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 \( (5.3 \pm 0.3 \mu g/mL) \) than in never-smokers \( (6.5 \pm 0.4 \mu g/mL) \). 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 \( 6.5 \pm 0.4 \mu g/mL; \) past smokers \( 5.6 \pm 0.3 \mu g/mL; \) current smokers \( 5.2 \pm 0.4 \mu g/mL; \) \( F=4.52; P=0.01 \)). 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; \( -14.5 \pm 0.6\% ; \) \( P<0.01 \)). 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.\(^20\)\(^-\)\(^22\) 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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foundering factors and without consideration of the sex difference in adiponectin level. 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 H₂O₂ 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 ethic 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. 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 homeostasis 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. 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/m²). 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 H₂O₂ 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. Seven days after the initiation of differentiation (as assessed by this criterion), 85% to 90% of the cells were judged to be differentiated. On day 7, the indicated concentrations of H₂O₂ 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 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 MiLiQ. 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-
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-\(\gamma\) (PPAR-\(\gamma\)) 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 \((5.1\pm0.2 \text{ vs. } 7.3\pm0.3 \, \mu \text{g/mL}; P<0.01)\), diabetes \((5.0\pm0.2 \text{ vs. } 6.2\pm0.3 \, \mu \text{g/mL}; P<0.01)\), and hyperlipidemia \((4.5\pm0.3 \text{ vs. } 5.8\pm0.2 \, \mu \text{g/mL}; P<0.01)\). We next performed multiple regression analysis including age, BMI, hypertension, diabetes, hyperlipidemia, and Ccr and revealed that adiponectin concentration in never-smokers was \(1.25\times\) higher than that in current smokers \((\text{never-smokers } 6.5\pm0.4 \, \mu \text{g/mL}; \text{ past smokers } 5.6\pm0.3 \, \mu \text{g/mL}; \text{ current smokers } 5.2\pm0.4 \, \mu \text{g/mL}; F=4.52; P=0.01)\).

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 \(7.0\pm1.5 \, \mu \text{g/mL}\). 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 \((-9.2\pm0.7\%)\) and 6 hours \((-13.1\pm1.2\%)\), and the maximum decrease was observed at 12 hours after smoking \((-14.5\pm0.6\%\); \(F=17.3\); \(P<0.01)\).

### Inhibitory Effects of H\(_2\)O\(_2\) and Nicotine on Expression and Secretion of Adiponectin in 3T3-L1 Adipocytes

We investigated the effect of H\(_2\)O\(_2\) and nicotine on the regulation of adiponectin secretion and gene expression in 3T3-L1 adipocytes. Incubation with H\(_2\)O\(_2\) 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\(_2\)O\(_2\) to reduce adiponectin mRNA expression and secretion into the media were antagonized by coinubcation with NAC (Figure 2). Secretion of adiponectin into the media was significantly reduced compared with control by nicotine.
at concentrations $\geq 10^{-6}$ 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$_2$O$_2$ 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$_2$O$_2$ (100 $\mu$mol/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 reduction23 and certain drugs such as PPAR-γ ligands,29 ACE inhibitors, and angiotensin II receptor blockers28 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$_2$O$_2$ are also normally produced during cellular oxidation reduction processes. Although our results showed significant cytotoxicity in adipocytes incubated with H$_2$O$_2$ at a concentration of 100 $\mu$mol/L, this cytotoxicity was significantly attenuated when they were cultured with NAC. Furthermore, H$_2$O$_2$ 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
β-adrenergic stimulation suppresses adiponectin gene expression.\textsuperscript{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,\textsuperscript{42} which is known to affect adiponectin concentration.\textsuperscript{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).\textsuperscript{44} The net effect of cigarette smoke on the function of adiponectin may be quite different from that of nicotine or H$_2$O$_2$ 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.\textsuperscript{26} On the other hand, the different localization of fat mass itself influences cardiovascular risk factors such as T-chol, TG, and HDL-chol.\textsuperscript{45} In our study, except for T-chol, the clinical characteristics were not significantly different among subjects (Table 1). Furthermore, the subjects

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Effects of H$_2$O$_2$ on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ treatment for each variable.}
\end{figure}
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.

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