelet phospholipid hydrolysis. Biochemistry 35:5822-5832, 1996

24. Schreiber E, Matthias P, Muller MM, et al: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 17:6419, 1989

25. Parhami F, Fang ZT, Fogelman AM, et al: Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. J Clin Invest 92:471-478, 1993

26. Li D, Mehta JL: Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. Circulation 101:2889-2895, 2000

27. Kobayashi K, Watanabe J, Umeda F, et al: Metabolism of oxidized glycated low-density lipoprotein in cultured bovine aortic endothelial cells. Horm Metab Res 27:356-362, 1995

28. Bucala R, Makita Z, Koschinsky T, et al: Lipid advanced glycosylation: pathway of lipid oxidation in vivo. Proc Natl Acad Sci USA 90:6434-6438, 1993

29. Yan SD, Schmidt AM, Anderson GM, et al: Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem 269:9889-9897, 1994

30. Bierhaus A, Illmer T, Kasper M, et al: Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. Circulation 96:2262-2271, 1997

31. Schmidt AM, Hori O, Chen JX, et al: Advanced glycation endproducts interacting with their endothelial receptor induced expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. J Clin Invest 96:1395-1403, 1995

32. Lopes-Virella MF, Klein RL, Lyons TJ, et al: Glycosylation of low-density lipoprotein enhances cholesteryl ester synthesis in human monocyte-derived macrophages. Diabetes 37:550-557, 1988

33. Khan BV, Parthasarathy SS, Alexander RW, et al: Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J Clin Invest 95:1262-1270, 1995

34. Sakurai T, Kimura S, Nakano M, et al: Oxidative modification of glycated low density lipoprotein in the presence of iron. Biochem Biophys Res Commun 177:433-439, 1991

35. Sugiyama S, Kugiyama K, Ogata N, et al: Biphasic regulation of transcription factor nuclear factor-κB activity in human endothelial cells by lysophosphatidylcholine through protein kinase C-mediated pathway. Arterioscler Thromb Vasc Biol 18:568-576, 1998

36. Takahara N, Kashiwagi A, Maegawa H, et al: lysophosphatidylcholine stimulates the expression and production of MCP-1 by human vascular endothelial cells. Metabolism 45:559-564, 1996

37. Ohara Y, Peteson TE, Kuo BZJF, et al: Lysophosphatidylcholine increases vascular superoxide anion production via protein kinase C activation. Arterioscler Thromb 14:1007-1013, 1994

38. Kugiyama K, Sugiyama S, Ogata N, et al: Burst production of superoxide anion in human endothelial cells by lysophosphatidylcholine. Atherosclerosis 143:201-204, 1999

39. Marumo T, Schini-Kerth VB, Fisslthaler B, et al: Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-κB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. Circulation 96:2361-2367, 1997

40. Satriano JA, Shuldiner M, Hora K, et al: Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-α and immunoglobulin G: Evidence for involvement of reduced nicotinamide adenine dinucle-

otide phosphate (NADPH)-dependent oxidase. J Clin Invest 92:1564-1571, 1993

41. Shi AH, Yoshinari M, Wakisaka M, et al: Lysophosphatidylcholine molecular species in low density lipoprotein of type 2 diabetes. Horm Metab Res 31:283-286, 1999

42. Nakano T, Raines EW, Abraham JA, et al: Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. Proc Natl Acad Sci USA 91:1069-1073, 1994

43. Jensen PE, Ohanian J, Stasbol-gron B, et al: Increase by lysophosphatidylcholine of smooth muscle Ca^{2+} sensitivity in a-toxin-permeabilized small mesenteric artery from the rat. Br J Pharmacol 117:1238-1244, 1996

44. Murohara T, Scalia R, Lefer AM: Lysophosphatidylcholine promotes p-selectin expression in platelets and endothelial cells: Possible involvement of protein kinase C activation and its inhibition by nitric oxide donors. Circ Res 78:780-789, 1996

45. Katsuyama K, Shichiri M, Marumo F, et al: NO inhibits cytokine-induced iNOS expression and NFκB activation by interfering with phosphorylation and degradation of IκB-α. Arterioscler Thromb Vasc Biol 18:1796-1802, 1998

46. Spiecker M, Peng HB, Liao JK: Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IκBα. J Biol Chem 272:30969-30974, 1997

47. Giugliano D, Marfella R, Coppola L, et al: Vascular effects of acute hyperglycemia in humans are reversed by L-arginine: Evidence for reduced availability of nitric oxide during hyperglycemia. Circulation 95:1783-1790, 1997

48. Heitzer T, Krohn K, Albers S, et al: Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with type II diabetic mellitus. Diabetologia 43:1435-1438, 2000

49. O'Byrne S, Forte P, Roberts LJ, et al: Nitric oxide synthesis and isoprostane production in subjects with type I diabetes and normal urinary albumin excretion. Diabetes 49:857-862, 2000