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Dilazep and fenofibric acid inhibit MCP-1 mRNA expression in glycoxidized LDL-stimulated human endothelial cells

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Abstract

We previously reported that glycoxidized low-density lipoprotein (glycoxidized LDL) enhanced monocyte chemoattractant protein-1 (MCP-1) mRNA expression through activation of nuclear factor-kappaB (NF-κB). Here we investigated the effects of dilazep, an anti-platelet agent, and fenofibric acid, an active metabolite of fenofibrate, on glycoxidized low-density lipoprotein-(LDL)-enhanced MCP-1 mRNA expression. Both 10 μg/ml dilazep and 100 μM fenofibric acid abrogated MCP-1 mRNA expression. ZM241385, an A2a adenosine receptor antagonist, partially inhibited the suppressive effect of dilazep. NF-κB activity was also suppressed by 1 μg/ml dilazep and 10 μM fenofibric acid. The antioxidative activity of these drugs on glycation to native LDL or oxidation to glycated LDL was measured using lipid peroxidation and lyso-phosphatidylcholine contents in LDL. Dilazep but not fenofibric acid exhibited antioxidative activity. Although the mechanisms of anti-atherogenic effects of the two drugs on glycoxidized LDL are different, both dilazep and fenofibric acid could potentially prevent atherosclerosis in diabetes mellitus.

Keywords: Diabetes mellitus; Atherosclerosis; LDL (low-density lipoprotein), glycoxidized; MCP-1 (monocyte chemoattractant protein-1); NF-κB (nuclear factor-κB); Umbilical vein endothelial cell, human

1. Introduction

The development and progression of atherosclerosis are accelerated in patients with diabetes mellitus and contribute to the increased morbidity and mortality of cardiovascular diseases (Tooke, 1996). Although the mechanisms of diabetic atherogenicity are not fully elucidated, low-density lipoprotein (LDL) modification is considered to be an important pathogenic factor. Glycation and oxidation are two lipoprotein modifications that are likely to occur under a diabetic state (Bowie et al., 1993; Cohen et al., 1993; Klein et al., 1995). Glucose autoxidation (Thornalley, 1985) and glycation of proteins (Gillery et al., 1988) lead to the generation of oxygen-free radicals, which can enhance lipid peroxidation and oxidation of LDL (Hunt et al., 1990; Kawamura et al., 1994; Tsai et al., 1994). In addition to being taken up by monocytes where it is involved in the formation of foam cells, oxidatively modified LDL is known to be cytotoxic to the endothelium and stimulant of monocyte adhesion and migration through the induction of expression of various adhesive molecules and chemoattractants (Cushing et al., 1990). Activated oxidative and glycoxidative pathways can enhance atherogenesis (Steinberg et al., 1989). Therefore, it is important to inhibit the atherogenicity of modified LDL to prevent atherosclerotic diseases in diabetes mellitus.

We previously reported that glycoxidized LDL enhanced the mRNA expression of monocyte chemoattractant protein-1 (MCP-1) in cultured human umbilical vein endothelial cells and that nitric oxide (NO) donor suppressed the increased MCP-1 mRNA expression (Sonoki et al., 2002). MCP-1 causes monocytes migration, and thus MCP-1 mRNA expression in endothelial cells has important implications for atherogenesis (Nelken et al., 1991;

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Yla-Herttuala et al., 1991). The present study was designed to investigate the effects of dilazep, an anti-platelet agent, and fenofibric acid, an active metabolite of fenofibrate, a lipid-lowering agent, on glycoxidized LDL-enhanced MCP-1 mRNA expression in human umbilical vein endothelial cells. Dilazep is used clinically for the treatment of kidney disease and ischemic heart disease and is reported to suppress tissue factor mRNA expression and its activity in human umbilical vein endothelial cells stimulated by tumour necrosis factor-α (TNF-α), thrombin, or phorbol 12-myristate 13-acetate (Deguchi et al., 1997), suggesting that it may have anti-atherogenic effects on endothelial cells. On the other hand, fenofibrate was recently found to prevent the progression of coronary artery disease in type 2 diabetic patients in the Diabetes Atherosclerosis Intervention Study (DAIS, Diabetes Atherosclerosis Intervention Study Investigators, 2001). Fibrates are known to be peroxisome proliferator-activated receptor (PPAR)-α ligands (Staels et al., 1998a) and evidence is accumulating that fibrates directly act on vascular cells and inhibit chronic inflammatory process occurring in atherosclerotic lesions by activating PPARα (Staels et al., 1998b; Marx et al., 1999; Xu et al., 2001).

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells were isolated from umbilical cord veins using 0.25% trypsin (Difco Laboratories, Detroit, MI) according to the method of Jaffe et al. (1973). Cells were grown in M199 medium supplemented with 10% foetal calf serum (Gibco, Life Technologies, Rockville, MD), 100 μg/ml heparin (Sigma, 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 containing 5% CO_2. The cells were used prior to the sixth passage.

2.2. Modification of LDL and its prevention by dilazep or fenofibric acid

LDL was isolated from human plasma using density-gradient ultra-centrifugation (Vieira et al., 1996). In brief, venous blood samples were collected into tubes containing ethylenediaminetetraacetic acid from consented healthy subjects 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 M HEPES, 0.4 M NaCl, and 0.4 M NaOH, pH 7.4, $d = 1.007$ g/ml). The tubes were ultra-centrifuged at 65,000 rpm for 3 h at 15 °C. The LDL fraction was aspirated and dialyzed by 0.15 M NaCl and 0.26 mM ethylenediaminetetraacetic acid, pH 7.4. Thereafter, the LDL solution was sterilized through a filter with a pore size of 0.45 μm (Millipore, Bedford, MA) and was stored at 4 °C under N_2 gas as native LDL.

Glycated LDL was obtained by incubating an aliquot of native LDL with 200 mM glucose at 37 °C for 3 days. Glycoxidized LDL was prepared by incubating glycated LDL with 5 μM $CuSO_4$ at 37 °C for 12 h after dialysis with phosphate-buffered saline (PBS). Dilazep (final concentration, 1–300 μg/ml, supplied by Kowa, Tokyo) or fenofibric acid (final concentration, 6.7 mM, supplied by Kaken, Tokyo) was added to native LDL or glycated LDL in advance. Thereafter, all LDL preparations were dialyzed with 0.15 M NaCl and 0.26 mM ethylenediaminetetraacetic acid, 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).

2.3. Measurement of lipid peroxidation in LDL

Lipid peroxidation was estimated by thiobarbituric acid-reactive substances method of Yagi (Yagi, 1976). 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 min. 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). Serial dilutions of 1,1,3,3-tetramethoxypropane (Sigma), which yields malondialdehyde, were used to construct a standard curve. Thiobarbituric acid-reactive substances were expressed as nmol malondialdehyde per mg LDL protein.

2.4. Measurement of lyso-phosphatidylcholine contents in LDL by electrospray ionisation liquid chromatography/mass spectrometry (ESI-LC/MS)

The method of measurement of lyso-phosphatidylcholine content was described recently by Sonoki et al. (2002). Briefly, lipids were extracted from 50 μl of concentrated LDL supplemented with 500 ng of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (Avanti Polar-Lipids, Alabaster, AL) as an internal standard according to the method of Bligh and Dyer (1959). Phospholipids were separated from the extracted lipids by the method of Kaluzny et al. (1985) using aminopropyl solid-phase extraction chromatography (BAKERBOND spe Columns; J.T. Baker, Phillipsburg, NJ). The phospholipids were immediately introduced into the electrospray mass spectrometer (LCQ™; ThermoQuest, Tokyo) via high-performance liquid chromatography (HPLC; LC-10, Shimazu, Kyoto). Reverse-phase HPLC was performed by injecting 20 μl of isolated phospholipids

in methanol into an STR-ODS analytical microcolumn (150 × 2.0 mm, 5 μM; Shimazu) at a flow rate of 0.3 ml/min and eluting with a mobile solvent of methanol/acetonitrile/deionized water (84:14:2, v/v/v). The mass spectrometer was operated in positive mode employing the "full-scan" function set from m/z 100 to 1000. Quantitative analysis of phospholipids was performed essentially as described by Han et al. (1996) using 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine as an internal standard. Phospholipids were quantitated based on their ion intensity relative to the internal standard. Liquid chromatography/mass spectrometry (LC/MS) chromatogram of LDL showed the peaks of palmitoyl-lyso-phosphatidylcholine, stearoyl-lyso-phosphatidylcholine, and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine as an internal standard, at retention time of 2.03, 2.50, and 5.54 min, respectively. The electrospray ionisation mass spectrum of LDL at a retention time of 2.03 min showed that palmitoyl-lyso-phosphatidylcholine had four masses, m/z 496.5, 497.5, 518.4, and 519.4, which matched the masses of the standard product of palmitoyl-lyso-phosphatidylcholine. The concentration of palmitoyl-lyso-phosphatidylcholine was calculated by integrating the relative intensities of the four masses for 10 min after comparison with the standard 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine. The concentration of stearoyl-lyso-phosphatidylcholine in each LDL was calculated in a similar fashion by integrating the relative intensities of the four masses of stearoyl-lyso-phosphatidylcholine; m/z 524.6, 525.5, 546.5, and 547.6. The coefficient of variation for EIS-LC/MS assay was 4.8% ($n = 12$) with 5 ng/ml 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine as the internal control. Standard curve experiments showed that the EIS-LC/MS method was linear over a wide range (0.1–5.0 ng/μl of palmitoyl- or stearoyl-lyso-phosphatidylcholine).

2.5. Measurement of MCP-1 mRNA expression

Human umbilical vein endothelial cells were incubated with or without 100 μg/ml of glycoxidized LDL in M199 containing 2% foetal calf serum for 4 h, and MCP-1 mRNA content was then measured by Northern blot analysis. In brief, total RNA was isolated from human umbilical vein endothelial cells by using Isogen (Nippon Gene, Tokyo). Total RNA (15 μg) was separated by electrophoresis and transferred to Hybond™-N+ nylon membranes (Amersham International, Buckinghamshire, UK). Hybridization was performed for 1 h 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, USA), which had been labelled with [γ-^{32}P] ATP (Amersham) by T4 polynucleotide kinase (Promega, Madison, WI). Autoradiography and quantitative analysis were performed using a Bio-Imaging Analyzer (Fuji Photo Film, Kanagawa, Japan).

2.6. Measurement of nuclear factor kappa B (NF-κB) activity

Human umbilical vein endothelial cells were incubated with or without 100 μg/ml of glycoxidized LDL in M199 medium containing 2% foetal calf serum for 2 h and nuclear extracts were then prepared by a modified method of Schreiber et al. (1989). In brief, 25 μl of 10% Nonidet P-40 (Sigma) was added to human umbilical vein endothelial cells suspended in 0.4 ml buffer. The mixture was centrifuged and the nuclear pellet was suspended in 50 μl of ice-cold buffer. The suspension was sonicated and shaken at 4 °C for 15 min. The supernatant of nuclear extracts spun at 13,000 × *g*, 4 °C for 5 min, was stored at −80 °C until use after determination of protein concentrations. Electrophoretic mobility shift assay (EMSA) was conducted as described previously (Sonoki et al., 2002). Nuclear protein (4 μg) was incubated in 10 μl of binding buffer [10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, and 0.05 mg/ml poly(dI–dC) (Pharmacia Biotech, Piscataway, NJ)] for 10 min at room temperature. Next, 1 μl of ^{32}P-labeled NF-κB oligonucleotide probe (Promega) was added, and the reaction mixture was incubated for 20 min at room temper-

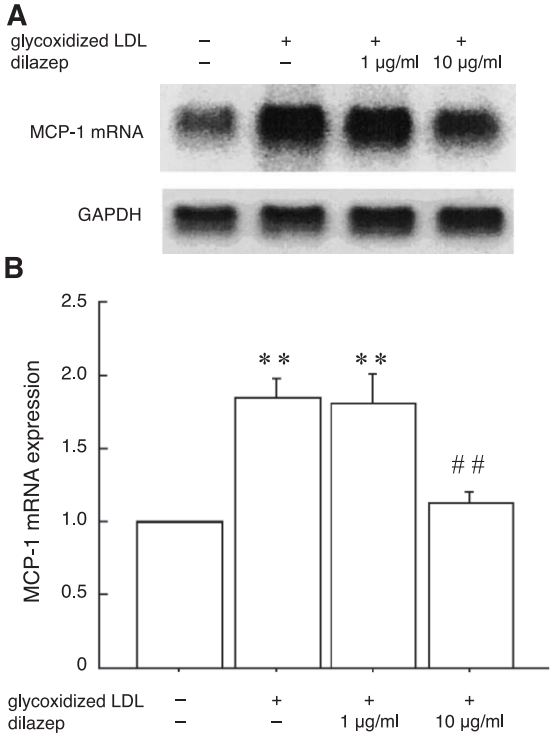

Fig. 1. (A) Effects of dilazep on MCP-1 mRNA expression induced by glycoxidized LDL in human umbilical vein endothelial cells. Cells were preincubated with or without dilazep at 1 or 10 μg/ml for 1 h and subsequently stimulated with or without 100 μg/ml glycoxidized LDL for 4 h. (B) MCP-1 mRNA expression relative to that of GAPDH when the expression in the basal state was considered to be 1.0. Data are mean ± S.E.M. of five experiments. $^{**}P < 0.01$ vs. basal state, $^{\#\#}P < 0.01$ vs. glycoxidized LDL.

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

2.7. Effects of dilazep and fenofibric acid on MCP-1 mRNA expression and NF-κB activity

Human umbilical vein endothelial cells were preincubated with dilazep at a final concentration of 1, 10 or 100 μg/ml for 1 h or fenofibric acid at a final concentration of 10, 100 or 200 μM for 16 h. The doses and incubation times of dilazep or fenofibric acid used were referred to the previous reports using human umbilical vein endothelial cells, i.e., dilazep inhibited tissue factor expression at doses of more than 1 μg/ml at 3 h incubation (Deguchi et al., 1997), 100 μM fenofibric acid inhibited adhesion molecules expression at 18 h incubation (Marx et al., 1999), and 50 μM fenofibric acid inhibited plasminogen activator inhibitor-1 expression at 14 h incubation (Nilsson et al., 1999).

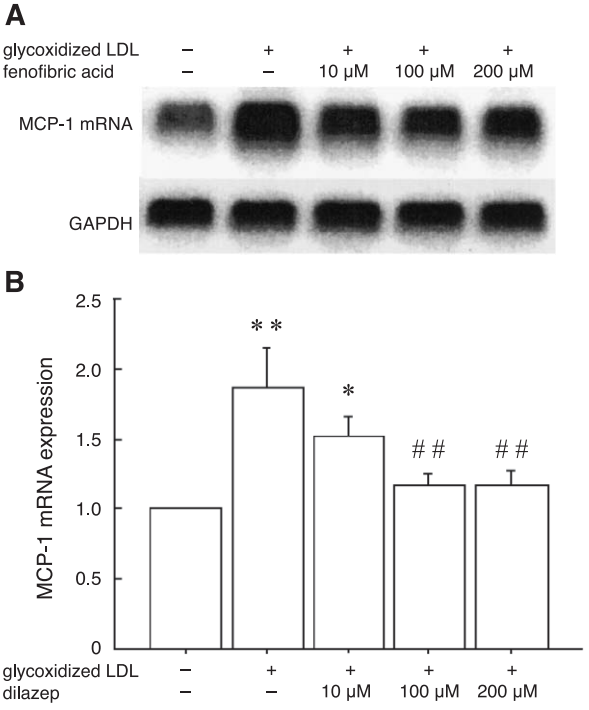

Fig. 2. (A) Effects of fenofibric acid on expression of MCP-1 mRNA induced by glycoxidized LDL in human umbilical vein endothelial cells. Cells were preincubated with or without fenofibric acid at 10, 100 and 200 μM for 16 h and subsequently stimulated with or without 100 μg/ml glycoxidized LDL for 4 h. (B) MCP-1 mRNA expression relative to that of GAPDH when the expression in the basal state was considered to be 1.0. Data are mean ± S.E.M. of six experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. basal state, $^{\#\#}P<0.01$ vs. glycoxidized LDL.

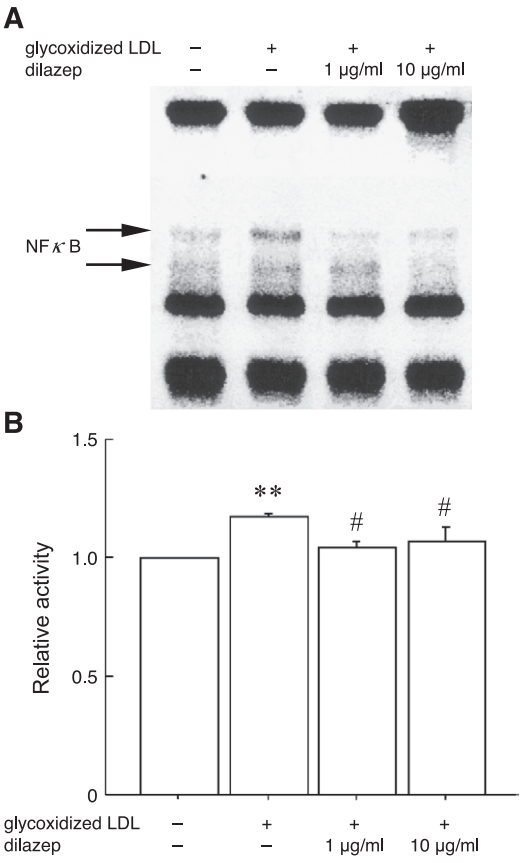

Fig. 3. (A) Effects of dilazep on NF-κB activation induced by glycoxidized LDL in human umbilical vein endothelial cells. NF-κB activity was determined by electrophoretic mobility shift assay. NF-κB-specific bands are indicated by two arrows. Cells were preincubated with or without dilazep for 1 h and subsequently stimulated with or without 100 μg/ml glycoxidized LDL for 2 h. (B) NF-κB activity expressed relative to the basal state. Data are mean ± S.E.M. of four experiments. $^{**}P<0.01$ vs. basal state, $^{\#}P<0.05$ vs. glycoxidized LDL.

The MCP-1 mRNA expression and NF-κB activity were determined as described above.

2.8. Effect of ZM241385, an A2a adenosine receptor antagonist, on MCP-1 mRNA expression

Dilazep is a nucleoside transport inhibitor and known to cause extracellular accumulation of adenosine, which exerts its biological action by binding adenosine receptors (Mustafa, 1979). Since human umbilical vein endothelial cells preferentially express A2a adenosine receptors (Feoktistov et al., 2002), we investigated the involvement of adenosine accumulation in the observed effect of dilazep on MCP-1 mRNA expression. For this purpose, 500 nM ZM241385 (Tocris Cookson, Bristol, UK), an A2a adenosine receptor antagonist, was added 1 h before incubation of glycoxidized LDL in the presence of 10 μg/ml dilazep or 10 μM adenosine (Sigma). At this concentration, adenosine was reported to be active via the A2a adenosine receptor in human umbilical vein endothelial cells (Wyatt et al., 2002).

2.9. Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Fisher's test to detect significant differences in multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

Glycoxidized LDL enhanced MCP-1 mRNA expression in human umbilical vein endothelial cells as reported previously by our group (Sonoki et al., 2002). Preincubation with 10 μg/ml of dilazep suppressed glycoxidized LDL-enhanced MCP-1 expression (Fig. 1), and preincubation with 100 μg/ml of dilazep inhibited GAPDH expression (data not shown). Furthermore, preincubation with fenofibric acid at 100 μM or higher concentration abrogated glycoxidized LDL-induced enhancement of MCP-1 mRNA expression (Fig. 2).

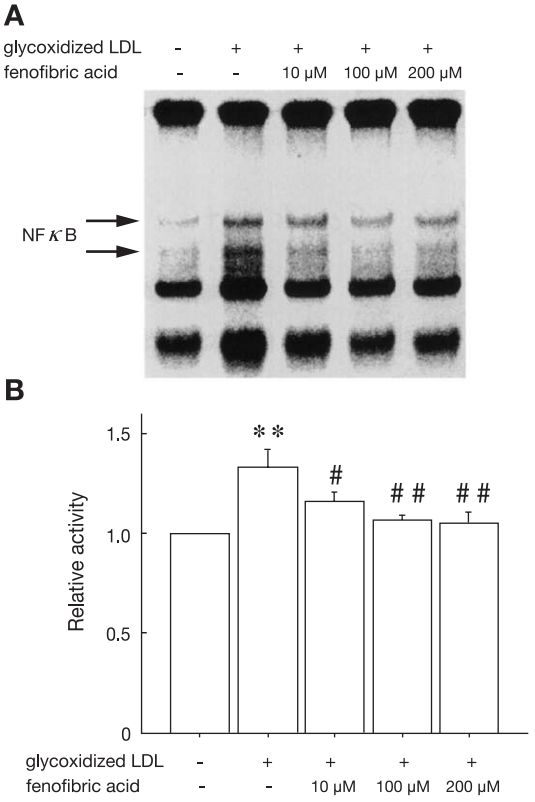

Fig. 4. (A) Effects of fenofibric acid on NF-κB activation induced by glycoxidized LDL in human umbilical vein endothelial cells. NF-κB activity was determined by electrophoretic mobility shift assay. NF-κB-specific bands are indicated by two arrows. Cells were preincubated with or without fenofibric acid for 16 h and subsequently stimulated with or without 100 μg/ml glycoxidized LDL for 2 h. (B) NF-κB activity expressed relative to the basal state. Data are mean ± S.E.M. of four experiments. $^{**}P<0.01$ vs. basal state, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. glycoxidized LDL.

Table 1
Antioxidative effects of dilazep on glycation to native LDL

	TBARS (nmol MDA/mg LDL protein)	Palmitoyl-lyso-PC (μg/mg LDL protein)	Stearoyl-lyso-PC (μg/mg LDL protein)
Native LDL	1.73 ± 0.34	2.72 ± 0.21	3.21 ± 0.27
Glycated LDL	1.87 ± 0.09	7.40 ± 0.24	6.02 ± 0.17
Dilazep during glycation			
1 μg/ml	1.84 ± 0.40	7.84 ± 0.16	6.32 ± 0.20
10 μg/ml	1.64 ± 0.47	6.44 ± 0.30	5.39 ± 0.48
100 μg/ml	1.59 ± 0.27	5.77 ± 0.75	5.64 ± 0.51
200 μg/ml	1.96 ± 0.49	4.86 ± 0.84[a]	4.57 ± 0.83
300 μg/ml	2.05 ± 0.33	4.37 ± 0.42[b]	3.85 ± 0.28[b]

Values are mean ± S.E.M. of four measurements.
[a]$P<0.05$, [b]$P<0.01$ vs. no treatment.
TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; lyso-PC, lyso-phosphatidylcholine.

Since NF-κB regulates MCP-1 mRNA expression, we investigated the effect of NF-κB activation by EMSA (Figs. 3 and 4). A NF-κB-specific band was identified by unlabeled NF-κB oligonucleotide probe (data not shown) as well as by the supershift assay using specific antibodies against p50 and p65 (data not shown). Glycoxidized LDL enhanced NF-κB activity in human umbilical vein endothelial cells, but preincubation with dilazep at 1 μg/ml or higher concentrations abrogated glycoxidized LDL-induced enhancement of NF-κB activity (Fig. 3). Similarly, fenofibric acid at 10 μM or higher concentrations prevented the stimulation of NF-κB activity (Fig. 4).

To further characterize the anti-atherogenic activities of dilazep and fenofibric acid, we investigated the antioxidative actions of these agents on glycation to native LDL or oxidation to glycated LDL. Oxidative parameters included lipid peroxidation, measured by thiobarbituric acid-reactive substances and two molecular species of

Table 2
Antioxidative effects of dilazep or fenofibric acid on oxidation to glycated LDL

	TBARS (nmol MDA/mg LDL protein)	Palmitoyl-lyso-PC (μg/mg LDL protein)	Stearoyl-lyso-PC (μg/mg LDL protein)
Glycoxidized LDL	7.55 ± 0.81	13.71 ± 1.10	10.73 ± 1.08
Dilazep during oxidation			
1 μg/ml	7.75 ± 0.52	14.01 ± 1.30	10.96 ± 1.82
10 μg/ml	6.68 ± 0.57	13.15 ± 1.43	10.52 ± 1.51
100 μg/ml	3.98 ± 0.22[a]	12.22 ± 0.23	9.44 ± 0.48
200 μg/ml	3.15 ± 0.15[a]	10.38 ± 0.21	8.50 ± 0.18
300 μg/ml	2.44 ± 0.30[b]	7.47 ± 0.86[a]	6.47 ± 0.37[a]
Fenofibric acid during oxidation			
6.7 mM	7.59 ± 0.48	13.15 ± 1.53	9.59 ± 0.98

Values are mean ± S.E.M. of four measurements.
[a]$P<0.05$, [b]$P<0.01$ vs. no treatment.
Abbreviations: as in Table 1.

lyso-phosphatidylcholine contents in LDL, i.e., palmitoyl-lyso-phosphatidylcholine and stearoyl-lyso-phosphatidylcholine. In glycation to native LDL (Table 1), dilazep had no effects on thiobarbituric acid-reactive substances, but significantly reduced palmitoyl-lyso-phosphatidylcholine content in LDL at 200 μg/ml and stearoyl-lyso-phosphatidylcholine at 300 μg/ml. In oxidation to glycated LDL (Table 2), dilazep significantly reduced thiobarbituric acid-reactive substances at 100 μg/ml as well as palmitoyl- and stearoyl-lyso-phosphatidylcholine contents at 300 μg/ml. On the other hand, fenofibric acid had no antioxidative activity on oxidation to glycated LDL even at high concentrations. As shown in Fig. 5, glycated LDL incubated with 300 μg/ml of dilazep during $CuSO_4$-oxidation no longer could enhance MCP-1 mRNA expression in human umbilical vein endothelial cells, while co-incubation with 6.7 mM fenofibric acid had no such effects.

As shown in Fig. 6, adenosine significantly suppressed glycoxidized LDL-stimulated MCP-1 mRNA expression, and the addition of ZM241385, the A2a adenosine receptor antagonist, totally abrogated the suppressive effect of adenosine. Dilazep also suppressed glycoxidized LDL-stimulated MCP-1 mRNA expression. However, ZM241385 partially inhibited this effect of dilazep.

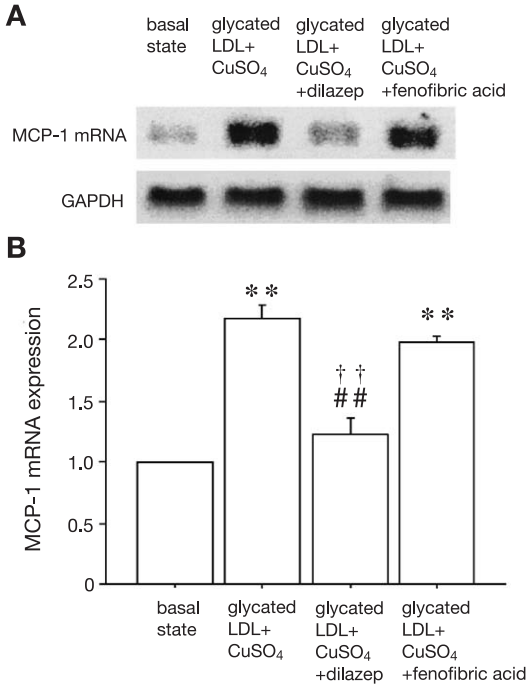

Fig. 5. (A) Northern blot analysis of MCP-1 mRNA in human umbilical vein endothelial cells incubated with glycated LDL subjected to $CuSO_4$-oxidization with or without dilazep or fenofibric acid. Cells were incubated for 4 h with or without 100 μg/ml glycated LDL after 5 μM $CuSO_4$-oxidization with or without 300 μg/ml dilazep or 6.7 mM fenofibric acid. (B) MCP-1 mRNA expression relative to that of GAPDH when the expression in the basal state was considered to be 1.0. Data are mean ± S.E.M. of three experiments. $^{**}P<0.01$ vs. basal state, $^{\#\#}P<0.01$ vs. glycated LDL + $CuSO_4$, $^{\dagger\dagger}P<0.01$ vs. glycated LDL + $CuSO_4$ + fenofibric acid.

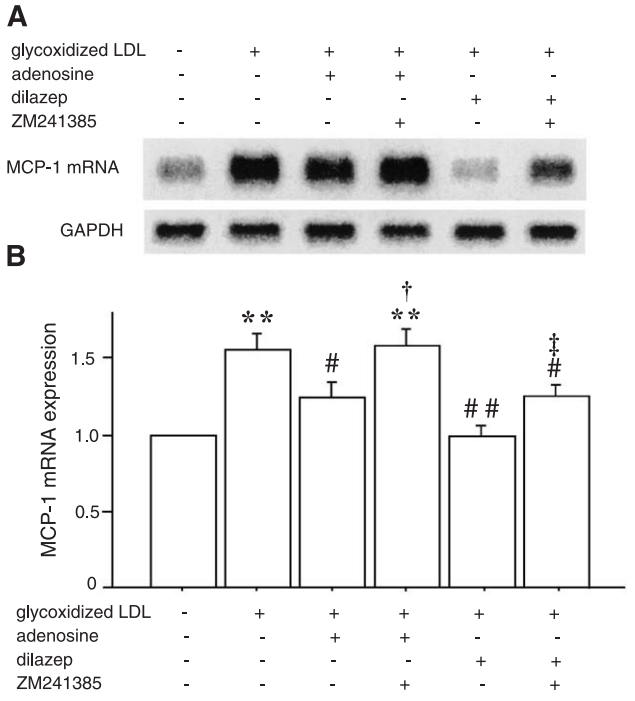

Fig. 6. (A) Northern blot analysis of MCP-1 mRNA in human umbilical vein endothelial cells incubated with glycoxidized LDL with 10 μM adenosine or 10 μg/ml dilazep in the presence or absence of 500 nM ZM241385, an A2a adenosine receptor antagonist. Cells were preincubated with 10 μM adenosine or 10 μg/ml dilazep with or without 500 nM ZM241385 and incubated with 100 μg/ml glycoxidized LDL for 4 h. (B) MCP-1 mRNA expression relative to that of GAPDH when the expression in the basal state was considered to be 1.0. Data are mean ± S.E.M. of four experiments. $^{**}P<0.01$ vs. basal state, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. glycoxidized LDL, $^{\dagger}P<0.05$ vs. glycoxidized LDL + adenosine, $^{\ddagger}P<0.05$ vs. glycoxidized LDL + dilazep.

4. Discussion

The major finding of the present study was that dilazep and fenofibric acid prevented the enhancement of MCP-1 mRNA expression induced by glycoxidized LDL through the suppression of NF-κB-DNA binding activity in human umbilical vein endothelial cells. Glycated LDL is known to increase the susceptibility to oxidative modification (Bowie et al., 1993). Oxidized LDL can modulate various biologic processes involved in early atherogenesis, including the adhesion and chemotaxis of monocytes to endothelial cells (Steinberg et al., 1989). Migration of circulating monocytes into the subendothelial space from the bloodstream is essential during the initial events of atherogenesis (Nelken et al., 1991; Yla-Herttuala et al., 1991). Immunohistochemically, MCP-1 was expressed in human atherosclerosis (Nelken et al., 1991), and glycoxidized LDL was demon-

strated in human atherosclerotic lesions including fatty streaks and plaques (Imanaga et al., 2000). Targeted disruption of the MCP-1 gene in mice deficient in LDL or apolipoprotein E receptor decreased atherosclerotic lesion formation (Gu et al., 1998; Aiello et al., 1999). In this context, the finding that dilazep and fenofibric acid suppressed glycoxidized LDL-enhanced MCP-1 mRNA expression in human umbilical vein endothelial cells might be clinically important. However, since the therapeutic blood concentrations of these drugs were reported to be around 1 μg/ml for dilazep (Schaumloffel and Prignitz, 1972) and 34 μM for fenofibric acid (Shepherd, 1994), care should be taken when interpreting the clinical significance of our results since the doses of dilazep and fenofibric acid used in our study were rather higher than the clinical doses.

NF-κB is a ubiquitous inducible transcription factor known to regulate the expression of genes of various chemokines including MCP-1 (Collins, 1993), cytokines, major histocompatibility complex molecules, growth factors, as well as adhesion molecules (Flohe et al., 1997). NF-κB is activated by phosphorylation and degradation of IκB, which allows its subsequent translocation to the nucleus where it binds to the DNA. It was reported that the oxidant status of the cytosol can activate IκB kinase complex, and antioxidant agents such as *N*-acetyl-L-cystein, pyrrolidine dithiocarbamate and α-tocopherol, can abrogate NF-κB activation (Flohe et al., 1997). Recently, the receptor for oxidized LDL has been identified on endothelial cells as lectin-like oxidized LDL receptor-1 (Sawamura et al., 1997). Cominacini et al. (2000) reported that oxidized LDL bound lectin-like oxidized LDL receptor-1 and induced production of reactive oxygen species and activation of NF-κB. In addition, recent studies reported enhancement of lectin-like oxidized LDL receptor-1 expression in the aortic endothelium of diabetic rats, and that very low-density lipoprotein (VLDL)/LDL subfraction of diabetic plasma directly enhanced the expression of lectin-like oxidized LDL receptor-1 in endothelial cells in vitro. These findings suggest that glycoxidized LDL may induce lectin-like oxidized LDL receptor-1 expression (Chen et al., 2001). In the present study, the concentration of dilazep required to prevent NF-κB activation was 1 μg/ml, while that to prevent the enhancement of MCP-1 mRNA expression was 10 μg/ml. This discrepancy may be explained by the shorter incubation time with glycoxidized LDL in the former, i.e., 3 h in the former vs. 5 h in the latter. Dilazep exhibited antioxidative action on glycoxidation and copper ion-induced oxidation in glycated LDL at >100 μg/ml. Our results are in line with the findings reported by Nakamura et al. (1998) who showed that dilazep suppressed free radical formation in glomeruli of rats with puromycin aminonucleoside nephrosis. Using electron spin resonance spectrometry, they showed that dilazep itself scavenged hydroxyl radicals. Therefore, the anti-oxidative activity of dilazep at its high concentrations may contribute to the suppression of NF-κB activation and MCP-1 mRNA expression in glycoxidized LDL-stimulated human umbilical vein endothelial cells.

Dilazep is known as a nucleoside transport inhibitor and causes extracellular accumulation of adenosine (Mustafa, 1979), which is produced by endothelial cells. It was reported that 1 μg/ml dilazep increased adenosine accumulation in the extracellular space fluid up to micromolar levels (Zhang et al., 1991). Adenosine exerts its biological actions by binding adenosine receptors coupled to guanine nucleotide-binding protein. Recent studies showed that adenosine can prevent NF-κB activation and TNF-α production in ischemic rat heart (Li et al., 2000). Other studies reported that adenosine deaminase inhibitors, which prevent the degradation of endogenous adenosine, inhibited cytokine release from human umbilical vein endothelial cells stimulated with interleukin-1β or TNF-α (Bouma et al., 1996), suggesting that adenosine is a modulator of inflammatory response. In addition, dilazep inhibited tissue factor mRNA expression in activated human umbilical vein endothelial cells through potentiation of adenosine activity (Deguchi et al., 1997). In the present study, ZM241385, an A2a adenosine receptor antagonist, partially counteracted the suppression of glycoxidized LDL-stimulated MCP-1 mRNA expression by dilazep (Fig. 6). Therefore, the suppressive action of dilazep is at least in part mediated by adenosine accumulation.

The lack of antioxidative activity by fenofibric acid as demonstrated in the present study is in agreement with the results of Chaput et al. (1999). However, fenofibrate has been shown to reduce the levels of small dense LDL (Chapman et al., 1998), and the latter is more susceptible to oxidative modification than large buoyant LDL (De Graaf et al., 1991). Therefore, fenofibrate is considered to have an indirect antioxidant effect on LDL in vivo. Fibrates exert a lipid-lowering effect by activating PPARα, which regulates the expression of various genes such as apolipoprotein A-I, apolipoprotein A-II, apolipoprotein C-III and lipoprotein lipase (Staels et al., 1998a). Human umbilical vein endothelial cells expressed PPARα mRNA, though such expression is relatively lower than in the liver (Inoue et al., 1998). Immunohistochemical staining of human carotid arteries revealed the expression of PPARα in the nuclei of endothelial cells (Marx et al., 1999). Recently, it is suggested that fibrates may exert a direct anti-atherogenic activity at the level of the vascular wall, which is independent of their lipid-lowering activity. PPARα ligands inhibited interleukin-1-induced interleukin-6 production as well as cyclooxygenase-2 expression and prostaglandin production by repressing NF-κB signalling in cultured human aortic smooth muscle cells (Staels et al., 1998b). Fenofibrate inhibited TNFα-induced expression of vascular cell adhesion molecule-1 and interleukin-6 production in human endothelial cells through inhibition of NF-κB activation (Marx et al., 1999; Xu et al., 2001). The present study showed that fenofibric acid suppressed glycoxidized LDL-induced enhancement of MCP-1 mRNA expression by inhibiting NF-κB activation in human

umbilical vein endothelial cells, extending the beneficial effects of fibrates on the atherogenicity under a diabetic milieu. Our in vitro findings provide support to the clinical efficiency of fenofibrate, which suppressed the progression of coronary atherosclerosis in type 2 diabetic patients in the DAIS study (Diabetes Atherosclerosis Intervention Study Investigators, 2001).

In conclusion, we have demonstrated in the present study that dilazep and fenofibric acid can suppress the upregulated NF-κB activity and MCP-1 mRNA expression in glycoxidized LDL-stimulated human umbilical vein endothelial cells. The suppressive effect of dilazep on MCP-I mRNA expression was partially inhibited by A2a adenosine receptor antagonist, and thus may be mediated at least in part by extracellular adenosine accumulation. The antioxidant property of prevention of copper ion oxidization to glycated LDL was seen in dilazep but not in fenofibric acid. Although the mechanisms of the anti-atherogenic effects of the two drugs on glycoxidized LDL are different, the present study suggests that dilazep and fenofibric acid could potentially prevent atherosclerosis in diabetes mellitus.

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