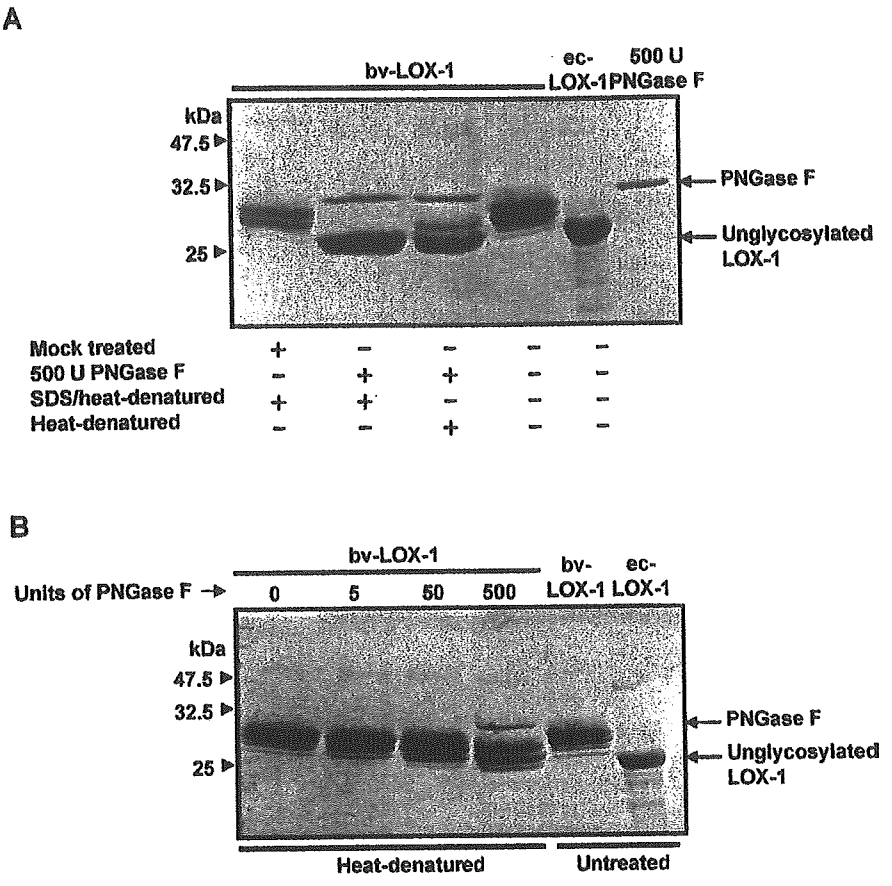

Figure 3 Glycosylation of bv-LOX-1

(**A**) Glycosylation of bv-LOX-1 was analysed using PNGase F. A 10 μg portion of SDS- and heat-denatured bv-LOX-1 and heat-denatured bv-LOX-1 were incubated for 16 h with 500 units of PNGase F, fractionated by SDS/12 % (w/v) PAGE and visualized using Coomassie Blue staining. Mock-treated SDS- and heat-denatured bv-LOX-1, untreated native bv-LOX-1 and ec-LOX-1 were also run as controls. (**B**) Samples (10 μg) of heat-denatured bv-LOX-1 were partially deglycosylated using different amounts of PNGase F (0–500 units) for 2 h and compared with untreated native bv-LOX-1 and ec-LOX-1. Samples were analysed by SDS/12 % (w/v) PAGE and visualised using Coomassie Blue staining. The predicted molecular mass of unglycosylated LOX-1 is 26 kDa.

mechanism of LOX-1 recognition of PS. Ca^{2+} titration in the phospholipid overlay assay showed a sigmoidal relationship, whereas control titration of Mg^{2+} (0–10 mM) showed little effect (Figure 5C). Bv-LOX-1 binding to PS was saturated in the presence of 10 mM Ca^{2+}; half-maximal binding was observed at 1.4 mM Ca^{2+} (Figure 5C).

LOX-1-mediated recognition of apoptotic cells is PS- and Ca^{2+}-dependent

Human LOX-1 is implicated in PS-mediated recognition of apoptotic bodies [12], and we thus tested the Ca^{2+}-dependence of this phenomenon using transfected HeLa cells expressing LOX-1-FLAG (Figure 6). Labelled annexin V detected external PS on apoptotic cells bound to HeLa cells expressing LOX-1-FLAG (Figure 6A). Apoptotic bodies were revealed by staining for both DNA and annexin V (Figure 6A). However, relatively small membrane fragments lacking DNA but derived from apoptotic cells also bound to both non-transfected and transfected HeLa cells in a non-specific manner (Figure 6A). To assess apoptotic-body binding to human LOX-1, the number of LOX-1-FLAG-expressing HeLa cells with one or more annexin V-positive apoptotic bodies containing DNA bound were counted under different incubation conditions (Figures 6B and 6C). The number of apoptotic bodies bound to each cell expressing LOX-1 ranged between 0 and 4. Binding of apoptotic bodies was decreased to 36 % (of control) in the presence of PS liposomes, but was not affected by PC liposomes, indicating PS-mediated recognition of apoptotic bodies (Figure 6B). Apoptotic-body binding to LOX-1-FLAG-expressing HeLa cells was decreased to 42 % (of control) in the presence of millimolar levels of EDTA (Figure 6C). Pre-incubation of LOX-1-FLAG-transfected cells with the JTX92 monoclonal antibody, which blocks LOX-1 binding to OxLDL [9,31,32], decreased apoptotic body binding to 32 % of control (Figure 6C). Pre-incubation with affinity-purified sheep anti-LOX-1 had a similar effect and reduced apoptotic body binding to 45 % (of control) (Figure 6C). We thus observed similar reductions in apoptotic-body binding in the presence of EDTA or LOX-1-specific antibodies, suggesting that Ca^{2+} is necessary for LOX-1-dependent binding to apoptotic bodies. Taken together, these data indicate that cellular LOX-1-mediated recognition of PS in biological membranes or particles is Ca^{2+}-dependent.

DISCUSSION

The present study shows that the LOX-1 scavenger receptor directly binds a key cellular phospholipid, PS, in a Ca^{2+}-dependent manner. This binding also occurs in biological membranes, as human cells expressing LOX-1 can recognize apoptotic bodies via

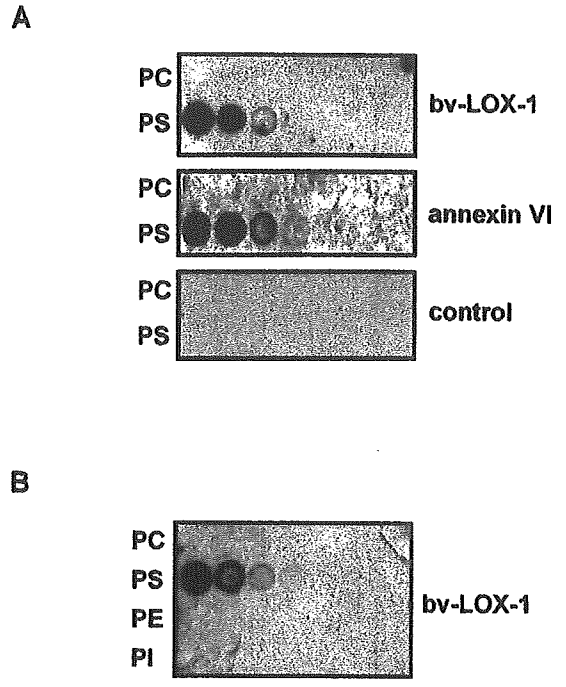

Figure 4 LOX-1 binding to phospholipids

(**A**) A protein lipid overlay assay was used to assess binding of bv-LOX-1 and control proteins to PC and PS. Serial dilutions (500, 250, 100, 50, 25, 10 and 5 pmol) of phospholipids were probed with 100 nM bv-LOX-1, 100 nM annexin VI or 100 nM control protein (His_6-tagged sTGN46), all in the presence of 2 mM Ca^{2+}. Bound protein was detected using affinity-purified antibodies, followed by species-specific HRP-conjugated antibodies and enhanced chemiluminescence. (**B**) Serial dilutions of PC, PS, PE and PI were probed with 100 nM bv-LOX-1 in the presence of 2 mM Ca^{2+}.

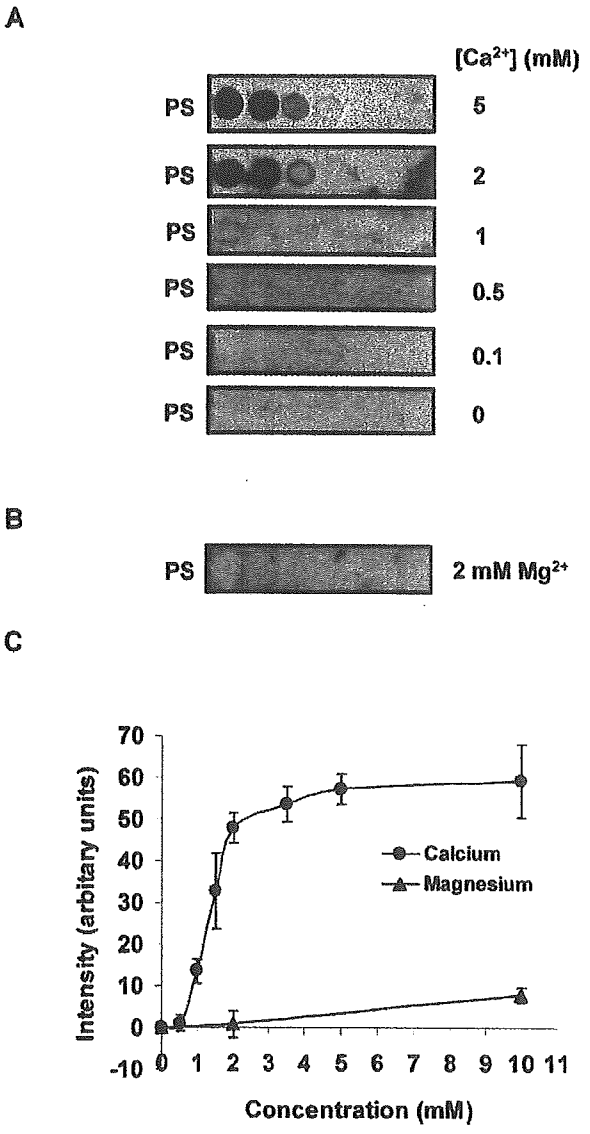

Figure 5 LOX-1 binds to PS in a Ca^{2+}-dependent manner

(**A**) Bv-LOX-1 (in the presence of 0–5 mM Ca^{2+}) was used to detect immobilized PS. Buffer containing no Ca^{2+} also contained 2 mM EDTA. (**B**) 100 nM bv-LOX-1 in the presence of 2 mM Mg^{2+} was tested for binding to immobilized PS. (**C**) Ca^{2+} and Mg^{2+} titration to quantify bv-LOX-1 binding to immobilized PS. Protein bound was quantified using densitometry. Results are means ± S.E.M. ($n = 3$) for three independent experiments.

LOX-1 in a PS- and Ca^{2+}-dependent mechanism. The recognition of PS by LOX-1 was maximal at physiological levels (> 1 mM) of Ca^{2+} ions, suggesting that native LOX-1 binds PS under extracellular conditions where millimolar Ca^{2+} levels are present. Bivalent Mg^{2+} was unable to substitute for Ca^{2+}, supporting the specificity for Ca^{2+} in ligand recognition.

How is PS bound by LOX-1? The C-type lectin-like domain of LOX-1 is the OxLDL-binding domain [16,21–24] and is highly homologous with the lectin-like domains of NK-cell receptors [16]. Our engineered recombinant, secreted, folded and N-glycosylated bv-LOX-1 protein, which lacks the cytoplasmic and transmembrane domains, contains both a 'neck' domain and the C-type lectin fold. Although it is possible the 80-residue 'neck' domain mediates PS binding rather than the 130-residue C-type lectin fold, the neck domain has no known homology with other proteins that mediate phospholipid recognition. It is more likely that the LOX-1 C-type lectin fold mediates PS recognition. Although the closely related NK cell receptors lack the Ca^{2+}-binding residues found in archetypal C-type lectins such as mannose-binding protein, it has been reported that the NK-cell receptor CD69 binds carbohydrate in a Ca^{2+}-dependent manner [33]. This, together with the fact that LOX-1 binds to PS in a Ca^{2+}-dependent manner, indicates that ligand recognition and Ca^{2+} binding to C-type lectins is more complex than predicted.

Our findings thus support a novel mechanism for LOX-1-mediated recognition of apoptotic bodies [12,13,34]. How is this linked with the well-characterized role for LOX-1 in high-affinity recognition of OxLDL? One possibility is that LOX-1 is a multifunctional sensor that recognizes both apoptotic bodies and OxLDL. The LOX-1 'neck' domain is unlikely to participate in ligand recognition, owing to its juxtaposition between the transmembrane domain (within the lipid bilayer) and the C-type lectin-like domain. Thus it is more likely that the LOX-1 C-type lectin fold binds to both PS and OxLDL; it has been shown that antibodies to this domain inhibit OxLDL recognition by LOX-1 [9,31,32]. It is well established that the oxidation or modification of LDL leading to formation of pro-atherogenic particles results in chemical changes on both the ApoB-100 (apolipoprotein B-100) protein moiety and phospholipids that form the particle [35,36]. Steric hindrance within a native LDL particle may prevent LOX-1-mediated recognition of PS moieties, thus contributing to a masking effect. However, oxidation of LDL particles could lead to a reorganization of ApoB-100 and phospholipid constituents, leading to presentation of hitherto 'masked' phospholipids to the aqueous medium, thus facilitating LOX-1-mediated recognition.

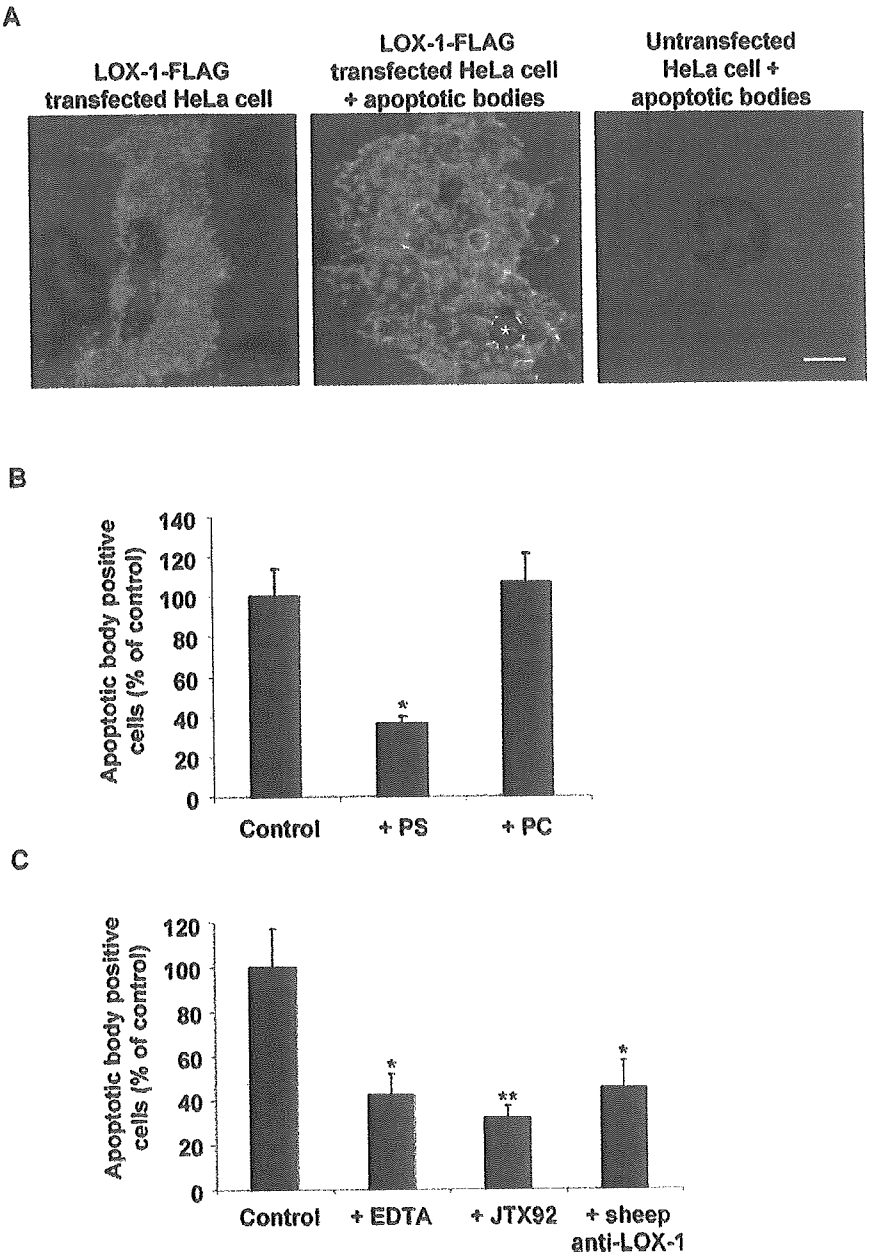

Figure 6 LOX-1-dependent binding to apoptotic bodies is Ca^{2+}-dependent

(**A**) Apoptotic-body binding to untransfected and LOX-1-FLAG transfected HeLa cells was analysed by immunofluorescence microscopy. Apoptotic bodies were incubated with untransfected or LOX-1-FLAG-transfected HeLa cells (plus Ca^{2+}) and exposed PS on bound apoptotic bodies was detected using AlexaFluor 594-labelled annexin V (red) prior to cell fixation. LOX-1-FLAG was detected using mouse anti-FLAG and secondary anti-mouse FITC conjugate (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole ('DAPI'; blue). The asterisk indicates a bound apoptotic body. Cell images represent projections of deconvolved three-dimensional stacks of 25–30 optical sections. The scale bar represents 10 μm. (**B**) HeLa cells expressing LOX-1-FLAG were pre-incubated with 100 μM PS or PC liposomes and assayed for apoptotic-body binding (in the presence of Ca^{2+}). The baseline control (100 %) is taken as apoptotic bodies bound to transfected cells in the absence of competing liposomes $\pm$ S.E.M. ($n = 3$ independent experiments). (**C**) HeLa cells expressing LOX-1-FLAG were assayed for apoptotic-body binding in the presence of EDTA (to complex bivalent cations), or the LOX-1-specific monoclonal antibody JTX92 (10 μg/ml) or purified sheep anti-LOX-1 (10 μg/ml). The baseline control (100 %) is taken as apoptotic bodies bound to transfected cells in the absence of competing EDTA or competing antibodies $\pm$ S.E.M. ($n = 6$ independent experiments). $^*P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

The PS phospholipid is known to be a constituent of the OxLDL particle [37,38], indicating that LOX-1-mediated recognition of PS-containing lipid particles is a likely possibility *in vivo*. LOX-1 bears no sequence similarity to annexins, indicating that PS binding is mechanistically different between these two proteins.

Is there a role for LOX-1-mediated recognition of apoptotic bodes *in vivo*? Programmed cell death is an essential feature of multicellular development in eukaryotes [39]. Apoptotic-body clearance is critical during body and tissue development in multicellular organisms. Macrophages and dendritic cells are key mediators of apoptotic-cell recognition and clearance. Interestingly, many of the mammalian scavenger receptors confer the ability to recognize apoptotic cells, especially in myeloid cells such as macrophages and monocytes [15]. The LOX-1 molecule is

found both on endothelial cells [1] and myeloid cells [4,40] raising the possibility that this receptor may also mediate recognition and clearance of apoptotic bodies by different cell types in mammals. The role of the endothelium in the recognition of apoptotic bodies has not been studied in depth, but this property appears to be conserved during mammalian evolution, since mouse, bovine and human endothelial cells all have this capacity [12,41–43]. It is clear that primary endothelial cells can bind apoptotic bodies in a LOX-1-dependent manner [12]. It is thus likely that LOX-1 is the receptor that mediates such endothelial recognition and clearance (phagocytosis) of apoptotic bodies.

In conclusion, our results show that the LOX-1 mammalian lectin that regulates vascular function can recognize a key cellular phospholipid, PS, *in vitro* on immobilized surfaces, but only in the presence of millimolar levels of Ca^{2+}. Human cells expressing LOX-1 also bind PS exposed on apoptotic bodies in a Ca^{2+}-dependent manner. Future studies will aim to map the site in LOX-1 that binds to Ca^{2+} and PS and determine how this can be reconciled with the structure of the extracellular domain.

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Hypoadiponectinemia is Associated With Coronary Artery Spasm in Men

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Background The relationship between adiponectin and coronary spastic angina (CSA), both of which are closely involved in coronary endothelial dysfunction, has not been elucidated.
Methods and Results Plasma adiponectin concentrations were examined in 55 men with CSA and 55 with chest pain syndrome (CPS). The plasma log-adiponectin levels were significantly lower in patients with CSA than with CPS (0.61±0.28 vs 0.80±0.21 μg/ml, p<0.0001). The prevalence of smoking was significantly higher in the CSA patients than in those with CPS (50.9% vs 29.1%, p=0.0195). In multiple logistic regression analysis, log-adiponectin (p=0.0008) and smoking (p=0.0210) were independent determinants of CSA.
Conclusions Hypoadiponectinemia is a potential risk factor for CSA in men, independent of smoking. (*Circ J* 2005; **69:** 1154–1156)

Key Words: Adiponectin; Coronary artery spasm; Smoking

Chronic coronary artery disease (CAD) is most commonly the result of obstruction of the coronary arteries by atheromatous plaques. However, coronary artery spasm, which is defined as an abnormal contraction of an epicardial coronary artery that results in myocardial ischemia, is also often observed and plays an important role in the pathogenesis of ischemic heart disease in Japan[1] Endothelial-derived nitric oxide (NO) is a key determinant of coronary artery spasm, and is synthesized by endothelial NO synthase (eNOS)[1]

Adiponectin has a protective action against the initiation and progression of atherosclerosis through its anti-inflammatory and anti-atherosclerotic effects[2,3] In cultured endothelial cells adiponectin also ameliorates the suppression of eNOS activity, leading to increased NO production[2] Patients with CAD have low levels of adiponectin[2] and clinically, low plasma levels of adiponectin have been also reported to be associated with vascular endothelial dysfunction in humans[4] However, the relationship between adiponectin and coronary artery spasm, both of which are closely involved in coronary endothelial dysfunction, has not been elucidated and so the hypothesis of the present study was that adiponectin may be associated with coronary spastic angina (CSA).

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Methods

We enrolled 110 Japanese men who underwent diagnostic cardiac catheterization at Kumamoto University Hospital (age: 63.6±9.9 years, mean±SD, range: 34–82 years). The group with CSA comprised 55 consecutive patients who fulfilled both inclusion criteria: (a) spontaneous anginal attacks associated with ST-segment elevation or depression on the ECG at rest; and (b) coronary artery spasm demonstrated angiographically by intracoronary infusion of acetylcholine (ACh)[5] The chest pain syndrome (CPS) group comprised 55 subjects in whom coronary artery spasm could not be provoked in any coronary artery by intracoronary infusion of ACh. The CPS group was matched for age and body mass index (BMI) with the CSA group. Incremental doses of ACh were infused into the left and right coronary arteries separately as described previously[5] Coronary artery spasm was defined as brief total or subtotal occlusion of the epicardial coronary arteries associated with chest pain and/or ischemic ST-segment changes on the electrocardiogram. Neither group had ≥25% organic coronary artery stenosis and none of the patients were taking any type of thiazolidinediones or calcium antagonists. Plasma adiponectin levels were determined by an enzyme-linked immunosorbent assay as described previously[6]

All data are expressed as mean±SD. Differences in frequencies were analyzed by the chi-square test. Differences in continuous variables between 2 groups were examined using an unpaired t-test. Because the distribution of the levels of plasma adiponectin, serum triglyceride, and C-reactive protein were skewed, logarithmically transformed values were used for statistical analysis. To examine the independent factors for CSA, multiple logistic regression analysis was performed including the variables that were significant in simple logistic regression analysis. A p-value <0.05 was considered statistically significant.

Table 1 Clinical and Biochemical Characteristics of the 2 Study Groups

	Coronary spastic angina (n=55)	Chest pain syndrome (n=55)	p value
Age, years	63.5±9.8	63.6±10.1	0.9847
Hypertension, n (%)	19 (34.5)	27 (49.1)	0.2461
Systolic blood pressure, mmHg	127±18	130±18	0.4137
Diastolic blood pressure, mmHg	79±10	77±11	0.4088
Pulse rate, beats/min	66±10	66±10	0.9123
Diabetes mellitus, n (%)	12 (21.8)	18 (32.7)	0.0868
Cigarette smoking, n (%)	28 (50.9)	16 (29.1)	0.0195
Fasting blood glucose, mg/dl	92.0±23.7	95.1±18.6	0.4491
HbA1c, %	5.5±1.3	5.9±1.1	0.0999
TC, mg/dl	189.4±31.2	192.4±33.6	0.7447
Log-TG, mg/dl	2.10±0.21	2.07±0.20	0.5576
HDL-C, mg/dl	48.8±13.7	55.7±20.2	0.0403
LDL-C, mg/dl	121.7±27.6	118.1±27.1	0.4998
Fibrinogen, mg/dl	324.7±75.7	308.7±63.6	0.2413
BMI, kg/m^2	23.8±2.96	23.8±3.08	0.9222
Creatinine, mg/dl	0.87±0.19	0.91±0.24	0.2396
Log-CRP, mg/dl	-1.10±0.35	-1.09±0.41	0.8974
Log-adiponectin, μg/ml	0.61±0.28	0.80±0.21	<0.0001
Family history of CAD, n (%)	14 (25.5)	10 (18.2)	0.4886

HbA1c, hemoglobin A1c; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; BMI, body mass index; CRP, C-reactive protein; CAD, coronary artery disease.

Table 2 Logistic Regression Analysis With Coronary Spastic Angina

Factor	Univariate			Multivariate		
	OR	95% CI	p value	OR	95% CI	p value
Age	1.000	0.962–1.038	0.9864			
Hypertension	0.547	0.254–1.179	0.1235			
Diabetes mellitus	0.574	0.245–1.346	0.2013			
Cigarette smoking	2.528	1.152–5.549	0.0208	2.757	1.165–6.524	0.0210
TC	0.998	0.986–1.010	0.7420			
Log-TG	1.761	0.270–11.48	0.5541			
HDL-C	0.976	0.953–1.000	0.0470	0.985	0.960–1.011	0.2555
LDL-C	1.005	0.991–1.019	0.4960			
Fibrinogen	1.003	0.998–1.009	0.2415			
BMI	1.006	0.888–1.140	0.9213			
Creatinine	0.340	0.055–2.081	0.2429			
Log-CRP	0.935	0.343–2.554	0.8962			
Family history of CAD	1.537	0.615–3.838	0.3576			
Log-adiponectin	0.032	0.005–0.204	0.0003	0.036	0.005–0.253	0.0008

OR, odds ratio; CI, confidence interval. All other abbreviations, see Table 1.

Results

Table 1 shows the clinical and biochemical characteristics of both groups. Plasma log-adiponectin levels were significantly lower in the CSA group compared with the CPS group (0.61±0.28 vs 0.80±0.21 μg/ml, p<0.0001). The prevalence of cigarette smoking was significantly higher in the CSA group than in the CPS group. High-density lipoprotein cholesterol (HDL-C) levels were significantly lower in the CSA group than in the CPS group.

Multiple logistic regression analysis revealed that hypoadiponectinemia is a predictive risk factor for coronary artery spasm, even after adjustment for cigarette smoking and HDL-C, which were significant in the simple logistic regression (Table 2).

Discussion

The plasma adiponectin level is considered to be a marker of atheromatous vascular changes[7] and smoking is a significant predictive risk factor for coronary artery spasm[8] but the relationship between adiponectin and coronary artery spasm has not been determined to date. In this study, we showed that plasma adiponectin levels were significantly lower in the CSA group than in the CPS group and that low levels of plasma adiponectin were closely related to the presence of CSA, independent of cigarette smoking.

Endothelial dysfunction together with reduced endothelial vasodilatory function and smooth muscle hypercontraction in coronary arteries may play an important role in the pathogenesis of coronary artery spasm[1] In particular, NO, which is recognized as an endothelium-derived relaxing factor, is deficient in patients with CSA[1] Cigarette smoking is considered to be a major risk factor for CSA[1] because the oxidative stress associated with cigarette smoking may be a source of free radicals, which may cause coronary artery spasm by reducing NO activity[1] On the other hand, a recent report indicates that adiponectin stimulates the production of NO in vascular endothelial cells[2] and furthermore, that hypoadiponectinemia is associated with impaired endothelium-dependent vasorelaxation, based on significantly re-

duced ACh-induced vasorelaxation in adiponectin-knockout mice compared with wild-type mice[4] In the present study, we demonstrated that adiponectin is a significant determinant of coronary artery spasm, irrespective of cigarette smoking, which suggests that the mechanism(s) by which hypoadiponectinemia impairs endothelium-dependent vasodilatation of the coronary arteries is different from that of cigarette smoking. Although the exact mechanism(s) of adiponectin-induced endothelium-dependent relaxation of coronary arteries is unknown, several potential mechanisms have been speculated. Hypoadiponectinemia is associated with insulin resistance and metabolic syndrome[9] and adiponectin may restore impaired endothelial vasomotor function through improvement of insulin resistance and its associated metabolic abnormalities. Adiponectin may also increase NO production in coronary vascular endothelial cells by activating adenosine monophosphate-activated protein kinase, which in turn promotes eNOS via activation of phosphatidylinositol 3-kinase-Akt-dependent pathways in endothelial cells![10]

The potential limitation of this study is that the subjects were all men, who have significantly lower plasma adiponectin concentrations than BMI-adjusted women[6,9] Further studies are warranted to evaluate the significance of plasma adiponectin concentrations in the prevalence of coronary artery spasm in women.

In conclusion, low adiponectin level is associated with CSA in men, independent of cigarette smoking, and hypoadiponectinemia is a potential important risk factor for CSA.

Acknowledgments

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Association of Hypoadiponectinemia With Smoking Habit in Men

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Abstract—Adiponectin is emerging as an important molecule in obesity, the metabolic syndrome, and cardiovascular disease. On the other hand, smoking habit is well known to be related to cardiovascular disease and hypertension. To examine the association between adiponectin concentration and smoking habit, we performed an epidemiological survey and an acute exposure test in humans and an experiment in adipocytes to elucidate the mechanism underlying the association between adiponectin and smoking. In the epidemiological study, we enrolled a total of 331 male subjects to examine chronic smoking exposure. Plasma adiponectin was significantly lower ($P=0.01$) in current smokers (5.3±0.3 μg/mL) than in never-smokers (6.5±0.4 μg/mL). A significant association between smoking and low adiponectin level was also confirmed in multiple regression analysis including age, body mass index, hypertension, diabetes, hyperlipidemia, and creatinine clearance (never-smokers 6.5±0.4 μg/mL; past smokers 5.6±0.3 μg/mL; current smokers 5.2±0.4 μg/mL; $F=4.52$; $P=0.01$). To examine the acute effect of smoking on adiponectin concentration for 12 hours, we measured plasma adiponectin level in 5 male never-smokers before smoking and 3, 6, and 12 hours after smoking, with the result that adiponectin showed a significant decrease after smoking (12 hours; −14.5±0.6%; $P<0.01$). In cultured mouse 3T3-L1 adipocytes, H_2O_2 and nicotine reduced the mRNA expression and secretion of adiponectin in a dose-dependent manner. Smoking habit is associated with adiponectin concentration in men, and its suppressive effect is mediated in part through direct inhibition of smoking on adiponectin expression in adipocytes. **(*Hypertension*. 2005;45:1094-1100.)**

Key Words: smoking ■ oxidative stress ■ risk factors ■ lipids ■ lipoprotein ■ metabolism

Cigarette smoking exacts a continuing toll on public health and is an established risk factor for hypertension and cardiovascular disease, and nonsmoking is a leading preventive strategy against coronary artery disease. Furthermore, cigarette smoking and its cessation are reported to alter lipid metabolism.[1–3] It is well established that smoking stimulates lipolysis in vivo. The lipolytic effect of smoking has been attributed to the nicotine component being mediated via release of catecholamines.[3] Nicotine, a major component of cigarette smoke, promotes inflammation[4] and progression of atherosclerotic lesions.[5,6] Furthermore, nicotine also has a direct effect on human adipose tissue.[7–9] On the other hand, oxidative stress has been shown to be a key phenomenon involved in the effects of smoking. Cigarette smoke contains a large amount of free radicals, which degrade NO released from the endothelium and also produce highly reactive intermediates, resulting in endothelial injury. Oxidative stress can damage many cell components, such as DNA, lipid membranes, and proteins, and lead to apoptosis and cell damage.[10,11]

Adiponectin, an adipose tissue–specific collagen-like factor, is abundantly present in plasma and possesses antiatherogenic properties. Adiponectin is emerging as an important molecule in obesity,[12] the metabolic syndrome,[13–15] cardiovascular disease,[16] lipid metabolism,[15] and hypertension.[17,18] In addition, adiponectin concentration is correlated independently with the vasodilator response to reactive hyperemia, and its concentration could be an independent parameter of endothelial function.[19] Endothelial dysfunction, an early marker of atherosclerosis, has been observed in chronic smokers as well as after acute cigarette smoking.[20,21] These results suggest that adiponectin may be a mediator between smoking and several diseases such as hypertension and coronary artery disease. Furthermore, smoking may directly regulate adiponectin concentration via lipolysis.

Although Miyazaki et al[22] reported that in subjects with coronary artery disease, smoking status was associated with reduced adiponectin concentration, using a small number of subjects, the association between plasma adiponectin and smoking status was evaluated without adjusting for con-

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founding factors and without consideration of the sex difference in adiponectin level.[23] Sex is an important confounding factor for evaluating adiponectin concentration, and the clinical importance of smoking habit in evaluating adiponectin concentration has not been fully elucidated. In the present study, we examined whether smoking habit is associated with a lower adiponectin level. First, we performed a cross-sectional study using a large number of subjects, including only males, to examine the chronic effect of smoking. Second, we performed an acute smoking exposure test in never-smokers and evaluated the effect for 12 hours. Finally, we demonstrated an inhibitory effect of H_2O_2 and nicotine on the expression and secretion of adiponectin in vitro.

Methods

Epidemiological Study (Chronic Effect of Smoking)

A total of 331 male subjects were selected from patients who were admitted and underwent medical investigation including a general check-up at Osaka University Hospital, Japan. All subjects enrolled in this study were Japanese. The study protocol was approved by the ethical committee of Osaka University, and all subjects gave written informed consent to participate in the study. All procedures followed were in accordance with the institutional guidelines of Osaka University. Smoking status was determined by interview on the day of measuring clinical parameters, and the subjects were divided into 3 groups according to smoking habit: never-smokers, past smokers (who had a history of habitual smoking but had quit), and current smokers. As a result, the numbers of never-smokers, past smokers, and current smokers were 79, 136, and 116, respectively. Hypertension was defined as systolic blood pressure (BP) of ≥140 mm Hg or diastolic BP of ≥90 mm Hg on repeated measurements, or receiving antihypertensive treatment. Diabetes mellitus was defined according to World Health Organization criteria.[24] Hyperlipidemia was defined as total cholesterol (T-chol) of >6.22 mmol/L, triglyceride (TG) of >2.26 mmol/L, or HDL cholesterol (HDL-chol) of <0.91 mmol/L. Ischemic heart disease was defined as a ≥75% organic stenosis of ≥1 major coronary artery, as confirmed by coronary angiography or a history of myocardial infarction or percutaneous transluminal coronary angioplasty. Renal failure was defined as fasting serum creatinine (Cr) concentration >176.8 μmol/L. Subjects with ischemic heart disease, chronic renal failure, nephrotic syndrome, overt congestive heart failure, valvular heart disease, secondary hypertension, or atrial fibrillation were excluded. Furthermore, no subjects receiving steroid therapy were included in this study.

Each subject was studied on the day after admission, in the morning after having abstained from alcohol, caffeine, and smoking, as well as food for 8 hours before the study. BP was measured by well-trained physicians, and venous blood was drawn from all subjects. Height and body weight were measured and body mass index (BMI) calculated. Plasma samples for subsequent assay were stored at −80°C. Insulin sensitivity was estimated using the homeostatic model assessment (HOMA) index (ie, plasma glucose level×(plasma insulin level/22.5). Brinkman index was calculated using the formula: number of cigarettes smoked per day×number of years of smoking. Plasma concentration of adiponectin was determined using a sandwich ELISA system (Adiponectin ELISA kit; Otsuka Pharmaceutical Co. Ltd.), as reported previously.[12] The parameters T-chol, TG, HDL-chol, and Cr levels were also determined. Urine samples were collected for 24 hours to evaluate Cr clearance (Ccr).

Acute Smoking Exposure Test

To examine the acute effect of smoking on adiponectin concentration, we measured plasma adiponectin level in 5 healthy volunteers who had never smoked (age 33 to 46 years; BMI 24.0±1.0 kg/m^2). All subjects were male and were coauthors included in this study, and the exclusion criteria of this study were the same as those described previously. After completion of the baseline study, all participants were asked to smoke a cigarette (1.1 mg nicotine; 14 mg tar) and were instructed to inhale. Before and 3, 6, and 12 hours after smoking, venous blood was drawn.

Effect of H_2O_2 and Nicotine on Expression and Secretion of Adiponectin In Vitro

3T3-L1 mouse preadipocytes were grown to confluence and induced to differentiate into adipocytes, as described previously.[25] Seven days after the initiation of differentiation (assessed by this criterion), 85% to 90% of the cells were judged to be differentiated. On day 7, the indicated concentrations of H_2O_2 with/without *N*-acetyl-L-cysteine (NAC) or nicotine (Sigma) were added to the media for 24 hours.

An aliquot of the media after 24 hours of stimulation was subjected to ELISA (Adiponectin ELISA kit; Otsuka Pharmaceutical Co. Ltd.) to detect the amount of adiponectin secreted.

Loss of 3T3-L1 adipocyte integrity was evaluated spectrophotometrically by measurement of lactate dehydrogenase (LDH) activity in the supernatant using a standard kit (LDH-Cytotoxic Test; Wako).

3T3-L1 adipocyte cellular protein samples were isolated using ISOGEN (Nippon Gene) according to manufacturer protocol. Adipocyte protein concentration was determined by colorimetric protein assay (detergent solubilization) using DC Protein Assay (Bio-Rad) according to manufacturer protocol. The relative secretion of adiponectin into the media was normalized to the amount of cellular protein in the same sample.

Total RNA from adipocytes was isolated using ISOGEN, treated with DNAse to prevent contamination with genomic DNA, and finally resuspended in diethylpyrocarbonate-treated MilliQ. Expression levels of adiponectin and 18S mRNA were quantified by real-time quantitative RT-PCR using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Inc.) according to manufacturer instructions. TaqMan probes and primers for adiponectin and 18S were Assay-on-Demand gene expression products (Applied Biosystems, Inc.). We used amplification of 18S ribosomal RNA in each of the stimulated conditions for sample normalization. The relative expression of adiponectin mRNA was normalized to the amount of 18S in the same mRNA sample using the standard curve method described by the manufacturer.

Statistical Analysis

Means or proportions of clinical characteristics and cardiovascular risk factors were computed for each smoking pattern. Continuous variables were expressed as mean±SEM. Differences between smoking status groups for variables including adiponectin concentration were analyzed by 1-way ANOVA and post hoc comparison (Dunnet's procedure). Unpaired *t* test was used to examine the differences in adiponectin between 2 groups. Pearson's correlation coefficients were used to assess the relationships between adiponectin and all other variables. Multiple regression models were used to assess the relationship between adiponectin concentration and smoking status after adjustment for potential confounding factors. The significance of differences in adiponectin levels before and after smoking was evaluated using repeated-measures ANOVA. In the in vitro study, differences were analyzed by unpaired *t* test. All *P* values were 2-sided, and those <0.05 were considered statistically significant. All calculations were performed using a standard statistical package (JMP 4.0; SAS Institute).

Results

Association of Plasma Adiponectin Concentration With Smoking Habit in Humans

The clinical and biochemical characteristics of the study subjects divided into 3 groups according to smoking habit are shown in Table 1. We first examined the association between smoking habit and adiponectin concentration. The concentra-

TABLE 1. Clinical Characteristics of Study Subjects

Variables	Never-Smokers	Past Smokers	Current Smokers
n	79	136	116
Brinkman index	0±0	792±53	742±58
Age, years	58.0±1.2	62.2±0.9*	57.5±1.0
BMI	23.6±0.3	23.7±0.3	23.2±0.3
Adiponectin, μg/mL	6.5±0.4	5.7±0.3	5.3±0.3*
Systolic BP, mm Hg	130±2	134±1	133±2
Diastolic BP, mm Hg	80±1	81±1	85±1*
Hypertension, %	66.7	71.0	73.9
Diabetes, %	10.3	15.9	20.0
Hyperlipidemia, %	27.9	30.0	38.0
T-chol, mmol/L	4.99±0.09	5.18±0.08	5.26±0.10*
TG, mmol/L	1.48±0.12	1.78±0.09	1.64±0.11
HDL-chol, mmol/L	1.48±0.05	1.45±0.04	1.41±0.04
HOMA index	1.7±0.3	2.0±0.3	2.1±0.4
Cr, μmol/L	82.0±2.5	80.6±1.8	76.3±2.2
Ccr, mL/min	85.7±3.7	82.4±2.6	83.5±3.2

Values are given as mean±SEM.
*$P<0.05$ compared with never-smokers for each parameter.

TABLE 2. Clinical Characteristics of Subgroups Without Medication and Diabetes

Variables	Never-Smokers	Past Smokers	Current Smokers
n	27	41	30
Brinkman index	0±0	850±94	554±74
Age, years	58.8±2.5	62.0±2.1	60.1±2.5
BMI	22.6±0.5	22.3±0.4	21.8±0.3
Adiponectin, μg/mL	8.3±0.8	7.1±0.6	6.1±0.7*
Systolic BP, mm Hg	117±4	125±3	128±4
Diastolic BP, mm Hg	74±3	76±2	79±3
Hypertension, %	14.8	17.1	16.1
Hyperlipidemia, %	31.8	29.4	34.8
T-chol, mmol/L	4.99±0.15	5.14±0.15	4.90±0.16
TG, mmol/L	1.49±0.20	1.48±0.18	1.64±0.22
HDL-chol, mmol/L	1.55±0.10	1.53±0.09	1.62±0.11
HOMA index	1.1±0.4	1.4±0.3	1.5±0.7
Cr, μmol/L	72.9±6.7	75.4±4.7	76.6±7.8
Ccr, mL/min	84.0±5.4	80.8±4.1	83.0±5.4

Values are given as mean±SEM.
*$P<0.05$ compared with never-smokers for each parameter.

tion of adiponectin was significantly lower in current smokers than in never-smokers ($P=0.01$). Furthermore, the concentration of adiponectin showed a tendency to be lower in past smokers than in never-smokers ($P=0.06$). Diastolic BP and T-chol in current smokers and age in past smokers were significantly higher than those in never-smokers ($P<0.05$). In addition, the kinds of drugs that influence adiponectin concentration, such as angiotensin II receptor blockers, angiotensin-converting enzyme (ACE) inhibitors, and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands, were not significantly different among the smoking status.

In the total subjects, adiponectin level was significantly associated with age ($r=0.38$; $P<0.01$), BMI ($r=-0.33$; $P<0.01$), and Ccr ($r=-0.36$; $P<0.01$). Furthermore, adiponectin level was significantly lower in patients with hypertension (5.1±0.2 versus 7.3±0.3 μg/mL; $P<0.01$), diabetes (5.0±0.2 versus 6.2±0.3 μg/mL; $P<0.01$), and hyperlipidemia (4.5±0.3 versus 5.8±0.2 μg/mL; $P<0.01$). We next performed multiple regression analysis including age, BMI, hypertension, diabetes, hyperlipidemia, and Ccr and revealed that adiponectin concentration in never-smokers was ≈1.25× higher than that in current smokers (never-smokers 6.5±0.4 μg/mL; past smokers 5.6±0.3 μg/mL; current smokers 5.2±0.4 μg/mL; $F=4.52$; $P=0.01$).

To exclude the effect of diabetes and drugs on adiponectin concentration, we next examined the effect of smoking habit on adiponectin concentration after excluding subjects with diabetes and subjects receiving any medication. The clinical and biochemical characteristics of these study subjects are shown in Table 2. Adiponectin concentration significantly increased with age ($r=0.41$; $P<0.01$) and HDL-chol ($r=0.43$; $P<0.01$) and decreased with BMI ($r=-0.50$; $P<0.01$), systolic BP ($r=-0.35$; $P<0.01$), diastolic BP ($r=-0.36$; $P<0.01$), TG ($r=-0.30$; $P<0.05$), HOMA ($r=-0.29$; $P<0.05$), and Ccr ($r=-0.41$; $P<0.01$). On the other hand, there was no significant association between adiponectin and T-chol ($r=-0.04$). Although clinical variables other than adiponectin concentration were not significantly different, adiponectin concentration was significantly lower in current smokers than in never-smokers ($P=0.04$).

Brinkman index was not associated with adiponectin concentration in the total subjects ($r=-0.05$) or in subjects without medication or diabetes ($r=-0.19$). However, in current smokers (n=116), the number of cigarettes smoked per day was inversely associated with adiponectin concentration ($r=-0.21$; $P<0.04$).

Effect of Acute Smoking Exposure on Plasma Adiponectin Concentration

The mean adiponectin level before smoking was 7.0±1.5 μg/mL. Percent changes in plasma concentration of adiponectin in response to smoking are shown in Figure 1. Acute smoking exposure produced a significant decrease in plasma level of adiponectin at 3 hours (−9.2±0.7%) and 6 hours (−13.1±1.2%), and the maximum decrease was observed at 12 hours after smoking (−14.5±0.6%; $F=17.3$; $P<0.01$).

Inhibitory Effects of H_2O_2 and Nicotine on Expression and Secretion of Adiponectin in 3T3-L1 Adipocytes

We investigated the effect of H_2O_2 and nicotine on the regulation of adiponectin secretion and gene expression in 3T3-L1 adipocytes. Incubation with H_2O_2 or nicotine reduced adiponectin mRNA expression and adiponectin secretion into the media in a dose-dependent manner (Figures 2 and 3). The effects of H_2O_2 to reduce adiponectin mRNA expression and secretion into the media were antagonized by coincubation with NAC (Figure 2). Secretion of adiponectin into the media was significantly reduced compared with control by nicotine

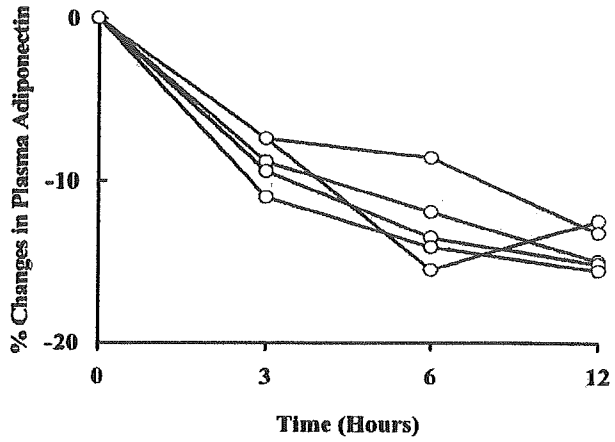

Figure 1. Percent changes in plasma adiponectin levels before and after smoking. Individual changes in adiponectin level were plotted. Adiponectin levels were expressed as percent change from initial values (n=5).

at concentrations $\geq 10^{-8}$ mol/L. We next studied the adipocyte protein concentration; the amount of adiponectin in the media was adjusted by each of the amount of cellular protein. As shown in Figures 2B and 3B, even after adjustment for protein amount, adiponectin secretion was significantly reduced by incubation with H_2O_2 or nicotine in a dose-dependent manner.

Cytotoxicity was also assessed by LDH leakage from adipocytes into the media. As shown in Figure 2C, H_2O_2 (100 μmol/L) significantly increased LDH release from adipocytes. When cultured in the presence of NAC (10^{-2} M), this increase was significantly attenuated. On the other hand, as shown in Figure 3C, treatment with nicotine also significantly increased leakage of LDH from adipocytes at concentrations $\geq 10^{-7}$ mol/L.

Discussion

The present study demonstrated that the plasma adiponectin concentration was significantly lower in male subjects who were current smokers than in never-smokers, and the association was observed even in subjects without diabetes and medication. Furthermore, multiple regression analysis including age, BMI, hypertension, diabetes, hyperlipidemia, and Ccr showed that adiponectin concentration was significantly lower in current smokers. Acute smoking exposure reduced adiponectin concentration significantly at 12 hours after smoking in never-smokers. In cultured 3T3-L1 adipocytes, oxidative stress and nicotine reduced the secretion and expression of adiponectin. These results suggest that smoking may decrease plasma adiponectin concentration in men.

In this study, even in subjects without diabetes and medication, the association between adiponectin concentration and clinical variables was in accordance with previous reports that adiponectin concentration was significantly associated with age,[18,26] BMI,[12] TG,[13] HDL-chol,[27] BP,[18] and insulin resistance indicated by HOMA.[14]

Although adiponectin concentration is decreased in several diseases,[12-14,16,18] the mechanisms that regulate plasma adiponectin concentration have not been fully elucidated. It has been reported that weight reduction[13] and certain drugs such as PPAR-γ ligands,[25] ACE inhibitors, and angiotensin II receptor blockers[28] increased the adiponectin concentration, a cytokine, tumor necrosis factor-α (TNF-α), reduced the expression of adiponectin in adipocytes,[25] and some human mutations of adiponectin affect plasma adiponectin concentration.[18,29] In this study, we demonstrated that smoking habit is also associated with adiponectin concentration. Furthermore, our finding of lower adiponectin levels in chronic smokers is in line with the fact that chronic smokers are insulin resistant.[30] Thus, our results may support investigation of the mechanisms of several disorders induced by smoking.

Smoking is known to be associated with increased oxidative stress. Reactive oxygen species such as H_2O_2 are also normally produced during cellular oxidation reduction processes. Although our results showed significant cytotoxicity in adipocytes incubated with H_2O_2 at a concentration of 100 μmol/L, this cytotoxicity was significantly attenuated when they were cultured with NAC. Furthermore, H_2O_2 decreased the expression and secretion of adiponectin from adipocytes in a dose-dependent manner. Previous reports have shown that oxidative stress disrupts activation of phosphatidylinositol 3-kinase (PI3K),[31,32] which is a key molecule in the secretion of adiponectin in 3T3-L1 adipocytes.[33] Thus, we propose the idea that oxidative stress induced by tobacco smoke decreases the secretion and expression of plasma adiponectin via inhibition of activation of PI3K in adipocytes.

Nicotine activates nicotinic acetylcholine (nACh) receptors, which belong to the family of ionotropic receptors consisting of 5 transmembrane subunits building up ion channels. nACh receptors are widely distributed throughout the central and peripheral nervous system and are involved in signal transmission at the skeletal neuromuscular junction, in autonomic ganglia, and in the brain.[34,35] Functional nACh receptors are expressed in adipocytes in mice,[36] and nicotine exerts direct stimulation of lipolysis via nACh receptors in human adipose tissue.[7-9] Thus, nicotine has the possibility of regulating adiponectin concentration directly. In our experiments, nicotine had a significant inhibitory effect at concentrations $\geq 10^{-7}$ mol/L, which can be found in the plasma of smokers.[37] Furthermore, our results also showed significant cytotoxicity in adipocytes incubated with nicotine at a concentration of 10^{-6} mol/L. These results could also be in accordance with previous reports that nicotine itself induces lipolysis by activating local nicotinic cholinergic receptors in adipose tissue.[7] Thus, our results indicate that nicotine in tobacco smoke decreases plasma adiponectin via inhibition of the secretion and expression of adiponectin in adipocytes.

Apart from nicotine and oxidative stress, there are several other possible mechanisms by which smoking habit may affect adiponectin concentration. It has been reported that smoking itself and tissue hypoxia elevate TNF-α,[38,39] a powerful proinflammatory cytokine and a mediator of inflammation, which is known to decrease adiponectin concentration.[25] These findings also support the idea that persistent production of TNF-α induced by chronic exposure to cigarette smoke may promote the development of hypoadiponectinemia. Furthermore, nicotine elicits release of the catecholamines epinephrine and norepinephrine,[40] and

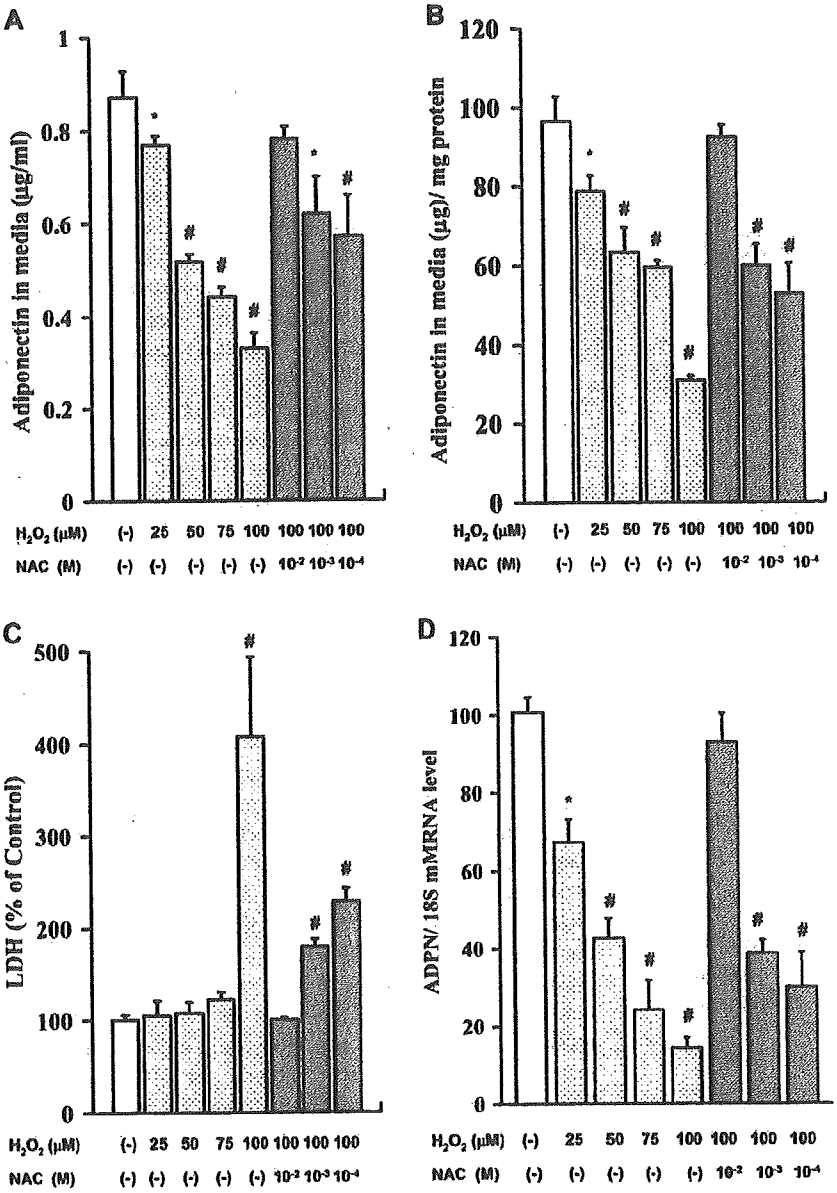

Figure 2. Effects of H_2O_2 on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of H_2O_2 with/without NAC on adiponectin secreted into media (A), adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent change relative to the level with 0 μmol/L H_2O_2 treatment. Values are given as mean±SEM (n=12 in each group). *P<0.05 and #P<0.01 compared with 0 μmol/L H_2O_2 treatment for each variable.

β-adrenergic stimulation suppresses adiponectin gene expression.[41]

With respect to cessation of habitual smoking, in this study, adiponectin level was between those of nonsmokers and current smokers, even after adjustment for confounding factors. These results suggest that the decreasing effect of smoking on adiponectin concentration might remain even after smoking cessation. Another reason is that even after smoking cessation, smoking-related damage persisted, such as endothelial dysfunction and continuing low-grade inflammation indicated by C-reactive protein,[42] which is known to affect adiponectin concentration.[19,43] To clearly confirm whether smoking cessation affects adiponectin concentration, a cohort study is required.

Because tobacco smoke consists of >4000 chemical constituents, it is impossible to predict the effect of nicotine and oxidative stress within this complex mixture of components. Although we showed that nicotine and oxidative stress have a potent inhibitory effect on adiponectin secretion, there are several other molecules in cigarette smoke that may be toxic to adipocytes (eg, cadmium, cotinine, and thiocyanate).[44] The net effect of cigarette smoke on the function of adiponectin may be quite different from that of nicotine or H_2O_2 alone. Another limitation is that this study was designed as a cross-sectional study rather than a randomized clinical trial or observational study. Furthermore, several important determinants of adiponectin level, such as body fat content and waist circumference, were not measured in our study. Instead of these measurements, we included HOMA and BMI in the analysis of this study. Previous reports have shown that body fat content, especially intra-abdominal fat, is a determinant of adiponectin level.[26] On the other hand, the different localization of fat mass itself influences cardiovascular risk factors such as T-chol, TG, and HDL-chol.[45] In our study, except for T-chol, the clinical characteristics were not significantly different among subjects (Table 1). Furthermore, the subjects

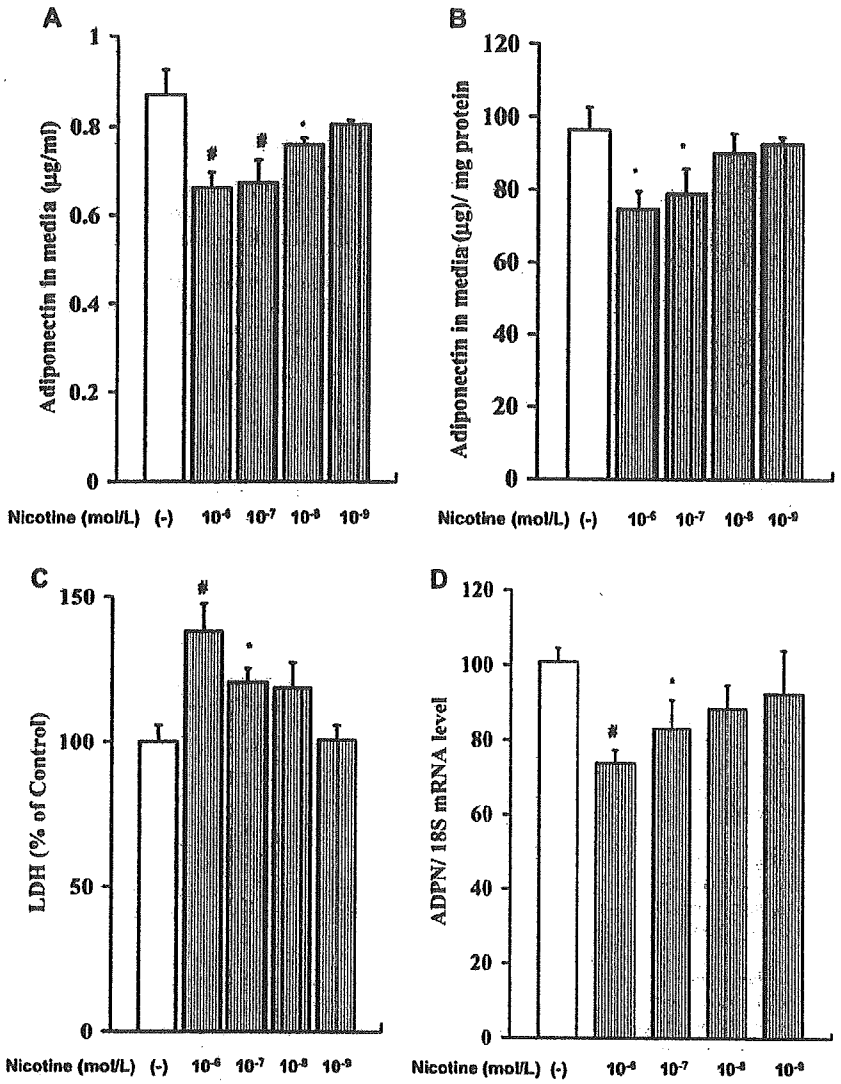

Figure 3. Effects of nicotine on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of nicotine on adiponectin secreted into media (A), adiponectin adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent change relative to the level with 0 mol/L nicotine treatment. Values are given as mean±SEM (n=12 in each group). *$P<0.05$ and #$P<0.01$ compared with 0 mol/L nicotine treatment for each variable.

included in this study were relatively lean, and obesity (BMI $\geq$30 kg/m^2) was present in only 2.5% of the total subjects. Thus, the effect of different fat distributions on adiponectin concentration among the groups may be relatively small in this study. On the other hand, our study could not provide a conclusion on the influence of "passive smoking" on adiponectin concentration. Further investigation is required to examine these effects.

In conclusion, our results demonstrated that smoking habit is associated with a lower adiponectin concentration in men. This reduction may be induced through a direct effect of oxidative stress and nicotine on adipocytes.

Acknowledgments

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AJH 2005; 18:949–957

Pioglitazone Improves Left Ventricular Diastolic Function in Patients With Essential Hypertension

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Background: Left ventricular (LV) hypertrophy and diastolic dysfunction, which are common cardiac consequences of hypertension, are modified by insulin resistance. The present study assessed the hypothesis that primary treatment of insulin resistance may reverse such cardiac changes in hypertensive patients.

Methods: A total of 30 patients with essential hypertension were enrolled in this study. In echocardiographic examinations, LV mass index, the peak velocity ratio of early diastolic to atrial filling (E/A), and the E-wave deceleration time (DcT) were determined. Insulin sensitivity test with steady-state plasma glucose (SSPG) method, oral glucose tolerance test, and blood samplings for measurement of adiponectin and matrix metalloproteinase (MMP)–2 were also performed. Six months after treatment with pioglitazone (30 mg/day), an insulin sensitizer, these examinations were repeated.

Results: Pioglitazone significantly increased E/A and decreased DcT, without a change in LV mass index. These improvements in diastolic properties were much greater in subjects with a marked (≧3.3 mmol/L) decrease in SSPG (n = 11) than the others (n = 19), although the decrease in glucose levels did not differ between the two groups. In addition, the changes in E/A and DcT were closely correlated with the decrease in SSPG. Pioglitazone treatment significantly elevated plasma adiponectin and MMP-2 levels, and the increase in MMP-2 was positively correlated with the increase in adiponectin.

Conclusions: The present findings demonstrate that pioglitazone improves LV diastolic function without LV mass regression in hypertensive patients in proportion to the amelioration of insulin resistance. These findings suggest that increased adiponectin and MMP may be involved in the beneficial effect of pioglitazone on diastolic function. Am J Hypertens 2005;18:949–957 © 2005 American Journal of Hypertension, Ltd.

Key Words: Ventricular function, diastole, insulin resistance, extracellular matrix, hypertension.

Left ventricular (LV) hypertrophy and LV diastolic dysfunction, which are common cardiac consequences of hypertension, are both independent risk factors for cardiovascular morbidity and mortality. Although LV hypertrophy is primarily considered an adaptation to the increased afterload, this structural adaptation of LV in hypertension is modified by several nonhemodynamic factors including metabolic factor.[1] Left ventricular diastolic dysfunction (abnormal relaxation) in hypertensive patients may result mainly from an increase in afterload (systemic arterial pressure) and LV hypertrophy. However, diastolic dysfunction is also affected by factors other than blood pressure (BP) and cardiac hypertrophy, especially by metabolic factors. We have shown that the presence of glucose intolerance and dyslipidemia (low high-density lipoprotein cholesterol and high triglycerides), which are components of the metabolic syndrome (insulin resistance syndrome), accelerates LV diastolic dysfunction in patients with treated essential hypertension.[2,3]

Thus, insulin resistance may unfavorably modify cardiac structure and function in hypertensive patients. In

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fact, there have been several reports that insulin resistance itself is related to LV hypertrophy and diastolic dysfunction in nondiabetic subjects with essential hypertension.[4–7] Furthermore, experimental studies have shown that thiazolidinediones, insulin-sensitizing and antidiabetic agents, inhibit cardiac hypertrophy and improve diastolic function through activation of peroxisome proliferator-activated receptor–γ (PPAR-γ).[8–12] However, despite the growing evidence in animal models, the beneficial cardiac effect of thiazolidinediones has not been clinically demonstrated in hypertensive subjects. To clarify whether cardiac structural and functional changes in hypertension can be improved by primary treatment of insulin resistance, the present study investigated the effect of one of the thiazolidinediones, pioglitazone, on LV hypertrophy and diastolic function in patients with essential hypertension.

Methods

Patients

A total of 30 Japanese outpatients with essential hypertension (11 men and 19 women; mean age, 66 ± 7 years) were enrolled in the present study. Patients with secondary hypertension, coronary heart disease, valvular heart disease, atrial fibrillation, congestive heart failure, or renal insufficiency (serum creatinine ≧106 μmol/L =1.2 mg/dL) were excluded from this study. Individuals with overt diabetes mellitus, ie, patients who were receiving hypoglycemic medication or whose fasting plasma glucose was ≧7.8 mmol/L (140 mg/dL) or hemoglobin A_{1c} (HbA_{1c}) was ≧7.0%, were also excluded from the study. All 30 subjects were receiving antihypertensive drugs, including combination therapy. Of these, 29 patients (97%) were treated with calcium channel blockers, 11 (37%) with β-blockers, 10 (33%) with renin-angiotensin system inhibitors, six (20%) with diuretics, and five (17%) with α_1-blockers, and 12 patients (40%) were receiving lipid-lowering drugs. All subjects gave their informed consent to participate in the present study. All procedures of the present study were carried out in accordance with institutional and national ethical guidelines for human study.

Study Protocol

Using a mercury sphygmomanometer, systolic and diastolic BP were measured by detecting the Korotkoff phase I and V sounds, respectively. The mean of three BP and heart rate measurements obtained on three different occasions with the subjects in a sitting position was calculated. Echocardiographic examinations, oral glucose tolerance test (OGTT), insulin sensitivity test, and blood samplings to determine fasting plasma glucose, HbA_{1c}, and serum levels of insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were also carried out. After these examinations, each patient received 30 mg of oral pioglitazone once daily in the morning for 6 months. The kinds and doses of antihypertensive and lipid-lowering drugs were unchanged during the study period. Except mild leg edema, no adverse effects of pioglitazone such as hypoglycemia, liver dysfunction, or heart failure were observed. After 6 months of treatment with pioglitazone, all examinations including echocardiography, insulin sensitivity test, and OGTT were repeated in all subjects.

Echocardiographic Measurement

Comprehensive two-dimensional echocardiography was performed using a cardiac ultrasound unit (Sonos 5500; Hewlett Packard, Andover, MA) as previously described.[2,3] Echocardiographic parameters were measured by the consensus of two experienced investigators who were blind to the metabolic data of the subjects. Measurements included inferior vena cava, left atrial dimension, interventricular septal thickness (IVSTd), posterior wall thickness (PWTd), LV diameter at end-diastole (LVDd), and LV diameter at end-systole (LVDs). Fractional shortening was calculated as (LVDd-LVDs)/LVDd. The LV relative wall thickness was calculated as (IVSTd+PWTd)/LVDd. The LV mass was estimated using the formula validated by Devereux and Reichek[13]: LV mass (g) = $1.04 \times [(IVSTd+PWTd+LVDd)^3-LVDd^3] - 13.6$. The LV mass was normalized for body surface area and expressed as LV mass index.

To assess LV diastolic function, the diastolic filling of LV (LV inflow) was examined using Doppler echocardiography.[2,3] The peak velocity of early diastolic filling (E) and the peak velocity of atrial filling (A) were recorded and the E to A ratio (E/A) was calculated. The deceleration time of the E wave (DcT) and the duration of the A wave (Ad) were also measured. After LV inflow velocities were examined, pulmonary venous flow velocities were obtained as previously described.[14] The peak forward flow velocities during ventricular systole (S) and diastole (D) and the peak reverse flow velocity at atrial contraction (PVa) were measured, and the S to D ratio (S/D) was calculated. The duration of the PVa wave (PVad) was also measured and the difference between the duration of the mitral A wave and the pulmonary reversal wave (Ad-PVad) was calculated.

Insulin Sensitivity Test

Glucose use in response to insulin was evaluated by a modified steady-state plasma glucose (SSPG) method after an overnight fast of at least 12 h, as previously reported.[15] The glucose value at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose use. High SSPG levels were taken to indicate peripheral insulin resistance.

Determinations of OGTT

A standard 75-g OGTT was performed after an overnight fast, and plasma glucose and serum insulin concentrations were determined at 0, 30, 60, and 120 min. The areas under the curve (AUC) of plasma glucose and serum insulin for 120 min (AUC for glucose and insulin) were calculated.

Table 1. Changes in clinical parameters before and after pioglitazone treatment

Characteristic	Before	After
Age (years)	66 ± 7	
Sex (male/female)	11/19	
Duration of hypertension (y)	20 ± 9	
Body weight (kg)	61.5 ± 9.6	62.8 ± 9.7*
Body mass index (kg/m^2)	25.0 ± 3.6	25.5 ± 3.6*
Systolic BP (mm Hg)	142 ± 9	142 ± 8
Diastolic BP (mm Hg)	79 ± 6	77 ± 7†
Heart rate (beats/min)	70 ± 7	67 ± 6*
Total cholesterol (mmol/L)	5.3 ± 0.8	5.3 ± 0.8
Triglycerides (mmol/L)	1.9 ± 1.5	1.5 ± 0.9†
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.5 ± 0.4*
Fasting glucose (mmol/L)	6.5 ± 1.0	5.9 ± 1.0*
Fasting insulin (mU/L)	7.7 ± 3.8	4.7 ± 1.7*
HbA_{1c} (%)	6.0 ± 0.7	5.8 ± 0.5*
AUC for glucose (mmol/L)	23.2 ± 4.6	19.0 ± 3.9*
AUC for insulin mU/L	122 ± 78	78 ± 36*
SSPG (mmol/L)	10.8 ± 2.0	8.0 ± 1.5*

AUC = area under the curve; BP = blood pressure; HDL = high-density lipoprotein; SSPG = steady-state plasma glucose.
Values are mean ± SD.
* $P < .01$, † $P < .05$, *v* before treatment.

Measurements of Adiponectin, Matrix Metalloproteinase–2, and Tissue Inhibitor of Metalloproteinase–1

In 15 of the present subjects, plasma levels of adiponectin, matrix metalloproteinase–2 (MMP-2), and tissue inhibitor of metalloproteinase–1 (TIMP-1) were determined before and 6 months after treatment with pioglitazone. The adiponectin concentration was measured by a sandwich enzyme-linked immunosorbent assay (Adiponectin ELISA Kit, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), as previously described.[16] Both MMP-2 and TIMP-1 concentrations were determined using enzyme immunoassays (SRL Inc., Tokyo, Japan).

Statistical Analysis

Values are expressed as mean ± SD. Unpaired *t* test was used for comparison between the two groups (groups 1 and 2). The significance of differences in various parameters before and after treatment with pioglitazone was evaluated with paired *t* test. Relationships between variables were assessed using univariate linear regression analyses and the Pearson correlation coefficient. Multiple regression analyses were performed to identify that the amelioration of insulin resistance by pioglitazone improves LV diastolic function independently of the changes in other metabolic factors. A value of $P < .05$ was accepted as statistically significant.

Results

Changes in clinical parameters before and 6 months after treatment with pioglitazone are shown in Table 1. Body weight and body mass index increased significantly with the administration of pioglitazone. Although systolic BP did not alter, diastolic BP slightly but significantly decreased, and heart rate also decreased after treatment. Serum triglycerides were decreased and HDL cholesterol was increased by pioglitazone, without a change in the total cholesterol level. Pioglitazone not only decreased glucose (fasting glucose, HbA_{1c}, and AUC for glucose on OGTT) and insulin (fasting insulin and AUC for insulin) levels, but also decreased the SSPG level; that is, insulin resistance was significantly improved by pioglitazone treatment.

Changes in echocardiographic parameters before and after treatment with pioglitazone are shown in Table 2. Inferior vena cava diameter, left atrial and ventricular dimensions, and LV systolic function (fractional shorten-

Table 2. Changes in echocardiographic parameters before and after pioglitazone treatment

	Before	After
Inferior vena cava (mm)	13.9 ± 3.3	13.8 ± 3.3
Left atrial dimension (mm)	36.8 ± 4.4	36.4 ± 3.9
IVSTd (mm)	10.6 ± 1.7	10.5 ± 1.4
PWTd (mm)	10.6 ± 1.5	10.5 ± 1.6
LVDd (mm)	44.1 ± 3.0	44.5 ± 3.0
LVDs (mm)	25.3 ± 3.3	24.8 ± 3.3
Fractional shortening	0.43 ± 0.05	0.45 ± 0.05
LV mass index (g/m^2)	117 ± 29	117 ± 28
Relative wall thickness	0.48 ± 0.07	0.47 ± 0.06
E wave velocity (m/sec)	0.72 ± 0.14	0.77 ± 0.14†
A wave velocity (m/sec)	0.91 ± 0.13	0.91 ± 0.12
E/A ratio	0.79 ± 0.15	0.86 ± 0.19*
DcT (msec)	256 ± 52	240 ± 39†
Duration of A wave (Ad) (msec)	150 ± 22	145 ± 14
S wave velocity (m/sec)	0.64 ± 0.10	0.61 ± 0.13
D wave velocity (m/sec)	0.38 ± 0.07	0.40 ± 0.12
S/D ratio	1.71 ± 0.33	1.58 ± 0.29†
PVa wave velocity (m/sec)	0.30 ± 0.08	0.27 ± 0.06
Duration of PVa wave (PVad) (msec)	131 ± 16	123 ± 16*
Ad-PVad (msec)	19 ± 24	22 ± 18

A = atrial filling; DcT = deceleration time of E wave; E = early diastolic filling; IVSTd = interventricular septal thickness; LV = left ventricular; LVDd = LV diameter at diastole; LVDs = LV diameter at systole; PVa = peak reverse flow velocity at atrial contraction; PWTd = posterior wall thickness.
Values are mean ± SD.
* $P < .01$, and † $P < .05$ *v* before treatment.

Table 3. Correlation between individual changes in metabolic parameters and left ventricular diastolic function before and after pioglitazone treatment

	Change in E/A Ratio		Change in DcT	
	r	*P*	*r*	*P*
Change in fasting glucose	0.173	0.364	0.258	0.170
Change in fasting insulin	−0.113	0.556	0.254	0.177
Change in HbA_{1c}	−0.101	0.598	0.238	0.208
Change in AUC for glucose	0.121	0.529	0.251	0.182
Change in AUC for insulin	−0.134	0.485	0.122	0.523
Change in SSPG	−0.402	0.027	0.431	0.017

Abbreviations as in Table 1 and 2.

ing) were not altered by pioglitazone administration. The LV wall thickness and two indices of LV hypertrophy (LV mass index and relative wall thickness) also did not change 6 months after administration of pioglitazone. As for LV diastolic filling properties, however, the peak velocity of the E wave and the E/A ratio were significantly increased and DcT was significantly shortened by treatment with pioglitazone. In pulmonary venous flow profiles, the S/D ratio and duration of the PVa wave (PVad) significantly decreased. The difference between mitral and pulmonary venous atrial flow duration (Ad-PVad) did not change before and after treatment.

To investigate whether the observed improvement in LV diastolic function was linked to the amelioration of insulin resistance, correlations between individual changes in metabolic parameters and LV diastolic function with pioglitazone treatment were examined in all subjects. The changes in the E/A ratio and DcT before and after administration of pioglitazone were not significantly correlated with the decrease in fasting glucose or insulin, AUC for glucose or insulin on OGTT, or HbA_{1c} (Table 3). However, the improvement of diastolic filling parameters, ie, the increase in the E/A ratio and shortening of DcT were significantly correlated with the decrease in SSPG (Table 3, Fig. 1). In addition, the association between individual changes in SSPG and diastolic parameters persisted after adjustment for the changes in glucose and insulin levels (Table 4).

Next, subjects were divided into two groups by the extent of improvement in insulin sensitivity after treatment with pioglitazone; subjects with a modest (<3.3 mmol/L = 60 mg/dL) decrease in SSPG (group 1, $n = 19$) and with a greater (≧3.3 mmol/L) decrease in SSPG (group 2, $n = 11$). In the comparison of baseline characteristics, no intergroup differences were found in any variable other than SSPG (Table 5). The decrease in SSPG with pioglitazone treatment was necessarily greater in group 2 than in group 1, but decreases in AUC for glucose and HbA_{1c} did not differ between the two groups (Fig. 2). The LV mass index was not altered by pioglitazone in either group. Both the increase in the E/A ratio and decrease in DcT were significantly greater in group 2 than in group 1.

In 15 subjects, plasma concentrations of adiponectin, MMP-2, and TIMP-1 were determined before and 6 months after treatment with pioglitazone. As shown in Figs. 3A and 3B, pioglitazone administration markedly

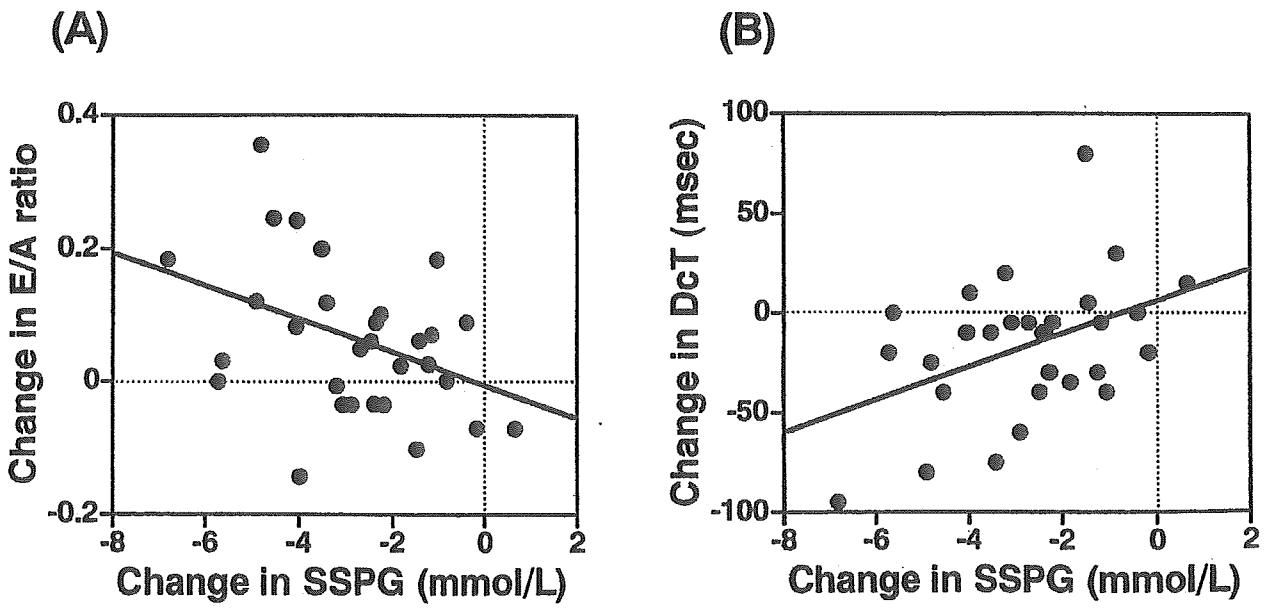

FIG. 1. Correlation between individual changes in insulin sensitivity and left ventricular (LV) diastolic function before and after treatment with pioglitazone. The change in steady-state plasma glucose (SSPG) significantly correlated with the change in E/A ratio (**A**, $r = -0.402$, $P = .027$) and with the change in DcT (**B**, $r = 0.431$, $P = .017$).

Table 4. Independent relation of the change in steady-state plasma glucose (SSPG) to the improvement of left ventricular diastolic function by multiple regression analysis

	Change in E/A Ratio		Change in DcT	
	β	P	β	P
Model 1				
Change in fasting glucose	0.139	0.432	0.298	0.082
Change in SSPG	−0.390	0.034	0.457	0.010
Model 2				
Change in fasting insulin	0.007	0.969	0.138	0.449
Change in SSPG	−0.404	0.038	0.390	0.039
Model 3				
Change in HbA_{1c}	−0.137	0.440	0.277	0.106
Change in SSPG	−0.413	0.026	0.455	0.011
Model 4				
Change in AUC for glucose	0.142	0.424	0.230	0.183
Change in SSPG	−0.409	0.027	0.419	0.019
Model 5				
Change in AUC for insulin	0.059	0.768	−0.090	0.647
Change in SSPG	−0.428	0.039	0.471	0.022

Abbreviations as in Table 1 and 2.

Considering the sample size of the present study, confounding factors (changes in fasting glucose, fasting insulin, HbA_{1c}, AUC for glucose, and AUC for insulin) were individually included.

elevated the adiponectin level (+270%) and also significantly increased the MMP-2 level (+16%). In addition, the percent increase in MMP-2 was closely correlated with the percent increase in adiponectin (Fig. 3C). The increase in plasma adiponectin or MMP-2 was partially associated with the change in E/A ratio or DcT (percent increase in adiponectin and change in DcT: $r = -0.554$, $P = .039$; increase in MMP-2 and change in E/A ratio: $r = 0.496$, $P = .072$). The plasma level of TIMP-1 did not significantly change before and after pioglitazone treatment (before, 62.1 ± 8.4 ng/mL; after, 62.8 ± 13.1 ng/mL).

Discussion

Several studies have shown that thiazolidinediones (pioglitazone and troglitazone) improve LV diastolic function in animal models such as rats with type 2 diabetes[10,11] and nondiabetic pigs after acute ischemia.[12] Only one study in human beings reported that troglitazone improved LV

Table 5. Comparison of baseline characteristics between the two study groups divided by the improvement in insulin sensitivity after pioglitazone treatment

	Group 1 (n = 19)	Group 2 (n = 11)	P
Age (y)	67 ± 7	65 ± 6	NS
Sex (male/female)	8/11	3/8	NS
Body weight (kg)	61.8 ± 10.8	61.1 ± 7.7	NS
Body mass index (kg/m²)	25.1 ± 3.8	24.7 ± 3.5	NS
Systolic BP (mm Hg)	142 ± 8	142 ± 10	NS
Diastolic BP (mm Hg)	78 ± 7	80 ± 5	NS
Heart rate (beats/min)	70 ± 7	70 ± 8	NS
Fasting glucose (mmol/L)	6.7 ± 1.1	6.1 ± 0.8	NS
Fasting insulin (mU/L)	7.0 ± 3.3	9.0 ± 4.4	NS
HbA_{1c} (%)	6.1 ± 0.7	5.8 ± 0.6	NS
AUC for glucose (mmol/L)	24.2 ± 5.0	21.6 ± 3.2	NS
AUC for insulin (mU/L)	103 ± 78	155 ± 72	NS
SSPG (mmol/L)	10.3 ± 1.8	11.8 ± 2.1	< .05
LV mass index (g/m²)	123 ± 32	107 ± 23	NS
E/A ratio	0.78 ± 0.09	0.83 ± 0.21	NS
DcT	254 ± 40	260 ± 71	NS

Abbreviations as in Table 1 and 2.

Group 1, subjects with a modest improvement in insulin sensitivity (modest decrease in SSPG); group 2, subjects with a greater improvement in insulin sensitivity (greater decrease in SSPG) after pioglitazone treatment.

Values are mean ± SD.