Adiponectin Specifically Increased Tissue Inhibitor of Metalloproteinase-1 Through Interleukin-10 Expression in Human Macrophages

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Background—Vascular inflammation and subsequent matrix degradation play an important role in the development of atherosclerosis. We previously reported that adiponectin, an adipose-specific plasma protein, accumulated to the injured artery and attenuated vascular inflammatory response. Clinically, high plasma adiponectin level was associated with low cardiovascular event rate in patients with chronic renal failure. The present study was designed to elucidate the effects of adiponectin on matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in human monocyte-derived macrophages.

Methods and Results—Human monocyte-derived macrophages were incubated with the physiological concentrations of human recombinant adiponectin for the time indicated. Adiponectin treatment dose-dependently increased TIMP-1 mRNA levels without affecting MMP-9 mRNA levels. Adiponectin also augmented TIMP-1 secretion into the media, whereas MMP-9 secretion and activity were unchanged. Time course experiments indicated that TIMP-1 mRNA levels started to increase at 24 hours of adiponectin treatment and were significantly elevated at 48 hours. Adiponectin significantly increased interleukin-10 (IL-10) mRNA expression at the transcriptional level within 6 hours and significantly increased IL-10 protein secretion within 24 hours. Cotreatment of adiponectin with anti–IL-10 monoclonal antibody completely abolished adiponectin-induced TIMP-1 mRNA expression.

Conclusions—Adiponectin selectively increased TIMP-1 expression in human monocyte-derived macrophages through IL-10 induction. This study identified, for the first time, the adiponectin/IL-10 interaction against vascular inflammation. (***Circulation.* 2004;109:2046-2049.**)

Key Words: proteins ■ glycoproteins ■ metalloproteinases ■ interleukins ■ inflammation

Current advances in basic science have illustrated the role of inflammation and its mechanisms, which contribute to atherosclerotic diseases. Inflammatory processes are not only associated with initiation and progression of atherosclerosis but are also responsible for acute thrombotic complications. Most coronary artery thrombi are triggered by the rupture of atherosclerotic plaque lesions, which are controlled by the balance between matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases [TIMPs]), mainly secreted from activated macrophages.[1] However, the precise mechanisms have not been elucidated fully. Adipose tissue secretes various bioactive molecules, termed adipocytokines, that directly contribute to obesity-linked metabolic and vascular diseases. Adiponectin is an adipocyte-specific plasma protein that we identified in a human adipose tissue cDNA library.[2] We have reported that physiological concentrations of human recombinant adiponectin suppressed tumor necrosis factor-α (TNF-α)–induced endothelial adhesion molecule expression, transformation from macrophage to foam cell, and TNF-α expression in macrophages.[3,4] Clinical hypoadiponectinemia was observed in patients with obesity, type 2 diabetes mellitus, and coronary artery disease.[5,6] Plasma adiponectin levels are an inverse predictor of cardiovascular outcomes among patients with end-stage renal disease.[7] Moreover, we recently found that C-reactive protein levels are negatively correlated with adiponectin levels in both human plasma and adipose tissue.[8] These data suggest that adiponectin has antiinflammatory properties and that adiponectin might regulate inflammatory responses at atherosclerotic lesions, in which MMPs and TIMPs are abun-

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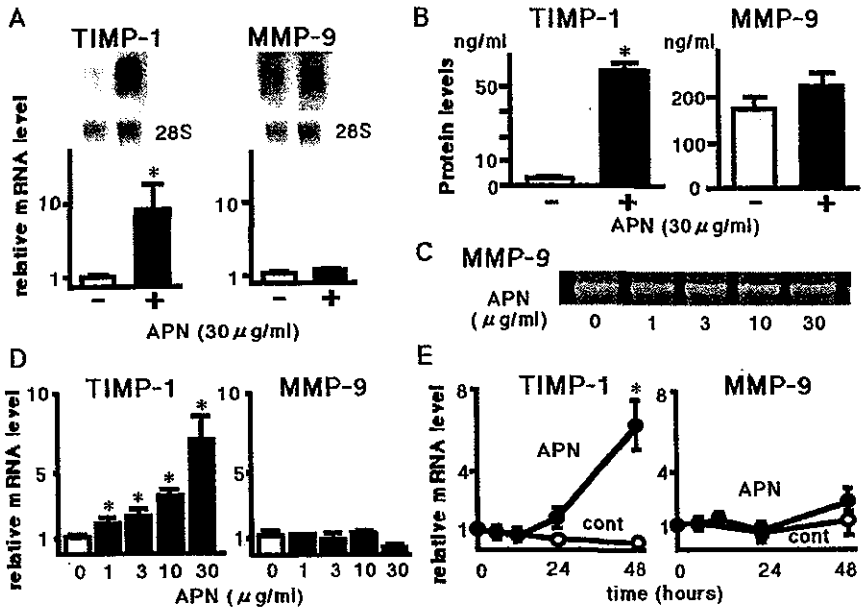

Figure 1. Effects of adiponectin (APN) on TIMP-1 and MMP-9 expressions in HMMs. HMMs were treated for 48 hours (A to D). mRNA levels were measured by real-time quantitative RT-PCR, as described in Methods (D and E). Data are mean±SD of 3 experiments. *$P<0.05$ vs control. A, top, Representative Northern blot of TIMP-1 and MMP-9; bottom, quantitative analysis of TIMP-1 and MMP-9 mRNA levels with Northern blot analyses, as described in Methods. B, TIMP-1 and MMP-9 protein levels secreted into the media measured by ELISA. C, Dose-response effects of adiponectin on MMP-9 activities in HMMs. The matrix-degrading activity of their supernatants was determined by zymography, as described in Methods. D, Dose-response effects of adiponectin on mRNA levels of TIMP-1 and MMP-9. E, Time course of TIMP-1 and MMP-9 mRNA levels. HMMs were treated with or without adiponectin (30 μg/mL) for the time indicated. Cont indicates control.

dantly present. This study was designed to elucidate the effects of adiponectin on expression of MMPs and TIMPs in human monocyte-derived macrophages (HMMs).

Methods

Materials

Mononuclear cells and recombinant human adiponectin were prepared as previously reported.[4] We used recombinant human interleukin-10 (IL-10) (Pepro Tech Ec. Inc), monoclonal anti-human IL-10 antibody (Genzyme Techne), and mouse IgG (Sigma). TIMP-1, MMP-9, and IL-10 concentrations of media were measured with enzyme-linked immunosorbent assay (ELISA) kits (Biotrac).

Gelatinolytic Zymography

Analysis of MMP-9 activity was performed by zymography according to the manufacturer's protocol (TEFCO).

Northern Blot Analysis

Total RNA was prepared by RNA-Trizol extraction (GIBCO) and treated with DNase I, then electrophoresed and transferred to a nylon membrane as previously described.[4] The membranes were hybridized with human TIMP-1 or MMP-9 cDNA probes labeled with [^{32}P]dCTP.

Reverse Transcription–Polymerase Chain Reaction

cDNA was produced with the use of the ThermoScript reverse transcription–polymerase chain reaction (RT-PCR) system (Invitrogen). Real-time PCR was performed as previously described.[8] Primers were 5′-CCTGTTGTTGCTGTGGCTGA-3′ and 5′-CATAACGCTGGTATAAGGTGGTCTG-3′ for human TIMP-1, 5′-GCTACCACCTCGAACTTTGACAG-3′ and 5′-TGCCGGATGCCATTCAC-3′ for human MMP-9, 5′-CTTGCTGGAGGACTTTAAGGGTT-3′ and 5′-GGAGTTCACATGCGCCTTG-3′ for human IL-10, and 5′-CAATGACCCCTTCATTGACCTC-3′ and 5′-AGCATCGCCCCACTTGATT-3′ for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mRNA levels of target genes were divided by those of GAPDH, a standard control gene, and normalized.

Cell Transfection and Measurement of Luciferase Activity

Human IL-10 promoter fragment (from −1044) was subcloned into the luciferase reporter vector with pGL3-Basic (Promega). For the transfection study, a human monocytic cell line (THP-1 cells; Riken Gene Bank) was used. Equivalent transcriptional efficacy was confirmed by cotransfecting the Renilla luciferase control vector, pRL-TK (Promega). THP-1 cells were transfected by the DEAE–dextran sulfate method, as previously reported.[4] After transfection, the cells were incubated with RPMI-1640 supplemented with 10% fetal calf serum for 18 hours and then treated with or without 30 μg/mL adiponectin for 6 hours. Luciferase activity was measured with a dual luciferase assay kit (Promega) and a luminometer.

Statistical Analysis

Data are presented as mean±SD. Differences were analyzed by Student unpaired t test. Between-group comparison of means was performed by ANOVA, followed by t tests. A level of $P<0.05$ was accepted as statistically significant.

Results

Effect of Adiponectin on MMP and TIMP Expression

Adiponectin treatment at 30 μg/mL for 48 hours significantly increased TIMP-1 mRNA levels in HMMs, whereas MMP-9 mRNA levels were unchanged, as shown by Northern blot analysis (Figure 1A). MMP-1, -2, and -3 and TIMP-2 expression levels were low and were unchanged with adiponectin treatment in HMMs (data not shown). We next investigated the TIMP-1 and MMP-9 protein levels secreted into the media. Adiponectin also augmented secreted levels of TIMP-1 without affecting secreted levels of MMP-9 (Figure 1B). Zymogram study revealed that adiponectin had no effect on MMP-9 activity (Figure 1C). The quantitative RT-PCR revealed that adiponectin dose-dependently increased TIMP-1 mRNA levels without affecting MMP-9 mRNA levels (Figure 1D). Time course experiments indicated that TIMP-1 mRNA levels started to increase at 24 hours of incubation with adiponectin and were significantly elevated at 48 hours of treatment. The MMP-9 mRNA levels were not changed over this period (Figure 1E).

Adiponectin Increased TIMP-1 Expressions via Upregulating IL-10 Expression

We next investigated the effect of adiponectin on IL-10 because the antiinflammatory cytokine was reported to in-

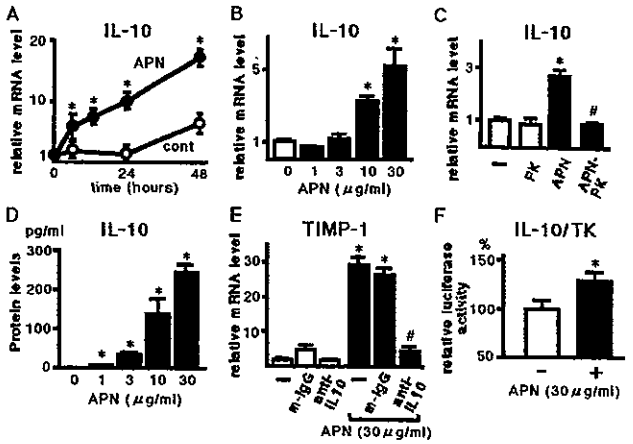

Figure 2. Adiponectin (APN) increased TIMP-1 expression in HMMs through IL-10 induction. The mRNA levels were measured by real-time quantitative RT-PCR, as described in Methods. HMMs were treated for 24 hours with the indicated concentrations of adiponectin (B to D). Data are mean±SD of 3 experiments. *$P<0.05$ vs control. A, Time course of effects of adiponectin on IL-10 mRNA levels. HMMs were treated with or without adiponectin (30 μg/mL) for the time indicated. Cont indicates control. B, Dose-response effects of adiponectin on IL-10 mRNA levels in HMMs. C, Effect of heat-digested adiponectin on IL-10 mRNA levels. Minus symbol indicates control; PK, heat-digested proteinase K; APN, adiponectin (30 μg/mL); and APN-PK, heat-digested recombinant adiponectin (30 μg/mL) with proteinase K. #$P<0.05$ vs APN. D, Dose-response effects of adiponectin on IL-10 protein levels secreted into the media, determined by ELISA. E, Inhibitory effect of anti-human IL-10 monoclonal antibody on adiponectin-induced TIMP-1 mRNA expression. Minus symbol indicates control. HMMs were treated with or without adiponectin (30 μg/mL) along with anti-human IL-10 monoclonal antibody (30 μg/mL) or control mouse (m) IgG (30 μg/mL) for 48 hours. #$P<0.05$ vs APN with mouse IgG. F, Effect of adiponectin on IL-10 promoter activity in THP-1 cells. TK indicates control vector (pRL-TK).

crease TIMP-1 expression without changing MMP-9 expression in HMMs.[9] Adiponectin treatment at 30 μg/mL significantly increased IL-10 mRNA expression within 6 hours (Figure 2A). Human recombinant adiponectin dose-dependently increased IL-10 mRNA levels of HMMs (Figure 2B). The induction of IL-10 mRNA expressions was abrogated with the use of the heat-digested recombinant adiponectin with proteinase K, indicating that the effect was not due to the contamination of recombinant protein (Figure 2C). Human recombinant adiponectin dose-dependently increased IL-10 protein levels secreted into the media (Figure 2D). Cotreatment of adiponectin with anti–IL-10 monoclonal antibody (30 μg/mL) completely abolished adiponectin-induced TIMP-1 mRNA expression compared with mouse IgG control (30 μg/mL) (Figure 2E). The promoter activity of human IL-10 standardized by control vector was significantly increased by adiponectin treatment in the transfected THP-1 cells (Figure 2F).

Adiponectin treatment did not change TIMP-1, MMP-9, and IL-10 mRNA levels in human aortic endothelial cells and human aortic smooth muscle cells (data not shown).

Discussion

In the present study, we identified, for the first time, the adiponectin-inducible antiinflammatory molecule, IL-10. Adiponectin rapidly upregulated IL-10 and subsequently increased TIMP-1 levels in HMMs. This effect of adiponectin is specific to HMMs among vascular component cells. It is generally accepted that MMPs and TIMPs play a crucial role in arteriosclerosis and plaque disruptions.[1] The balance between MMPs and TIMPs determines the actual metalloproteinase activities and controls the extracellular matrix degradation. We focused on MMP-9 and TIMP-1 because they have been dominantly secreted from HMMs. In this study, adiponectin selectively increased the expression of TIMP-1 in both mRNA and protein levels, whereas the mRNA, protein levels, and activities of MMP-9 were not changed in HMMs.

We have reported that adiponectin suppressed stimulated vascular cellular response in vitro, and overexpression of adiponectin with recombinant adenovirus suppressed the development of atherosclerosis in apolipoprotein E–deficient mice.[3,4,10] However, adiponectin-inducible molecules have not been identified. In the present study we found that adiponectin selectively upregulated TIMP-1 expression in HMMs. TIMP-1 mRNA levels started to increase at 24 hours of incubation with adiponectin, suggesting that adiponectin-stimulated TIMP-1 induction was an indirect effect. Because IL-10 was reported to increase TIMP-1 expression without changing MMP-9 expression in HMMs, we next focused on IL-10.[9]

Adiponectin has a variety of antiinflammatory functions against atherosclerosis. Therefore, we hypothesized that adiponectin may modulate the inflammatory response through a multifunctional paracrine factor, IL-10. Adiponectin increased IL-10 mRNA expression within 6 hours. This effect preceded TIMP-1 mRNA expression, and anti–IL-10 monoclonal antibody completely blocked adiponectin-induced TIMP-1 mRNA expression. Moreover, the promoter activity of human IL-10 was significantly increased by adiponectin treatment. These data suggest that adiponectin-induced IL-10 production is at least partly due to the enhanced IL-10 transcription in HMMs. Clinically, both hypoadiponectinemia and low IL-10 plasma concentration are independently reported to be associated with acute coronary syndrome.[6,11,12] These findings suggest the importance of the adiponectin/IL-10 interaction against vascular inflammation in vivo, although further study will be necessary to elucidate the precise mechanism in vivo.

In conclusion, adiponectin selectively increased TIMP-1 expression in HMMs through IL-10 induction. The adiponectin/IL-10 interaction will provide important information for understanding the pathogenesis of atherosclerosis.

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Adiponectin I164T Mutation Is Associated With the Metabolic Syndrome and Coronary Artery Disease

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OBJECTIVES This study examined the association of mutations in adiponectin gene with the prevalence of coronary artery disease (CAD).

BACKGROUND Coronary artery disease is a major cause of mortality in the industrial countries. Adiponectin gene locus, chromosome 3q27, is the candidate site for CAD. We have reported that adiponectin has antiatherogenic and antidiabetic properties, and that the plasma levels negatively correlated with body mass index (BMI) are significantly low in patients with CAD or type 2 diabetes.

METHODS The study subjects were 383 consecutive patients with angiographically confirmed CAD and 368 non-CAD subjects adjusted for age and BMI in the Japanese population. Single nucleotide polymorphisms (SNPs) in the adiponectin gene were determined by Taqman polymerase chain reaction (PCR) method or a PCR-based assay for the analysis of restriction fragment length polymorphism. The plasma adiponectin concentration was measured by enzyme-linked immunosorbent assay.

RESULTS Among SNPs, the frequency of I164T mutation was significantly higher in CAD subjects (2.9%) than in the control (0.8%, $p < 0.05$). The plasma adiponectin levels in subjects carrying the I164T mutation were significantly lower than in those without the mutation, and were independent of BMI. In contrast, SNP94 and SNP276, which are reported to be associated with an increased risk of type 2 diabetes, were associated neither with CAD prevalence nor with plasma adiponectin level. Subjects with I164T mutation exhibited a clinical phenotype of the metabolic syndrome.

CONCLUSIONS The I164T mutation in the adiponectin gene was a common genetic background associated with the metabolic syndrome and CAD in the Japanese population. (J Am Coll Cardiol 2004;43:1195–200) © 2004 by the American College of Cardiology Foundation

Cardiovascular disease is a major cause of morbidity and mortality in industrial countries. Both environmental and genetic factors contribute to the development of cardiovascular disease (1). Among various adipocyte-derived bioactive substances, adipocytokines, dysregulated production of leptin, tumor necrosis factor (TNF)-α, and plasminogen activator inhibitor type 1 is closely associated with increased cardiovascular mortality and morbidity (2–6). Adiponectin is an adipocyte-specific adipocytokine, which we identified in the human adipose tissue complementary DNA library (7). The mouse homologue of adiponectin was identified as ACRP30 and AdipoQ (8,9). Hypoadiponectinemia (low plasma adiponectin level) has been identified in patients with coronary artery disease (CAD) (10) and type 2 diabetes, and is a predictor of cardiovascular outcome in patients with end-stage renal failure (11). Plasma adiponectin rapidly accumulates in the subendothelial space of an injured human artery (12). We have reported that human recombinant adiponectin suppresses endothelial adhesion molecule expression, vascular smooth muscle cell proliferation, and macrophage-to-foam cell transformation as well as TNF-α production by macrophages in vitro (13,14). Recently, we reported that the adiponectin-knockout mice exhibited enhanced neointimal thickening after vascular injury (15). In addition, we and others demonstrated that adiponectin treatment improved insulin resistance and glucose metabolism in diabetic mice model (16–18). These findings suggest that adiponectin has both antiatherogenic and antidiabetic properties and acts as an endogenous mediator of vascular and metabolic diseases.

We have previously identified several mutations of the adiponectin gene, including missense mutations (R112C, I164T, R221S, and H241P) in the globular domain and the G/T single nucleotide polymorphism at nucleotide 94

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Abbreviations and Acronyms

BMI	= body mass index
CAD	= coronary artery disease
HbA1C	= hemoglobin A1C
HDL-chol	= high-density lipoprotein cholesterol
HOMA	= homeostasis model assessment
PCR	= polymerase chain reaction
SNP	= single nucleotide polymorphism
T-chol	= total cholesterol
TG	= triglyceride
TNF	= tumor necrosis factor

(SNP94) in the Japanese population (19,20). Among these mutations, the I164T mutation correlated with type 2 diabetes (19); SNP94 was reported to be associated with type 2 diabetes and obesity (21,22). A weak association was observed between SNP94 and plasma adiponectin levels in French Caucasians, although no significant association was found in the Japanese population (23). Recently, SNP at position 276 (SNP276) was reported to be associated with type 2 diabetes (21); SNP276 was associated with plasma adiponectin levels in French Caucasians and only in obese Japanese subjects (21,23). In addition, the haplotype identified by SNP94 and SNP276 was related with obesity and other features of the insulin resistance syndrome in Caucasians (24). A susceptibility locus for type 2 diabetes was mapped on chromosome 3q27, which harbors the adiponectin gene (25). A genome-wide scan for CAD replicated linkage with the metabolic syndrome on the region 3q27, suggesting that adiponectin might be one of the candidate genes susceptible for the metabolic syndrome-linked CAD (26). Although the metabolic syndrome includes insulin resistance, it is very important to elucidate the genetic contribution of adiponectin in the development of CAD.

In the present study, we investigated the frequency and the clinical significance of I164T, SNP94, and SNP276 of adiponectin gene in consecutive CAD patients and age- and body mass index (BMI)-matched non-CAD subjects.

METHODS

Study subjects. Consecutive 383 CAD patients were recruited from the inpatients who were admitted to Osaka University Hospital. The criteria for CAD were a 75% ≤ organic stenosis of at least one segment of a major coronary artery confirmed by coronary angiogram. The control subjects were selected from people who received medical check in Osaka University Hospital or our affiliated hospitals. In these latter subjects, it was unethical to perform coronary angiography to rule out the presence of asymptomatic CAD. Therefore, the following inclusion criteria were used: no history of angina or other atherosclerotic vascular diseases, and normal exercise electrocardiogram stress testing. They were matched with CAD patients for age and BMI. All patients and subjects enrolled in this study were Japanese and gave written informed consent. This study was approved by the Ethics Committee of Osaka University.

Laboratory methods. Venous blood was drawn from all patients and control subjects after an overnight fast. Plasma samples were kept at −80° centigrade for subsequent assay. Plasma concentration of adiponectin was evaluated by a sandwich ELISA system (Adiponectin ELISA Kit, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) as previously reported (27). Serum total cholesterol (T-chol) and triglyceride (TG) concentrations were determined by an enzymatic method. High-density lipoprotein cholesterol (HDL-chol) was also measured by an enzymatic method after heparin and calcium precipitation. Plasma glucose was measured by a glucose oxidase method. The value of hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography. Insulin resistance was assessed by homeostasis model assessment (HOMA) (insulin resistance index = [fasting glucose (mmol/l) × fasting insulin (U/ml)]/22.5 (28). Body mass index was calculated as weight/height^2.

Definitions of risk factors. Diabetes mellitus was defined according to World Health Organization criteria, and/or having received treatment for diabetes mellitus (29). Dyslipidemia was defined as a T-chol concentration >5.69 mmol/l, a TG concentration >1.69 mmol/l, an HDL-chol concentration <1.03 mmol/l, and/or having received treatment for dyslipidemia. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or having received treatment for hypertension. We did not exclude the subjects under medical treatment for diabetes mellitus, dyslipidemia, and hypertension.

DNA extraction and genotyping. Genomic DNA was prepared from frozen whole blood with the use of a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, California). We determined the missense mutation I164T and the SNP276 of adiponectin gene by the TaqMan (Roche Molecular Systems Inc., Pleasanton, California) polymerase chain reaction (PCR) chemistry method as previously described (30). The TaqMan probe is a fluorogenic probe that consists of an oligonucleotide labeled with both a fluorescent reporter dye and a quenched dye. The fluorescent reporter dye, such as VIC and FAM (Applied Biosystems Inc., Foster City, California), is covalently linked to the 5′ end of the nucleotide. Each of the reporters is quenched by minor groove binder, typically located at the 3′ end. The following primers were used for the missense mutation I164T: a forward primer, 5′-AACATTCCTGGGCTGTACTACTTTG-3′; a reverse primer, 5′- GGCTGACCTTCACATCCTTCATA-3′; a T-allele-specific probe, 5′-VIC-ACCACATCA-CAGTCTA-MGB-3′; a C-allele-specific probe, 5′-FAM-CCACACCACAGTCT-MGB-3′. The following primers were used for the G/T SNP at position 276: a forward primer, 5′-AGAATGTTTCTGGCCTCTTTCATC-3′; a reverse primer, 5′- TTCTCCCTGTGTCTAGGCCTTAGT-3′; a G-allele-specific probe, 5′-FAM-CTATATGAAGGCAT-TCATTA-MGB-3′; T-allele-specific probe, 5′-VIC-

Table 1. Clinical Characteristics of Control Subjects and CAD Patients

	Control Subjects (n = 368)	CAD Patients (n = 383)	p Value
Age, yrs	62.3 ± 0.6	63.0 ± 0.4	NS
Gender, M/F	240/128	270/113	NS
Adiponectin, μg/ml	7.7 ± 0.2	6.1 ± 0.2	< 0.001
BMI, kg/m^2	23.8 ± 0.2	24.1 ± 0.2	NS
Family history of diabetes mellitus, n (%)	(15.8)	(18.5)	NS
Diabetes mellitus, n (%)	58 (10.3)	71 (48.0)	< 0.001
FPG, mmol/l	38 ± 0.04	184 ± 0.14	< 0.001
	5.40	6.67	
HbA1c, %	5.11 ± 0.04	6.09 ± 0.08	< 0.001
Dyslipidemia, n (%)	179 (48.6)	259 (67.6)	< 0.001
T-chol, mmol/l	5.23 ± 0.05	5.29 ± 0.05	NS
TG, mmol/l	1.57 ± 0.05	1.77 ± 0.06	<0.05
HDL-chol, mmol/l	1.52 ± 0.03	1.19 ± 0.02	< 0.001
Hypertension, n (%)	272 (73.9)	264 (68.9)	NS
SBP, mm Hg	134.6 ± 1.0	132.9 ± 0.9	NS
DBP, mm Hg	80.1 ± 0.7	75.4 ± 0.8	< 0.001

Data represent means ± SE.
BMI = body mass index; CAD = coronary artery disease; DBP = diastolic blood pressure; FPG = fasting plasma glucose; HbA1c = hemoglobin A1C; HDL-chol = high-density lipoprotein cholesterol; SBP = systolic blood pressure; T-chol = total cholesterol.

AAACTATATGAAGTCATTCATTA-MGB-3′. The fluorescence level of PCR products was measured with the ABI PRISM 7200 Sequence Detector (Applied Biosystems, Inc.). We determined the SNP94 in exon 2 of adiponectin gene by a PCR-based assay for the analysis of restriction fragment length polymorphism as previously described (20).

Statistical methods. For continuous variables, results are presented as mean ± SE. Differences in continuous parameter, such as BMI, between two groups were calculated by the Student *t* test, and differences in continuous parameter, such as plasma adiponectin level, among more than three groups were evaluated by analysis of variance. Because plasma adiponectin level, HOMA, and TG were skewed, these three parameters were log-transformed before analysis, and the parameters presented were back-transformed. Categorical variables were presented using frequency counts, and intergroup comparisons were analyzed by chi-square test. A level of $p < 0.05$ was accepted as statistically significant. All calculations were performed using a standard statistical package (JMP for Macintosh, version 4.0, SAS Institute Inc., Cary, North Carolina).

RESULTS

The clinical characteristics of CAD patients and non-CAD control subjects are shown in Table 1. The mean plasma adiponectin level in CAD patients was significantly lower than the control ($p < 0.001$), as we described previously (10). Patients with CAD had significantly higher levels of fasting plasma glucose, HbA1c, TG, numbers of diabetes mellitus, dyslipidemia, and lower levels of HDL-chol and diastolic blood pressure than the control group. There were no significant differences in age, gender, BMI, number of family history for diabetes, T-chol, systolic blood pressure, and number of hypertension between the two groups.

The frequency of I164T mutation in CAD patients (11 [2.9%] of 383) was significantly higher than that in non-CAD subjects (3 [0.8%] of 368, $p < 0.05$) (Table 2). All subjects with the mutation were heterozygotes. In contrast to this mutation, no significant differences in the distribution of SNP94 and SNP276 genotypes were observed between the two groups. The plasma adiponectin levels in subjects carrying the I164T mutation (3.2 ± 0.5 μg/ml) were significantly lower than in subjects without the mutation (6.9 ± 0.2 μg/ml, $p < 0.0001$) (Fig. 1A), although no

Table 2. Frequency of Mutation and Polymorphism in Adiponectin Gene

		Control Subjects	CAD Patients	
n		368	383	p Value
I164T, n (%)		3 (0.8)	11 (2.9)	<0.05
	G/G	29 (7.9)	33 (8.6)	
SNP94, n (%)	G/T	148 (40.2)	140 (36.6)	NS
	T/T	191 (51.9)	210 (54.8)	
	G/G	190 (51.6)	185 (48.3)	
SNP276, n (%)	G/T	149 (40.5)	164 (42.8)	NS
	T/T	29 (7.9)	34 (8.9)	

CAD = coronary artery disease; SNP = single nucleotide polymorphism.

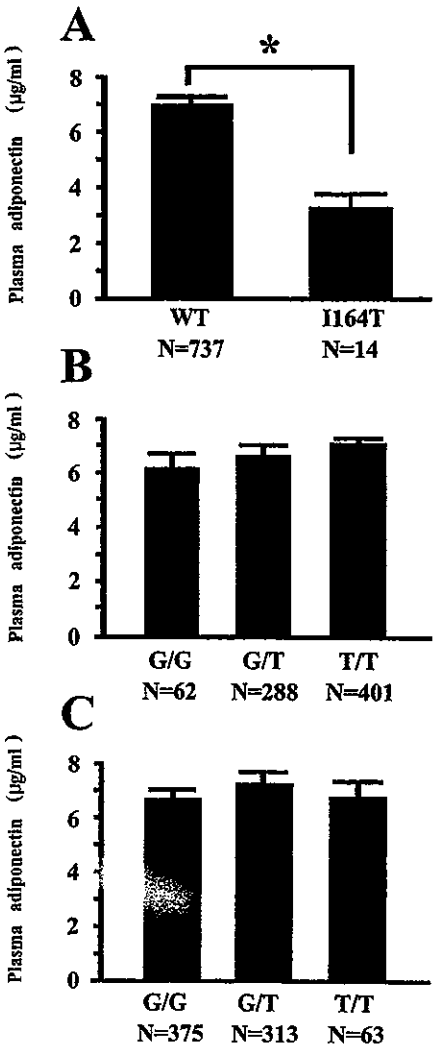

Figure 1. Association of I164T mutation, SNP94, and SNP276 with plasma adiponectin concentrations. (A) Plasma adiponectin levels in the subjects with wild type (WT) or I164T mutation in adiponectin gene. (B) Relationship between SNP94 genotypes and plasma adiponectin levels. (C) Relationship between SNP276 genotypes and plasma adiponectin levels. Columns and vertical bars denote mean and SE of the indicated sample numbers. *$p < 0.05$ vs. WT.

significant difference was observed in BMI between the subjects with and without I164T mutation (24.4 ± 1.2 vs. 24.0 ± 0.1 kg/m^2). The plasma adiponectin levels in subjects with the mutation were markedly low in both CAD and control groups (2.9 ± 0.6 vs. 4.3 ± 1.2 μg/ml, respectively), and did not correlate with BMI. The negative correlation between plasma adiponectin levels and BMI was observed in subjects without the mutation (data not shown). These data indicated that hypoadiponectinemia in subjects with the mutation was independent of BMI. The plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP94 were 6.2 ± 0.6, 6.6 ± 0.2, and 7.1 ± 0.2 μg/ml, respectively (Fig. 1B). The plasma adiponectin level in the subjects having G allele at SNP94 tended to be lower, but it was not statistically significant. On the other hand, no differences were observed in plasma adiponectin levels of the subjects with G/G, G/T, and T/T allele at SNP276 (6.6 ± 0.2, 7.2 ± 0.2, and 6.7 ± 0.5 μg/ml, respectively) (Fig. 1C).

As shown in Table 3, all subjects carrying I164T had at least one risk factor including diabetes mellitus, hypertension, and dyslipidemia. Six (case 4 to 8, and 11) of the 11 CAD patients with the I164T mutation and 75 of 372 wild type CAD patients had all three metabolic abnormalities, which is a key feature of the metabolic syndrome. The percentage of the subjects with all three metabolic abnormalities was significantly higher in I164T mutation (54.5%) than that in wild type (20.2%) ($p < 0.01$). Nine (case 4 to 8 and 11 to 14) of 14 subjects with I164T mutation had diabetes mellitus, and cases 13 and 14 had received insulin treatment. However, except three cases (3, 4, and 8), six diabetic I164T patients had no apparent insulin resistance assessed by HOMA-insulin resistance (IR) compared with CAD patients (n = 383, HOMA-IR; 2.4 ± 0.2). In addition, there were no differences in HOMA-IR levels between nondiabetic I164T subjects (case 1 to 3, 9, and 10) and control subjects (n = 368, HOMA-IR; 1.8 ± 0.1).

DISCUSSION

In the present study, we found that the I164T mutation of adiponectin gene was associated with CAD prevalence and hypoadiponectinemia in the Japanese population. In contrast, the genotypes of SNP94 and SNP276, which were reported to be present in type 2 diabetes, influenced neither the prevalence of CAD nor the plasma adiponectin level.

Table 3. Clinical Profile of the Subjects With I164T Mutation

Case Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Age, yrs	53	65	78	52	59	59	61	69	71	72	65	67	70	73
Gender	M	M	F	M	M	M	M	M	M	M	F	F	F	F
Plasma adiponectin, μg/ml	2.7	6.7	3.7	0.4	2.7	2.8	2.6	3.7	3.5	0.9	4.4	2.0	1.6	7.2
BMI, kg/m^2	23.6	ND	24.0	27.0	25.4	29.2	25.6	21.7	19.2	23.8	34.1	25.0	19.0	19.2
FPG, mmol/l	3.5	4.7	5.3	7.6	16.6	5.8	8.2	8.6	5.2	5.4	6.3	6.1	4.8	7.5
FIRI, μU/ml	8.0	4.0	5.0	13.0	5.7	6.0	3.0	10.9	6.2	4.8	6.4	10.2	3.5	2.3
HOMA-IR	1.8	0.8	1.2	3.3	4.2	1.5	2.3	4.2	1.4	1.2	1.4	2.8	0.7	0.8
Number of risk factors*	1	2	2	3	3	3	3	3	1	1	3	2	2	2
Coronary artery disease	-	-	-	AP	AP	AP	AP	MI	AP	MI	AP	AP	AP	AP

*Risk factors: diabetes mellitus, hypertension, and dyslipidemia.
AP = angina pectoris; BMI = body mass index; FIRI = fasting immunoreactive insulin; FPG = fasting plasma glucose; HOMA-IR = homeostatis model assessment of insulin resistance; MI = myocardial infarction.

Importantly, all subjects carrying I164T in the present study including CAD and non-CAD subjects had at least one or more metabolic disorders including diabetes mellitus, hypertension, and dyslipidemia. Among CAD patients, the prevalence of the metabolic syndrome was significantly higher in I164T mutation than that in wild type. These findings suggest that the I164T mutation of adiponectin gene is associated with the development of the metabolic syndrome-linked CAD. Importantly, the severe hypoadiponectinemia in subjects with the I164T mutation was independent of BMI. Recently, we have demonstrated that intimal thickening was accelerated in mechanically injured arteries of adiponectin knockout mice, and that adenovirus-mediated supplement of adiponectin completely abolished the enhanced neointimal formation (15). These results suggest that hypoadiponectinemia directly contributes to abnormal vascular remodeling. Therefore, the I164T mutation plays a pivotal role in the development of atherosclerosis.

We have reported that the plasma adiponectin levels were significantly low in subjects with obesity (27), diabetes mellitus (31), and hypertension (32). In addition, we reported that plasma adiponectin level was predictive of the development of type 2 diabetes in the Pima Indian population (33). These observations suggest that the plasma adiponectin levels might be closely associated with the development of the metabolic syndrome. In adiponectin knockout mice, glucose metabolism was normal under standard diet, and severe insulin resistance, hyperglycemia, and hypertension were developed after two weeks' feeding of atherogenic diet (18,34). In the present study, all subjects carrying I164T had at least one or more coronary risk factors. However, HOMA-IR levels of nondiabetic I164T mutation were no different than those of control subjects. These results suggest that the hypoadiponectinemia caused by I164T mutation might lead to diabetes mellitus, hypertension, and atherosclerosis only under overnutrition in the modern industrialized countries.

A recent study demonstrated that the I164T mutation was not found in the type 2 diabetic and obese French Caucasian subjects and that the genotypes of SNP94 and SNP276 affected plasma adiponectin levels (23). Higher plasma adiponectin levels were associated with the T allele of SNP94 and the G allele of SNP276 in Caucasians (23). We and others demonstrated that the I164T mutation was observed in the Japanese population (19,21). In the present study, the G allele of SNP94 tended to be associated with lower plasma adiponectin levels, and SNP276 did not correlate with plasma adiponectin levels in CAD and non-CAD Japanese subjects whose mean BMI were approximately 24 kg/m^2. Recently, the genotypes of SNP276 were reported to be associated with plasma adiponectin levels only in the obese subgroup of Japanese subjects (21). These differences between the French and Japanese populations may be due to ethnic background, although a larger population study is required to elucidate the discrepancy.

In the current study, three of the 14 subjects with the I164T mutation did not suffer from CAD, although they had at least one coronary risk factor and markedly low plasma adiponectin level. The follow-up study will be necessary to clarify whether the non-CAD subjects with I164T mutation develop CAD in the future.

In summary, we demonstrated that the I164T mutation of adiponectin gene affects CAD prevalence and the clustering of multiple risk factors for atherosclerosis. Our results indicate that screening the common genetic background of hypoadiponectinemia is helpful in evaluating the risk of the metabolic syndrome and CAD.

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Glucose Enhances Human Macrophage LOX-1 Expression
Role for LOX-1 in Glucose-Induced Macrophage Foam Cell Formation

Ling Li, Tatsuya Sawamura, Geneviève Renier

Abstract—Lectin-like oxidized LDL receptor-1 (LOX-1) is a newly identified receptor for oxidized LDL that is expressed by vascular cells. LOX-1 is upregulated in aortas of diabetic rats and thus may contribute to the pathogenesis of human diabetic atherosclerosis. In this study, we examined the regulation of human monocyte-derived macrophage (MDM) LOX-1 expression by high glucose and the role of LOX-1 in glucose-induced foam cell formation. Incubation of human MDMs with glucose (5.6 to 30 mmol/L) enhanced, in a dose- and time-dependent manner, LOX-1 gene and protein expression. Induction of LOX-1 gene expression by high glucose was abolished by antioxidants, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB), and activated protein-1 (AP-1) inhibitors. In human MDMs cultured with high glucose, increased expression of PKCβ$_2$ and enhanced phosphorylation of extracellular signal-regulated protein kinase 1/2 was observed. Activation of these kinases was inhibited by the antioxidant *N*-acetyl-L-cysteine (NAC) and by the PKCβ inhibitor LY379196. High glucose also enhanced the binding of nuclear proteins extracted from human MDMs to the NF-κB and AP-1 regulatory elements of the LOX-1 gene promoter. This effect was abrogated by NAC and PKC/MAPK inhibitors. Finally, high glucose induced human macrophage-derived foam cell formation through a LOX-1–dependent pathway. Overall, these results demonstrate that high glucose concentrations enhance LOX-1 expression in human MDMs and that this effect is associated with foam cell formation. Pilot data showing that MDMs of patients with type 2 diabetes overexpress LOX-1 support the relevance of this work to human diabetic atherosclerosis. (***Circ Res.* 2004;94:892-901.**)

Key Words: macrophages ■ lectin-like oxidized low-density lipoprotein receptor-1 ■ glucose ■ diabetes ■ foam cell formation

The prevalence, incidence, and mortality from all forms of cardiovascular diseases are increased in patients with diabetes.[1] Among the cardiovascular risk factors documented in diabetes, hyperglycemia appears as an independent risk factor for diabetic macrovascular complications.[2] Mechanisms through which hyperglycemia may promote the development of diabetic cardiovascular disease include glycoxidation and lipoxidation, increased oxidative stress, and protein kinase C (PKC) activation.[3–8] One of the earliest events in atherogenesis is the accumulation of oxidized LDL (oxLDL) in the intima and the subsequent uptake of this modified lipoprotein by macrophages, leading to foam cell formation.[9] One limiting factor for oxLDL uptake by endothelial cells is lectin-like oxLDL receptor-1 (LOX-1), a newly identified vascular receptor for oxLDL.[10–12] Accumulating evidence indicates a key role for LOX-1 in atherogenesis. First, uptake of oxLDL by endothelial cells through LOX-1 induces endothelial dysfunction. Second, the two main LOX-1 ligands, oxLDL and advanced glycation end products (AGE), are implicated in the pathogenesis of atherosclerosis.[5,9] Third, expression of LOX-1 by vascular cells, including endothelial cells and macrophages, is enhanced by proatherogenic factors.[13–15] Finally, LOX-1 is expressed in vivo in the aortas of animals with proatherogenic settings[16,19,20] and is upregulated in early human atherosclerotic lesions.[21]

LOX-1 expression is increased in the endothelium and aortas of diabetic rats[16] and thus may play a role in atherogenesis associated with diabetes. Evidence that AGE induce LOX-1 expression in cultured endothelial cells[16] and macrophages[22] supports a primary role for these products in modulating vascular LOX-1 expression in diabetes.

On the basis of these results and given the key role for macrophages as precursors of foam cells in the vascular wall,[9] the present study was aimed at investigating the regulation of human macrophage LOX-1 expression by hyperglycemia and the role of this receptor in glucose-induced macrophage foam cell transformation.

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Materials and Methods

Reagents

See the online data supplement, available at http://circres.ahajournals.org, for details about reagents.

Cell Culture

Freshly isolated human monocytes[23] or THP-1 monocytes were differentiated into macrophages in vitro and treated with high glucose (see the online data supplement for details).

Analysis of mRNA Expression

Northern Blot Analysis

LOX-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in THP-1 monocyte-derived macrophages (MDMs) (10×10^6) was analyzed by hybridization with [^{32}P] dCTP-labeled human LOX-1 and GAPDH cDNA probes (see the online data supplement for details).

Polymerase Chain Reaction Analysis

Total RNA for use in the polymerase chain reaction (PCR) reaction was extracted from human MDMs (2×10^6/mL) by an improvement of the acid-phenol technique of Chomczynski and Sacchi.[24] cDNA was synthesized from RNA and amplified by synthetic primers specific for human LOX-1 and GAPDH (see the online data supplement for details).

Western Blot

LOX-1, mitogen-activated protein kinase (MAPK), and PKC-β_2 expression in human MDMs was analyzed by Western blot analysis using specific antibodies (see the online data supplement for details).

DNA Binding Assay

Nuclear proteins were isolated from THP-1 MDMs, and their binding to consensus sequences of the LOX-1 promoter nuclear factor-κB (NF-κB) and activated protein-1 (AP-1)–enhancing elements was assessed by DNA retardation electrophoretic mobility shift assay[25,26] (see the online data supplement for details).

DNA Probes

See the online data supplement for details about DNA probes.

Uptake of Dil-oxLDL by Human MDMs

Native LDL (density, 1.019 to 1.063) was isolated from plasma obtained from healthy donors by sequential ultracentrifugation[27] and extensively dialyzed for 24 hours at 4°C against 5 mmol/L Tris/50 mmol/L NaCl to remove EDTA. Oxidation of LDL was performed by incubating native LDL (2 mg of protein per mL) at 37°C for 20 hours in serum-free RPMI 1640 containing 7.5 μg/mL $CuSO_4$. Oxidation of LDL was monitored by measuring the amount of thiobarbituric acid–reactive substances and by electrophoretic mobility on agarose gel. OxLDL was labeled with Dil as described previously.[28] Uptake of DiI-oxLDL by human MDMs was assessed by fluorescence microscopy and determination of fluorescence at 520/564 nm (see the online data supplement for details). Results were normalized to total cell protein concentrations.[29]

Quantification of Cytosolic AGE in MDMs

The total AGE content present in the cytosolic extracts of glucose-treated MDMs was determined by competitive ELISA. Results were expressed as B/Bo (see the online data supplement for details).

Patients

The study group comprised 7 patients with type 2 diabetes and 12 healthy control subjects. The patients with diabetes were recruited from the Notre-Dame Hospital outpatient clinic and gave written consent to participate in this study. The patients had a mean (±SEM) age of 65±3 years, body mass index of 32±2 kg/m^2, fasting glucose of 9.4±1.2 mmol/L, triglyceride level of 3.26±1.21 mmol/L, LDL cholesterol level of 3.08±0.31 mmol/L, and serum glycohemoglobin of 0.072±0.006. All patients were treated with glyburide and metformin. None of the patients was primarily insulin dependent. One patient was hypertensive and was treated with enalapril, and one had macroangiopathy and microalbuminuria. Control subjects were recruited from the hospital staff and relatives. They had a mean (±SEM) age of 38±4 years, body mass index of 23±1 kg/m^2, fasting glucose of 5.0±0.1 mmol/L, triglyceride level of 1.30±0.20 mmol/L, and LDL cholesterol level of 3.50±0.40 mmol/L. Subjects who had infectious or inflammatory conditions or cardiac, renal, or pulmonary decompensated diseases or who were treated with antiinflammatory or antioxidant drugs were excluded from the study.

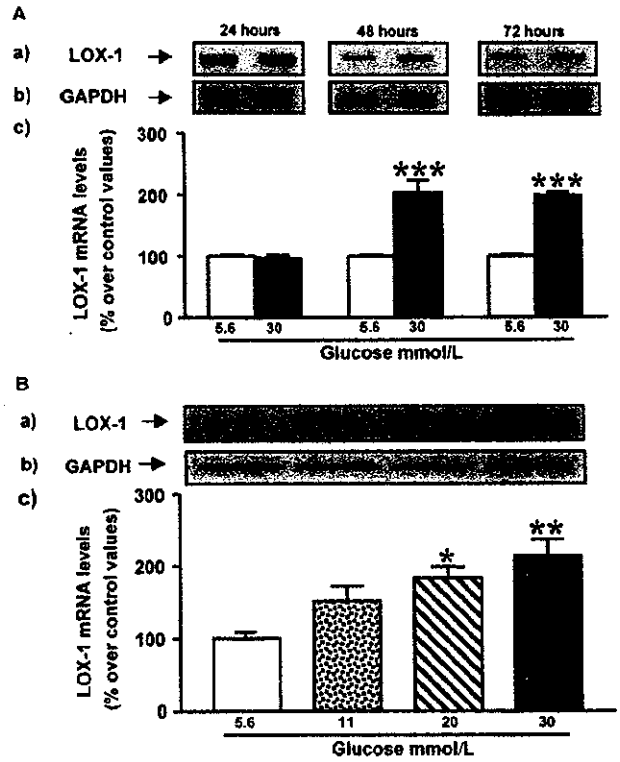

Figure 1. Time- and dose-dependent effect of high glucose on LOX-1 mRNA levels in human MDMs. Cultured human MDMs were incubated for 24 to 72 hours (A) or 48 hours (B) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by reverse transcriptase (RT)-PCR (A and B). LOX-1 mRNA levels (a) were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar (c) represent the mean±SEM of 6 (A and B) different experiments. *P<0.05, **P<0.01, ***P<0.001 vs 5.6 mmol/L glucose.

Determination of Cell Viability

Cell viability after treatment with the different agents under study was assessed by trypan blue exclusion and was consistently found to be >90%.

Statistical Analysis

Values are expressed as mean±SEM. Data were analyzed by one-way ANOVA followed by the Tukey test. P<0.05 was considered statistically significant.

Results

Effect of D-Glucose on Human MDM LOX-1 mRNA Expression

Incubation of human MDMs for 24 to 72 hours with 5.6 or 30 mmol/L D-glucose increased, in a time-dependent manner,

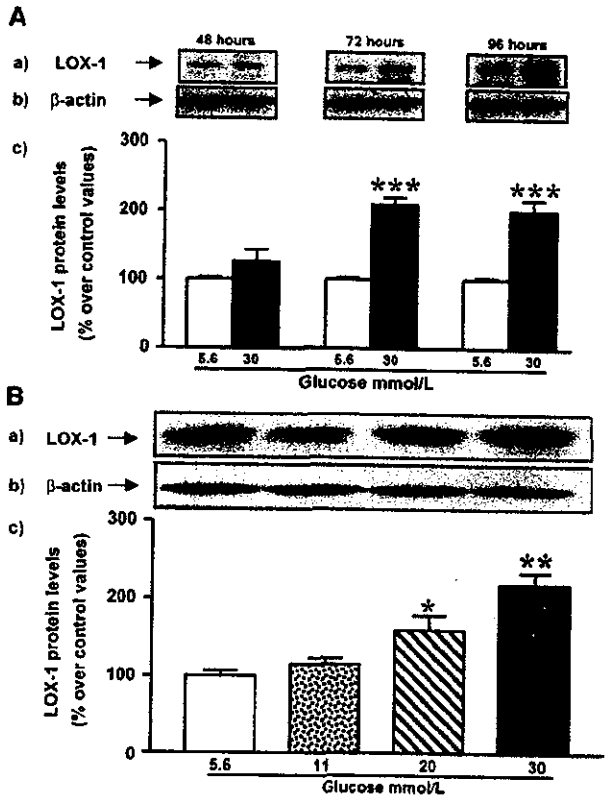

Figure 2. Time- and dose-dependent effect of high glucose on LOX-1 protein expression in human MDMs. Human MDMs were cultured for 48 to 96 hours (A) or 72 hours (B) with 5.6 to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 membrane protein expression was determined by Western blot analysis (a). LOX-1 protein levels were normalized to the levels of β-actin protein (b). Data illustrated on the graph bar (c) represent the mean±SEM of 6 (A) and 3 (B) different experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs 5.6 mmol/L glucose.

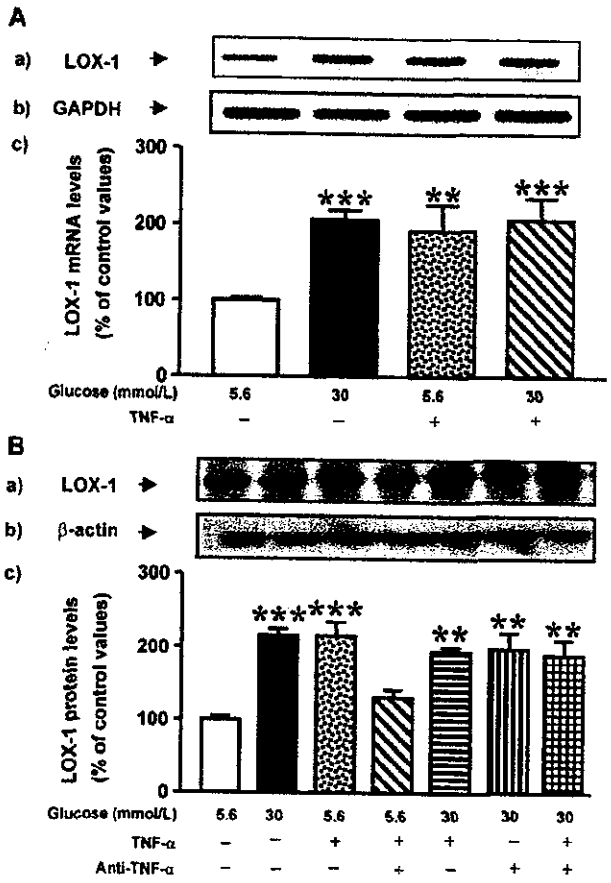

Figure 3. Effect of high glucose on TNF-α–induced macrophage LOX-1 mRNA and protein expression. Human MDMs were cultured for 24 hours (A) or 48 hours (B) in 5.6 or 30 mmol/L glucose environment and then treated for an additional 24 hours with TNF-α (5 ng/mL) in the presence or absence of anti–TNF-α antibodies (10 μg/mL) (B). At the end of the incubation period, cells were lysed and LOX-1 mRNA (A) and membrane protein expression (B) were determined by RT-PCR and Western blot analysis, respectively. LOX-1 mRNA and protein levels were normalized to the levels of GAPDH mRNA (Ab) or β-actin protein (Bb). Data illustrated on the graph bar (Ac and Bc) represent the mean±SEM of 4 different experiments. **$P<0.01$, ***$P<0.001$ vs 5.6 mmol/L glucose.

macrophage LOX-1 gene expression. Maximal effect was observed from 48 to 72 hours (Figure 1Aa). Under these experimental conditions, no modulation of the mRNA expression of GAPDH, used as internal control, was observed (Figure 1Ab). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA, are shown in Figure 1Ac. The stimulatory effect of D-glucose on human MDM LOX-1 mRNA expression was dose-dependent, with maximal effect occurring between 20 and 30 mmol/L glucose (Figure 1Ba). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed (Figure 1Bb). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA, are shown in Figure 1Bc. Incubation of human MDMs with L-glucose or mannitol (30 mmol/L) did not enhance LOX-1 mRNA expression (LOX-1 mRNA expression [% of control values]: L-glucose, 98±5; mannitol, 107±6).

Effect of D-Glucose on Human MDM LOX-1 Protein Expression

Treatment of human MDMs with 30 mmol/L D-glucose enhanced LOX-1 protein expression in these cells. This effect was observed from 72 to 96 hours (Figure 2Aa). Under these experimental conditions, no modulation of β-actin, used as internal control, was observed (Figure 2Ab). LOX-1 protein levels normalized to the levels of β-actin protein are illustrated in Figure 2Ac. Incubation of human MDMs for 72 hours with increasing D-glucose concentrations (5.6 to 30 mmol/L) enhanced, in a dose-dependent manner, LOX-1 protein expression in these cells (Figure 2Ba). LOX-1 protein levels normalized to the levels of β-actin (Figure 2Bb) are illustrated in Figure 2Bc. No stimulatory effect of mannitol (30 mmol/L) on macrophage LOX-1 protein expression was observed (LOX-1 protein expression [% of control values]: mannitol, 109±9).

Role of AGE in the Induction of MDM LOX-1 by High Glucose

To evaluate whether intracellular AGE formation may play a role in the induction of MDM LOX-1 expression by high

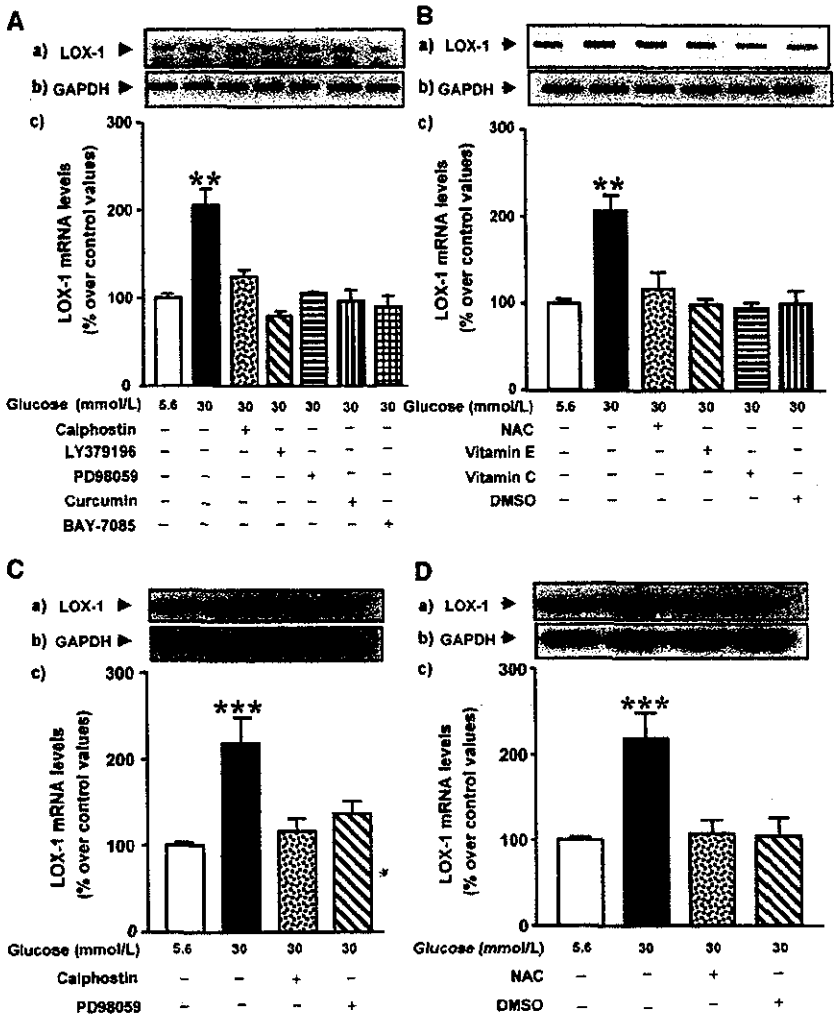

Figure 4. Effect of PKC, MAPK, NF-κB, and AP-1 inhibitors (A and C) and antioxidants (B and D) on glucose-induced LOX-1 mRNA levels. Human (A and B) and THP-1 (C and D) MDMs were pretreated for 1 hour with the PKC inhibitor calphostin C (0.1 μg/mL), the specific PKCβ inhibitor LY379196 (30 nmol/L), the MAPK inhibitor PD98059 (50 μmol/L), the NF-κB inhibitor BAY 11-7085 (40 μmol/L), the AP-1 inhibitor curcumin (10 μmol/L), or the antioxidants NAC (10 mmol/L), vitamin E (50 μmol/L), vitamin C (10 μmol/L), and DMSO (0.5%) and then exposed for 48 hours to 30 mmol/L glucose. At the end of the incubation period, cells were lysed and LOX-1 mRNA was analyzed by RT-PCR (Aa and Ba) or Northern blot analysis (Ca and Da). LOX-1 mRNA levels were normalized to the levels of GAPDH mRNA (b). Data illustrated on the graph bar represent the mean±SEM of 7 (Ac and Bc) and 6 (Cc and Dc) different experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs 5.6 mmol/L glucose.

glucose, the levels of cytosolic glycated proteins present in MDMs exposed to high glucose for 24 to 48 hours were determined. Regardless of the glucose concentrations used, levels of glycated proteins in MDMs consistently fell below the minimum concentration of AGE detected by this assay, ie. <0.25 ng AGE/μg protein (B/Bo: glucose at 24 hours [in mmol/L], 5.6: 2.5±0.7; 10: 3.9±1.3; 20: 3.4±0.8; 30: 3.9±0.7; glucose at 48 hours [in mmol/L], 5.6: 3.7±1.7; 10: 2.9±1.2; 20: 3.3±0.3; 30: 2.3±0.5). Although nonglycated BSA (50 ng/mL), used as negative control, failed to inhibit antiserum binding (B/Bo, 1.1), competition for antibody binding was observed with methylglyoxal- and glucose-derived AGE-BSA (50 ng/mL) used as positive controls (B/Bo, 0.53 and 0.57, respectively).

Effect of High Glucose on Tumor Necrosis Factor-α–Induced MDM LOX-1 Expression

One pathophysiological stimulus relevant to atherosclerosis in diabetes is tumor necrosis factor-α (TNF-α).[30,31] Because this cytokine stimulates LOX-1 expression in vascular cells[13,17] and is released by monocytic cells in response to high glucose and AGE,[32–35] we determined the modulatory effect of TNF-α on human MDM LOX-1 expression under normal and high glucose conditions. As shown in Figures 3A and 3B, TNF-α–treated human MDMs cultured under normoglycemic conditions express similar levels of LOX-1 gene and protein expression as high glucose–treated cells. Levels of LOX-1 expression elicited by this cytokine did not differ when human MDMs were cultured in high glucose conditions. The effect of TNF-α alone on LOX-1 protein expression was blocked by an anti–TNF-α antibody (Figure 3B).

Signaling Pathways Involved in Glucose-Induced Human MDM LOX-1 Gene Expression

To identify the signaling pathways involved in the stimulatory effect of high glucose on LOX-1 gene expression, human MDMs were pretreated for 2 hours with PKC, MAPK, tyrosine kinase, NF-κB, or AP-1 inhibitors before exposure to glucose. As shown in Figure 4A, the pan-specific PKC inhibitor calphostin C (0.1 μg/mL) and the PKCβ inhibitor LY379196 (30 nmol/L) totally abrogated glucose-induced macrophage LOX-1 gene expression. A similar effect was observed when the cells were preincubated with the MAPK inhibitor PD98059 (50 μmol/L), the AP-1 inhibitor curcumin (10 μmol/L), or the NF-κB inhibitor BAY 11-7085 (40 μmol/L)[36] (Figure 4A). In contrast, tyrosine kinase inhibitors

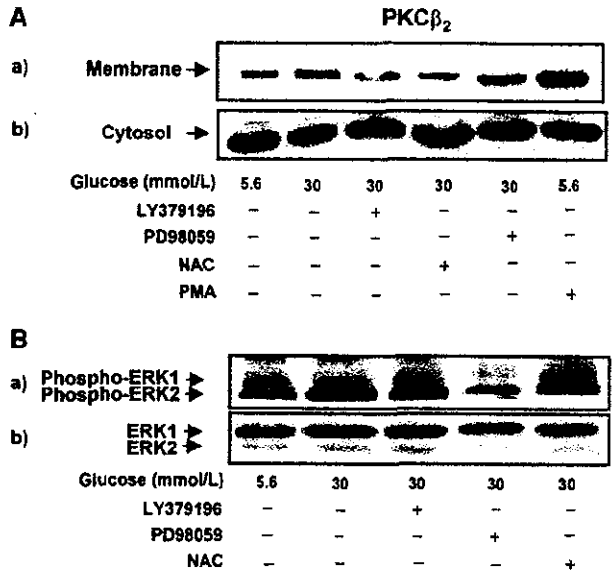

Figure 5. Effect of high glucose on PKC and MAPK activation in THP-1 MDMs. Modulatory effect of NAC and PKC/MAPK inhibitors. THP-1 MDMs were pretreated for 1 hour with NAC (10 mmol/L), LY379196 (30 nmol/L), or PD98059 (50 μmol/L) and then incubated for 48 hours with 30 mmol/L glucose. A, Membrane (a) and cytosolic (b) fractions were assayed for PKCβ2 expression by Western blot analysis. Cells stimulated with PMA (0.5 μmol/L) for 30 minutes were used as positive control. B, Phosphorylation of ERK1/2 was assessed by Western blot using phospho-specific ERK1/2 antibody (a) or specific ERK1/2 antibody (b). Representative blots are shown.

did not affect this parameter (data not shown). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed (Figure 4Ab). LOX-1 mRNA levels, normalized to the levels of GAPDH mRNA, are presented in Figure 4Ac. Because diabetes and high glucose induce increased oxidative stress,[37] we next determined the role of oxidative stress in the regulation of LOX-1 gene expression by glucose. As shown in Figure 4B, preincubation of human MDMs with various antioxidants, including *N*-acetyl-L-cysteine (NAC) (10 mmol/L), vitamin E (50 μmol/L), vitamin C (10 μmol/L), and DMSO (0.5%), totally prevented the stimulatory effect of high glucose on LOX-1 gene expression. Involvement of these signaling events was confirmed in THP-1 MDMs by demonstrating that PKC and MAPK inhibitors as well as antioxidants abolished glucose-induced LOX-1 mRNA expression in these cells (Figures 4C and 4D). Having established the relevance of THP-1 cells to human MDMs, we next assessed using these cells the sequential events leading to glucose-induced PKC/MAPK activation. As shown in Figure 5, treatment of THP-1 cells for 48 hours with high glucose induced PKCβ2 (Figure 5A) and extracellular signal-regulated protein kinase (ERK) 1/2 (Figure 5B) activation, as assessed by Western blot analysis. Glucose-induced activation of these kinases was reduced by NAC (Figures 5A and 5B). Furthermore, ERK1/2 activation in glucose-treated macrophages was inhibited by LY379196 (Figure 5B), thereby identifying MAPK as downstream targets of PKC.

Effect of High Glucose Concentrations on the Binding of Nuclear Proteins to the Regulatory NF-κB and AP-1 Sequences of the LOX-1 Gene Promoter

Exposure of THP-1 MDMs to a high glucose environment increased the binding of nuclear proteins to the NF-κB (Figure 6) and AP-1 (Figure 7) consensus sequences of the human LOX-1 promoter. These binding complexes were specifically competed in the presence of a 1000-molar excess of the unlabeled NF-κB or AP-1 oligonucleotides and were significantly decreased by BAY 11-7085 (Figure 6) or curcumin (Figure 7). Nuclear protein binding was additionally inhibited in the presence of antibodies against p50 and p65 (Figure 6) or c-fos and c-Jun (Figure 7). In contrast, irrelevant antibodies or competitors did not alter glucose-induced NF-κB and AP-1 activation, thus confirming the specificity of the inhibition documented in these electrophoretic mobility shift assays (Figures 6 and 7). Preincubation of THP-1 cells with NAC, PKC, and MAPK inhibitors also suppresses the nuclear binding to the NF-κB and AP-1 sequences of the LOX-1 gene promoter (Figures 6 and 7).

Role of LOX-1 in Mediating Glucose-Induced Human MDM Foam Cell Formation

To evaluate whether increased expression of LOX-1 by high glucose resulted in enhanced uptake of oxLDL by human MDMs, these cells were treated for 48 hours with 5.6 or 30 mmol/L glucose, and then incubation was pursued for an additional 24-hour period in the presence of saturating amounts (20 μg/mL) of antibodies to CD36, SR-A, LOX-1, or IgG_1. At the end of the incubation period, cells were exposed for 3 hours to DiI-oxLDL (80 μg/mL) in the presence or absence of excess unlabeled oxLDL. Incubation of human MDMs with high glucose in the presence of anti-CD36 and anti–SR-A antibodies led to enhanced uptake of oxLDL by these cells, as assessed by fluorescence microscopy (Figure 8A) and measurement of extracted DiI-oxLDL (Figure 8B). This effect was abrogated by incubating human MDMs with excess unlabeled oxLDL or with anti–LOX-1 antibody. In contrast, exposure of these cells to anti-IgG_1 did not affect glucose-induced MDM foam cell formation (Figures 8A and 8B).

Levels of LOX-1 mRNA in MDMs of Patients With Diabetes

MDMs of patients with type 2 diabetes demonstrated a significant increase in LOX-1 mRNA levels compared with those isolated from control subjects (LOX-1 mRNA levels [%], controls: 100±7; patients with diabetes, 169±25; $P<0.01$).

Discussion

Despite the recent evidence linking experimental diabetes with increased vascular LOX-1 expression,[10] only few studies have examined the regulation of LOX-1 by metabolic factors dysregulated in diabetes. The present study demonstrates for the first time that high glucose increases human macrophage LOX-1 expression, both at gene and protein levels. These results together with our preliminary observations that

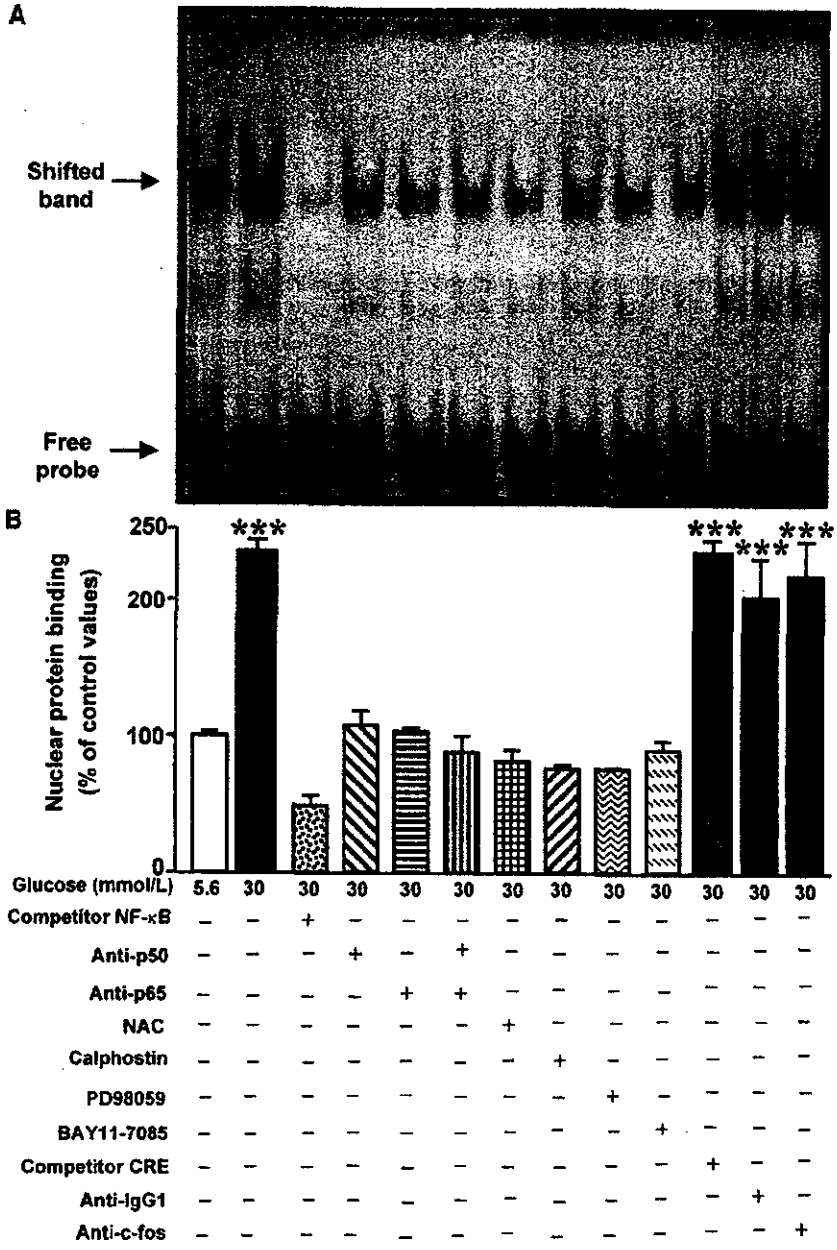

*Figure 6. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDMs to the NF-κB sequence of the LOX-1 gene promoter. THP-1 MDMs were pretreated or not for 1 hour with NAC (10 mmol/L), calphostin C (0.1 μg/mL), PD98059 (50 μmol/L), or BAY 11-7085 (40 μmol/L) and then exposed for 24 hours to 5.6 or 30 mmol/L glucose. Nuclear proteins isolated from these cells were incubated with end-labeled double-stranded oligonucleotide containing the NF-κB sequence of the LOX-1 promoter in the presence or absence of 1000-fold molar excess of unlabeled NF-κB or CRE DNA probe (competitor). In some experiments, nuclear proteins were incubated in the presence of anti-p50, anti-p65, anti-IgG_1, or anti-c-fos antibodies. Retardation was assessed by gel electrophoresis. A, Data represent the results of 1 representative experiment out of 4. B, Graph bar showing the results of 4 independent experiments. ***$P<0.001$ vs 5.6 mmol/L glucose.*

MDMs of diabetic patients exhibit increased LOX-1 gene expression suggest a role of hyperglycemia in the regulation of vascular LOX-1 in human diabetes. In macrophages that do express multiple scavenger receptors,[38] >50% of the uptake of oxLDL seems to occur via CD36,[39] whereas SR-A shares the rest with several other scavenger receptors, including LOX-1. Because high glucose enhances macrophage CD36 expression,[40] it is tempting to postulate that upregulation of macrophage scavenger receptors in response to glucose may play a role in the pathogenesis of atherosclerosis in human diabetes.

It has been previously shown that AGE enhance LOX-1 mRNA expression in cultured aortic endothelial cells and human macrophages.[16,22] On the basis of the time course and concentration of glucose required to modulate macrophage LOX-1 expression, we speculated that generation of AGE might be responsible for LOX-1 induction in glucose-treated MDMs. However, arguing against this hypothesis, we did not ascertain the presence of AGE in these cells over the time course required to modulate LOX-1 gene expression. Considering the short incubation period of macrophages with high glucose, lack of intracellular AGE detection may be related to this in vitro variable. Alternatively, characteristics relating to the sensitivity of the ELISA and the specificity of the anti-AGE-RNAse antiserum used in this assay[41] may account for these negative results.

Interestingly, we found that the extent of stimulation of macrophage LOX-1 expression achieved by glucose was comparable to that elicited by TNF-α and that these two stimuli did not synergize for macrophage LOX-1 induction. Because glucose and AGE stimulate TNF-α secretion,[32–35] one possible explanation for this observation is that induction

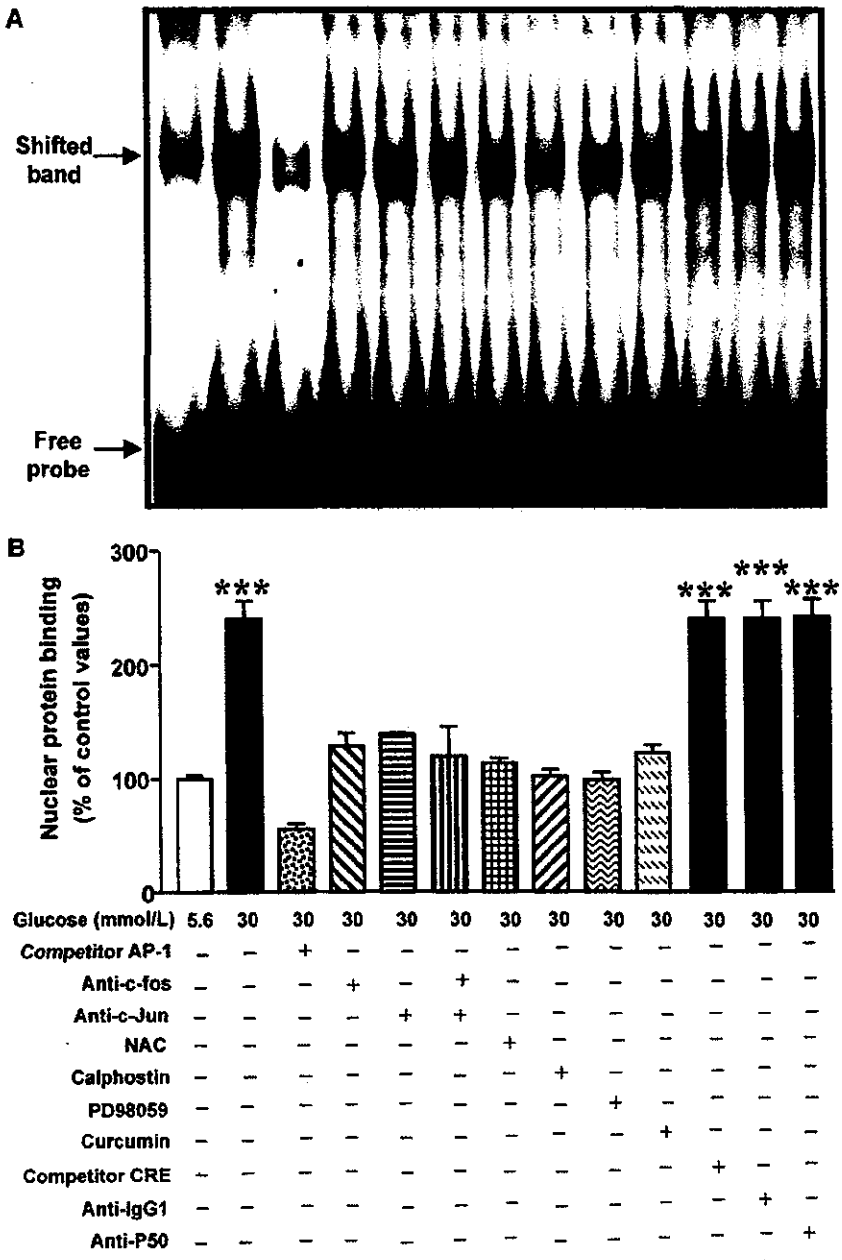

Figure 7. Effect of high glucose on the binding of nuclear proteins extracted from THP-1 MDMs to the AP-1 sequence of the LOX-1 gene promoter. THP-1 MDMs were pretreated or not for 1 hour with NAC (10 mmol/L), calphostin C (0.1 μg/mL), PD98059 (50 μmol/L), or curcumin (10 μmol/L) and then exposed for 24 hours to 5.6 or 30 mmol/L glucose. Nuclear proteins isolated from these cells were incubated with end-labeled double-stranded oligonucleotide containing the AP-1 sequence of the LOX-1 promoter in the presence or absence of 1000-fold molar excess of unlabeled AP-1 or CRE DNA probe (competitor). In some experiments, nuclear proteins were incubated in the presence of anti–c-fos, c-Jun, anti-IgG_1, or anti-p50 antibodies. Retardation was assessed by gel electrophoresis. A, Data represent the results of 1 representative experiment out of 4. B, Graph bar showing the results of 4 independent experiments. ***$P<0.001$ vs 5.6 mmol/L glucose.

of LOX-1 by glucose involves TNF-α. However, this hypothesis is not supported by our finding that immunoneutralization of TNF-α does not affect glucose-induced LOX-1 expression. Alternatively, glucose and TNF-α may regulate macrophage LOX-1 through one major and possibly identical pathway. Like TNF-α, glucose is a well-known activator of NF-κB and AP-1[42–46] and may therefore induce, through the activation of these factors, the transcription of the LOX-1 gene. Consistent with this, we found that glucose increases the LOX-1 mRNA levels in macrophages and enhances the binding of nuclear proteins to the NF-κB and AP-1 regulatory sequences of the LOX-1 promoter.[47] Although final proof for a role for NF-κB and AP-1 as functional responsive elements involved in the transcriptional activation of the LOX-1 gene would require promoter-reporter gene assays, these data support a role for these transcriptional factors in the regulation of LOX-1 gene expression by glucose.

Regulation of LOX-1 gene expression is redox sensitive.[14] Therefore, reactive oxygen species (ROS) generated by glucose in vascular cells[6,7,46] may represent key intermediates in the regulation of LOX-1 gene expression by this metabolic factor. Evidence linking glucose-induced oxidative stress with activation of PKC and MAPK in vascular cells[6–8,48,49] additionally supports a role of these kinases in the control of LOX-1 expression by hyperglycemia. In line with these hypotheses, we found that antioxidants and PKC/MAPK inhibitors abolish glucose-induced macrophage LOX-1 mRNA levels, thus implicating ROS and kinases as signaling molecules in this effect. Our findings that antioxidants suppressed glucose-induced PKC/MAPK activation and that

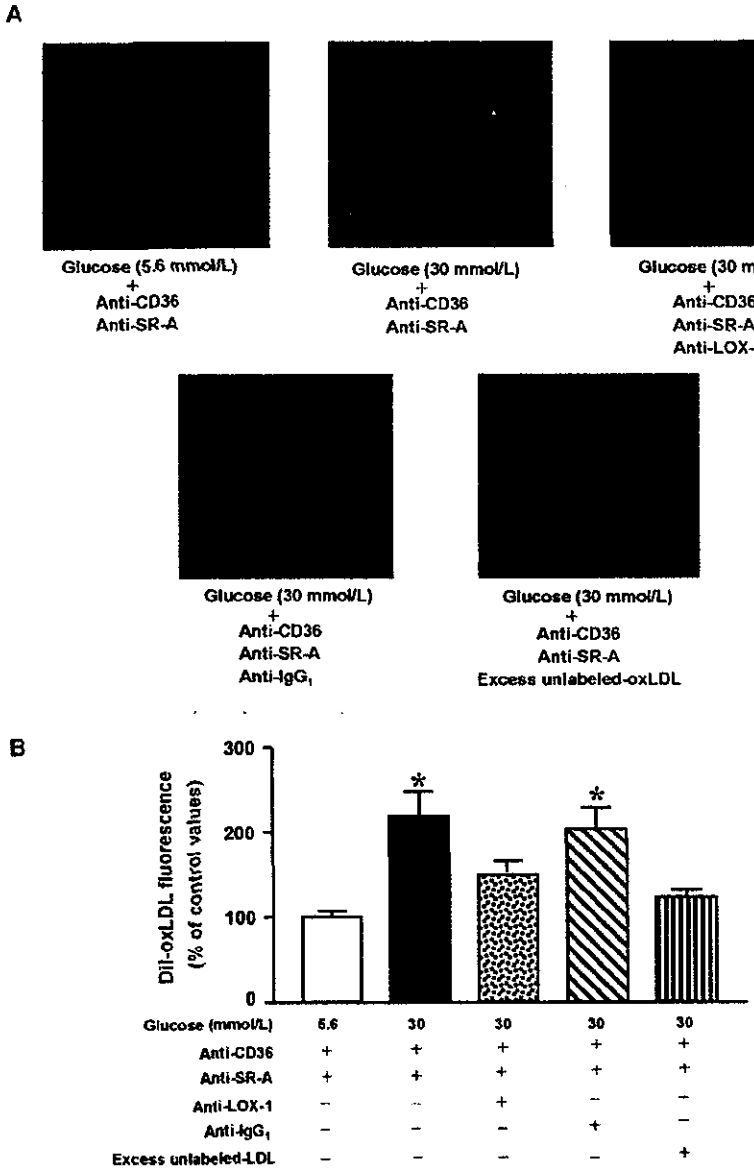

Figure 8. Effect of high glucose on oxLDL uptake by human MDMs. Role of LOX-1. Human MDMs were treated for 48 hours with 5.6 or 30 mmol/L glucose, and then incubation was pursued for an additional 24-hour period in the presence of saturating amounts (20 μg/mL) of antibodies to CD36, SR-A, LOX-1, or IgG_1. At the end of the incubation period, cells were exposed for 3 hours to Dil-oxLDL (80 μg/mL) in the presence or absence of excess unlabeled oxLDL. After washing, fluorescence of Dil was detected in cytoplasm of MDMs by fluorescence microscopy (A) or measured at 520/564 nm (B). Data illustrated on the graph bar represent the mean±SEM of 4 independent experiments. *$P<0.05$ vs 5.6 mmol/L glucose.

PKC inhibition abolished glucose-induced MAPK activation support the hypothesis that glucose-induced kinase activation involves oxidative stress and that MAPKs act in this signaling cascade as intermediate molecules transducing signals from PKC to macrophage LOX-1. Convincing data also indicate a role for oxidative stress and kinases in NF-κB and AP-1 activation.[7,42,46,49–51] In accordance with these results, we found that antioxidants as well as PKC/MAPK inhibitors block glucose-induced NF-κB and AP-1 activation, thus identifying these transcriptional factors as downstream ROS and kinase targets. Taken together, these results indicate that increased production of intracellular ROS and activation of PKC/MAPK pathways are initial signaling events in the regulation of LOX-1 gene by glucose that are required for subsequent activation of NF-κB and AP-1.

Accumulation of cholesterol-loaded foam cells in the arterial intima is a hallmark and key event of early atherogenesis. Evidence that incubation of macrophages in high glucose conditions leads to increased intracellular accumulation of cholesterol ester[52] suggests a role for hyperglycemia in foam cell formation. Like other scavenger receptors, LOX-1 is highly expressed in macrophages present in human atherosclerotic lesions[21] and thus may play a role in macrophage foam cell formation. The present study demonstrates for the first time that increased LOX-1 surface expression in glucose-treated macrophages is associated with enhanced uptake of oxLDL by these cells, suggesting thereby a new role for LOX-1, that of mediating glucose-induced foam cell formation. Importantly, such a role for LOX-1 in foam cell formation was only evident after functional blockade of CD36. It is widely believed that much of the oxLDL uptake by human macrophages occurs via CD36.[39] Although the quantitative contribution of CD36 in glucose-induced foam cell formation is unknown, it has been shown that glucose-induced macrophage CD36 expression correlates with a 10-fold increase in CD36-mediated oxLDL uptake,[40] thus suggesting a major role of this receptor in glucose-induced foam cell

formation. In the present study, we reported a 2-fold increase in non-CD36/non–SR-A–mediated oxLDL uptake in glucose-treated macrophages that was only partly reduced by an anti-LOX-1 antibody. Although these results demonstrate a role of LOX-1 in glucose-induced foam cell formation, they do not argue for a major contribution of LOX-1 in this process. Consistent with this idea, one recent study failed to demonstrate a key role of LOX-1 in the progression of macrophages to foam cells in vitro.[53] Nevertheless, extrapolation of in vitro results to the in vivo situation is hazardous, and additional studies are needed to assess the functional significance of increased LOX-1 expression on foam cell formation in vivo.

In summary, the present study demonstrates that high glucose enhances human MDM LOX-1 expression in vitro and that this effect is associated with foam cell formation. Our preliminary results showing increased MDM LOX-1 expression in human diabetes support the relevance of this work to the human setting.

Acknowledgments

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