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

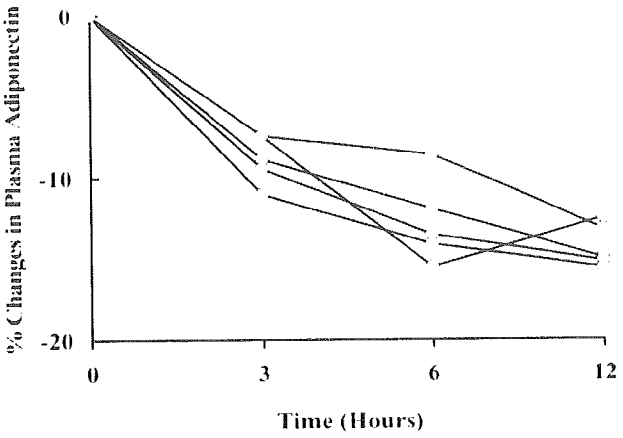

Figure 1. Percent changes in plasma adiponectin levels before and after smoking. Individual changes in adiponectin level were plotted. Adiponectin levels were expressed as percent change from initial values (n=5).

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

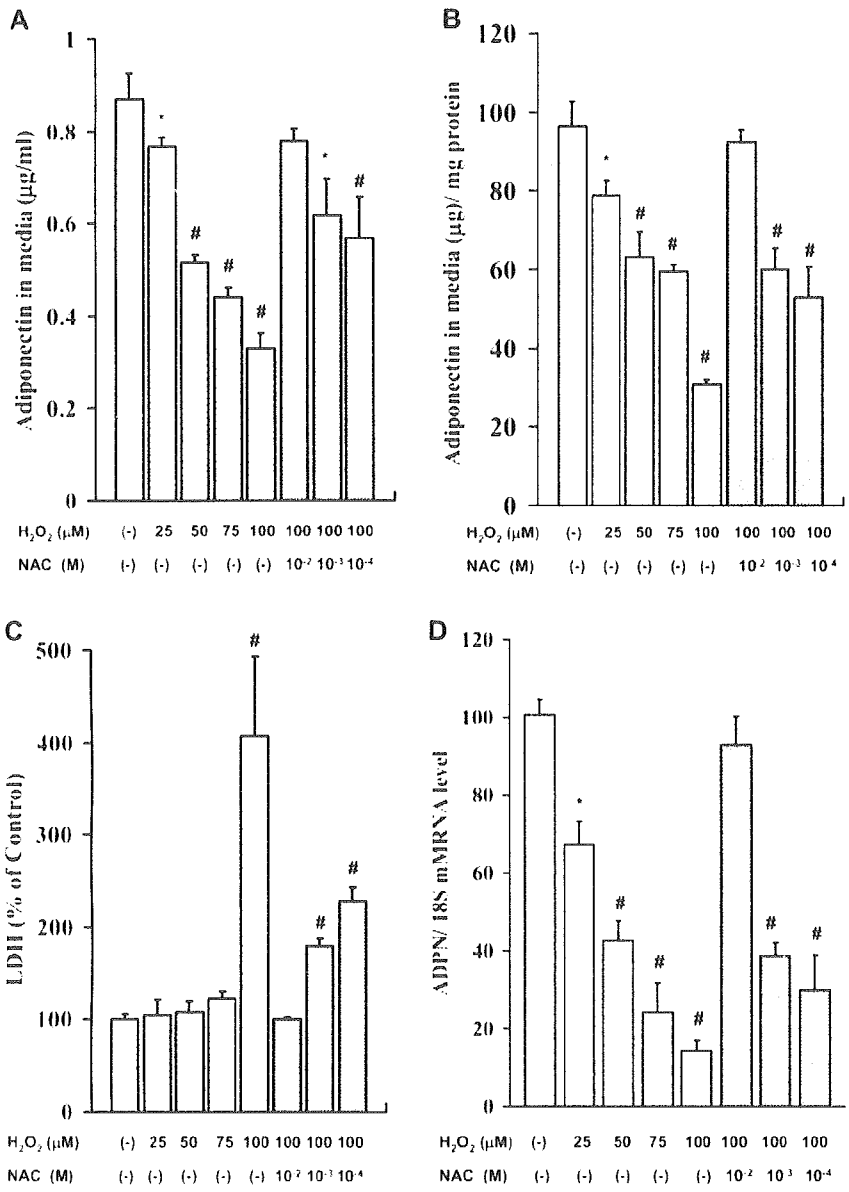

Figure 2. Effects of H_2O_2 on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of H_2O_2 with/without NAC on adiponectin secreted into media (A), adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent 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.[11]

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

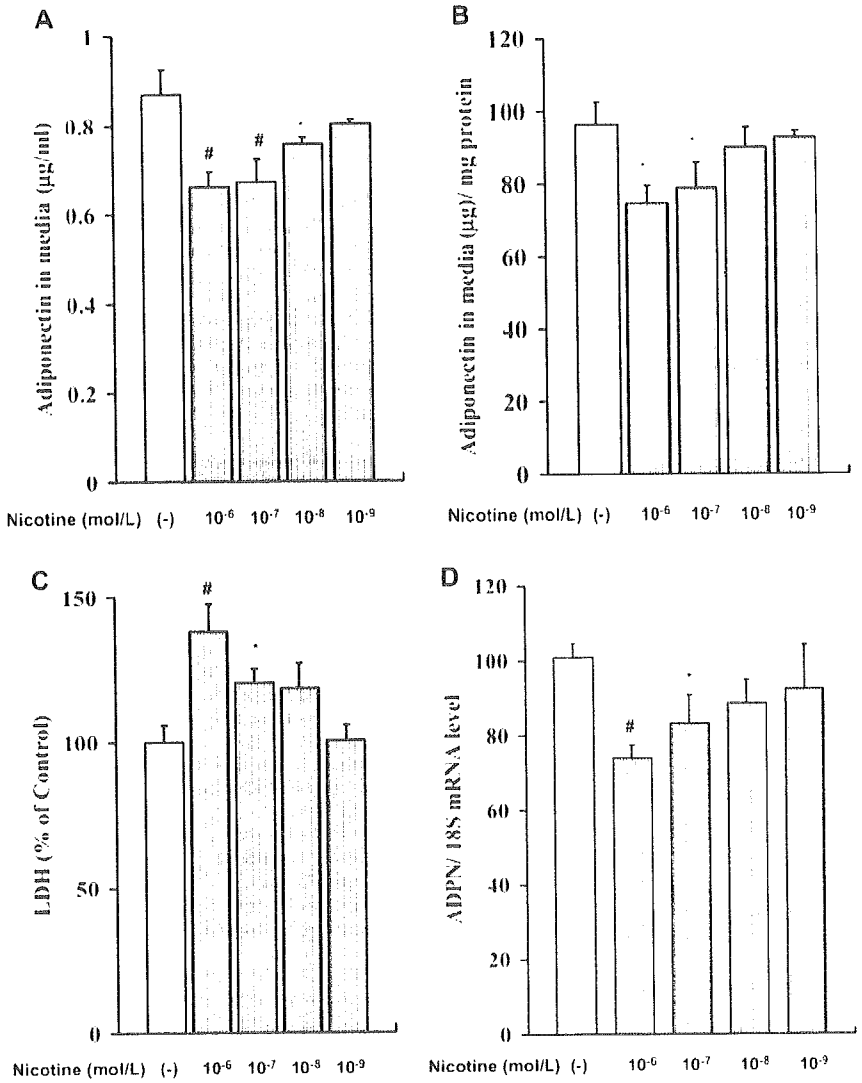

Figure 3. Effects of nicotine on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of nicotine on adiponectin secreted into media (A), adiponectin adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent 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 ≧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.

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Resting metabolic rate is an important predictor of serum adiponectin concentrations: potential implications for obesity-related disorders[1–3]

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ABSTRACT

Background: Little is known about the regulation of adiponectin. Animal studies suggest local regulation by adipocytokines or alterations in energy expenditure, and studies in humans suggest regulation by alcohol intake and ethnicity.

Objective: To identify regulators of adiponectin in humans, we measured resting metabolic rate (RMR), serum adiponectin, glucose, insulin, triacylglycerol, alcohol intake, and anthropometric indexes in 457 white patients with overweight or obesity.

Design: A cross-sectional design was used, and multivariate regression analysis was performed with adiponectin as the dependent variable and potential predictors as independent variables.

Results: Simple linear analyses showed significant associations between adiponectin and sex, with a standardized coefficient of −0.38 (women compared with men) and an explanation of variation of the model (R^2) of 14%; age (0.21; 4%); RMR (−0.52; 27%); fat-free mass (−0.40; 16%); fat mass (−0.16; 2%); visceral fat (−0.24; 6%; computed tomography at L4–L5); fasting triacylglycerol (−0.28; 8%); and insulin resistance (−0.38; 14%; homeostasis model assessment). Adiponectin and alcohol were not associated (−0.04; 0%). Multivariate analyses, which allowed adjustment for confounding, showed that RMR is the most important predictor of adiponectin (−0.31; 29%), followed successively by insulin resistance (−0.16; 31%; model containing RMR and insulin resistance), fat mass (0.20; 34%), age (0.34; 35%), visceral fat (−0.34; 40%), and fasting triacylglycerol (−0.12, 41%).

Conclusions: Low resting metabolism (RMR) is associated with high serum adiponectin. We speculate that subjects with low RMR, who are theoretically at greater risk of obesity-related disorders, are especially protected by adiponectin. *Am J Clin Nutr* 2005;82:21–5.

KEY WORDS Basal metabolism, adiponectin, obesity, metabolic syndrome X, insulin resistance, body constitution

INTRODUCTION

Recent progress in obesity research has shown that adipocytes are not merely fat-storing cells but that they secrete a variety of hormones, cytokines, growth factors, and other bioactive substances, conceptualized as adipocytokines. A disturbance of regulation of these adipocytokines contributes to the pathogenesis of obesity-related disorders such as insulin resistance, type 2 diabetes, dyslipidemia, endothelial dysfunction, and vascular disease (1). In particular, the adipocytokine adiponectin has been shown to play an important role; it exerts insulin-sensitizing and antiatherogenic effects (2). The adiponectin knockout (KO) mice, for example, exhibited severe diet-induced insulin resistance with reduced insulin receptor substrate-1–associated phosphatidylinositol 3 kinase activity in muscle. The KO mice also showed neointimal thickening in response to vascular injury and hypertension induced by salt diet. These phenotypes in KO mice were reversed by viral-mediated production of adiponectin (3). A causal relation between decreased plasma adiponectin and insulin resistance and atherosclerosis has been suggested in humans as well (4, 5).

Unfortunately, we do not sufficiently know how adiponectin concentration is regulated. Local effects of tumor necrosis factor-α, interleukin-6, β-adrenoceptor agonist, glucocorticoids (6), specific receptors on the adipocyte, environmental effects such as alcohol intake, and genetic effects, eg, ethnicity, have been suggested (7, 8). In addition, energy expenditure might affect adiponectin, as suggested by animal models (9). In general, energy expenditure is required to maintain basic physiologic functions (eg, heart beat, muscle contraction, respiration); to metabolize, digest, and store consumed food; and to perform physical activity (10). A relation between physical activity and adiponectin could not be established by some recent investigators (11, 12).

To explore the role of energy expenditure in the regulation of adiponectin, we measured basal metabolism or resting metabolic rate (RMR), the energy expenditure required to maintain basic physiologic functions, as well as other potential regulators or confounders and explored their relation with serum adiponectin in overweight or obese patients.

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SUBJECTS AND METHODS

Patients with overweight or obesity were recruited from the outpatient clinic of the Department of Diabetology, Metabolism, and Clinical Nutrition of the University Hospital, Antwerp, Belgium. Patients with endocrine disorders (eg, hypothalamopituitary disorders, hypothyroidism, Cushing's syndrome), genetic (dysmorphic) disorders, obesity, or diabetes mellitus type 2 defined according to World Health Organization criteria (13) were excluded. The study was performed according to the standards on human experimentation in accordance with the Helsinki Declaration of 1975 as revised in 1983. Smoking and alcohol habits were estimated by careful questioning, and individuals were divided into dichotomous categories. Individuals who had quit smoking were defined as nonsmokers, and individuals who did not use alcohol on a daily basis were defined as nonalcohol drinkers.

Anthropometry and resting metabolic rate

Height was measured to the nearest 0.5 cm, body weight was measured with a digital scale to the nearest 0.1 kg, body mass index (in kg/m^2) was calculated, and percentage of body fat and fat-free mass (FFM; in kg) were assessed by bioimpedance (SEAC SFB3; SEAC, Brisbane, Australia) as described by Lukaski et al (14). Visceral and subcutaneous fat were assessed by a computerized tomography scan at the L4–L5 level, determined according to the technique described by Van der Kooy and Seidell (15) and Kvist et al (16). The RMR is the amount of energy expended when an adult organism is awake but resting, fasting, and at thermal neutrality. RMR was measured and the respiratory quotient was calculated as $\dot{V}CO_2/\dot{V}O_2$, as reported previously (17). In short, RMR was measured by indirect calorimetry with a ventilated hood system (Deltatrac; Datex, Helsinki, Finland). Subjects stayed overnight at the metabolic ward of the University Hospital Antwerp, and RMR was measured in the morning on awakening after an overnight fast. Oxygen consumption and carbon dioxide production in expired air were measured each minute for 30 min after a 10-min equilibration period. Energy expenditure was calculated with the equation of de Weir (18). In addition to energy expenditure, substrate oxidation was calculated with the equations of Lusk (19).

Laboratory analyses

A fasting blood sample was drawn for measurements of glucose, insulin, triacylglycerol, and adiponectin. Plasma glucose was measured with the glucose oxidase method (on Vitros 750 XRC; Ortho Clinical Diagnostics Inc, Rochester, NY). Fasting triacylglycerol was measured on Vitros 750XRC (Ortho Clinical Diagnostics, Johnson & Johnson, Raritan, NJ). Insulin was measured by a radioimmunoassay with the use of Pharmacia Insulin RIA (Pharmacia Diagnostics, Uppsala, Sweden). This assay shows 41% cross-reactivity with proinsulin. Insulin resistance was calculated by the homeostasis model assessment (HOMA) method with the use of fasting plasma glucose and insulin concentrations. Assuming that normal-weight subjects <35 y have an insulin resistance of 1, the value for insulin resistance can be assessed by the following equation:

$$\text{Fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose (mmol/L)}/22.5 \quad (1)$$

Results from the HOMA method correlate well with measurements obtained by means of the euglycemic clamp technique. The CV is reported to be between 7.8% and 11.7% (20, 21). Plasma adiponectin concentration was measured by enzyme-linked immunosorbent assay (Otsuka Pharmaceutical Co, Tokushima, Japan) as described previously (22).

Statistical analysis

Study population characteristics and anthropometric and laboratory measurements were presented as proportions or median values with their range and the 25th and 75th percentile. To show the associations between adiponectin and anthropometric measurements, after control for confounding influence of sex and age, partial Pearson's correlation coefficients were presented. A P value < 0.05 was regarded as statistically significant. To explore the relation between adiponectin and potential variables,

TABLE 1
Study population characteristics and anthropometric and laboratory measurements[1]

	Median	Minimum–maximum	25th–75th percentile
Age (y)	41	18–75	32–50
Weight (kg)	106	60–166	95–119
Height (m)	1.68	1.41–1.97	1.62–1.74
BMI (kg/m^2)	38	22–60	34–42
FFM (kg)	51	35–92	46–60
Total fat mass (kg)	53	20–92	44–62
RMR (kcal/24 h)	1875	1030–3120	1672–2127
RMR/FFM (kcal · kg^{-1} · 24 h^{-1})	35	26–47	33–38
Respiratory quotient	0.76	0.70–1.00	0.74–0.79
Subcutaneous fat (cm^2)	605	218–1026	504–709
Visceral fat (cm^2)	156	34–554	111–209
Fasting serum glucose (mg/dL)	82	63–110	77–89
Fasting serum insulin (μU/mL)	17	0.2–82	12–24
Calculated insulin resistance (HOMA)	3.5	0.04–18.2	2.3–5.2
Fasting serum triacylglycerol (mg/dL)	138	46–568	103–185
Fasting serum adiponectin (μg/mL)	8.3	1.5–31.8	5.8–11.5

[1] n = 457. Women composed 75% of the study population. FFM, fat-free mass; RMR, resting metabolic rate; HOMA, homeostasis model assessment.

TABLE 2
Serum adiponectin concentrations according to tertiles of the resting metabolic rate (RMR) per fat-free mass (FFM)[1]

	Tertile of RMR/FFM (kcal · kg^{-1} · 24 h^{-1})		
	1st (< 34)	2nd	3rd (> 37)
Serum adiponectin (μg/mL)			
Women (n = 175)	10.7	10.2	8.2
Men (n = 52)	7.7	5.8	5.0

[1] The interaction of sex and tertile was not significant, $P = 0.73$. The main effects of sex ($P = 0.008$) and of tertile of RMR/FFM ($P = 0.001$) were significant.

linear regression analysis was applied. Linear regression analysis provides insight into linear associations and allows adjustment for the influence of potential confounders. If appropriate, variables were normalized by transformation into their natural logarithm to improve the plots of residual analyses. Results were expressed as standardized coefficients and R^2, or proportion of variation "explained" by the predictor of interest. Analyses were performed with the SPSS-PC software, version 11.0.1 (SPSS Inc, Chicago, IL).

RESULTS

Four hundred fifty-seven patients were recruited with a median body mass index of 38 (range: 22-60), and 75% were women. The population characteristics and anthropometric and laboratory measurements are shown in **Table 1**.

Men as well as women with a low RMR expressed per kilogram FFM have higher adiponectin concentrations (**Table 2**). This association between adiponectin and RMR is further explored in **Table 3**, which confirms a negative correlation between adiponectin and RMR (-0.36, $P < 0.001$), after control for confounding influence of age and sex. Also shown in Table 3 are significant inverse correlations of adiponectin to insulin resistance, triacylglycerol, and various anthropometric measurements, of which the strongest is visceral fat (-0.38, $P < 0.001$). The RMR is also strongly correlated to insulin resistance, triacylglycerol, and various anthropometric indicators, of which the strongest is FFM (0.73, $P < 0.001$), as has clearly been established previously (10, 23).

To further explore the regulation of serum adiponectin and to allow control for confounding, linear regression models were built with adiponectin as the dependent variable and potential predictors and confounders as independent variables, as shown in **Table 4**. In the simple linear analyses, various variables were associated with adiponectin: sex, age, RMR, FFM, fat mass, visceral fat, fasting triacylglycerol, and insulin resistance. Adiponectin was not associated with alcohol in the present study, which might be the result of a limitation in precision of assessment of alcohol intake.

TABLE 4
Results from simple linear regression analyses with serum adiponectin as dependent variable and various potential predictors as independent variables

Potential predictors in the model	β	SE	P	R^2 of model
Sex (female vs male)	−0.38	0.05	0.001	0.14
Age (y)	0.21	0.002	0.001	0.04
Resting metabolic rate (kcal/24 h)	−0.52	0.000	0.001	0.27
Fat-free mass (kg)	−0.40	0.002	0.001	0.16
Fat mass (kg)	−0.16	0.002	0.001	0.02
Visceral fat (cm^2)	−0.24	0.000	0.001	0.06
Fasting triacylglycerol (mg/dL)	−0.28	0.000	0.001	0.08
Insulin resistance (HOMA)[1]	−0.38	0.009	0.001	0.14
Alcohol (no vs yes)	−0.04	0.048	0.933	0.00

[1] HOMA, homeostasis model assessment.

On the basis of the results of Table 4, one multivariate regression model was built by using the stepwise regression procedure. The strongest correlate appeared to be the RMR, which explained 29% of the variation of adiponectin (**Table 5**). The second strongest correlate appeared to be insulin resistance, which explained, together with the RMR, 31% of the variation of adiponectin, followed successively by fat mass (34%), age (35%), visceral fat (40%), and fasting triacylglycerol (41%). These results clearly show that adiponectin and RMR are strongly and inversely associated. Additional adjustment for potential confounders, such as age, visceral fat, or HDL cholesterol (data not shown), did not affect the relation between adiponectin and RMR significantly. Using the same variables in a backward procedure resulted in similar findings (data not shown). The results of these multivariate analyses also show the importance of fasting triacylglycerol concentrations in regulation of adiponectin. The model that included RMR, insulin resistance, fat mass, age, visceral fat, and fasting triacylglycerol explained 41% of the variation of adiponectin (Table 5). When HDL cholesterol was included in the model, fasting triacylglycerol would be excluded by the stepwise

TABLE 3
Partial Pearson's correlation coefficients between adiponectin and various potential predictors, adjusted for sex and age[1]

	Adiponectin	RMR	Total fat mass	FFM	BMI	Visceral fat	Subcutaneous fat	Insulin resistance
RMR	−0.36[2]							
Total fat mass	−0.15[3]	0.65[2]						
FFM	−0.23[2]	0.73[2]	0.47[2]					
BMI	−0.23[3]	0.67[2]	0.88[2]	0.57[2]				
Visceral fat	−0.38[2]	0.50[2]	0.49[2]	0.41[2]	0.55[2]			
Subcutaneous fat	−0.07	0.43[2]	0.79[2]	0.30[2]	0.73[2]	0.27[2]		
Insulin resistance	−0.28[2]	0.37[2]	0.28[2]	0.26[2]	0.32[2]	0.32[2]	0.23[3]	
Fasting triacylglycerol	−0.26[2]	0.28[2]	0.08	0.09	0.12[3]	0.16[3]	0.01	0.11

[1] RMR, resting metabolic rate; FFM, fat-free mass.
[2] $P < 0.001$.
[3] $P < 0.05$.

TABLE 5
Results from stepwise multivariate linear regression analyses with serum adiponectin as dependent variable and potential predictors as independent variables[1]

Predictive variables in one model	β	SE	P	R^2 of model
Resting metabolic rate (kcal/24 h)	−0.31	0.000	0.001	0.29
Insulin resistance (HOMA)[2]	−0.16	0.013	0.009	0.31
Fat mass (kg)	0.20	0.002	0.002	0.34
Age (y)	0.34	0.003	0.001	0.35
Visceral fat (cm^2)	−0.34	0.001	0.001	0.40
Fasting triacylglycerol (mg/dL)	−0.12	0.000	0.034	0.41

[1] Potential predictors are sex, age, resting metabolic rate, fat-free mass, fat mass, visceral fat, fasting triacylglycerol, and insulin resistance.
[2] HOMA, homeostasis model assessment.

procedure. Because triacylglycerol and HDL cholesterol are inversely and undeniably associated and because increased triacylglycerol concentrations more clearly explain their contribution to the development of the metabolic syndrome (24), fasting triacylglycerol instead of HDL cholesterol was included in the final model.

DISCUSSION

We report here on a significant link between serum adiponectin concentrations and RMR. The present study shows high serum adiponectin concentrations in subjects with low RMR. We speculate that protection by adiponectin against obesity-related disorders is especially important for subjects with low RMR. Theoretically, subjects with low RMR are at increased risk of developing these disorders; a larger portion of their daily food intake is stored as fat, in the situation of similar calorie intake. However, the literature reports discrepant findings between RMR and the risk of developing obesity (10, 23, 25). We speculate that this discrepancy in the literature can be explained by a difference in magnitude of protection mechanisms; high risk can be tempered by a better protection by adiponectin.

A disadvantage of the present cross-sectional study is that it does not allow definite conclusions with respect to cause and effect. RMR might affect adiponectin or vice versa. Previous animal studies showed an increase in adiponectin after exposure to cold and suggest a link, not only between adiponectin and thermogenesis but also between adiponectin and genetic-, instead of nutrition-induced obesity (9). Other rodent studies suggest regulation of energy expenditure by adiponectin (26, 27). The latter might be the case as well, but analyses in the present study were performed to test the hypothesis that adiponectin is regulated by energy expenditure. It is, however, also possible that a third underlying unknown factor, eg, genetics, affects both energy expenditure and adiponectin.

In the present study, neither physical activity nor thermogenesis was measured. A link between adiponectin and physical activity is unlikely (11, 12). Except for the previously mentioned report on adiponectin and thermogenesis (9), which represents a maximum 10% of the RMR, to the best of our knowledge, our present study is the first to correlate RMR and adiponectin concentrations.

The present study confirms the earlier described link between adiponectin and triacylglycerol concentrations (28, 29). Increased concentrations of free fatty acid (FFA) and triacylglycerol may ultimately cause an abnormal triacylglycerol storage, which results, in turn, in an increased FFA flux from adipose tissue to nonadipose tissue, which participates in and amplifies many of the fundamental derangements of the metabolic syndrome (24). A recent report mentioned a decrease of adiponectin concentrations after lowering of FFA (30) and suggested an influence of adiponectin on FFA and triacylglycerol (31); thus, a feedback mechanism may exist.

The present study also shows that the inverse association between adiponectin and visceral fat increases after adjustment for fat mass (Table 5), which implies that adiponectin, exclusively secreted by the fat cell, is especially compromised in subjects with visceral obesity (32). This finding, together with the well-known inverse relation between adiponectin and insulin resistance, fit in the same framework and confirm earlier findings on the important interplay between adiponectin and the pathogenesis of the metabolic syndrome (33).

In conclusion, an inverse association between basal metabolism and serum adiponectin concentrations was shown. This inverse association might point to protection by adiponectin against obesity-related disorders particularly when low RMR is present, which itself is theoretically associated with development of obesity (and related disorders).

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LFG was the main investigator, who coordinated and monitored the study. JBR performed the statistical analyses. All authors participated in evaluating the results and in the writing and editing of the manuscript. None of the authors had any financial conflicts related to the work.

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Serum Soluble Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Levels Are Elevated in Acute Coronary Syndrome: A Novel Marker for Early Diagnosis

Kazutaka Hayashida, Noriaki Kume, Takatoshi Murase, Manabu Minami, Daisuke Nakagawa, Tsukasa Inada, Masaru Tanaka, Akira Ueda, Goro Kominami, Hirofumi Kambara, Takeshi Kimura and Toru Kita

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Serum Soluble Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Levels Are Elevated in Acute Coronary Syndrome

A Novel Marker for Early Diagnosis

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Background—Markers of cardiac injury, including troponin-T (TnT), are used to diagnose acute coronary syndrome (ACS); however, markers for plaque instability may be more useful for diagnosing ACS at the earliest stage. Lectin-like oxidized LDL receptor-1 (LOX-1) appears to play crucial roles in the pathogenesis of atherosclerotic plaque rupture and ACS onset. LOX-1 is released in part as soluble LOX-1 (sLOX-1) by proteolytic cleavage.

Methods and Results—We examined serum sLOX-1 levels in 521 patients, consisting of 427 consecutive patients undergoing coronary angiography, including 80 ACS patients, 173 symptomatic coronary heart disease patients, 122 patients with significant coronary stenosis without ischemia, and 52 patients without apparent coronary atherosclerosis plus 34 patients with noncardiac acute illness and 60 patients with noncardiac chronic illness. Time-dependent changes in sLOX-1 and TnT levels were analyzed in an additional 40 ACS patients. Serum sLOX-1 levels were significantly higher in ACS than the other groups and were associated with ACS as shown by multivariable logistic regression analyses. Given a cutoff value of 1.0 ng/mL, sLOX-1 can discriminate ACS from other groups with 81% and 75% of sensitivity and specificity, respectively. sLOX-1 can also discriminate ACS without ST elevation or abnormal Q waves and ACS without TnT elevation from non-ACS with 91% and 83% of sensitivity, respectively. Peak values of sLOX-1 in ACS were observed earlier than those of TnT.

Conclusions—sLOX-1 appears to be a useful marker for early diagnosis of ACS. (***Circulation.*** **2005;112:812-818.**)

Key Words: angina ■ atherosclerosis ■ lipoproteins ■ myocardial infarction ■ receptors

Acute coronary syndrome (ACS) is one of the major causes of mortality and morbidity in developed countries. Accurate diagnosis of ACS at the earliest stage would improve prognosis through appropriate treatment without delay. ACS appears to be provoked by a rupture of lipid-rich atheromatous plaques, followed by thrombus formation.[1,2] Several diagnostic tests such as echocardiography,[3] radioisotope scintigraphy,[4] and measurement of circulating levels of troponin-T (TnT)[5,6] and the MB isoform of creatine kinase (CPK)[7] have been used to detect ischemic myocardial damage in clinical practice; however, none of these markers directly indicates plaque instability or rupture before myocardial damage becomes apparent. Such markers for plaque instability or rupture would establish the diagnosis of ACS at the earliest stage and may predict the onset. Several serum markers, including high-sensitivity C-reactive protein (hs-CRP),[8] oxidized LDL (Ox-LDL),[9] and soluble forms of membrane proteins such as CD40 ligand (CD40L),[10,11] ICAM-1,[12,13] and E-selectin,[12,13] were reported to be associated with ACS or acute myocardial infarction. Although soluble CD40L has recently been shown to be correlated with prognosis after ACS,[14] none of these markers has been established as a diagnostic marker of ACS at the earliest stage.

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LDL-lowering therapy has been shown to decrease the incidence of ACS and other atherosclerosis-related diseas-

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es.[15-18] In addition, the importance of oxidatively modified LDL has been demonstrated in this process.[19,20] In fact, plasma Ox-LDL levels have been shown to be elevated in patients with ACS.[9] Effects of Ox-LDL on vascular cells in atherosclerotic progression and plaque rupture appear to be mediated by its receptors.[21] Lectin-like oxidized LDL receptor-1 (LOX-1) is a receptor with an expression that is not constitutive but dynamically inducible by proinflammatory stimuli, angiotensin II, and Ox-LDL, which are risk factors for ACS.[22-28] In human atherosclerotic lesions, LOX-1 is expressed prominently by intimal smooth muscle cells and lipid-laden macrophages in the advanced plaques.[29] Furthermore, LOX-1 plays an important role in Ox-LDL–induced apoptosis of vascular smooth muscle cells[30,31] and production of matrix metalloproteinases,[32] which may directly be linked to plaque rupture. LOX-1 is also expressed on the surface of activated platelets,[33] which may also be involved in thrombus formation after plaque rupture.

LOX-1 expressed on the cell surface can be proteolytically cleaved at its membrane proximal extracellular domain and released as soluble forms (sLOX-1).[34] Therefore, we have established a specific and sensitive assay to measure concentrations of sLOX-1 in human sera. The present report shows that serum sLOX-1 levels are elevated in ACS from its early stage, suggesting its usefulness as an early diagnostic marker of ACS.

Methods

Patient Sample

We enrolled 427 patients who underwent diagnostic coronary angiography (CAG) at the cardiovascular center and 34 patients who visited the emergency department and immediately were hospitalized in the Osaka Red Cross Hospital because of severe noncardiac acute diseases such as infectious diseases, trauma, and asthmatic fit and 60 patients with chronic problems in the outpatient department of internal medicine. All subjects were consecutively identified. All patients in this study gave written informed consent. Consecutive patients undergoing CAG were assigned to 4 groups depending on CAG findings and clinical features. Fifty-two patients whose CAG did not show any apparent atherosclerotic lesions were assigned to the group of patients with intact coronary. One hundred twenty-two patients who had documented coronary atherosclerosis by CAG but had been free of episodes of angina or documented cardiac ischemia for at least 3 months were assigned to the group of patients with controlled coronary heart disease (CHD). One hundred seventy-three patients who had significant coronary stenosis and ischemic symptoms (stable angina) and required elective coronary artery revascularization procedures such as percutaneous coronary intervention (PCI) or CABG were assigned to the group of patients with ischemic CHD. Eighty patients presented with ACS, which was defined as acute onset of prolonged chest pain or discomfort accompanied by ST-segment elevation or depression evolving into pathological Q waves or T-wave inversion and emergency CAG-documented total occlusion or marked delayed filling of a coronary artery. Among ACS patients, those without ST-segment elevation or pathological Q waves were defined as non–Q-wave ACS (NQ-ACS).

In another group of 40 ACS patients, serum sLOX-1 and TnT were serially measured on admission (at 4.4±4.2 hours after onset), immediately after emergency PCI, and at days 1, 3, 5, and 7. Patients with symptomatic peripheral vascular diseases were excluded from this study.

This study, carried out in accordance with the principles of the Declaration of Helsinki, was approved by local ethics committees.

Measurement of sLOX-1 and Other Serum Markers

Serum samples were collected at coronary angiography for patients undergoing CAG or at time of visit for patients with acute illness and chronic illness. In a time-dependent analysis, serum samples were collected serially at the indicated time periods. These samples were stored at −80 C until assays were performed. Serum sLOX-1 levels were determined by a sandwich ELISA using 2 different human LOX-1–specific antibodies. Antibodies were obtained after purification of serum from 2 different rabbits that had been immunized with a recombinant protein corresponding to the extracellular domain of human LOX-1. One of these antibodies was used to coat the plates; the other was fragmented into Fab' and labeled with horseradish peroxidase for enzymatic detection. Standard curves were obtained by use of a recombinant protein corresponding to the extracellular domain of human LOX-1. Intra-assay and interassay coefficients of variation were 2.0% to 11.8% and 0.0% to 8.1%, respectively. The lower limit of the detection for sLOX-1 was 0.5 ng/mL. All assays were carried out by personnel who had no knowledge of the clinical diagnosis of the patients. Measurement of diluted serum samples by the same ELISA (see the Figure in the online-only Data Supplement) and immunoprecipitation followed by immunoblotting (data not shown) showed comparable results, indicating the accuracy and reliability of this ELISA for sLOX-1. Levels of hs-CRP and TnT were determined on the same serum samples as those for sLOX-1 by commercially available electrochemiluminescent immunoassay kit (F. Hoffmann–La Roche Ltd. and particle-enhanced immunonephelometry (Dade Behringer Ltd), respectively.

Statistical Analysis

We performed statistical analysis using Stat-View, version 5, and SPSS. The 1-way ANOVA was used to compare clinical continuous variables with the Tukey-Kramer test for multiple comparisons and 2-way cross-tabulation with the χ^2 test for binary variables, when appropriate, to compare differences between groups. When sLOX-1 was undetectable by ELISA, the sLOX-1 level was assigned 0. Levels of sLOX-1 did not distribute normally; therefore, the Kruskal-Wallis and Dunn's tests were used for multiple comparisons. Association between sLOX-1 and hs-CRP, LDL cholesterol, HDL cholesterol, triglycerides, or TnT was evaluated by Spearman's rank correlation coefficient. Multivariable logistic regression analysis was performed to assess the correlation between ACS and age, gender, hypertension, diabetes, smoking, LDL cholesterol, HDL cholesterol, triglycerides, hs-CRP, or sLOX-1.[35] Transformed values of hs-CRP in logarithm were used as variables for statistical analyses. Time profiles of serum sLOX-1 and TnT levels were analyzed after conversion of the individual's serial sLOX-1 levels into relative ratios to each individual's maximum value by 1-way repeated-measures ANOVA and multiple comparisons with Bonferroni's test. Receiver-operating characteristic (ROC) analysis was also carried out on the levels of sLOX-1 and hs-CRP for ACS and ACS without apparent ST elevation or pathological Q waves (NQ-ACS) separately. This analysis plots the true-positive fraction (sensitivity) against the false-positive fraction (1−specificity) by changing the cutoff value for the test. Areas under the ROC curves indicate the relative accuracy of diagnostic tests.[36] All probability values are 2 sided. Values of $P < 0.05$ were considered statistically significant.

Results

Clinical Characteristics of the Study Samples

Table 1 summarizes age, gender, conventional cardiovascular risk factors, and lipid profiles in each group of patients, as well as the combined non-ACS patients, undergoing CAG. Patient characteristics, including age, gender, and incidence of hypertension, diabetes, and hypercholesterolemia, were comparable between the ACS group and the combined non-ACS CAG, except that the

TABLE 1. Characteristics of Consecutive CAG Patients

Characteristics	Intact Coronary	Controlled CHD	Ischemic CHD	Non-ACS CAG	ACS
Patients, n	52	122	173	Subtotal, 347	80
Age (mean±SD), y	66±9	66±10	67±9	67±10	64±12
Male sex, n (%)	32 (62)	89 (73)	128 (74)	249 (67)	59 (74)
Risk factors, n (%)					
Hypertension	22 (42)	65 (53)	82 (47)	168 (48)	30 (38)
Diabetes	8 (15)†	43 (35)	63 (36)	114 (33)	26 (33)
Smoking	19 (37)	61 (50)	57 (33)‡	136 (39)*	43 (54)*
Hypercholesterolemia	14 (27)	47 (39)	84 (49)§	144 (41)	27 (34)
Lipid profile (mean±SD), mg/dL					
LDL cholesterol	122±38	125±35	121±35	121±36	122±35
HDL cholesterol	50±16	45±13	45±14	46±14¶	41±11¶#
Triglycerides	137±103	141±63	132±52	136±68	140±75
hs-CRP (mean±SD), ng/mL	3.10±0.75	3.09±0.65	3.11±0.87	3.10±0.78¶	3.41±0.87‖¶

Values for hs-CRP were transformed in logarithm of 10. One-way ANOVA was followed up with Tukey-Kramer pairwise comparisons among means.

*$P<0.01$ for comparison with combined all non-ACS and ACS with 2-way cross-tabulation with χ^2 test.

†$P<0.05$ for comparison with controlled CHD, ischemic CHD, and ACS.

‡$P<0.05$ for comparison with controlled CHD and ACS.

§$P<0.05$ for comparison with intact coronary and ACS.

‖$P<0.05$ for comparison with intact coronary, controlled CHD, and ischemic CHD.

¶$P<0.001$ for comparison between non-ACS CAG and ACS with t test.

#$P<0.05$ for comparison with intact coronary.

ACS group showed higher smoking rate and lower HDL cholesterol levels (Table 1). Table 2 compares the patient characteristics among ACS, non-ACS CAG, and noncardiac acute and chronic illness groups. Patient characteristics were comparable between the ACS and combined non-ACS group, except that HDL cholesterol levels were significantly lower and the incidence of smoking habits was significantly higher in ACS than in the combined all non-ACS group (Table 2), as shown in CAG groups alone (Table 1).

TABLE 2. Characteristics of All Enrolled Patients

Characteristics	Noncardiac Chronic Illness	Noncardiac Acute Illness	Non-ACS CAG	Combined All Non-ACS	ACS
Patients, n	60	34	347	Subtotal, 441	80
Age (mean±SD, y	67±16	54+18†	67±10	66±13	64±12
Male sex, n (%)	18 (30)‖	21 (62)	249 (67)	288 (65)	59 (74)
Risk factors, n (%)					
Hypertension	16 (27)	9 (26)	168 (48)§	193 (44)	30 (38)
Diabetes	5 (8)§	2 (6)§	114 (33)	121 (27)	26 (33)
Smoking	9 (15)‖	13 (38)	136 (39)	158 (36)*	43 (54)*
Hypercholesterolemia	25 (42)	6 (18)†	144 (41)	175 (40)	27 (34)
Lipid profile (mean±SD), mg/dL					
LDL cholesterol	127±30	101±37†	121±36	122±36	122±35
HDL cholesterol	59±18	56±17	46±14	49±16¶	41±11‡¶
Triglycerides	147±106	120±168	136±68	136±86	140±75
hs-CRP (mean±SD), ng/mL	3.14±0.58	4.17±1.02†	3.10±0.78	3.19±0.83	3.41±0.87#

Values for hs-CRP were transformed in logarithm of 10. One-way ANOVA was followed up with Tukey-Kramer pairwise comparisons among means.

*$P<0.01$ for comparison with combined all non-ACS and ACS with 2-way cross-tabulation with χ^2 test.

†$P<0.05$ for comparison with chronic illness, non-ACS CAG, and ACS.

‡$P<0.05$ for comparison with acute illness, chronic illness, and non-ACS CAG.

§$P<0.05$ for comparison with non-ACS CAG and ACS.

‖$P<0.05$ for comparison with chronic illness, non-ACS CAG, and ACS.

¶$P<0.001$ for comparison between combined all non-ACS and ACS with t test.

#$P<0.05$ for comparison with non-ACS CAG.

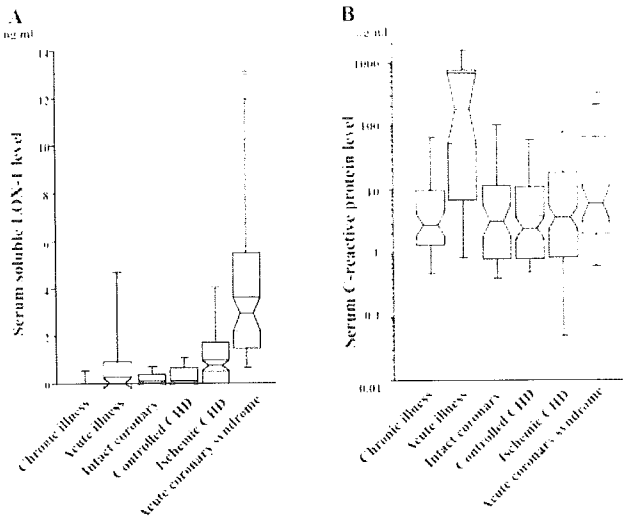

Figure 1. Serum sLOX-1 and hs-CRP levels. In 427 consecutive patients who underwent CAG, consisting of 80 with ACS, 173 with symptomatic CHD (ischemic CHD), 122 with coronary atherosclerosis without ischemia (controlled CHD), and 52 without apparent coronary atherosclerosis (intact coronary) plus 34 with noncardiac acute illness (acute illness) and 60 patients with noncardiac chronic illness (chronic illness), serum LOX-1 (A) and hs-CRP (B) levels were determined and are indicated in box plots. Center horizontal lines indicate median values; inner trapezoidal boxes, 95% CIs for medians; upper and lower edges of outer boxes, 25th and 75th percentiles; and lower and upper bars, 10th and 90th percentiles. *Statistically significant differences among the 6 groups by Kruskal-Wallis test with Dunn's test (A) and 1-way ANOVA with Tukey-Kramer test (B) ($P<0.05$). **Significant differences among 4 CAG groups by 1-way ANOVA with Tukey-Kramer test ($P<0.05$).

Serum sLOX-1 Levels

As shown in Figure 1A, serum sLOX-1 levels were remarkably higher in ACS (median, 2.91 ng/mL; range, <0.5 to 170 ng/mL) when compared among 6 groups including intact coronary (median, <0.5 ng/mL; range, <0.5 to 1.3 ng/mL), controlled CHD (median, <0.5 ng/mL; range, <0.5 to 3.4 ng/mL), ischemic CHD (median, 0.73 ng/mL; range, <0.5 to 14.0 ng/mL), acute noncardiac illness (median, <0.5 ng/mL; range, <0.5 to 6.4 ng/mL), and chronic illness (median, <0.5 ng/mL; range, <0.5 to 3.3 ng/mL). Serum sLOX-1 can discriminate ACS from other CAG groups ($\chi^2=88.2$, $P<0.001$), given a cutoff value of 1.0 ng/mL, with 81% sensitivity and 75% specificity (Table 3).

Lipid Profiles, Conventional Cardiovascular Risk Factors, hs-CRP, and sLOX-1

Serum hs-CRP levels were significantly higher in the ACS than non-ACS groups when compared among 4 CAG groups alone (Table 1 and Figure 1B). Levels of hs-CRP in patients with noncardiac acute illness were significantly higher than in any of other groups because this group contained acute inflammatory diseases (Figure 1B and Table 2). Although levels of hs-CRP in patients with ACS were significantly higher than in any of other groups when compared among CAG patients alone, ACS did not show statistically significant difference in serum hs-CRP levels when compared among all the 6 groups, including noncardiac acute and chronic illness groups (Figure 1B and Table 2).

TABLE 3. Sensitivity and Specificity of sLOX-1 and hs-CRP for ACS Among CAG Patients

	sLOX-1	hs-CRP	TnT
Non-ACS CAG (n=347)			
Positive, n	86	91	...
Specificity	75	74	...
All ACS (n=80)			
Positive, n	65	36	54
χ^2	88.2	12	...
P	<0.001	<0.001	...
Sensitivity	81	45	68
NQ-ACS (n=23)			
Positive, n	21	9	11
χ^2	43.2	1.7	...
P	<0.001	0.22	...
Sensitivity	91	39	48
ACS with TnT negative at the time of visit (n=24)			
Positive, n	20	3	...
χ^2	37.8	3.1	...
P	<0.001	0.2	...
Sensitivity	83	13	...

Cutoff values were 1.0 ng/mL for sLOX-1, 4 μg/mL for hs-CRP, and 0.03 ng/mL for TnT. ACS patients with <0.03 ng/mL TnT determined at the time of visit were defined as cases with TnT negative at the time of visit. χ^2 was determined by the Yates continuity-corrected χ^2 test, and probability values were obtained by comparison with non-ACS patients.

Significant inverse correlation was found between sLOX-1 and HDL cholesterol levels (Spearman's $\rho=-0.17$; $P<0.01$). However, no significant correlation was found between sLOX-1 and either LDL cholesterol (Spearman's $\rho=-0.02$; $P=0.68$) or triglyceride (Spearman's $\rho=-0.01$, $P=0.89$) levels. We also examined the association between sLOX-1 levels and other cardiovascular risk factors such as hypertension, diabetes, and smoking among all enrolled patients. No significant differences were found in sLOX-1 levels between those with and without hypertension, diabetes, or smoking.

Multivariable logistic regression analyses of all patients (Cox and Snell's $R^2=0.263$) showed that sLOX-1 was associated with ACS (odds ratio, 1.51; 95% CI, 1.35 to 1.70; $P<0.001$). Levels of hs-CRP, HDL cholesterol, and smoking habits also were significantly associated with ACS (odds ratio, 1.40, 0.96, and 2.07; 95% CI, 1.00 to 1.94, 0.94 to 0.98, and 1.08 to 3.96; $P<0.05$, $P<0.01$, and $P<0.05$, respectively). However, no significant correlation was found between sLOX-1 and hs-CRP levels among all patients and patients with ACS alone (Spearman's $\rho=0.01$ and -0.06; $P=0.81$ and $P=0.58$, respectively).

sLOX-1 as a Diagnostic Marker of ACS

Figure 2 shows ROC curves for the levels of sLOX-1 and hs-CRP in all 80 ACS patients (Figure 2A) and 24 patients with ACS without ST elevation or abnormal Q waves at the time of visit (NQ-ACS) (Figure 2B) compared with the 347

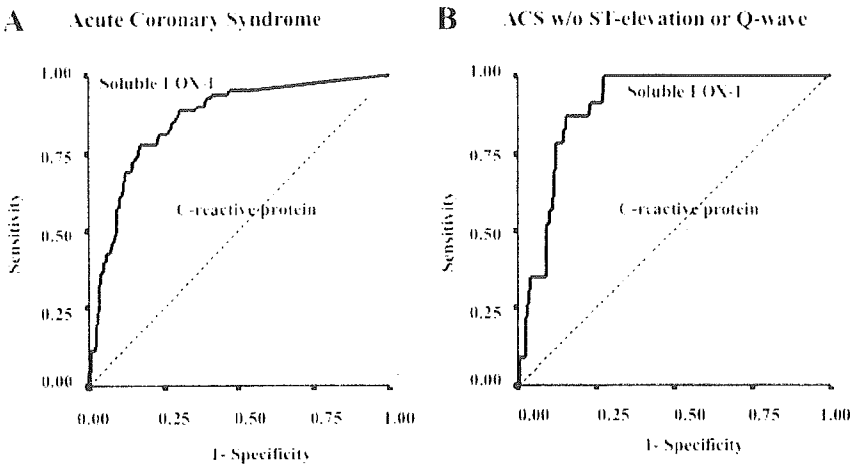

Figure 2. ROC curves of sLOX-1 and hs-CRP for diagnosis of ACS (A) and ACS without ST elevation or abnormal Q-waves (NQ-ACS; B) among consecutive patients undergoing coronary angiography. True-positive fraction (sensitivity as *y* axis) was plotted vs false-positive fraction (1 − specificity as *x* axis) by changing cutoff values for test.

non-ACS CAG patients as a reference group. In all ACS patients, the areas below the curves were 0.86 (95% CI, 0.81 to 0.90) for sLOX-1 and 0.62 (95% CI, 0.55 to 0.69) for hs-CRP. In patients with NQ-ACS, the areas below the curves were 0.90 (95% CI, 0.86 to 0.94) for sLOX-1 and 0.63 (95% CI, 0.52 to 0.74) for hs-CRP. These differences between sLOX-1 and hs-CRP (0.24 and 0.27; 95% CI, 0.20 to 0.28 and 0.21 to 0.33, respectively) are statistically significant ($P<0.05$) in both all ACS and NQ-ACS patients. Given a cutoff value of 1.0 ng/mL for sLOX-1, serum sLOX-1 can significantly discriminate ACS patients from non-ACS patients (non-ACS CAG) among consecutive patients undergoing coronary angiography ($P<0.001$) and showed 81% sensitivity and 75% specificity for the diagnosis of ACS (Table 3). In contrast, an hs-CRP cutoff value of 4 μg/mL, which had comparable specificity (74%), showed lower sensitivity (45%) for the diagnosis of ACS. Values of sLOX-1 at the time of visit efficiently discriminated patients with NQ-ACS ($P<0.001$) from non-ACS CAG with 91% sensitivity; however, sensitivity of TnT (cutoff value, 0.03 ng/mL) for diagnosis of NQ-ACS was 48%. Moreover, sLOX-1 showed 83% sensitivity for diagnosis of ACS even in patients with negative TnT (<0.03 ng/mL) at the time of visit (Table 3).

Time-Dependent Changes in sLOX-1 Concentrations After the Onset of ACS

Serum sLOX-1 and TnT were serially measured in consecutive 40 ACS patients. Figure 3A indicates relative values of serum sLOX-1 and TnT compared with the highest values among serial blood samples obtained from each individual patient. Peak levels of sLOX-1 were observed on admission or after PCI ($P<0.01$). In contrast, the highest TnT values were observed around day 1, which is consistent with previous reports ($P<0.01$).[37,38] In addition, no significant correlation was found between peak levels of sLOX-1 and CPK (Spearman's $\rho=0.28$; $P=0.10$) or TnT (Spearman's $\rho=0.20$; $P=0.20$; Figure 3B).

Discussion

Rupture of atheromatous plaques, followed by thrombus formation, is considered a crucial step in the pathogenesis of ACS. Atherosclerotic plaques with abundant lipid-laden macrophages and activated smooth muscle cells in the intima appear to be prone to rupture.[39] In such vulnerable plaques, LOX-1 is expressed prominently by smooth muscle cells and macrophages and contributes to apoptosis of smooth muscle cells[29–31] and production of matrix metalloproteinases.[32] Under these conditions, enhanced protease activities may cleave

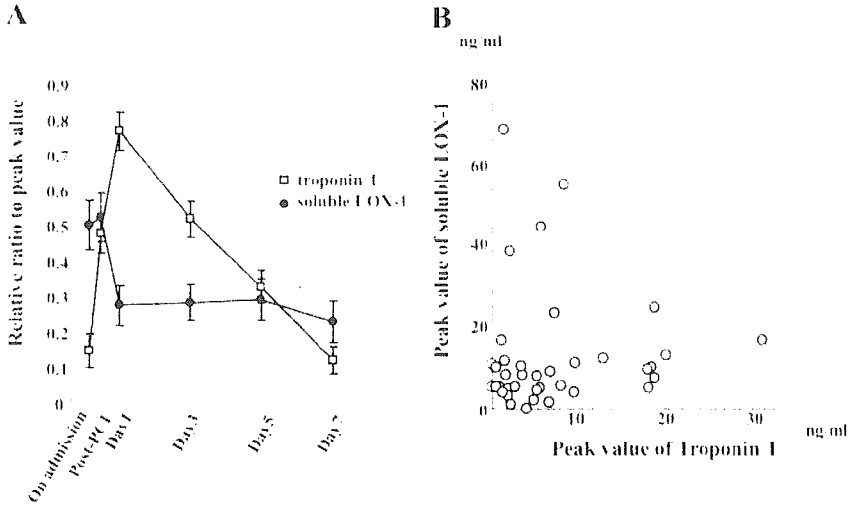

Figure 3. Time-dependent changes in sLOX-1 and TnT levels after onset of ACS (A) and comparison between peak values of sLOX-1 and TnT (B). Blood samples were collected on admission, immediately after PCI (post-PCI), and on days 1, 3, 5, and 7 from 40 ACS patients undergoing emergency PCI. Relative ratios (mean ± SEM) to peak value of each individual patient are indicated (●, sLOX-1; ○, TnT). Statistically significant correlation was not found between peak values of sLOX-1 and TnT during these periods (Spearman's $\rho=0.20$; $P=0.20$).

sLOX-1 from the surface of these vascular cells in which LOX-1 is abundantly expressed, although proteases responsible for LOX-1 cleavage have not been fully identified. Additionally, in the process of thrombus formation after plaque rupture, LOX-1 expression on the surface of platelets may also be abundant by thrombotic activation,[33] as is the case for CD40L.[14] However, LOX-1 can also bind activated platelets[40]; therefore, sLOX-1 might not be liberated from the surface of activated platelets. In fact, we did not observe significant differences in sLOX-1 levels between plasma and serum samples or high levels of circulating sLOX-1 in typical patients with disseminated intravascular coagulation (data not shown). Moreover, LOX-1 expression can be inducible in cardiac myocytes by norepinephrine or endothelin,[11] which may be upregulated by proinflammatory stimuli or ischemia. LOX-1 on the cell surface of cardiac myocytes might possibly be another source of sLOX-1.

Although LOX-1 expression was prominent in atherosclerotic lesions[29] and remarkably inducible by proinflammatory stimuli,[23,25,26] serum sLOX-1 did not reflect just general inflammation or atherosclerotic lesion sizes but rather instability of atherosclerotic plaques. In fact, sLOX-1 was elevated in the acute phases of ACS, but not in general acute inflammatory diseases in which serum hs-CRP levels were high (Figure 1). In addition, serum sLOX-1 levels were not significantly correlated with those of the inflammatory marker hs-CRP or numbers of affected coronary arteries (data not shown). Although a recent report has shown that CRP can induce LOX-1 expression,[42] LOX-1 can also be induced by a variety of biological stimuli, and regulation of LOX-1 cleavage may not be so correlated with CRP. Circulating Ox-LDL levels, which might be mildly oxidized, have been reported to be elevated in ACS, although its sensitivity or specificity for the diagnosis of ACS was not demonstrated.[9,43] The antibodies used in our ELISA can be bound to sLOX-1 in the presence of Ox-LDL; in fact, the addition of Ox-LDL to sLOX-1 samples did not affect the results of our sLOX-1 ELISA (see the Table in the online-only Data Supplement). Therefore, Ox-LDL in serum does not appear to interfere with the results of our sLOX-1 ELISA.

In addition, sLOX-1 did not show any correlation with TnT (Figure 3B) or CPK, suggesting that sLOX-1 is not a marker for cardiac necrosis or injury. Furthermore, peak time of sLOX-1 in serum was earlier than that of TnT (Figure 3A). This is quite reasonable because plaque instability or rupture precedes cardiac necrosis or ischemic injury and suggests that sLOX-1 appears to be a suitable serum marker for early diagnosis of ACS, especially NQ-ACS without severe cardiac necrosis or damage. In fact, sLOX-1 showed higher sensitivity for early detection of NQ-ACS than TnT or hs-CRP did (Table 3). Moreover, even in ACS patients without significant elevation of TnT levels (<0.03 ng/mL) at the time of visit, 86% of these TnT-negative patients showed sLOX-1 levels >1.0 ng/mL (Table 3), indicating the usefulness of sLOX-1 measurement, in addition to TnT, at the very early stage.

We currently do not know exactly when serum sLOX-1 levels begin to increase before the onset of ACS; however, sLOX-1 levels at the time of visit showed almost the peak values for each patient (Figure 3A), suggesting that serum sLOX-1 levels may begin to rise before the onset of ACS. Further large-scale prospective studies will tell us more about the value of serum sLOX-1 for predicting ACS onset.

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Original Article

Polymorphisms in Four Genes Related to Triglyceride and HDL-cholesterol Levels in the General Japanese Population in 2000

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We studied the association of six common polymorphisms of four genes related to lipid metabolism with serum lipid levels. We selected single-nucleotide polymorphisms (SNPs) in the genes for cholesteryl ester transfer protein (*CETP*), lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), and apolipoprotein CIII (*APOC3*), and studied 2267 individuals randomly selected from the participants of Serum Lipid Survey 2000. There was a significant association of *CETP* polymorphism (D442G, Int14 +1 G → A, and TaqIB), *LPL* polymorphism (S447X), and *LIPC* polymorphism (–514 → CT) with HDL-cholesterol levels. We also found a significant association of *LPL* polymorphism (S447X) and *APOC3* polymorphism (SstI) with triglyceride levels. This is the largest database showing the association of common genetic variants in lipid metabolism with serum lipid levels in the general Japanese population. Further study is necessary to elucidate the role of these gene polymorphisms in cardiovascular events. *J Atheroscler Thromb, 2005; 12: 240–250.*

Key words; Hyperlipidemia, Polymorphism, Cholesterol ester transfer protein, Lipoprotein lipase, Triglyceride lipase, Apolipoprotein CIII

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Introduction

Hyperlipidemia is a major risk factor for coronary artery disease (CAD) (1). In contrast to the sharp decline in both serum cholesterol levels and mortality from CAD in the United States and Western Europe, remarkable increases