Atherogenic Role of Lysophosphatidylcholine in Low-Density Lipoprotein Modified by Phospholipase A_2 and in Diabetic Patients: Protection by Nitric Oxide Donor

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

The aim of our study was to investigate the atherogenic role of lysophosphatidylcholine (lyso-PC) in low-density lipoprotein (LDL) under diabetic environment. Expression of monocyte chemoattractant protein-1 (MCP-1) mRNA and nuclear factor-kappa B (NF-κB)–DNA binding activity were determined in human umbilical vein endothelial cells (HUVEC) incubated with native or glycoxidized LDL, LDL modified by phospholipase A_2 (PLA_2) and LDL isolated from diabetic patients. Lyso-PC contents in LDL were measured using electrospray ionization–liquid chromatography/mass spectrometry (ESI-LC/MS). Lyso-PC contents were higher in glycoxidized LDL and PLA_2-treated LDL compared with native LDL. Glycoxidized LDL and enrichment of lyso-PC by PLA_2 treatment resulted in upregulation of MCP-1 mRNA expression through increased NF-κB activity in HUVEC. Moreover, LDL isolated from diabetics contained more lyso-PC than that from nondiabetic subjects, and induced higher MCP-1 mRNA expression and NF-κB activity in HUVEC. In both in vitro and human studies, palmitoyl- and stearoyl-lyso-PC contents correlated with MCP-1 expression and NF-κB activity. Preincubation with 4-ethyl-2-hydroxyimino-5-nitro-3-hexenamide, a NO donor, abrogated increased expression of MCP-1 mRNA and high NF-κB activity induced by PLA_2-treated LDL and by LDL isolated from diabetic patients. Our results suggest that lyso-PC contents in LDL play an important role in atherogenesis under diabetic condition, which could be prevented by increased availability of vascular NO.

ATHEROSCLEROSIS IS accelerated in patients with diabetes mellitus, and the resultant cardiovascular disease is the leading cause of death.[1] Although the mechanisms behind diabetic macroangiopathy are not fully understood, increasing body of evidence indicates that oxidized low-density lipoprotein (LDL) plays a key role in the development and progression of atherosclerosis.[2] Oxidized LDL induces the expression of adhesion molecules, leukocytes chemotaxis factors, and growth factors and the suppression of nitric oxide (NO) release, leading to endothelial dysfunction, an important pathogenic factor of atherosclerotic diseases.[3]

Oxidation of LDL is associated with increased lipid peroxides of its constituents, fragmentation of apolipoprotein B100, and increased anionic charge on the particles. In addition, during oxidative modification of LDL, there is extensive conversion of phosphatidylcholine (PC) to lysophosphatidylcholine (lyso-PC), which is catalyzed by a phospholipase A_2 (PLA_2) activity present in LDL, probably intrinsic to apolipoprotein B.[4] Lyso-PC inhibits endothelial-dependent relaxation and induces the gene expression of growth factors and adhesion molecules in endothelial cells.[5] We and other investigators reported that lyso-PC contents of LDL were increased in diabetic patients compared with nondiabetic subjects.[6,7] Recently, we reported that LDL subjected to both glycation and oxidation in vitro, ie, glycoxidized LDL enhanced monocyte chemoattractant protein-1 (MCP-1) mRNA expression through activation of transcription factor nuclear factor-kappa B (NF-κB) in human umbilical vein endothelial cells (HUVEC).[8] MCP-1 plays a major role in the recruitment of monocytes into vessel walls, which is thought to be one of the earliest events in atherogenesis.[9] In addition, we found that lyso-PC contents in LDL significantly correlated with MCP-1 mRNA expression and NF-κB activation in HUVEC.[8] Therefore, it is conceivable that increased lyso-PC contents in LDL may be associated with accelerated atherosclerosis in diabetes mellitus.

The present study was designed to further clarify the role of lyso-PC in atherogenesis under diabetic conditions. Firstly, native or glycoxidized LDL was treated with PLA_2 in vitro to increase its lyso-PC contents and then examined the effects of PLA_2-modified LDL on the MCP-1 mRNA expression and DNA binding activity of NF-κB in HUVEC. Secondly, we studied lyso-PC contents in LDL isolated from diabetic patients or nondiabetic control, and compared the effects of diabetic and control LDL on MCP-1 mRNA expression and NF-κB activity in HUVEC. Lastly, NO suppressed MCP-1 mRNA expression and NF-κB activation in HUVEC stimulated by glycoxidized LDL.[8] Thus, we investigated whether a NO donor protected MCP-1 mRNA expression and NF-κB activation induced by PLA_2-modified LDL or by diabetic LDL.

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 April 6, 2002; accepted September 28, 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.

0026-0495/03/5203-0007$30.00/0
doi:10.1053/meta.2003.50049

MATERIALS AND METHODS

Cell Cultures

HUVEC were isolated from umbilical cord veins using 0.25% trypsin (Difco Laboratories, Detroit, MI) according to the method of Jaffe et al[10] with minor modifications.[8] Cells were grown in M199 medium supplemented with 10% 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, Tokyo, Japan) in a humidified atmosphere of 5% CO_2-95% air. The cells were used prior to the 6th passage.

Isolation and Modification of LDL

LDL (d = 1.019 to 1.063 g/mL) was isolated from plasma using density-gradient ultracentrifugation according to methods of Vieira et al,[11] as we described previosly.[8] Glycoxidized LDL was prepared by incubating an aliquot of LDL isolated from healthy volunteers with 200 mmol/L glucose at 37°C for 3 days, and then, after the dialysis with 4 L of phosphate-buffered saline (PBS) 3 times, by incubating glycated LDL with 1 μmol/L $CuSO_4$ at 37°C for 12 hours. Native LDL and glycoxidized LDL were dialyzed by 0.15 mol/L NaCl and 0.26 mmol/L EDTA, pH 7.4, sterilized with a 0.45 μm pore-size filter, and then stored at 4°C under N_2 gas until use. For PLA_2 modification, native LDL and glycoxidized LDL were concentrated and dialyzed with 1,000-fold volume of 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, Bedford, MA) 3 times. After addition of 8 μL of 0.1 mmol/L Tris-HCl, pH 7.4, and 10 mmol/L $CaCl_2$ containing 8 U of snake venom PLA_2 (*Crotalus adamanteus*; Worthington Biochemical Corp, Lakewood, NJ), they were incubated at 37°C for 2 hours.[12] Similarly, mildly PLA_2-modified LDL was obtained by incubating native or glycoxidized LDL with 0.1 U of PLA_2 for 20 minutes. The protein concentration of LDL preparations was determined by a protein assay kit with Coomassie Brilliant Blue (Nacalai Tesque, Kyoto, Japan) using bovine serum albumin as a standard.

Subjects

Eight patients with type 2 diabetes mellitus and 8 nondiabetic control subjects matched for age and body mass index were recruited from the Second Department of Internal Medicine at Kyushu University Hospital. Diabetes mellitus was diagnosed according to the criteria of the World Health Organization.[13] Subjects who had hyperlipidemia (total cholesterol > 5.69 mmol/L or triglyceride > 1.70 mmol/L) or renal insufficiency (serum creatinine > 97.24 μmol/L), those taking lipid-lowering agents and vitamin supplements, and cigarette smokers were excluded from the study. At the time of the study, 4 of 8 diabetic patients were treated with sulfonylurea, while the other patients were treated with insulin. None of the subjects had history of ischemic heart disease, cerebrovascular disease, peripheral vascular disease, or abnormal electrocardiogram. Venous blood was collected into tubes containing EDTA after an overnight fast. LDL was isolated and was used for the determination of oxidation parameters and biological effects on HUVEC as described below.

Oxidation Parameters of LDL

The degree of oxidation of LDL was evaluated by electrophoretic mobility, lipid peroxidation, and lyso-PC contents as we previously reported.[8] Electrophoresis of lipoprotein was performed to measure the mobility of LDL using a commercial kit (Titan Gel Lipoproteins; Helena Laboratories, Saitama, Japan). The degree of lipid peroxidation was estimated by measuring the amounts of thiobarbituric acid–reactive substances (TBARS) in LDL using the method of Yagi[14] with minor modifications. Serial dilutions of 1,1,3,3-tetramethoxypropane (Sigma), which yields malondialdehyde (MDA), was used to construct the standard curve. The extent of LDL modification was expressed as nanomoles of MDA per milligram of LDL protein. Lyso-PC was measured by electrospray ionization–liquid chromatography/mass spectrometry (ESI-LC/MS). In brief, LDL was concentrated and dialyzed with HEPES buffer by centrifuging in an Ultrafree-15 centrifugal filter, and total lipids were extracted according to the method of Bligh and Dyer[15] after addition of internal standard, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC; Avanti Polar-Lipids, Alabaster, AL). Then, phospholipids were separated from the extracted lipids by the method of Kaluzny et al[16] using aminopropyl solid-phase extraction chromatography (Bakerbond spe Columns; J. T. Baker Inc, Phillipsburg, NJ). Phospholipids were immediately introduced into the electrospray mass spectrometer (LCQ; ThermoQuest, Tokyo, Japan) via high-performance liquid chromatography (HPLC; LC-10, Shimazu, Kyoto, Japan). Quantitative analysis of phospholipids was performed essentially as described by Han et al.[17] The coefficient of variation for ESI-LC/MS assay was 4.8% (n = 12) with 5 ng/mL DMPC as the internal control. Standard curve experiments showed that ESI-LC/MS method was linear over a wide range (0.1 to 0.5 ng/μL of palmitoyl- or stearoyl-lyso-PC).

Measurement of NF-κB Activity

HUVEC were incubated with or without 100 μg/mL LDL in M199 medium containing 2% fetal calf serum (FCS) for 2 hours. Electrophoretic mobility shift assay (EMSA) was performed as we previously reported.[8] Briefly, nuclear extracts proteins (4 μg) prepared by a modified method of Schreiber et al[18] were incubated in 10 μL of binding buffer for 10 minutes, and 1 μL of ^{32}P-labeled NF-κB oligonucleotide probe (Promega, Madison, WI) was added and incubated for 20 minutes. All samples were electrophoresed on 6% polyacrylamide gels. Gel contents were dried and autoradiographed by the Bio-Imaging Analyzer (Fuji Photo Film Co, Kanagawa, Japan). The NF-κB–specific band was determined by competition assays of 100-fold concentrations of unlabeled oligonucleotides and supershift assay using antibodies against p50 and p65 (Santa Cruz Biotechnologies, Santa Cruz, CA). The density of NF-κB–specific band was expressed relative to that in the basal state.

Measurement of MCP-1 mRNA Expression

Cells were incubated with or without 100 μg/mL LDL in M199 containing 2% FCS for 4 hours. MCP-1 mRNA contents were measured by Northern blot analysis. Briefly, total RNA (15 μg) isolated from HUVEC was separated by electrophoresis and transferred to Hybond-N+ nylon membranes (Amersham International plc, Buckinghamshire, UK). 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) (5,000 Ci/mmol; Amersham) by T4 polynucleotide kinase (Promega). Autoradiography and quantitative analysis were performed with a Bio-Imaging Analyzer (Fuji Photo Film Co, Kanagawa, Japan). MCP-1 mRNA density was corrected for by GAPDH density, and expressed relative to the value in the basal state.

Effect of NO Donor on MCP-1 mRNA Expression and NF-κB Activity

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 LDL. The expression of MCP-1 mRNA and NF-κB activity was determined as mentioned above.

Statistical Analysis

Data are presented as the mean ± SEM. Differences between groups were examined for statistical significance using the unpaired Student's t test. Correlation coefficients were assessed by Pearson's correlation. A P value less than .05 denoted the presence of a statistically significant difference.

RESULTS

Study of LDL Modified In Vitro

Electrophoretic mobility, TBARS, and palmitoyl- and stearoyl-lyso-PC were significantly increased in glycoxidized

Table 1. Parameters of Oxidation of LDL Modified With PLA_2 In Vitro

	nLDL			goLDL		
	Untreated	Mildly PLA_2-Treated	PLA_2-Treated	Untreated	Mildly PLA_2-Treated	PLA_2-Treated
Electrophoretic mobility (mm)	6.9 ± 0.1	8.1 ± 0.6	11.8 ± 0.9†‖	11.6 ± 0.4‡	12.1 ± 0.9†**	14.5 ± 0.9‡§
TBARS (nmol MDA/mg protein)	1.74 ± 0.30	1.11 ± 0.55	0.36 ± 0.02†	7.03 ± 1.91*	6.04 ± 2.43	3.81 ± 0.91**
Palmitoyl-lyso-PC (μg/mg protein)	4.31 ± 1.09	9.24 ± 2.25	40.80 ± 6.74†#	10.43 ± 1.36*	13.41 ± 1.42†	39.58 ± 7.70†¶‖
Stearoyl-lyso-PC (μg/mg protein)	3.02 ± 0.54	6.62 ± 1.41	39.33 ± 7.66†#	5.93 ± 0.81*	8.94 ± 0.59‡§	36.39 ± 8.84†§‖

NOTE. Values are means ± SEM of 4 experiments.

Abbreviations: nLDL, native LDL; goLDL, glycoxidized LDL; PLA_2, phospholipase A_2.

*$P < .05$, †$P < .01$, ‡$P < .001$ *v* nLDL; §$P < .05$, ¶$P < .01$ *v* goLDL; ‖$P < .05$, #$P < .01$ *v* mildly PLA2-treated; **$P < .05$ *v* similarly treated nLDL.

LDL compared with native LDL (Table 1). PLA_2 modification of native LDL or glycoxidized LDL significantly increased electrophoretic mobility and palmitoyl- and stearoyl-lyso-PC contents, respectively. On the other hand, PLA_2 treatment reduced TBARS in native LDL and glycoxidized LDL. Mild PLA_2 modification of native or glycoxidized LDL did not significantly affect its oxidation parameters except stearoyl-lyso-PC in glycoxidized LDL.

NF-κB–DNA binding activity was significantly increased in HUVEC incubated with glycoxidized LDL compared with that in cells incubated with native LDL (Fig 1A, 1.27 ± 0.04 *v* 1.12 ± 0.03, $P < .05$). PLA_2 modification of native LDL and glycoxidized LDL significantly increased NF-κB–DNA binding activity (PLA_2-modified native LDL: 1.28 ± 0.05, *v* native LDL, $P < .05$; PLA_2-modified glycoxidized LDL: 1.53 ± 0.07, *v* glycoxidized LDL, $P < .05$). Preincubation with NOR3, a NO donor, significantly attenuated NF-κB–DNA binding activity in HUVEC incubated with native or glycoxidized LDL and PLA_2-modified native or glycoxidized LDL. Mild PLA_2 modification of native or glycoxidized LDL did not significantly affect NF-κB-DNA binding activity (mildly PLA_2-treated native LDL: 1.20 ± 0.06, mildly PLA_2-treated glycoxidized LDL: 1.32 ± 0.04).

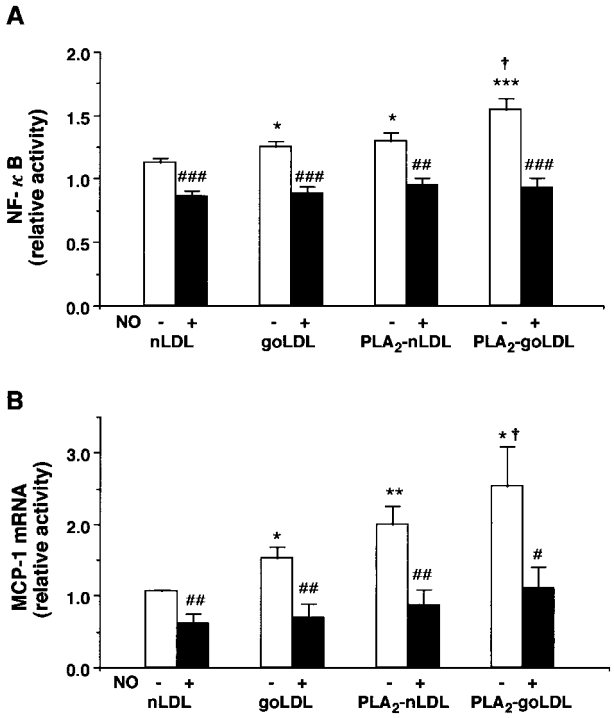

Fig 1. NF-κB–DNA binding activity measured by electrophoretic mobility shift assay (A) and MCP-1 mRNA expression measured by Northern blot analysis (B) after incubation of HUVEC with native LDL (nLDL), glycoxidized LDL (goLDL), and PLA_2-treated native LDL (PLA_2-nLDL) and glycoxidized LDL (PLA_2-goLDL). □, Activity without preincubation with NOR3 (NO donor); ■, activity with NOR3 preincubation. Data are mean ± SEM of 5 experiments. *$P < .05$, **$P < .01$, ***$P < .001$ *v* nLDL, #$P < .05$, ##$P < .01$, ###$P < .001$ *v* without NOR3, †$P < .05$ *v* goLDL.

MCP-1 mRNA expression was significantly higher in glycoxidized LDL compared with native LDL (Fig 1B, 1.55 ± 0.17 *v* 1.06 ± 0.02, $P < .05$). PLA_2 treatment on native or glycoxidized LDL significantly increased MCP-1 mRNA expression (PLA_2-modified native LDL: 2.11 ± 0.22, *v* native LDL, $P < .001$; PLA_2-modified glycoxidized LDL: 2.49 ± 0.43, *v* glycoxidized LDL, $P < .05$). Preincubation with the NO donor significantly diminished MCP-1 mRNA expression in native and glycoxidized LDL as well as in PLA_2-modified native and glycoxidized LDL. There were no significant differences among the groups after NO donor supplementation. Mild PLA_2 modification of native or glycoxidized LDL showed no significant changes in MCP-1 mRNA expression (mildly PLA_2-treated native LDL: 1.26 ± 0.22, mildly PLA_2-treated glycoxidized LDL: 1.57 ± 0.24).

NF-κB–DNA binding activity correlated with palmitoyl-lyso-PC contents (Fig 2A, $r = 0.52$, $P < .01$) and stearoyl-lyso-PC contents (Fig 2B, $r = 0.48$, $P < .05$). Furthermore, MCP-1 mRNA expression also correlated with palmitoyl-lyso-PC contents (Fig 2C, $r = 0.73$, $P < .0001$) and stearoyl-lyso-PC contents (Fig 2D, $r = 0.71$, $P < .0001$).

Study in Diabetic Patients

Table 2 summarizes the clinical characteristics of patients with type 2 diabetes mellitus and nondiabetic control subjects. Fasting plasma glucose was significantly higher in diabetic patients, but total cholesterol, triglyceride, high-density lipoprotein (HDL)-cholesterol, and calculated LDL-cholesterol were not different between diabetic and nondiabetic subjects.

Parameters of oxidation in LDL isolated from nondiabetic and diabetic subjects are shown in Table 3. Electrophoretic mobility and TBARS values were not different in LDL of nondiabetic and diabetic subjects. However, palmitoyl- and

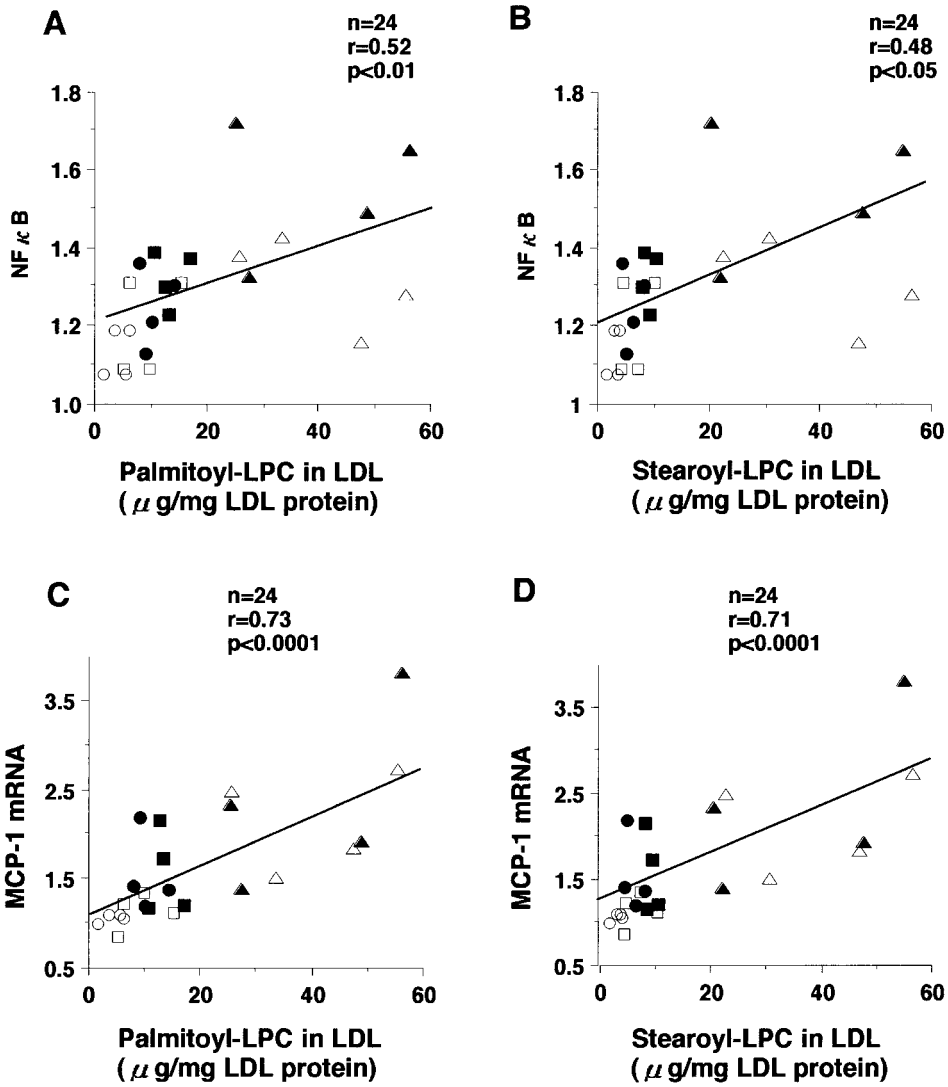

Fig 2. Correlations between NF-κB–DNA binding activity with palmitoyl-lyso-PC (A) and stearoyl-lyso-PC (B) and that of MCP-1 mRNA expression with palmitoyl-lyso-PC (C) and stearoyl-lyso-PC (D). ○, nLDL; ●, goLDL; □, mildly PLA_2-treated nLDL; ■, mildly PLA_2-treated goLDL; △, PLA_2-treated nLDL; ▲, PLA_2-treated goLDL.

stearoyl-lyso-PC contents in LDL were significantly higher in diabetic patients than in control subjects.

NF-κB–DNA binding activity was slightly but significantly higher in HUVEC incubated with diabetic LDL compared with those incubated with control LDL (Fig 3A, 1.12 ± 0.02 *v* 1.02 ± 0.03, $P < .05$). MCP-1 mRNA expression was also higher in HUVEC incubated with diabetic LDL compared to control LDL (Fig 3B, 1.19 ± 0.04 *v* 1.05 ± 0.02, $P < .05$). Preincubation with NOR3 significantly attenuated NF-κB–DNA binding activity as well as MCP-1 mRNA expression in HUVEC incubated with diabetic LDL but not with control LDL.

Table 2. Chacteristics of Nondiabetic Controls and Diabetic Patients

	Controls	Diabetic Patients
n (male/female)	8 (4/4)	8 (5/3)
Age (yr)	58 ± 2	59 ± 3
Body mass index (kg/m^2)	22.1 ± 1.0	23.7 ± 2.1
Fasting plasma glucose (mg/dL)	91 ± 2	203 ± 15*
Total cholesterol (mg/dL)	196 ± 5	195 ± 14
Triglyceride (mg/dL)	111 ± 17	108 ± 11
HDL-cholesterol (mg/dL)	52 ± 2	52 ± 3
Calculated LDL-cholesterol (mg/dL)	118 ± 4	129 ± 9

NOTE. Values are means ± SEM. LDL-cholesterol was calculated by Friedewald's formula.

*$P < .001$ *v* control.

Table 3. Parameters of Oxidation of LDL in Diabetic Patients

	Controls	Diabetic Patients
Electrophoretic mobility (mm)	7.6 ± 0.2	7.9 ± 0.3
TBARS (nmol MDA/mg protein)	1.49 ± 0.09	1.66 ± 0.11
Palmitoyl-lyso-PC (μg/mg protein)	4.53 ± 0.14	5.68 ± 0.33†
Stearoyl-lyso-PC (μg/mg protein)	3.82 ± 0.23	4.47 ± 0.11*

NOTE. Values are means ± SEM. The number of subjects is shown in Table 1.

*$P < .05$, †$P < .01$ *v* control.

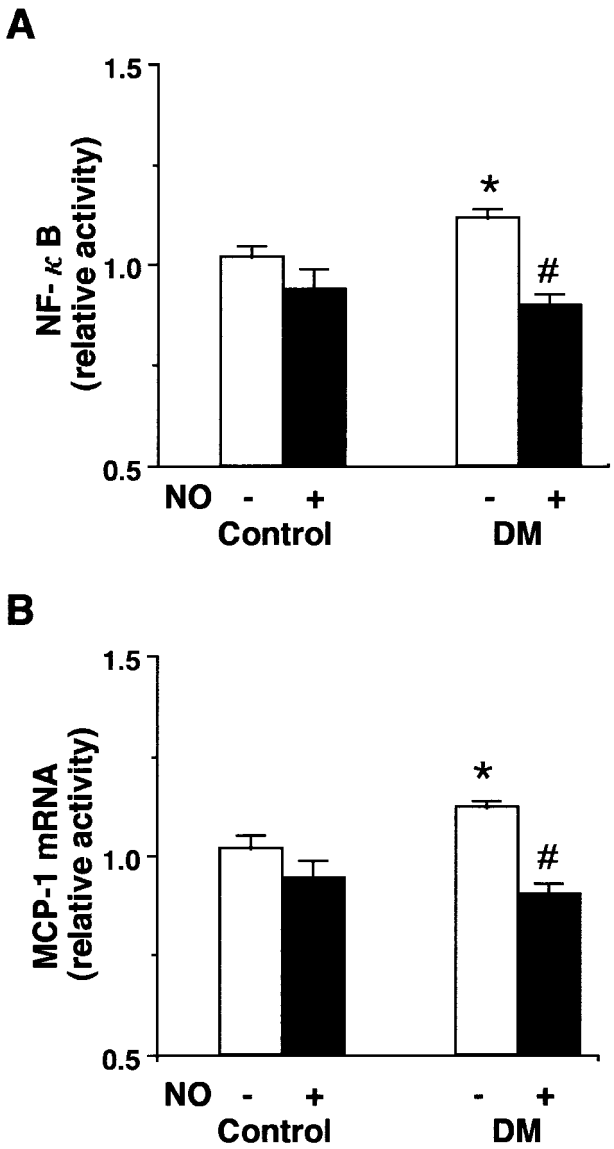

Fig 3. NF-κB–DNA binding activity measured by electrophoretic mobility shift assay (A) and MCP-1 mRNA expression measured by Northern blot analysis (B) after incubation of HUVEC with LDL isolated from diabetic patients (n = 8) and nondiabetic control subjects (n = 8). □, Activity without preincubation with NOR3 (NO donor); ■, activity with NOR3. Data are mean ± SEM. *$P < .05$ *v* nondiabetic control, #$P < .05$ *v* without NOR3.

NF-κB–DNA binding activity correlated significantly with palmitoyl-lyso-PC (Fig 4A, $r = 0.59$, $P < .05$) and stearoyl-lyso-PC levels (Fig 4B, $r = 0.59$, $P < .05$). MCP-1 mRNA expression also correlated with palmitoyl-lyso-PC (Fig 4C, $r = 0.72$, $P < .01$) and stearoyl-lyso-PC levels (Fig 4D, $r = 0.62$, $P < .01$).

DISCUSSION

The present study demonstrated that lyso-PC enrichment by PLA_2 treatment significantly increased NF-κB–DNA binding activity and MCP-1 mRNA expression in HUVEC, and lyso-PC contents in LDL correlated significantly with NF-κB activity and MCP-1 mRNA expression. These in vitro findings are consistent with the in vivo observation, ie, LDL isolated from diabetic patients contained more lyso-PC compared with that from nondiabetic control, showed enhanced MCP-1 mRNA expression through increased activity of NF-κB–DNA binding, and significant correlations of lyso-PC contents with NF-κB activity and MCP-1 mRNA expression were seen. In addition, the NF-κB activation and enhanced MCP-1 mRNA expression were suppressed by NOR3, a NO donor in HUVEC stimulated with PLA_2-treated LDL or diabetic LDL.

Lyso-PC, which is considered a major constituent of oxidized LDL, is produced by PLA_2 activity intrinsic to apolipoprotein B100 of LDL.[4] Recent reports indicate that lyso-PC is mainly produced by hydrolyzing oxidized PC with LDL-associated platelet-activating factor acetylhydrolase, a member of the PLA_2 family,[19] with a selective release of the peroxidized unsaturated fatty acid at the sn-2 position of oxidized PC. Takahara et al[7] reported that diabetic patients had 2.8 times higher lyso-PC contents in IDL and LDL fractions compared with nondiabetic subjects despite similar serum lipid profiles in the 2 groups. The increase in lyso-PC contents seen in our diabetic patients was smaller than their results (Table 3). The reason for this difference may be due to the different method used for lyso-PC measurements, ie, thin-layer chromatography for Takahara's and ESI-LC/MS for ours, or due to the use of IDL fraction. However, they also showed that lyso-PC content correlated with MCP-1 mRNA expression in HUVEC, although they did not report the correlation between lyso-PC contents and NF-κB activity. Our results suggest that even a minor elevation in lyso-PC content of LDL may pose atherogenetic activity in endothelial cells at least in diabetic patients.

Lyso-PC itself increased the DNA-binding activity of NF-κB partly through a protein kinase C (PKC)-mediated pathway[20] and stimulated MCP-1 gene expression in HUVEC.[21] In addition, lyso-PC upregulated lectin-like receptor for oxidized LDL (LOX-1),[22] the first cloned receptor for oxidized LDL on endothelial cells. In this regard, oxidized LDL uptake through LOX-1 increases intracellular oxidative stress and enhances the activation of NF-κB in endothelial cells, suggesting that LOX-1 could be considered as a molecule linking oxidized LDL and atherogenesis. Lyso-PC shows diverse biological activities relative to the length of its acyl group at the sn-1 position. Stearoyl-lyso-PC upregulates heparin-binding/epidermal growth factor (HB-EGF) mRNA in monocytes but palmitoyl-lyso-PC does not.[23] In contrast, palmitoyl-lyso-PC increases Ca^{2+} sensitivity through a PKC-dependent mechanism in rat mesenteric arteries, but stearoyl-lyso-PC does not.[24] In the present study, however, our results showed no differences in the effects of palmitoyl- and stearoyl-lyso-PC contents in LDL on MCP-1 mRNA expression and NF-κB activity in HUVEC when 2 molecular species of lyso-PC were measured by ESI-LC/MS.

PLA_2 modification, not mildly, used in the present study seems to convert almost all PC to palmitoyl- or stearoyl-lyso-PC in LDL. Lyso-PC content in PLA_2-treated LDL was much higher than in that in LDL isolated from diabetic patients, and a wider range of correlation was observed between lyso-PC content and MCP-1 mRNA expression or NF-κB activity. PLA_2 modification decreased TBARS in

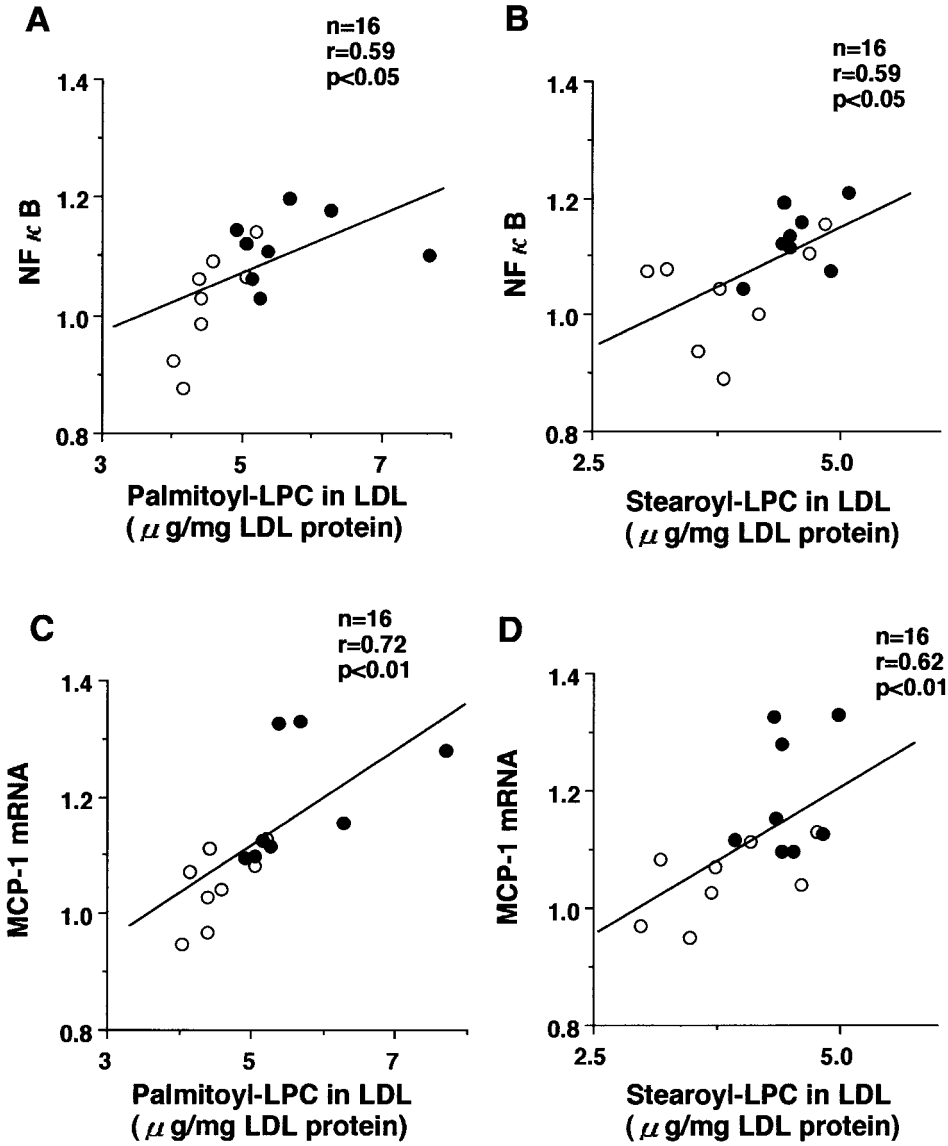

Fig 4. Correlations between NF-κB–DNA binding activity with palmitoyl-lyso-PC (A) and stearoyl-lyso-PC (B) and that of MCP-1 mRNA expression with palmitoyl-lyso-PC (C) and stearoyl-lyso-PC (D). ○, Nondiabetic control; ●, diabetic patients.

native LDL and glycoxidized LDL, because lyso-PC includes no polyunsaturated fatty acids that produce lipid peroxides and MDA.[25] On the other hand, PLA_2 modification increased electrophoretic mobility of LDL as reported by Kleinman et al..[26] This change was probably due to the free carboxyl group of free fatty acid molecule released from the sn-2 residues of PC. PLA_2 modification reduces phospholipids contents in particle surface of LDL and decreased the size of LDL.[26] Such LDL is called small dense LDL, and is known to be a risk factor for atherosclerotic cardiovascular diseases as a component in diabetic dyslipidemia.[27] In this context, it is interesting that PLA_2-modified LDL increased MCP-1 mRNA expression in HUVEC in the present study. When considering the importance of lyso-PC and small dense LDL in atherogenesis, PLA_2 inhibitors could be viewed as potentially promising therapeutic agents for the prevention of atherosclerosis.[28]

Our results also showed that preincubation with a NO donor completely protected against enhancement of MCP-1 mRNA expression and NF-κB activity induced by increased lyso-PC contents in PLA_2-treated LDL and diabetic LDL. So far, two mechanisms have been reported by which NO may inhibit NF-κB action, namely, NO suppressed NF-κB activation by inducing and stabilizing the NF-κB inhibitor, IκB-α[29] or by S-nitrosylation of NF-κB p50 subunit, the cysteine 62 residue of which is used for specific contacts with κB motif DNA.[30] Although the mechanism of the protective effect of NO donor noted in our study remains to be elucidated, enhancement of NO delivery may prevent increased MCP-1 expression in patients with diabetes mellitus, who are known to have reduced vascular NO availability.[31]

In conclusion, we have demonstrated in the present study that further enrichment of lyso-PC in glycoxidized LDL by PLA_2 resulted in upregulation of MCP-1 mRNA expression

through increased NF-κB activity in HUVEC. Moreover, LDL isolated from patients with diabetes mellitus contained more lyso-PC than that from nondiabetic subjects, and induced greater MCP-1 mRNA expression and NF-κB activity in HUVEC. In both in vitro and human studies, palmitoyl- and stearoyl-lyso-PC contents correlated positively with MCP-1 mRNA expression and NF-κB activity in HUVEC. Preincubation with NO donor abrogated overexpression of MCP-1 mRNA and increased NF-κB activity. The present study reemphasized the importance of lyso-PC contents in LDL for atherogenesis in diabetes mellitus, which may be prevented by increasing vascular NO availability.

REFERENCES

1. Kannel WB, McGee DL: Diabetes and cardiovascular disease: The Framingham Study. JAMA 24:2.35-2.38, 1978
2. Yla-Herttula S: Oxidized LDL and atherosclerosis. Ann NY Acad Sci 30:134-137, 1999
3. Berliner JA, Heinecke JW: The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 20:707-727, 1996
4. Parthasarathy S, Barnett J: Phospholipase A2 activity of low density lipoprotein: Evidence for an intrinsic phospholipase A2 activity of apoprotein B-100. Proc Natl Acad Sci USA 87:9741-9745, 1990
5. McIntyre TM, Zimmerman GA, Prescott SM: Biologically active oxidized phospholipids. J Biol Chem 274:25189-25192, 1999
6. Si 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
7. Takahara N, Kashiwagi A, Nishio Y, et al: Oxidized lipoproteins found in patients with NIDDM stimulate radical-induced monocyte chemoattractant protein-1 mRNA expression in cultured human endothelial cells. Diabetologia 40:662-670, 1997
8. Sonoki K, Yoshinari M, Iwase M, et al: Glycoxidized low-density lipoprotein enhances MCP-1 mRNA expression in HUVEC. Relation to lysophosphatidylcholine contents and inhibition by nitric oxide donor. Metabolism 51:1135-1142, 2002
9. Nelken NA, Coughin SR, Gordon D, et al: Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 88: 1121-1127, 1991
10. Jaffe EA, Nachman RL, Becker CG, et al: Culture of human endothelial cells derived from umbilical veins. J Clin Invest 52:2745-2756, 1973
11. 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
12. Quinn MT, Parthasarathy S, Steinberg D: Lysophosphatidylcholine: A chemotactic factor for human monocytes and its potential role in atherogenesis. Proc Natl Acad Sci USA 85:2805-2809, 1988
13. Alberti KGMM, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Provisional report of a WHO conclusion. Diabet Med 15:539-553, 1998
14. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med 15:212-216, 1976
15. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917, 1959
16. 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
17. 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
18. 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
19. Sakurai T, Yamamoto Y, Baba N, et al: Phospholipase A_2 activity in non-glycated and glycated low density lipoproteins. Biochim Biophys Acta 1301:85-89, 1996
20. 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
21. 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
22. Aoyama T, Fujiwara H, Masaki T, et al: Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. J Mol Cell Cardiol 31:2101-2114, 1999
23. 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
24. Jensen PE, Ohanian J, Stausbol-Gron B, et al: Increase by lysophosphatidylcholines of smooth muscle Ca^{2+} sensitivity in alpha-toxin-permeabilized small mesenteric artery from the rat. Br J Pharmacol 117:1238-1244, 1996
25. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acide reaction. Anal Biochem 95:351-358, 1979
26. Kleinman Y, Krul ES, Burnes M, et al: Lipolysis of LDL with phospholipase A_2 alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells. J Lipid Res 29:729-743, 1988
27. Sobenin IA, Tertov VV, Orekhov AN: Atherogenic modified LDL in diabetes. Diabetes 45:S35-39, 1996 (suppl 3)
28. Yedgar S, Lichtenberg D, Schnitzer E: Inhibition of phospholipase A_2 as a therapeutic target. Biochim Biophys Acta 1488:182-187, 2000
29. Peng HB, Libby P, Liao JK: Induction and stabilization of IκBα by nitric oxide mediates inhibition of NF-κB. J Biol Chem 270:14214-14219, 1995
30. Matthews JR, Botting CH, Panico M, et al: Inhibition of NF-κB DNA binding by nitric oxide. Nucleic Acids Res 24:2236-2242, 1996
31. 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