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, 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 PKC 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. Among the cardiovascular risk factors documented in diabetes, hyperglycemia appears as an independent risk factor for diabetic macrovascular complications. 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. 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. One limiting factor for oxLDL uptake by endothelial cells is lectin-like oxLDL receptor-1 (LOX-1), a newly identified vascular receptor for oxLDL. 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. Third, expression of LOX-1 by vascular cells, including endothelial cells and macrophages, is enhanced by proatherogenic factors. Finally, LOX-1 is expressed in vivo in the aortas of animals with proatherogenic settings and is upregulated in early human atherosclerotic lesions.

LOX-1 expression is increased in the endothelium and aortas of diabetic rats and thus may play a role in atherogenesis associated with diabetes. Evidence that AGE induce LOX-1 expression in cultured endothelial cells and macrophages 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, 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 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) (10×10⁶) was analyzed by hybridization with [³²P] 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 (2×10⁶/mL) by an improvement of the acid-phenol technique of Chomczynski and Sacchi. 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, 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 (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 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 CuSO₄. 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. Uptake of Dil-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.

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/m², fasting glucose of 9.4±1.2 mmol/L, triglyceride level of 3.26±1.21 mmol/L, LDL cho-

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,
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 illustrated 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 (Figure 2Ac) are 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.

**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
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 pathophysiologic stimulus relevant to atherosclerosis in diabetes is tumor necrosis factor-α (TNF-α).32–31 Because this cytokine stimulates LOX-1 expression in vascular cells13,17 and is released by monocytes 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 4](http://circres.ahajournals.org/Download)
Membrane (a) and cytosolic (b) fractions were assayed for PKC and then incubated for 48 hours with 30 mmol/L glucose. A, /H9262 induce increased oxidative stress,37 we next determined the present in Figure 4Ac. Because diabetes and high glucose levels, normalized to the levels of GAPDH mRNA, are experimental conditions, no modulation of the mRNA expres-

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 mmol/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. 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 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,16 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
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, >50% of the uptake of oxLDL seems to occur via CD36, whereas SR-A shares the rest with several other scavenger receptors, including LOX-1. Because high glucose enhances macrophage CD36 expression, 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. 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 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, one possible explanation for this observation is that induction
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 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. 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. Therefore, reactive oxygen species (ROS) generated by glucose in vascular cells 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 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
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. 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 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 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 in 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. 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, thus suggesting a major role of this receptor in glucose-induced foam cell formation.

**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 IgG1. 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.
formation. In the present study, we reported a 2-fold increase in non-C3D36/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. 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.

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References


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**METHODS-SUPPLEMENTS**

**Reagents**

Fetal calf serum (FCS) was purchased from Wisent (St Bruno, Quebec). RPMI 1640 medium, phenylmethylsulfonyl fluoride (PMSF), Nonidet-P-40 (NP-40), Hank’s balanced salt solution (HBSS), penicillin-streptomycin, salmon sperm DNA, sodium dodecyl sulfate (SDS), phorbol myristate acetate (PMA) and Trizol reagent were obtained from Gibco BRL (Burlington, Ontario, Canada). D-glucose, L-glucose, mannitol, bovine serum albumin (BSA) fraction V, human lipoprotein-deficient serum (LPDS), dihydrochloride dimethyl sulphoxide (DMSO), isopropanol, curcumin, vitamin E, vitamin C and dithiothreitol (DTT) were purchased from Sigma. Antibodies against CD36, β-actin, c-fos, c-Jun, p50, p65, extracellular signal-regulated protein kinase (ERK) 1/2 and PKCβ2 were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TNF-α and IgG1 neutralizing antibody were purchased from R&D Systems (Minneapolis, MN). Anti-SR-A and anti-phospho-ERK 1/2 antibodies were bought from Serotec (Serotec, UK). Monoclonal antibody to human LOX-1 was provided by Dr. Sawamura (Osaka, Japan). Purified rabbit polyclonal antibody raised against AGE-modified RNAse was kindly provided by Dr Friguet (Paris, France). Ficoll and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Amersham Biosciences. Formaldehyde solution and CuSO4 was obtained from Fischer. [32p] dCTP (specific activity 3,000 Ci/mmol) was provided by ICN Biochemicals. Calphostin C, PD98059, N-acetyl-L-cysteine (NAC), BAY-7085 and aminoguanidine were obtained from Calbiochem (La Jolla, CA). Vectashield
mounting medium was obtained from Vector Laboratories (CA, USA). 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) was bought from Molecular Probes. LY379196 was kindly provided by Eli Lilly (Indiana, USA).

**Cell culture**

Peripheral blood mononuclear cells were isolated from healthy control subjects by density centrifugation using Ficoll, allowed to aggregate in the presence of FCS, then further purified by the rosetting technique. After density centrifugation, highly purified monocytes (85-90%) were recovered. Human monocyte purity was assessed by flow cytometry (FACScan, Becton Dickinson) using phycoerythrin-conjugated anti-CD14 monoclonal antibody (Becton Dickinson). Purified monocytes (2x10^6/ml) were cultured in RPMI medium supplemented with penicillin-streptomycin and 20% (vol/vol) autologous serum for 8 days to allow differentiation into monocyte-derived macrophages (MDM). To assess the in vitro effect of high glucose on MDM LOX-1 expression, differentiated MDM were incubated for different time periods in fresh RPMI medium containing 5.6 to 30mmol/L glucose and supplemented with 20% autologous serum. The glucose RPMI media were prepared by adding to glucose-free RPMI medium appropriate amounts of glucose to make up the desired final glucose concentrations.

The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). THP-1 monocytes (10x10^6/ml) were cultured in RPMI medium containing 25 mmol/L HEPES buffer, L-
glutamine, 10% FCS and 100 µg/ml penicillin-streptomycin. THP-1 cells were differentiated into MDM by treatment with 0.5 µmol/L PMA for 2 days.

**Analysis of mRNA expression**

- **Northern Blot Analysis**

  THP-1 MDM (10x10^6/ml) were lysed with Trizol reagent. Total RNA was isolated and 25 µg of total RNA were separated on a 1.2% agarose gel containing 2.2 mol/L formaldehyde. The blots were prehybridized for 8h with prehybridization buffer. The mRNA expression was analyzed by hybridization with [\(^{32}\)P] dCTP-labeled human LOX-1 and GAPDH cDNA probes. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). mRNA expression was quantified by high-resolution optical densitometry (Alpha Imager 2000, Packard Instruments, Meriden, CT).

- **Polymerase Chain Reaction Analysis**

  Cells were lysed with TRIzol reagent and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 µg oligodT (Pharmacia) for 5 min at 98°C then by incubating the mixture with reverse transcription buffer for 60 min at 37°C. The cDNA obtained was amplified by using 0.8 µmol/L of two synthetic primers specific for human LOX-1 (5’-TTACTCTCCATGGTGTTGCC-3’) (5’-AGCTTTTCTDCTTGGTTGCC-3’) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5’-
AGTCTTTCTGGGTGGCAGTGATGG-3’), used as internal standard in the PCR reaction mixture. A 193-base pair human LOX-1 cDNA fragment and a 456-base pair human GAPDH cDNA fragment were amplified enzymatically by 22 and 19 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000, Packard Instrument Company). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve.

**Western blot for LOX-1 and MAPK**

Human or THP-1 MDM protein extracts (15 µg) were applied to 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 V for 60 min. Non-specific binding was blocked with 5% BSA for 1h at room temperature. After washing with phosphate-buffered salt solution (PBS)-Tween 0.1%, blots were incubated overnight with anti-LOX-1, -ERK1/2, -phospho-ERK1/2 or -β-actin antibodies. After further wash, membranes were incubated for 1h at room temperature with a horseradish peroxidase-conjugated donkey anti-mouse IgG (1/5000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham).

**Western blot for PKC-β2 isoform**

After appropriate treatment, THP-1 MDM were recovered and homogenized (Dounce : 15 strokes) in 500 µl of ice-cold buffer A (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l ethylene glycol-bis tetraacetic acid
(EGTA), 25 μg/ml aprotinin, and 25 μg/ml leupeptin). The membrane and cytosolic fractions were separated by ultracentrifugation (100,000 g for 30 minutes at 4°C). After recovery of high-speed supernatants containing cytosolic PKC, the corresponding membrane pellets were homogenized in 500 μl of buffer A containing 0.5% Triton X-100. Protein extracts (10 μg) were then applied to PAGE and PKC-β2 expression was analyzed by western blot analysis using specific PKC-β2 antibody.

**DNA binding assay**

THP-1 MDM (10x10^6/ml) were collected, washed with cold PBS, and lysed in 1 ml ice-cold buffer A (15 nmol/L KCl, 2 mmol/L MgCl₂, 10 mmol/L HEPES, 0.1% PMSF, and 0.5% NP-40). After 10-min incubation on ice, lysed cells were centrifuged, and the nuclei were washed with buffer A without NP-40. The nuclei were then lysed in a buffer containing 2 mol/L KCl, 25 mmol/L HEPES, 0.1 mmol/L EDTA, and 1 mmol/L DTT. After a 15 min incubation period, a dialysis buffer (25 mmol/L HEPES, 1 mmol/L DTT, 0.1% PMSF, 2 μg/ml aprotinin, 0.1 mmol/L EDTA, and 11% glycerol) was added to the nuclei preparation. Nuclei were collected by centrifugation for 20 min at 13,000 rpm. Aliquots (50 μl) of the supernatants were frozen at -80°C, and protein concentration was determined. DNA retardation electrophoretic mobility shift assay (EMSA) was performed as previously described by Fried and Crothers.² Briefly, 5 μg nuclear extracts were incubated for 15 min in the presence of 5X binding buffer (125 mmol/L HEPES, pH 7.5, 50% glycerol, 250 mmol/L NaCl, 0.25% NP-40, and 5 mmol/L DTT) in the presence or absence of 200 ng relevant or irrelevant antibodies (anti-p50, anti-p65, anti-c-fos or anti-c-Jun, anti-
IgG1). End-labeled double-stranded consensus sequences of the LOX-1 promoter NFκB or AP-1-enhancing elements (20,000 cpm per sample) were then added to the samples for 30 min. Samples were analyzed on a 4% nondenaturating PAGE containing 0.01% NP-40. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins isolated from THP-1 MDM with a labeled DNA probe in the presence of a 1,000-molar excess of unlabeled relevant (NF-κB, AP-1) or irrelevant (cyclic AMP-response element (CRE)) DNA probe.

DNA probes

The cDNA probes for detection of human LOX-1 were prepared by the PCR technique. Two synthetic primers spanning bases 151-170 and 804-823 of the LOX-1 cDNA were used to enzymatically amplify a 673-bp region of the LOX-1 probe. The cDNA probe for GAPDH was purchased from ATCC. Double-stranded oligonucleotides containing the NF-κB (5' - CGTCTGCCCTTCCCCTCTCT-3'; 5'-GAGAAGAGGGGAAAGG-3') or the AP-1 (5'-CGTCTGCCCTTCCCCTCTCT-3'; 5'-GAGAAGAGGGGAAAGG-3') consensus sequences of the human LOX-1 gene promoter were synthesized with the aid of an automated DNA synthesizer. After annealing, the oligonucleotides were labeled with [γ-32P] ATP using the Boehringer-Mannheim 5' end-labeling kit (Indianapolis, IN). A double-stranded oligonucleotide containing the CRE consensus sequence of the murine peroxisome proliferator-activated receptor alpha (5'-CTGGCACGGGGCGCGTGG-3'; 5'-ACCGCCTACGGCGCCGCCTG-3') was used as irrelevant competitor in the EMSA.
**Uptake of Dil-oxLDL by human MDM**

To examine cellular uptake of oxLDL, human MDM were seated in 8-well cultureslides (FALCON®) and incubated for 3h in medium containing 5% of LPDS with Dil-labeled oxLDL (80 µg/ml) in the presence or absence of a 500-fold excess of unlabeled oxLDL. At the end of the incubation period, cells were washed, mounted on coverslips and examined by fluorescence microscopy. To measure amounts of Dil-oxLDL accumulated in cells, Dil was extracted by isopropanol and the fluorescence was determined at 520/564 nm.

**Quantification of cytosolic AGE in MDM**

The total AGE content present in the cytosolic extracts of glucose-treated MDM was determined by competitive ELISA. Briefly, BSA was glycated in vitro as previously described and each well of a 96-well microtitre plate (Nunc, Denmark) was coated overnight with 1 µg glycated BSA (AGE-BSA), washed with PBS 0.05% Tween 20, then incubated with PBS containing 6% skimmed milk. After washing, serial dilutions of AGE-BSA as competing antigen were added, followed by the addition of purified rabbit polyclonal antibody (1/5000) raised against AGE-modified RNAse as described earlier. After a 2h-incubation period, wells were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG. After addition of the substrate, absorbance was measured at 405nm on an ELISA microplate reader. Results were expressed as B/Bo calculated as experimental OD-background OD (no antibody) / total OD (no competitor)-background OD, versus ng AGE-BSA. Modified albumins, including methylglyoxal (1mmol/L)-
derived AGE-BSA, low and high (50 and 500 mmol/L)-derived AGE-BSA were used as positive controls. Non glycated BSA was used as negative control.

**REFERENCE**