Soluble lectin-like oxidized low density lipoprotein receptor-1 in type 2 diabetes mellitus

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Abstract The lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) can be proteolytically cleaved and released as soluble forms (sLOX-1). We have determined serum sLOX-1 in type 2 diabetes and evaluated the effect of glucose and advanced glycation end products (AGEs) on sLOX-1 in vitro and in vivo. Endothelial cells were incubated with glucose or AGEs, and sLOX-1 in cell medium was measured. Serum sLOX-1 was measured in 219 diabetic patients and 187 controls by ELISA. The effect of lowering glucose and AGEs on sLOX-1 was determined in 38 poorly controlled diabetic patients after improvement in glycemic control. Incubation of endothelial cells with AGE-BSA led to a dose-dependent increase in sLOX-1, whereas the effect of glucose on sLOX-1 was less marked. Serum sLOX-1 was 9% higher in diabetic patients compared with controls ($P < 0.01$). In the poorly controlled patients, serum sLOX-1 decreased by 12.5% after improvement in glycemic control ($P < 0.05$). The magnitude of reduction in sLOX-1 correlated with the improvement in hemoglobin A1c and AGEs but not with the reduction in oxidized LDL. sLOX-1 level is increased in type 2 diabetes. Both glucose and AGEs are important determinants of LOX-1 expression, and lowering glucose and AGEs leads to a reduction in sLOX-1.—Tan, K. C. B., S. W. M. Shiu, Y. Wong, L. Leng, and R. Bucala. **Soluble lectin-like oxidized low density lipoprotein receptor-1 in type 2 diabetes mellitus. *J. Lipid Res.* 2008.** 49: **1438–1444.**

Supplementary key words advanced glycation end products • oxidized LDL • soluble receptors

The lectin-like oxidized low density lipoprotein receptor (LOX-1), a class E scavenger receptor, is a newly identified oxidized LDL (oxLDL) receptor mainly expressed by endothelial cells (1). It is also expressed by macrophages and vascular smooth muscle cells (2). LOX-1 has been implicated in vascular inflammation and atherosclerotic plague formation and destabilization (3, 4). LOX-1 expression has been demonstrated in animal and human atherosclerotic lesions (5, 6), and binding of oxLDL to LOX-1 in endothelial cells leads to activation of nuclear factor-κB, increases monocyte adhesion, and causes endothelial dysfunction (7–9).

Diabetes is associated with a high cardiovascular risk, and recent evidence suggests that LOX-1 may play a role in atherogensis associated with diabetes. LOX-1 is a receptor with an expression that is not constitutive but dynamically inducible (10), and LOX-1 is upregulated by a number of pathophysiological stimuli, including oxLDL, shear stress, inflammatory cytokines, and angiotensin II (3, 4, 11). In addition, it has been reported that LOX-1 expression can be increased by glucose both in macrophages and in endothelial cells (12, 13). LOX-1 is an endothelial receptor for advanced glycation end products (AGEs) (14), and AGEs can induce LOX-1 expression in cultured endothelial cells and macrophages (15, 16). Animal studies have shown that diabetes leads to a marked upregulation of LOX-1 expression in the vasculature, and LOX-1 expression was significantly increased in diabetic rat aorta (15). Whether LOX-1 expression is increased in human diabetes is unclear. Pilot data from a very small group of patients with type 2 diabetes have shown that LOX-1 mRNA level was significantly increased in monocyte-derived macrophages (13).

LOX-1 is expressed on the cell surface and can be proteolytically cleaved at its membrane proximal extracellular domain and released as soluble forms (sLOX-1), which can be measured in the serum (17). Hence, the serum level of sLOX-1 may reflect LOX-1 expression, and serum sLOX-1 has been shown to be elevated in patients with acute coronary syndrome (18). Since LOX-1 expression can be upregulated by glucose and by AGEs in vitro (12–16), we have determined whether this was associated with an increase in the release of the soluble forms of the receptor in conditioned medium. We further investigated whether serum sLOX-1 concentration was increased in patients with type 2 diabetes mellitus and evaluated the impact of lowering glucose and AGEs by improving glycemic control on serum sLOX-1 concentration.

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METHODS

In vitro study

To investigate the effect of AGEs and glucose on sLOX-1 production in endothelial cells, human aortic endothelial cells (Clonetics, San Diego, CA) were grown to confluence in EGM-1 Bullet Kit Medium, and passages 5 to 8 were used throughout the experiments. Confluent human aortic endothelial cells were kept overnight with serum-free, supplement-free EGM-1 medium and subsequently treated with different concentrations of AGE-BSA (0–250 μg/ml), BSA, or glucose (0–50 mM). After 24 h of incubation, cells and medium were then harvested and cellular LOX-1 expression and concentration of sLOX-1 in medium were measured by Western blot analysis. To determine whether sLOX-1 was associated with changes in endothelial cell function, soluble intercellular adhesion molecule-1 (sICAM-1) in medium was measured by ELISA (R&D) according to the manufacturer's instructions.

AGE-BSA was prepared by incubating 20 mg/ml BSA (fraction V, low endotoxin; Invitrogen) with 0.5 M glucose in PBS, pH 7.2, at 37°C under sterile conditions. After 90 days of incubation, unbound low-molecular-weight products were removed by extensive dialysis against PBS. The degree of glycation on BSA (AGE-BSA fluorescence) was measured by spectrofluorometric detection at excitation of 370 nm and emission of 440 nm (19).

Western blot analysis was performed to measure cellular LOX-1 and sLOX-1 in medium. AGE-BSA- and glucose-treated endothelial cells were lysed in cold homogenization solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 μg/ml leupeptin, and 1 mM aprotinin) while the conditioned medium was concentrated by Centricon 10 (Amicon). Cell lysates and conditioned medium were then mixed with sample running buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, and 0.02% bromophenol blue) followed by 10% SDS-PAGE and were finally electrotransferred onto polyvinylidene difluoride membranes (Millipore). Nonspecific binding was blocked overnight by 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). The blocked membrane was incubated with a mouse monoclonal antibody specific to human LOX-1 (R&D; 1:500 dilutions with 1% nonfat milk in TBST) for 3 h at room temperature. After washing with TBST a few times, the secondary horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Sigma Chemical Co., St. Louis, MO) was added and incubated for another 1 h at room temperature. Antigenic LOX-1 in cell lysates and medium sLOX-1 detection was performed by the ECL Plus protocol (Amersham) according to the manufacturer's instructions.

Clinical study

To determine whether serum sLOX-1 level was increased in diabetes, a cross-sectional study was performed. Consecutive patients with type 2 diabetes mellitus attending the diabetes clinics at Queen Mary Hospital were invited to participate. Patients on insulin therapy were eligible if they had been previously managed with diet and an oral agent at some point and had no known history of diabetic ketoacidosis. Patients with deranged renal and/or liver function were excluded. A total of 219 type 2 diabetic patients were recruited. Fasting blood samples and clinical data were collected. One hundred eighty-seven nondiabetic controls of similar age were recruited from the local community. The study protocol was approved by the local Institutional Review Board, and written informed consent was obtained from all subjects.

To investigate the impact of improving glycemic control on serum sLOX-1, 38 Chinese type 2 diabetic patients who were on metformin and/or sulfonylurea at less than half-maximum dose with suboptimal glycemic control [hemoglobin A1c (HbA1c) ⩾ 7.5%] were recruited. The dose of sulfonylurea was increased, and if fasting blood glucose level remained >7.0 mmol/l at 3 months, the dose of sulfonylurea was increased further. The dosages of metformin and any lipid-lowering and/or antihypertensive agents were kept unchanged throughout the study period. Fasting blood samples were taken at baseline and at the end of 6 months. In addition to indices of glycemic control and serum AGEs, oxLDL also was measured to determine whether any changes in sLOX-1 may be related to oxLDL, a major ligand of LOX-1.

To measure sLOX-1, 96-well enzyme immunoassay microtiter plates (Costar) were coated with antigen, the N-terminal synthetic peptide of human LOX-1 (R&D; 50 ng/ml in 50 μl per well) in coating buffer (0.1 M sodium bicarbonate, pH 9.6) overnight at 4°C. Wells were then washed extensively five times with washing buffer (PBS containing 0.1% Tween 20) and incubated at room temperature for 2 h. After rinsing five times, 50 μl of one-fourth diluted serum samples was added, followed by 50 μl of mouse anti-human LOX-1 monoclonal antibody (R&D; 1:10,000 diluted in PBS containing 1% BSA). Plates then were incubated for 3 h at room temperature with gentle agitation. After another five washes, 100 μl/well horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2,000 in dilution with PBS containing 1% BSA) was added and incubated for 1 h. After further washing steps, 100 μl/well 3,3′,5,5′-tetramentylbenzidine substrate (TMB substrate kit; Pierce Rockford, IL) was added. The color reaction was finally stopped by the addition of 50 μl/well 2 M sulfuric acid. Wells then were analyzed at 450 nm by an ELISA reader. A standard curve of 450 nm absorbance versus the known concentrations of LOX-1 peptide to LOX-1 antibody was constructed, and the results of serum LOX-1 levels were expressed in ng/ml. The standard curve was linear between 25 and 80 ng/ml, and the average recovery was 102% (ranging from 89% to 114%). All samples were run in triplicate. The intra-assay and interassay precision of this sLOX-1 specific competitive ELISA were 6.1% and 7.7%, respectively.

Serum AGEs were measured by a competitive ELISA developed in-house using a well-characterized polyclonal rabbit antiserum raised against AGE-RNase as described previously (20). The polyclonal anti-AGEs antibody recognizes N^{ε}-(carboxymethyl) lysine as well as the major nonfluorescent, AGE cross-linked arginine-lysine imidazole and therefore detects pathologically relevant AGEs (21). The intra-assay and interassay coefficients of variation were 2.5% and 7.4%, respectively. Plasma C-reactive protein (CRP) was measured by a high-sensitivity particle-enhanced immunoturbidimetric assay (Roche Diagnostics, GmbH, Mannheim, Germany) using anti-CRP mouse monoclonal antibodies coupled to latex microparticles. Plasma total cholesterol and triglyceride were determined enzymatically on a Hitachi 912 analyzer (Roche Diagnostics). HDL-cholesterol was measured using a homogenous method with polyethylene glycol-modified enzymes and α-cyclodextrin. LDL-cholesterol was calculated by the Friedewald equation. HbA1c was measured in whole blood using ion-exchange high-performance liquid chromatography with the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad Laboratories, Inc.). Plasma oxLDL was determined by ELISA (Mercodia AB, Uppsala, Sweden), which was a solid-phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies were directed against separate antigenic determinants on the oxidized apolipoprotein B molecule.

Results are expressed as means and SD or as median and interquartile range if the distribution of the data was found to be skewed. Data that were not normally distributed were logarithmically transformed before analyses were made. Comparisons between diabetic patients and controls were done using indepen-

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dent sample t-test, and Pearson's correlations were used to test the relationship between variables. A paired-sample t-test was used to compare parameters in diabetic subjects before and after improving glycemic control.

RESULTS

The effect of AGE-BSA or glucose on LOX-1 expression in endothelial cells was investigated in vitro, and analysis of cell lysates demonstrated that stimulation of cells with AGE-BSA or glucose increased LOX-1 expression (**Fig. 1A, B**). Western blot analysis of medium showed that sLOX-1 was released from endothelial cells after stimulation with AGE-BSA or glucose. Incubation of endothelial cells with AGE-BSA led to a dose-dependent increase in sLOX-1 in cell culture medium (**Fig. 2A**). The effect of glucose on sLOX-1 was less marked, and we only observed a significant increase in sLOX-1 concentration at high glucose concentration (Fig. 2B). In AGE-BSA-stimulated endothelial cells, sICAM-1 concentration in cell culture medium also increased in a dose-dependent manner, and the changes correlated with that of sLOX-1 ($r = 0.64$, $P = 0.03$). No significant changes in sICAM-1 were observed in glucose-stimulated cells.

The relationship between AGEs, glucose, and sLOX-1 in patients with type 2 diabetes mellitus was then determined in a cross-sectional study. The clinical characteristics of the diabetic patients and controls are shown in **Table 1**. In the diabetic patients, 9% were on sulfonylurea monotherapy, 12% on metformin monotherapy, 47% on a combination of sulfonylurea and metformin, 11% on insulin monotherapy, and 21% on insulin and metformin. Fasting plasma glucose, HbA1c, serum AGEs, and CRP were significantly increased in diabetic patients compared with controls. Serum sLOX-1 was also significantly elevated in diabetic patients, and the differences remained significant ($P < 0.01$) after adjusting for possible confounders including age,

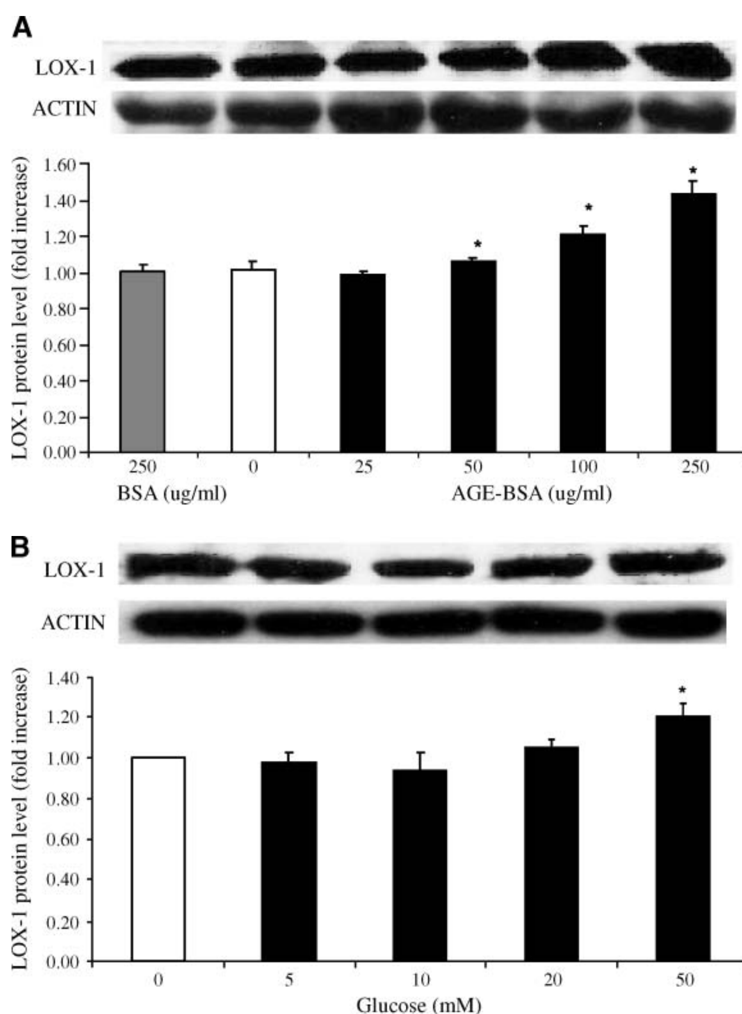

Fig. 1. Effects of AGE-BSA (A) and glucose (B) on lectin-like oxidized low density lipoprotein receptor (LOX-1) protein expression in cell lysates of human aortic endothelial cells. Cells were incubated with different concentrations of AGE-BSA (with BSA as a control, depicted as a gray bar) or glucose for 24 h. At the end of the incubation period, LOX-1 protein was measured in cell lysates by Western blot. Data represent means ± SEM of four different experiments. * $P < 0.05$ versus control.

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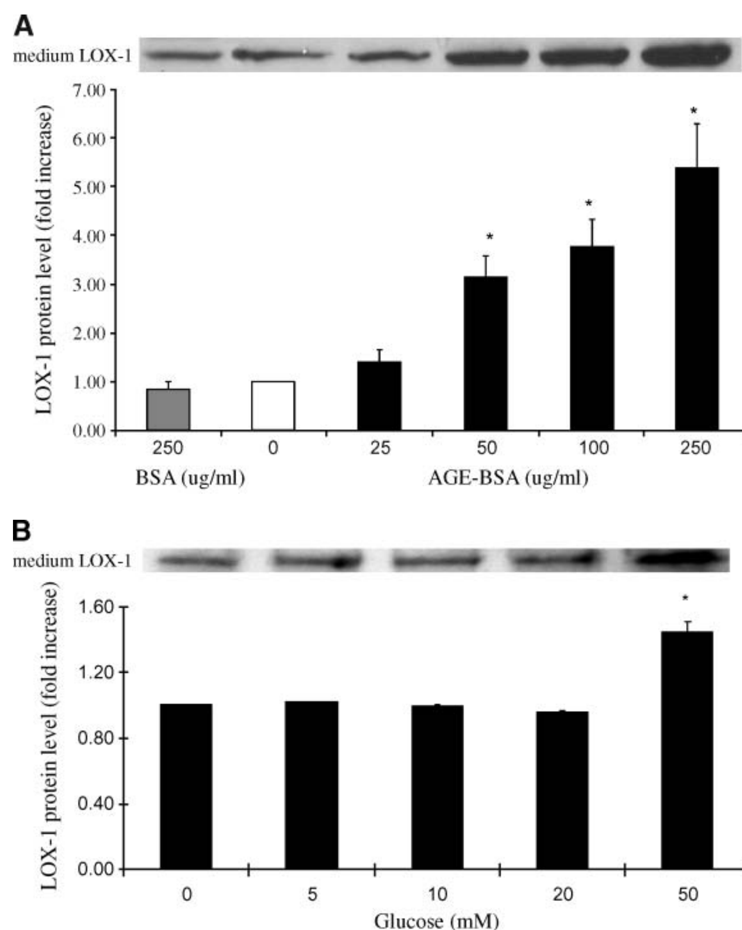

Fig. 2. Effects of AGE-BSA (A) and glucose (B) on LOX-1 protein levels in medium of human aortic endothelial cells. Cells were incubated with different concentrations of AGE-BSA (with BSA as a control, depicted as a gray bar) or glucose for 24 h. At the end of the incubation period, LOX-1 protein was measured in cell culture medium by Western blot, showing a 35 kDa band corresponding to soluble forms of LOX-1 (sLOX-1). Data represent means ± SEM of four different experiments. * $P < 0.05$ versus control.

gender, body mass index, smoking, systolic blood pressure, the presence of cardiovascular disease, CRP, and triglyceride levels. The analysis was repeated after excluding subjects on lipid-lowering agents, and serum sLOX-1 remained significantly higher in diabetic subjects. The type of antidiabetic agents had no significant effect on serum sLOX-1. We also determined whether blockade of the renin-angiotenisin system has any effect on sLOX-1 level, as angiotensin II has been shown to stimulate LOX-1 expression. Diabetic subjects receiving angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonist had significantly lower serum sLOX-1 levels than those not on those medications [105.0 (89.6–124.0) ng/ml vs. 114.0 (94.3–163.7); respectively; $P = 0.01$]. On univariate analysis, serum log(sLOX-1) correlated only with serum AGEs ($r = 0.16$, $P = 0.02$) in the diabetic patients, and no correlation was seen with age, body mass index, systolic or diastolic blood pressure, fasting glucose, plasma lipids, or log(CRP). There was a trend toward a weak correlation with HbA1c. In the control group, no correlation was seen between serum sLOX-1 and AGEs or any other metabolic parameters.

The impact of improving glycemic control on serum sLOX-1 concentration was evaluated in 38 diabetic subjects, and the results are shown in **Table 2**. As expected, plasma glucose, HbA1c, and AGEs improved significantly at the end of 6 months. Although there were no changes in plasma lipids or CRP, plasma oxLDL decreased significantly and serum sLOX-1 also decreased significantly. The magnitude of reduction in sLOX-1 correlated with the improvement in HbA1c and AGEs (**Fig. 3A, B**) but not with the reduction in oxLDL ($P = 0.65$).

DISCUSSION

LOX-1 is increasingly being viewed as a mediator of endothelial or vascular dysfunction (4). Previous in vitro studies have shown that posttranslational processing of bovine LOX-1 in cultured cells generates both membrane-bound and soluble polypeptides (17, 22). Proteolytic cleavage of LOX-1 within a basic stretch of residues close to the transmembrane region releases a soluble form of the receptor containing the C-type lectin-like domain. Since the level of soluble receptors in circulating blood may reflect the expression of membrane proteins and disease activities, sLOX-1 may be a potential biomarker of vascular disease.

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TABLE 1. Clinical characteristics and serum sLOX-1 in controls and diabetic patients

Characteristic	Control (n = 187)	Diabetic (n = 219)
Male/female (%)	52/48	44/56
Age (years)	47.7 ± 7.8	54.3 ± 8.9[a]
Duration of diabetes (years)	—	11.4 ± 7.1
Body mass index (kg/m^2)	24.6 ± 3.3	25.6 ± 3.6[b]
Waist circumference (cm)	81.3 ± 9.3	87.6 ± 9.7[a]
Smokers (%)	13	11
Hypertension (%)	—	54
Cardiovascular disease (%)	—	6
Lipid-lowering agents (%)	—	16
Angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonist (%)	—	32
Systolic blood pressure (mmHg)	115 ± 17	130 ± 17[a]
Diastolic blood pressure (mmHg)	75 ± 10	77 ± 9[b]
Total cholesterol (mmol/l)	5.11 ± 0.82	5.25 ± 1.09
Triglyceride (mmol/l)	1.00 (0.78–1.50)	1.30 (0.90–1.96)[a]
LDL-cholesterol (mmol/l)	3.09 ± 0.76	3.27 ± 0.97[b]
HDL-cholesterol (mmol/l)	1.43 ± 0.38	1.23 ± 0.32[a]
Hemoglobin A1c (%)	5.70 ± 0.79	8.47 ± 1.72[a]
Fasting glucose (mmol/l)	4.90 ± 0.81	8.89 ± 2.69[a]
AGEs (U/ml)	3.51 ± 1.22	4.25 ± 1.07[a]
sLOX-1 (ng/ml)	102.5 (85.2–119.6)	111.4 (91.7–155.2)[a]
CRP (mg/l)	0.93 (0.43–1.89)	1.32 (0.70–2.65)[a]

AGEs, advanced glycation end products; CRP, C-reactive protein; sLOX-1, soluble forms of lectin-like oxidized low density lipoprotein receptor-1. Values are means ± SD or median (interquartile range).
[a] $P < 0.01$ versus controls.
[b] $P < 0.05$ versus controls.

TABLE 2. Glycemic control, serum AGEs, and sLOX-1 concentrations at baseline and 6 months

Characteristic	Baseline	6 Months
Male/female (n)	18/20	—
Age (years)	57.4 ± 9.6	—
Duration of diabetes (years)	9.0 ± 3.8	—
Smokers (%)	8.0	—
Body mass index (kg/m^2)	25.8 ± 3.9	25.3 ± 4.2
Systolic blood pressure (mmHg)	126 ± 15	122 ± 14
Diastolic blood pressure (mmHg)	79 ± 8	75 ± 9[a]
Total cholesterol (mmol/l)	4.81 ± 0.86	4.76 ± 1.04
Triglyceride (mmol/l)	1.20 (0.90–1.60)	1.10 (0.80–1.75)
LDL-cholesterol (mmol/l)	2.92 ± 0.81	2.80 ± 0.83
HDL-cholesterol (mmol/l)	1.29 ± 0.37	1.31 ± 0.44
Hemoglobin A1c (%)	9.1 ± 1.5	8.0 ± 0.8[a]
Fasting glucose (mmol/l)	9.2 ± 2.6	7.9 ± 1.4[a]
AGEs (U/ml)	5.54 ± 1.36	5.12 ± 1.30[a]
oxLDL (mU/l)	49.7 ± 15.7	45.7 ± 14.4[b]
sLOX-1 (ng/ml)	112.6 (79.5–132.5)	98.1 (71.7–116.1)[b]
CRP (mg/l)	1.06 (0.52–2.92)	1.05 (0.68–2.11)

Values are means ± SD or median (interquartile range).
[a] $P < 0.01$ versus baseline.
[b] $P < 0.05$ versus baseline.

At present, there are only scanty data on sLOX-1 in human disease states, and a recent study in human subjects has shown that serum sLOX-1 levels are elevated significantly in patients with acute coronary syndrome (18). We report for the first time that the serum sLOX-1 level is elevated in patients with type 2 diabetes mellitus and that there is a correlation between AGEs and sLOX-1. This is in keeping with the findings from both in vitro and animal studies showing that LOX-1 expression is increased by the hyperglycemic milieu (12, 13, 15). Moreover, we have confirmed that both glucose and AGEs can increase the level of sLOX-1 in conditioned medium of cultured endothelial cells. The effect of AGEs on LOX-1 and sLOX-1 was more evident than that of glucose in our experiments, and we only observed an increase in LOX-1 and sLOX-1 with extremely high glucose levels in vitro. Chen et al. (15) also reported a similar finding, in that the effect of AGE-BSA on LOX-1 expression was more marked than that of high glucose.

Type 2 diabetes is associated with increased oxidative stress and subclinical inflammation (23, 24). Plasma concentrations of oxLDL and markers of inflammation like CRP have been shown to be increased in type 2 diabetes mellitus (25, 26). Since LOX-1 expression can also be in-

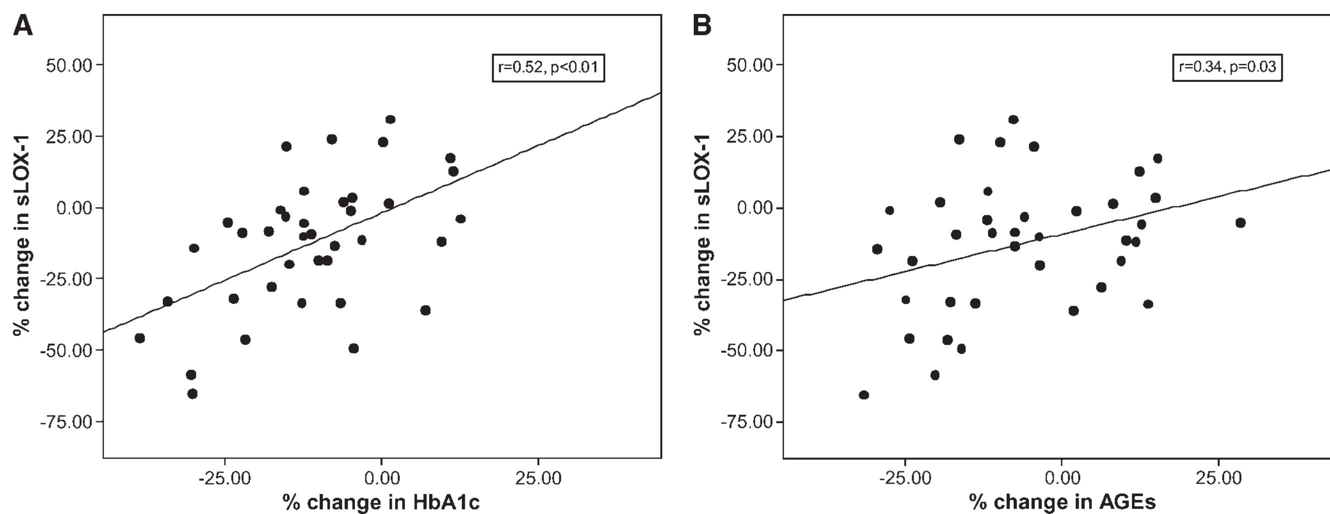

Fig. 3. Correlation between the magnitude of change in serum sLOX-1 with hemoglobin A1c (HbA1c; A) and AGEs (B).

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duced by factors like oxLDL, shear stress, inflammatory cytokines, and angiotensin II (3, 4, 11), glucose and AGEs may not be the main determinants of circulating sLOX-1 in patients with diabetes. In order to evaluate the major determinants of sLOX-1 levels in patients with type 2 diabetes and the contribution of glucose and AGEs in vivo, we proceeded to measure sLOX-1 in a group of poorly controlled type 2 diabetic subjects before and after improvement of their glycemic control. At the end of 6 months, HbA1c dropped form a mean of 9.1% to 8%, and this was accompanied by an ~13% reduction in serum sLOX-1 levels. Despite the improvement in glycemic control and a significant reduction in oxLDL, the plasma CRP level did not change. The degree of reduction in sLOX-1 was mainly associated with the magnitude of improvement in HbA1c and AGEs, and no association between the changes in sLOX-1 and oxLDL could be seen. Our results, therefore, suggest that both glucose and AGEs play an important role in the increase in sLOX-1 levels observed in poorly controlled type 2 diabetic patients in vivo.

The increase in sLOX-1 in diabetic patients suggests that LOX-1 expression was increased, and this might contribute to the development of endothelial dysfunction in diabetes (4). Although we cannot determine the site of origin of these soluble receptors in our study, increased LOX-1 expression in the vasculature has been shown in animal studies. In diabetic rats, LOX-1 expression was increased significantly in the aorta, and immunohistochemistry revealed that the most distinctive staining of LOX-1 was in the endothelial cells (15). In addition to endothelial cells, LOX-1 also is expressed in smooth muscle cells and monocytes/macrophages, and overexpression of LOX-1 in monocyte-derived macrophages in type 2 diabetic patients also has been described (13). The mechanism whereby LOX-1 is posttranslationally processed and secreted in response to cardiovascular damage is as yet unknown. Hayashida et al. (18) have shown that enhanced thrombin and matrix metalloproteinase activities are implicated in membrane LOX-1 cleavage to generate sLOX-1. The number of subjects with cardiovascular disease in our cross-sectional study was small, and we could not address the issue of whether sLOX-1 concentration is increased even further in diabetic subjects with cardiovascular disease. Additional studies are required to evaluate the potential of sLOX-1 as a vascular disease biomarker in humans.

In conclusion, the serum level of sLOX-1 is increased in patients with type 2 diabetes mellitus. Both glucose and AGEs are important determinants of LOX-1 expression in diabetes mellitus, and lowering glucose and AGEs by improving glycemic control can lead to a reduction in circulating sLOX-1.

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