tions. Read et al [10] found that thoroughly masticating food rather than merely swallowing it increased plasma glucose concentrations after the ingestion of 4 kinds of carbohydrate (sweet corn, potato, rice, and apple) in 6 healthy subjects, mostly because of improved digestibility and absorption. However, no variables other than the plasma glucose concentration during the early postprandial period were examined. The aim of the present study is to evaluate the effect of thorough mastication on postprandial plasma glucose concentrations. We used a mixed-nutrient meal of hamburger steak and rice as the test meal. Hamburger steak is a kind of processed meat, the frequent consumption of which is reported to increase the risk of type 2 diabetes [11]. The hamburger steak and rice used in this study were both readily swallowed without thorough mastication.

2. Subjects and methods

2.1. Subjects

A total of 26 volunteers (17 men and 9 women; mean age, 38.9 ± 11.5 [SD] years [range, 25-71 years]; mean body mass index [BMI], 21.8 ± 2.8 kg/m^2 [range, 16.8-26.8 kg/m^2]) participated in the study. Sixteen had normal glucose tolerance (NGT), 6 were first-degree relatives of type 2 diabetic patients, 2 had impaired glucose tolerance (IGT), and 2 had mild type 2 diabetes mellitus without pharmacotherapy. None of the subjects were taking medication known to influence glucose concentration. Subjects were classified into 2 groups, one with NGT (NGT group) and the other with a predisposition to diabetes (predisposed group), which comprised IGT, mild type 2 diabetes, and first-degree relatives of type 2 diabetic patients, and underwent 1 session of each mastication procedure. Fourteen of the 16 NGT subjects and all 10 subjects in the predisposed group underwent 75-g oral glucose tolerance test (OGTT). Subjects with fasting plasma glucose (FPG) of less than 5.6 mmol/L and HbA$_{1c}$ of less than 5.0% and/or with FPG of less than 6.1 mmol/L and 2-hour glucose of less than 7.8 mmol/L in OGTT by 1998 World Health Organization diagnostic criteria [12] were classified as NGT. IGT and type 2 diabetes also were defined according to World Health Organization criteria. Table 1 shows the clinical characteristics of the NGT and the predisposed groups. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Kyoto University, and was conducted in accordance with the Declaration of Helsinki. All subjects gave written informed consent.

Table 1
Clinical characteristics of the 26 subjects

	NGT group	Predisposed group
n (male/female)	16 (9/7)	10 (8/2)
Definition	16 NGT	6 First-degree relatives, 2 IGT, 2 type 2 diabetes
Age (y)	35.6 ± 2.1	44.1 ± 4.4
BMI (kg/m^2)	21.2 ± 0.6	23.0 ± 1.0
HbA$_{1c}$ (%)	4.6 ± 0.1	5.4 ± 0.4
FPG (mmol/L)	5.2 ± 0.1	5.8 ± 0.2
Fasting insulin (pmol/L)	57.4 ± 5.0	49.5 ± 4.3
Total cholesterol (mmol/L)	5.1 ± 0.2	5.0 ± 0.2
HDL cholesterol (mmol/L)	1.8 ± 0.1	1.5 ± 0.1
Triglyceride (mmol/L)	1.0 ± 0.1	1.0 ± 0.1

Data are means (of 2 sessions) ± SE. There were no significant differences between the NGT and the predisposed groups. HDL indicates high-density lipoprotein.

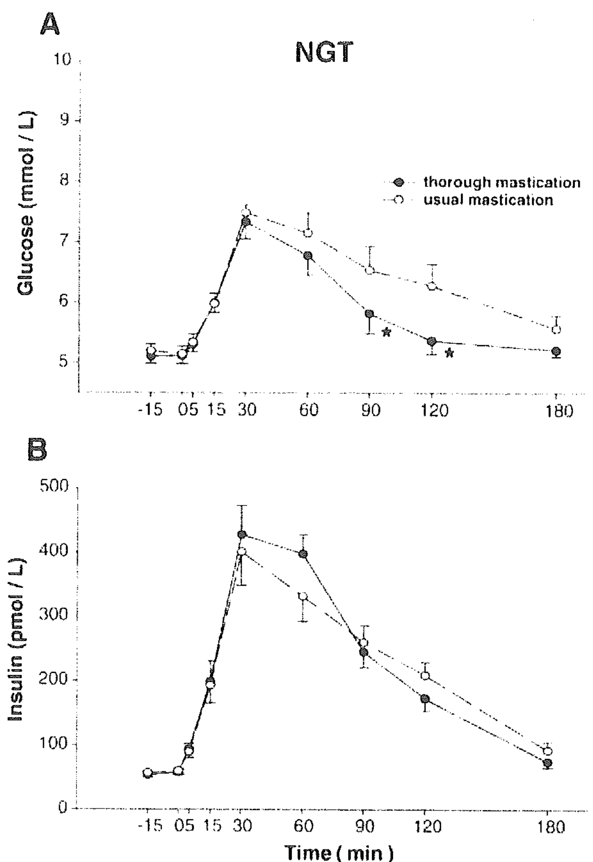

Fig. 1. Plasma glucose (A) and serum insulin (B) concentrations in the NGT group at various time points in usual and thorough mastication. Data are means ± SE, n = 16, *P < .05.

2.2. Experimental protocol

The study was a crossover experiment that included 52 sessions. After overnight fasting for at least 12 hours, the subjects began each eating session at 8:00 AM. A butterfly needle was inserted into an antecubital vein to draw blood samples at −15 minutes and was kept open by a slow drip of physiological saline. Immediately after a blood sample was drawn at 0 minute, the test meal of 130 g hamburger steak of 962 kJ (230 kcal) (Tokiwa Kanpo Pharmaceutical, Osaka, Japan) and 100 g rice of 649 kJ (155 kcal) (Hagoromo Foods, Shizuoka, Japan), with a total energy content of 1611 kJ (385 kcal) comprising 51%, 15%, and 34% carbohydrate, protein, and fat, respectively, began. Each

food item was sealed in a retort pouch and heated in a standard microwave oven for 2 minutes before the meal. The hamburger steak and rice were divided into 8 equal portions. Each subject underwent both mastication procedures. In the "usual mastication" sessions, the subjects took 16 teaspoonfuls of food, chewing each teaspoonful for 10 seconds before swallowing. In the "thorough mastication" sessions, each teaspoonful was swallowed only after 30 seconds of chewing. The rate of mastication was maintained at about 1 cycle per second in each session. Thus, thorough mastication involved 3-fold more bites than usual mastication. As the difference in the time taken eating might be a confounding factor, the subjects in usual mastication paused for 20 seconds after every 10 seconds, during which they were permitted to drink nonenergetic water, equalizing the duration of all meals at 8 minutes. The succession of mastication procedure was randomized for each subject. The average duration of the experiment was 9.9 days for male subjects. Female subjects participated only during the follicular phase of the menstrual cycle, with the interval fixed at 4 weeks to reduce variations in insulin sensitivity [13].

Blood samples for glucose and insulin were withdrawn at −15 and 0 minute before each meal and at 5, 15, 30, 60, 90, 120, and 180 minutes after each meal.

2.3. Analytical methods

Plasma glucose was measured by the glucose oxidase method using a Hitachi Automatic Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured in duplicate using LS regand Eiken insulin (Eiken, Tokyo, Japan) by automatic chemiluminescence enzyme immunoassay analyzer BCS 600 (SRL, Tokyo, Japan). The cross-reactivity to proinsulin, C-peptide, and split insulin was 0.01%, 0%, and 0%, respectively.

2.4. Data analysis

Values are expressed as mean ± SE unless otherwise noted. Statistical analysis was performed using StatView 5.0 (Abacus Concepts, Berkeley, CA). The area under the curve (AUC) was calculated according to the trapezoid rule. FPG and fasting serum insulin concentrations are the average of 2 premeal values (−15 and 0 minute). The insulinogenic index (II) [14], the ratio of the incremental serum insulin to plasma glucose concentration during the first 30 minutes after glucose ingestion calculated by OGTT (II_{OGTT}), has been commonly used as a measure of early-phase insulin secretion [15-18] since it was proposed by Seltzer et al [19] in 1967. In this study, the II during the first 30 minutes after meal tolerance test (MTT) (II_{MTT}) was calculated as the serum insulin concentration (30 − 0 minute)/plasma glucose concentration (30 − 0 minute) (pmol/mmol), and II_{MTT} and II_{OGTT} were compared. To estimate differences between 2 means, Student paired *t* test was performed with paired variates. To compare unpaired variates, Student unpaired *t* test with equal variances or Welch test with unequal variances was used. Multiple comparisons between differences among individual time points were done by analysis of variance (repeated measures) followed by Student *t* test with Bonferroni correction. Pearson *r* was used to evaluate univariate correlations. $P < .05$ was considered statistically significant.

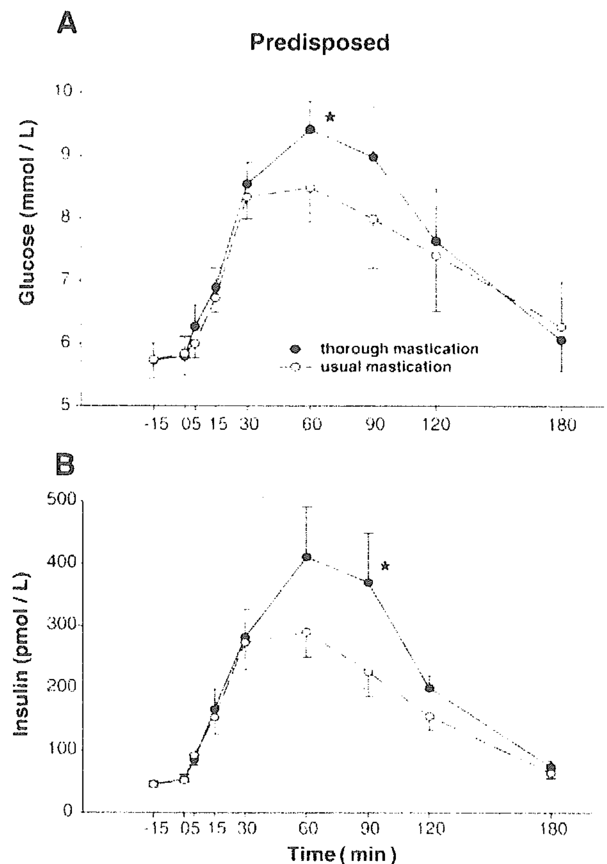

Fig. 2. Plasma glucose (A) and serum insulin (B) concentrations in the predisposed group at various time points in usual and thorough mastication. Data are means ± SE, n = 10. *$P < .05$.

Table 2
Comparison of the total AUCs (−15 to 180 minutes) for glucose and insulin in the NGT and the predisposed groups

	Glucose AUC ([mmol/L] · h) (−15 to 180 min)	Insulin AUC ([pmol/L] · h) (−15 to 180 min)
NGT group		
Usual mastication	20.6 ± 0.8	722.5 ± 60.3
Thorough mastication	19.1 ± 0.6*	722.5 ± 50.9
Predisposed group		
Usual mastication	23.7 ± 1.7	574.7 ± 65.3
Thorough mastication	24.9 ± 1.5**	755.5 ± 91.8**

Data are means ± SE.
* $P < .05$, significantly different from usual mastication in each group.
** $P < .01$, significantly different from usual mastication in each group.

3. Results

All 26 subjects completed all sessions of the test meals. Fig. 1 shows the plasma glucose and serum insulin concentrations in the NGT group in usual and thorough mastication. The plasma glucose concentration in both masticatory procedures increased in the first 30 minutes to 7.4 and 7.3 mmol/L, respectively. On the other hand, plasma glucose in thorough mastication decreased more rapidly than in usual mastication and was significantly reduced at 90 and 120 minutes (90 minutes, 5.8 ± 0.3 vs 6.5 ± 0.4 mmol/L, $P < .05$; 120 minutes, 5.4 ± 0.2 vs 6.3 ± 0.4 mmol/L, $P < .05$) (Fig. 1A). The AUC for glucose in the NGT group from −15 to 180 minutes was significantly less in thorough mastication than in usual mastication ($P = .017$) (Table 2). Insulin secretion was increased in thorough mastication from 5 minutes to nearly 90 minutes (the major difference occurring at 60 minutes: 397.5 ± 29.4 vs 332.2 ± 39.5 pmol/L) (Fig. 1B). The AUC for insulin in the NGT group from 90 to 180 minutes was significantly less in thorough mastication than in usual mastication (228.9 ± 20.1 vs 268.3 ± 23.0 [pmol/L] · h, $P = .044$). The total AUCs for insulin in the 2 mastication procedures were the same (Table 2).

The data on the predisposed group are shown in Fig. 2. During the first 30 minutes, both plasma glucose and serum insulin concentrations showed a similar pattern in the 2 mastication procedures. At 60 minutes, there was a significantly higher glucose response in thorough mastication than in usual mastication (9.4 ± 0.45 vs 8.4 ± 0.55 mmol/L, $P < .05$) (A), as well as a significantly higher insulin response at 90 minutes (370.9 ± 78.9 vs 226.0 ± 40.2 pmol/L, $P < .05$) (B). The AUCs for both glucose ($P = .008$) and insulin ($P = .002$) in the predisposed group were increased significantly in thorough mastication compared with usual mastication (Table 2).

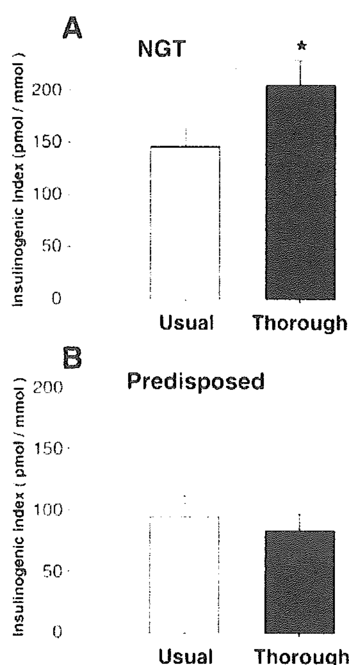

Fig. 3. II_{MTT} in the NGT group (n = 16) (A) and the predisposed group (n = 10) (B) during the first 30 minutes after meal in usual and thorough mastication. Data are means ± SE. *$P < .05$.

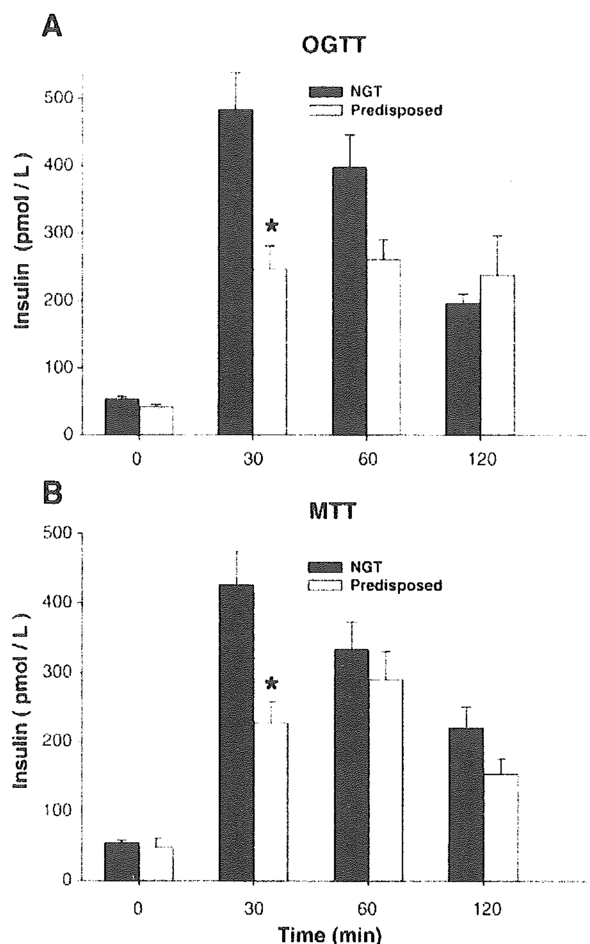

Fig. 4. Serum insulin concentrations in the NGT group (black bars, n = 14) and the predisposed group (gray bars, n = 10) measured by OGTT (A) and MTT in usual mastication (B) at 4 time points. Data are means ± SE. *$P < .05$.

Fig. 3 shows the II measured by MTT for the 2 mastication procedures in the NGT (A) and the predisposed group (B). In the NGT group, II_{MTT} in thorough mastication was significantly higher than in usual mastication (205.0 ± 27.6 vs 145.6 ± 17.7 pmol/mmol, $P = .02$) (Fig. 3A). On the other hand, there was no significant difference in II_{MTT} between the 2 mastication procedures in the predisposed group (B).

Fig. 4 shows the serum insulin concentrations at 4 time points in OGTT (A) and MTT in usual mastication (B) in the NGT group (n = 14) and the predisposed group (n = 10) in subjects who underwent both OGTT and MTT. The

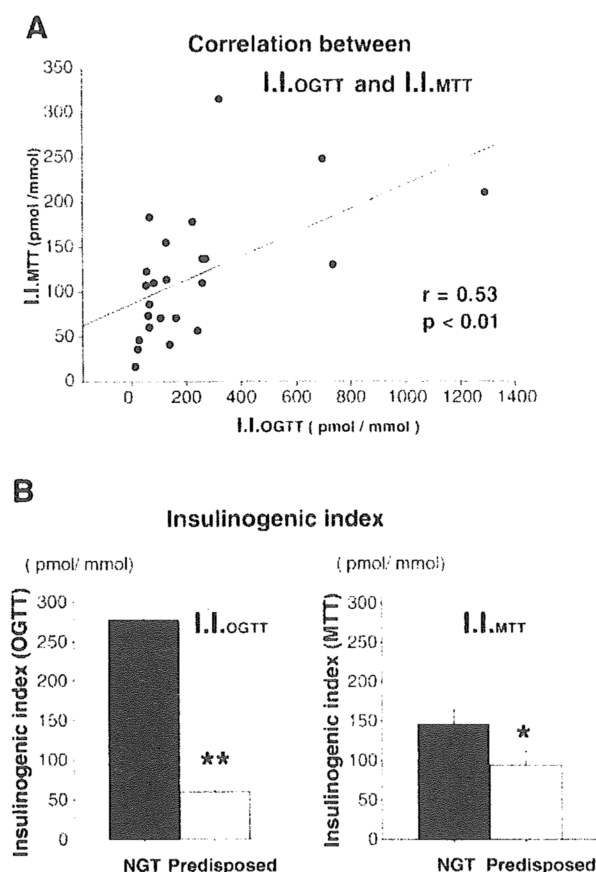

Fig. 5. A, Correlation between the II estimated from OGTT and MTT in usual mastication in 24 subjects who underwent both OGTT and MTT. B, II_{OGTT} (II during the first 30 minutes after OGTT) and II_{MTT} (II during the first 30 minutes after MTT) in usual mastication in the NGT group (n = 14) and the predisposed group (n = 10). Data are means ± SE, *$P < .05$, **$P < .01$.

serum insulin concentrations in the NGT and the predisposed groups showed a general similarity in both OGTT and MTT. The fasting serum insulin concentrations in both groups were similar in both tests. The peak serum insulin concentration occurred at 30 minutes in the NGT group and at 60 minutes in the predisposed group in both tests. The serum insulin concentration at 30 minutes was significantly higher in the NGT group than in the predisposed group in both tests (OGTT, 482.9 ± 55.2 vs 246.8 ± 34.4 pmol/L, $P < .05$; MTT, 426.2 ± 48.1 vs 227.4 ± 30.9 pmol/L, $P < .05$). There was no significant difference in the serum insulin concentration between the 2 groups at 60 and 120 minutes in both tests.

Fig. 5 shows the correlation between II_{OGTT} and II_{MTT} in usual mastication (A) and the IIs (B). II_{MTT} was significantly correlated with II_{OGTT} in the 24 subjects who underwent both tests ($r = 0.53$, $P < .01$) (A). II_{OGTT} (276.6 ± 28.0 vs 60.1 ± 11.3 pmol/mmol, $P = .004$) and II_{MTT} (145.8 ± 17.8 vs 94.0 ± 17.6 pmol/mmol, $P = .046$) were significantly higher in the NGT group than in the predisposed group (B).

4. Discussion

In this study, we compared the effects of thorough mastication on postprandial glucose and insulin secretion in subjects with NGT and subjects predisposed to type 2 diabetes. Thorough mastication was especially effective in reducing the postprandial plasma glucose concentrations in the NGT group, probably because of greater early-phase insulin secretion.

Surprisingly, the AUC for glucose was significantly less in thorough mastication compared with usual mastication, without an increase in the AUC for insulin. Mastication breaks food into small pieces, stimulates salivation, and mixes food with salivary enzymes, improving hydrolysis of carbohydrates in the mouth and stomach [10] and enhancing glycemic and insulinemic responses. Thus, thorough mastication should be expected to increase both postprandial plasma glucose and serum insulin concentrations. However, regardless of the mastication procedure, the plasma glucose and serum insulin concentration reached a peak at 30 minutes in the NGT group. In addition, II_{MTT} in thorough mastication was significantly higher than in usual mastication. Apparently, NGT subjects have sufficient early-phase insulin secretory capacity to lower the plasma glucose concentration after the more rapid absorption of glucose in thorough mastication. Thorough mastication was especially effective in NGT subjects in potentiating insulin secretion from 5 minutes (the cephalic-phase) to 90 minutes, resulting in lower plasma glucose concentrations after 30 minutes. Insulin secretion from 90 to 180 minutes was thus reduced, resulting in the same AUC for insulin in the 2 mastication procedures in the NGT group. Thus, the present study suggests that people with NGT can reduce postprandial plasma glucose concentrations by masticating food thoroughly.

In contrast to the NGT group, thorough mastication was not effective in reducing the postprandial plasma glucose concentration in the predisposed group. Compared with usual mastication, the glycemic response in thorough mastication was significantly enhanced at 60 minutes, and the insulinemic response was enhanced at 90 minutes. In addition, the AUCs for both glucose and insulin in thorough mastication were significantly greater than in usual mastication. In addition, in contrast to the NGT group, there was no significant difference in the II between the 2 mastication procedures in the predisposed group. The fact that thorough mastication did not potentiate insulin secretion in the predisposed group during the first 30 minutes suggests inability of the beta-cells to respond promptly to glucose stimulation. Thorough mastication might be expected to promote satiation with reduced food intake in ordinary life. Food intake can be reduced by a number of monoamines acting on noradrenaline, serotonin, dopamine, and histamine receptors within the hypothalamus [20]. The rate of 40 masticating cycles per minute has been shown to increase the firing rate of serotonergic neurons in cats [21]. Moreover, thorough mastication enhances satiation

independently of energy expenditure by activating neuronal histamine in the hypothalamus [22]. Accordingly, thoroughly masticating food might also benefit similarly predisposed individuals in daily life.

Early-phase insulin secretion is known to be disturbed in patients with type 2 diabetes, IGT, and normoglycemic first-degree relatives of patients with type 2 diabetes [14,23-27], so first-degree relatives of type 2 diabetic patients were included in the predisposed group. Early-phase insulin secretion in both OGTT and MTT was significantly less in the predisposed group than in the NGT group. Thus, the correlation between II_{MTT} and II_{OGTT} observed in the present study suggests that II_{MTT} calculated by the same formula as II_{OGTT} can be used as an index of early-phase insulin secretion. The II_{MTT} in the NGT and the predisposed groups of the present study were clearly different, most probably because of the difference in early-phase insulin secretion, which may underlie the altered postprandial plasma glucose concentrations. Early-phase insulin secretion is commonly referred to in both OGTT and MTT analyses [15-18]. Although the relation between the first-phase insulin response to intravenous glucose challenge and the early insulin response to oral glucose has been investigated recently [28-30], further studies are required to distinguish first- and second-phase insulin secretion sufficiently for in vivo comparison of mastication procedures. Because the plasma glucose concentrations increased gradually in this study, we used the term *early-phase insulin secretion*.

Thorough mastication was found to reduce the postprandial plasma glucose concentration mainly in the NGT group. Although the most important substance in physiological regulation of insulin release is glucose, incretin hormones (gastric inhibitory peptide [GIP] and glucagon-like peptide 1 [GLP-1]) also play important roles in postprandial insulin secretion in healthy subjects [7]. GIP and GLP-1 are released from the gut to the portal vein and are diluted when entering the systemic circulation. Only 10% to 15% of GLP-1 reaches systemic circulation and the pancreas in the intact form [31,32]. In the present study, thorough mastication elicited at most a 1.2-fold increase in the peripheral serum insulin concentration at 60 minutes in the NGT group compared with usual mastication. In a rodent study, intraportal injection of a pharmacological dose of GLP-1 was reported to evoke a peak of only a 2-fold increase in the peripheral insulin response to portal glucose compared with the control condition [33]. Accordingly, the slight change in GIP and GLP-1 in systemic circulation that may correspond to the difference in peripheral serum insulin concentrations in the NGT group would be difficult to detect by peripheral blood sampling. Further studies are required to determine whether differences in the rate of mastication affect incretin concentrations. In addition, other hormones, including glucagon, growth hormone, and cortisol, and other nutrients (amino acids) are also involved in insulin secretion upon meal ingestion [30,34]. We also measured the plasma arginine concentration. Insulin secretion is stimulated by amino acids after the digestion of protein in meal [35]. The AUC of incremental arginine from 0 to 120 minutes in thorough mastication was significantly greater than in usual mastication (data not shown). Thus, increased absorption of arginine in thorough mastication is at least partly responsible for the increased insulin secretion in NGT subjects.

The pancreas has rich innervation from both the sympathetic and parasympathetic nervous system. Sympathetic fibers are found primarily in the splanchnic nerve, whereas parasympathetic fibers are found in the vagus nerve [36]. According to the study of Rasmussen et al [37] and our previous report [38], neural factors play important roles in the normal pattern of insulin secretion. There are 2 neural stages before the late enteric stage when nutrients are absorbed [37]. During the cephalic phase in thorough mastication, the release of acetylcholine is considered to be stimulated more strongly than in usual mastication through activation of vagal-efferent fibers. Thus, during the early enteric phase, when the neurons of the enteric nervous system are activated by nutrients entering the intestine [37], thorough mastication may promote stronger release of cholecystokinin by augmenting gastric emptying. Accordingly, acetylcholine and cholecystokinin might contribute to potentiating early-phase insulin secretion in thorough mastication in the NGT group. In contrast, in the predisposed group, potentiation of early-phase insulin secretion in thorough mastication was not observed, most likely because of the poor response of the beta-cell to neural stimulation.

In addition, mastication mixes food particles with saliva. In studies comparing normal and diabetic subjects, the flow rate of saliva, the volume of saliva secreted per minute, is diminished significantly in diabetic patients [39]. Diabetic neuropathy may well account for this decrease [40]. Because the concentration of amylase in diabetic subjects has been reported to be lower [40], higher [39], and similar [41] to healthy subjects, whether improved exposure of food particles to amylase in saliva affects postprandial plasma glucose concentrations in persons with NGT remains to be determined.

Although the predisposed group was composed of 3 subgroups, first-degree relatives of type 2 diabetic patients, IGT, and type 2 diabetes, it was clearly distinguished from the NGT group in terms of early-phase insulin secretion. However, further investigation of the effect of thorough mastication in each of the subgroups with more samples would be informative.

In conclusion, in the present study, thorough mastication elicited lower postprandial plasma glucose concentrations than usual mastication in the NGT group, most probably because of the potentiation of early-phase insulin secretion. In contrast, in the predisposed group, thorough mastication did not potentiate early-phase insulin secretion and elicited higher postprandial plasma glucose concentrations.

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Three measures of tumor necrosis factor α activity and insulin resistance in nonobese Japanese type 2 diabetic patients

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Abstract

The aim of the present study was to investigate the relationship between insulin resistance and tumor necrosis factor α (TNF-α) as well as soluble TNF receptors (sTNF-R), body mass index (BMI), leptin, adiponectin, and serum lipid profile including triglycerides in nonobese Japanese patients with type 2 diabetes. A total of 88 nonobese Japanese type 2 diabetic patients were studied. The duration of diabetes was 11.0 ± 0.8 years. In conjunction with BMI, glycosylated hemoglobin (HbA1c), fasting concentrations of plasma glucose, serum lipids (triglycerides, high-density lipoprotein cholesterol, and total cholesterol), serum leptin, serum adiponectin, serum TNF-α, and soluble TNF receptors (sTNF-R1 and sTNF-R2) were also measured. Insulin resistance was estimated by the insulin resistance index of homeostasis model assessment. Insulin resistance was positively correlated with BMI, triglycerides, leptin, and total cholesterol and negatively correlated with adiponectin and high-density lipoprotein cholesterol. In contrast, insulin resistance was not associated with TNF-α, nor sTNF-R (sTNF-R1 and sTNF-R2) in our diabetic patients. There was no significant relationship between the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) and BMI, serum triglycerides, leptin, or adiponectin in these patients. From these results, it can be concluded that peripheral levels of TNF-α system activity are not a major factor responsible for insulin resistance in nonobese Japanese type 2 diabetic patients.
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1. Introduction

Type 2 diabetes mellitus is a heterogeneous syndrome characterized by insulin resistance and/or defective insulin secretion [1]. In contrast to white populations, nonobese Japanese patients with type 2 diabetes are unique in that they are divided into 2 variants: one with insulin resistance and the other with normal insulin sensitivity [2-9]. The former group is characterized by higher body mass index (BMI), higher triglycerides, higher leptin, and lower adiponectin as compared with the latter group. Whereas serum leptin level is shown to be associated with subcutaneous fat area, serum concentrations of triglycerides and adiponectin are linked to visceral fat areas in nonobese Japanese type 2 diabetic patients [7-9]. Thus, the adipose tissue linked substances are hypothesized to be associated with insulin resistance in nonobese Japanese type 2 diabetic patients.

Tumor necrosis factor α (TNF-α) is 1 of the most important candidates expressed in human adipocytes [10]. Adipocytes of obese subjects are reported to have higher rates in TNF-α messenger RNA expression and TNF-α protein production as compared with those of nonobese subjects, thus resulting in a greater serum TNF-α concentration in obese subjects [11-13]. The increase in TNF-α messenger RNA levels is positively correlated to the degree

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of hyperinsulinemia, and weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity [11-13]. It remains, however, unsolved whether the relationships between serum TNF-α and insulin resistance are caused by or are a result of obesity itself. Furthermore, it is suggested that glucose is proinflammatory and may potentially induce TNF-α. To address this, we recruited nonobese well-controlled Japanese type 2 diabetic patients carefully stratified by their resistance to insulin and explored the relationships between insulin resistance and the TNF-α system (serum TNF-α, serum-soluble TNF receptors). This is the first documented case where peripheral levels of TNF-α system activity (TNF-α, soluble TNF receptors) are not a major factor responsible for the evolution of insulin resistance, at least not in nonobese Japanese type 2 diabetic patients.

2. Subjects and methods

Eighty-eight nonobese Japanese type 2 diabetic patients who visited Kansai-Denryoku Hospital were enrolled for the present study. Type 2 diabetes mellitus was diagnosed based on the World Health Organization criteria [14]. The patients showed no evidence of acute infectious illness at the time of the study. The duration of diabetes was 11.0 ± 0.8 years (mean ± SEM) (range, 1-35 years). Seventy-six of 88 diabetic patients were taking sulfonylureas (gliclazide), and the rest were treated on a dietary regimen with no medication to alter blood glucose level. No patients have received insulin therapy. All subjects had ingested at least 150 g of carbohydrate for the 3 days preceding the study. None of the subjects had significant renal, hepatic, or cardiovascular disease. Patients did not consume alcohol or perform heavy exercise for at least 1 week before the study. Blood pressure was also measured.

Blood was drawn in the morning after a 12-hour fast. Plasma glucose was measured with glucose oxidase method. The triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were also measured. Serum insulin was measured using a 2-site immunoradiometric assay (Insulin Riabead II, Dainabot, Osaka, Japan). Coefficients of variation were 4% for insulin greater than 25 μU/mL and 7% for insulin less than 25 μU/mL. Serum leptin and adiponectin concentrations were measured with a radioimmunoassay kit (Linco Research, St Charles, Mo) as described previously [7,8]. The intra-assay and interassay coefficients of variation were less than 5% for leptin and adiponectin. Serum TNF-α concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-α immunoassay kit, R&D Systems, Inc, Minneapolis, Minn), and serum concentrations of sTNF-R1 and sTNF-R2 were measured by enzyme-linked immunosorbent assay (BIOTRAK, Amersham Life Sciences, Uppsala, Sweden), as described previously [15]. The limits of sensitivity for TNF-α, sTNF-R1, and sTNF-R2 were 0.5, 25, and 50 pg/mL, respectively. Samples for insulin, leptin, adiponectin, and TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were prepared, frozen, and stored at −70°C until the assay.

The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) was calculated with the following formula: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5 [16]. The HOMA-IR value of normal tolerant subjects was 1.6 ± 0.9 (mean ± SD), and we defined the values greater than 2.5 as an insulin-resistant state and the values less than 2.5 as an insulin-sensitive state [2,5,17]. The threshold value (2.5) for insulin resistance in our study is similar to that (2.77) in nonobese subjects with no metabolic disorders reported by Bonora et al [18]. It may be argued that the use of sulfonylureas in patients with diabetes might significantly affect the estimate of insulin resistance by HOMA, as these drugs are known to decrease fasting plasma glucose without substantially changing fasting plasma insulin [19]. It seems, however, unlikely because Bonora et al [20] and Emoto et al [21] showed that in the validation studies of HOMA, the correlation of insulin sensitivity measured by such method and that measured by the glucose clamp was not substantially different in diet-treated and sulfonylurea-treated type 2 diabetes. Another problem is that pancreatic B-cell function per se might affect HOMA-IR in Japanese type 2 diabetic patients because these patients are accompanied by mild impairments in pancreatic B-cell function [2]. In our present study, however, fasting C-peptide level was greater than 0.8 ng/mL, indicating that their pancreatic function is not severely impaired. Therefore, we used HOMA-IR in diet-treated and sulfonylurea-treated diabetic patients, taking into account pancreatic insulin secretion.

Table 1
Clinical characteristics in insulin-resistant and insulin-sensitive diabetic patients

	Insulin-resistant	Insulin-sensitive	*P*
No. of subjects	32	56	
Age (y)	61.9 ± 1.7	63.2 ± 1.1	.252
Men/women	25/7	38/18	.155
HOMA-IR	3.58 ± 0.22	1.58 ± 0.07	<.001
Diabetes duration (y)	10.7 ± 1.5	11.2 ± 0.8	.376
Smoking (no/yes)	25/7	42/14	.307
SU/diet	27/5	49/7	.343
BMI (kg/m^2)	23.7 ± 0.3	22.4 ± 0.3	.003
HbA1c (%)	7.4 ± 0.2	6.8 ± 0.1	.007
Triglycerides (mg/dL)	153 ± 12	104 ± 5	<.001
Total cholesterol (mg/dL)	214 ± 6	198 ± 5	.026
Leptin (ng/mL)	6.4 ± 0.8	4.7 ± 0.4	.018
HDL-C (mg/dL)	54 ± 2	61 ± 2	.012
Adiponectin (μg/mL)	10.7 ± 1.1	16.9 ± 1.6	.005
Fasting glucose (mg/dL)	150 ± 4	135 ± 3	.003
Fasting insulin (μU/mL)	9.8 ± 0.6	4.7 ± 0.2	<.001
Systolic blood pressure (mm Hg)	139 ± 3	135 ± 3	.107
Diastolic blood pressure (mm Hg)	86 ± 2	79 ± 1	.001
TNF-α (pg/mL)	3.70 ± 0.49	3.15 ± 0.19	.107
sTNF-R1 (pg/mL)	1132 ± 55	1208 ± 55	.185
sTNF-R2 (pg/mL)	2025 ± 88	2073 ± 67	.333

3. Statistical analysis

Data are presented as mean values ± SEM. Statistical analyses were conducted using the StatView 5 system (Statview, Berkeley, Calif). The mean values of the 2 groups were compared with Student t test. Spearman rank correlation coefficient analysis was also performed to calculate a correlation. $P < .05$ was considered as significant.

4. Results

The subjects studied were all Japanese type 2 diabetic patients (63 men and 25 women) with an age range of 43 to 84 years (62.8 ± 1.0 years) and a BMI of 17.1 to 26.7 kg/m^2 (21.0 ± 0.8 kg/m^2). They were all nonobese [22]. The fasting plasma glucose was 141 ± 3 mg/dL, and glycosylated hemoglobin (HbA1c) was 7.0% ± 0.1%. Fasting insulin level was 6.56 ± 0.39 μU/mL. Serum triglycerides, total cholesterol levels, and HDL-C levels were 121 ± 6, 204 ± 4, and 59 ± 2 mg/dL, respectively. Serum leptin and adiponectin concentrations were 5.3 ± 0.4 ng/mL and 14.6 ± 1.2 pg/mL, respectively. There was a wide variation in insulin resistance calculated by HOMA in our diabetic patients (range, 0.51-7.17; mean ± SD, 2.30 ± 0.15). Thirty-two (36%) of 88 patients had HOMA-IR greater than 2.5, indicating that they are insulin-resistant [4,5]. On the other hand, serum TNF-α, soluble TNF-R1(sTNF-R1), and soluble TNF-R2 (sTNF-R2) were 3.35 ± 0.22 (range, 1.6-15.7), 1180 ± 43 (range, 699-2920), and 2055 ± 56 pg/mL (range, 1250-3860 pg/mL), respectively.

Table 1 shows the clinical profile between insulin-resistant and insulin-sensitive type 2 diabetic patients. Compared with insulin-sensitive type 2 diabetic patients, insulin-resistant patients had significantly higher levels of BMI, HbA1c, triglycerides, total cholesterol, leptin, and diastolic blood pressure and lower concentrations of HDL-C and adiponectin. No significant difference was observed in age, sex, duration of diabetes, smoking, systolic blood pressure, and the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) between the 2 groups.

Table 2
Correlation of TNF-α, sTNF-R1, and sTNF-R2 to measures of variables in diabetic patients

	TNF-α		sTNF-R1		sTNF-R2	
	r	P	r	P	r	P
BMI	−0.062	.563	−0.013	.904	−0.159	.137
Systolic blood pressure	0.042	.712	0.208	.682	0.154	.177
Diastolic blood pressure	0.136	.233	0.006	.956	−0.009	.940
HbA1c	0.028	.790	−0.031	.769	−0.128	.233
Fasting glucose	−0.067	.948	−0.073	.496	−0.161	.133
Fasting insulin	0.048	.653	0.026	.811	−0.012	.908
HOMA-IR	0.026	.806	−0.008	.938	−0.061	.571
Triglycerides	0.082	.442	0.011	.920	−0.041	.705
Leptin	−0.204	.059	−0.004	.968	−0.093	.389
Adiponectin	−0.188	.089	0.111	.314	0.148	.180

The correlation between the 3 measures of TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) and the factors associated with insulin resistance (BMI, systolic blood pressure, diastolic blood pressure, HbA1c, fasting glucose, fasting insulin, HOMA-IR, triglycerides, leptin, and adiponectin) was next investigated in our diabetic patients (Table 2). Peripheral levels of the 3 measures of the TNF-α system (TNF-α, sTNF-R1, and sTNF-R2) were not associated with these variables.

5. Discussion

Type 2 diabetes is a heterogenous syndrome characterized by insulin resistance and/or defective insulin secretion [1]. There seems to be racial difference in insulin resistance in type 2 diabetes. Haffner et al [23] surveyed the prevalence of white type 2 diabetic patients and found that 92% of type 2 diabetic patients were insulin-resistant. Chaiken et al [24] reported that 60% of type 2 diabetic patients with BMI less than 30 kg/m^2 were insulin-resistant in African-American populations. We recently demonstrated that 40% of type 2 diabetic patients are insulin-resistant in nonobese Japanese type 2 diabetic patients [4,5]. Whereas the patients with type 2 diabetes already manifest some elements of inflammation, the intriguing feature that nonobese Japanese type 2 diabetic patients are divided into 2 variants enables us to explore whether some inflammatory markers such as TNF-α participated in the worsening of insulin resistance. We therefore investigated TNF-α and sTNF-R in nonobese Japanese type 2 diabetic patients stratified into 2 different groups: one with insulin resistance and the other with normal insulin sensitivity.

The reason why Japanese type 2 diabetic patients are not always associated with insulin resistance is unclear, but it may be due to the fact that mean BMI in our type 2 diabetic patients is 21.0 kg/m^2 less than that in white populations (average BMI 30 kg/m^2). Chang et al [25] recently reported that only 23.6% of Korean type 2 diabetic patients are insulin-resistant. Their mean level of BMI was 22.6 kg/m^2.

Using HOMA-IR and/or minimal model analysis, we have investigated the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients [2-9]. Whereas BMI and triglycerides are considered to be the most important factors responsible for the evolution of insulin resistance, regional abdominal adipose tissue distribution per se contributes to insulin resistance in nonobese Japanese type 2 diabetic patients [19]. In contrast to white and African-American populations, subcutaneous and visceral fat areas are independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [26,27]. Not only serum triglycerides but also serum leptin and adiponectin levels are shown to be associated with insulin resistance in our populations [4,5,7,8]. Serum triglycerides level is positively correlated to visceral fat area [9]. Serum leptin level is positively correlated to subcutaneous fat areas, whereas serum adiponectin level is negatively correlated to

visceral fat areas [7,8]. Furthermore, we recently demonstrated that inflammation per se is independently associated with insulin resistance in nonobese Japanese type 2 diabetic patients [6]. We subsequently found that C-reactive protein, 1 of the inflammatory markers, is not only associated with insulin resistance but also with BMI and adipocytokine such as leptin and adiponectin (data not shown). Thus, the factors underlying insulin resistance in nonobese Japanese type 2 diabetic patients are hypothesized to be linked to adipose tissue related insulin resistance.

Another candidate that is associated with adipose tissue related insulin resistance is TNF-α, a potent proinflammatory cytokine [10]. Hotamisligil and Spiegelman [28] were the first workers who proposed that TNF-α represents a key mediator of obesity-linked insulin resistance. Overexpression of TNF-α from adipose tissue is shown in different rodent models of obesity. Dandona et al [12] showed that plasma concentration of TNF-α is increased among obese subjects, and it decreases with weight loss. In vitro studies have shown that TNF-α inhibits insulin-stimulated glucose uptake in adipocytes in vitro by decreasing phosphorylation of the insulin receptor [29].

In the present study, we used serum TNF-α, soluble TNF-R1, and soluble TNF-R2 as an index of TNF-α system activity since peripheral levels of TNF receptor remain elevated for a longer time than TNF-α itself and reflect the degree of TNF-α activation more accurately than the measurement of TNF-α itself. Using the 3 measures of TNF-α system activity, we first demonstrated that TNF-α system activity is not responsible for insulin resistance, at least not in nonobese Japanese type 2 diabetic patients. This is a surprising finding because TNF-α is suggested to have a key role in the assessment of insulin resistance of obese and type 2 diabetic patients [10,28]. Thus, the reason why we could not find the relationship between insulin resistance and peripheral levels of TNF-α system in our patients is not known, but it may be due to the difference in clinical characteristics studied. The previous studies supporting the relationship between insulin resistance and TNF-α are derived from the studies dealing with the obese diabetic patients [10-13]. Obese subjects are shown to have higher concentration of TNF-α than nonobese subjects. Moreover, adipose tissue TNF-R2 messenger RNA is shown to be correlated with BMI and hyperinsulinemia in obese diabetic patients. Weight loss is accompanied by a decrease in serum TNF-α concentration and an increase in insulin sensitivity.

On the other hand, there is some literature supporting our present finding that peripheral levels of TNF-α system activity are not associated with insulin resistance in human subjects. Kellerer et al [30] found no correlation between plasma TNF-α and insulin resistance in the offspring of type 2 diabetic patients. Two investigators [31,32] have shown that administration of antibodies or antagonists to TNF-α have not improved insulin sensitivity in insulin-resistant individuals. Zavarotoni et al [33] recently demonstrated that differences in TNF-α activity do not appear to contribute to the marked variation in insulin action that occurs in healthy individuals. Ghanim et al [34] very recently showed that TNF-α is not related to HOMA-IR in obese subjects. Thus, it may be speculated that adipose tissue–linked TNF-α system activity might function locally at the level of the adipocyte in a paracrine or autocrine fashion in our study's diabetic patients. Alternatively, adipose tissue may not play a major role in the determination of peripheral levels of TNF-α system activity in our nonobese, well-controlled, unique Japanese type 2 diabetic patients.

In summary, we demonstrated for the first time that although the number of patients with type 2 diabetes is limited, peripheral levels of TNF-α system activity do not appear to be a major explanation of the mechanisms underlying insulin resistance at least in nonobese well-controlled Japanese type 2 diabetic patients. This idea can be inferred from our present study that peripheral levels of TNF-α system activity are not associated with serum leptin and adiponectin which are another index of insulin resistance in human beings [10].

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Expression of lectin-like oxidized LDL receptor-1 in smooth muscle cells after vascular injury

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Abstract

Lectin-like oxidized LDL receptor-1 (LOX-1) is an oxidized LDL receptor, and its role in restenosis after angioplasty remains unknown. We used a balloon-injury model of rabbit aorta, and reverse transcription-polymerase chain reaction revealed that LOX-1 mRNA expression was modest in the non-injured aorta, reached a peak level 2 days after injury, and remained elevated until 24 weeks after injury. Immunohistochemistry and in situ hybridization showed that LOX-1 was not detected in the media of non-injured aorta but expressed in both medial and neointimal smooth muscle cells (SMC) at 2 and 24 weeks after injury. Low concentrations of ox-LDL (10 μg/mL) stimulated the cultured SMC proliferation, which was inhibited by antisense oligonucleotides of LOX-1 mRNA. Double immunofluorescense staining showed the colocalization of LOX-1 and proliferating cell nuclear antigen in human restenotic lesion. These results suggest that LOX-1 mediates ox-LDL-induced SMC proliferation and plays a role in neointimal formation after vascular injury.
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Keywords: LOX-1; Angioplasty; Restenosis; Vascular smooth muscle cell

Oxidized low-density lipoprotein (ox-LDL) is believed to play a key role in cellular dysfunction, as well as cholesterol accumulation and subsequent foam-cell transformation in macrophages and phenotypically modulated vascular smooth muscle cells (SMC) [1]. Lectin-like oxidized LDL receptor-1 (LOX-1), a new class of oxidized LDL receptors, is expressed by intimal SMC and macrophages in advanced atheroscrelotic lesions, as well as vascular endothelial cells covering early atherosclerotic lesions [2,3]. Therefore, LOX-1 is thought to play a role in the atherogenesis and the pathogenesis of atherosclerotic plaque rupture [4] and inflammatory intramyocardial vasculopathy [5].

Percutanous coronary intervention (PCI) is an important procedure for the treatment of coronary stenosis. Sirolimus-eluting and polymeric paclitaxel-eluting stents strongly suppress neointimal hyperplasia [6,7]. However, a meta-analysis of randomized clinical trials of drug-eluting stents reported that the rate of angiographic restenosis after drug-eluting stents is 8.9% [8]. Restenosis after PCI is still a significant problem. Identifying the molecules involved in the restenosis after PCI will help to develop new strategies for the prevention of restenosis.

Although roles of LOX-1 in atherosclerotic plaque stability have been suggested [4], its role in restenosis after PCI remains unknown. Therefore, we investigated LOX-1 expression in the neointima and media of the rabbit aorta

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after balloon injury, as well as human lesions after PCI. Furthermore, in order to address the role of LOX-1 in neointimal hyperplasia after balloon injury, we performed cell culture experiments.

Methods

Rabbit aorta model. Male Japanese white rabbits weighing 3.0–3.5 kg were used and all surgical procedures on rabbits were carried out under general anesthesia with sodium pentobarbital (40 mg/kg), ketamine (2 mg/kg), and xylazine (8 mg/kg). The balloon-injury model of the rabbit aorta was created as reported previously [9]. At various time points (6, 12, and 24 h, 2, 5, and 7 days, and 2, 4, 8, 16, and 24 weeks after balloon injury), animals were anesthetized with sodium pentobarbital (40 mg/kg), ketamine (2 mg/kg), and xylazine (8 mg/kg), sacrificed with overdose sodium pentobarbital, and the aorta was carefully removed. For immunohistochemical study, three portions of the injured aorta were fixed with 10% neutral buffered formalin for 24 h and then embedded in paraffin. The specimens were cut into 10-μm thick sections and fixed to glass slides. For isolation of RNA and protein, the adventitia was stripped away from the aorta in PBS with the use of fine forceps and then snap-frozen in liquid nitrogen. Sham-operated animals were used as controls. The investigation conforms to Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the principles outlined in the Declaration of Helsinki. This study was carried out in accordance with the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

Immunohistochemical staining. Immunohistochemical staining was performed as described previously [10]. After deparaffinization and hydration of specimens, endogenous peroxidase activity was blocked and the specimen was fixed by immersion in 0.3% H_2O_2 in methanol for 20 min. Immunohistochemistry was performed using a mouse monoclonal antibody against rabbit LOX-1, a mouse monoclonal antibody against rat muscle actin (HHF-35, Enzo Diagnostics), and a mouse monoclonal antibody against human ox-LDL (donated by Dr. Itabe, Showa University, Tokyo, Japan), by use of the labeled streptavidin–biotin complex method (Histofine SAB-PO kit, Nichirei). An anti-rabbit LOX-1 monoclonal antibody was produced by immunizing mice with a recombinant rabbit LOX-1 extracellular domain and subsequent screening of the hybridomas. Immunoreactivity was confirmed by immunostaining of cells transfected with full-length rabbit LOX-1 cDNA. After blocking with 10% rabbit serum, slides were incubated overnight with a primary antibody at 4 °C in a moisture chamber. Slides were washed with Tris-buffered saline (TBS) and incubated with a biotinylated secondary antibody at room temperature for 30 min. After washing with TBS, slides were incubated with streptavidin at room temperature for 30 min and visualized with 3,3′-diaminobenzidine. The specificity of the immunoreaction was evaluated in comparison with negative control specimens in which irrelevant mouse IgG was used instead of primary antibody.

Preparation of protein extracts. Proteins were extracted for Western blot analysis as reported previously [11]. Briefly, rabbit aorta was ground to a fine powder under liquid nitrogen and incubated in ice-cold 0.1% Triton lysis solution [mmol/L: Hepes 10 (pH 7.4), sodium pyrophosphate 50, NaF 50, EDTA 5, EGTA 5, and NaCl 50; and 100 mmol/L Na_3VO_4, 0.1% Triton X-100, 500 mmol/L PMSF, and 10 mg/mL leupeptin] for 30 min. Insoluble matter was removed by centrifugation, and the protein concentration was measured by a bicinchoninic acid assay (Pierce).

Western blot analysis. Western blotting was performed with a NuPAGE Electrophoresis System (Novex) as reported previously [9]. Briefly, 10-μg protein samples were resuspended in reduced sample buffer and then electrophoresed on a 4–12% Bis-Tris gel (Novex) with Mops running buffer; blotted to nitrocellulose; and sequentially probed with polyclonal goat antisera raised against human LOX-1 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology) was then added, and secondary antibody was detected by autoradiography using enhanced chemiluminescence (ECL Plus, Amersham).

Reverse transcription-polymerase chain reaction (RT-PCR) for LOX-1 mRNA. Frozen rabbit aortas were ground to a fine powder under liquid nitrogen, and total RNA was isolated by the guanidium thiocyanate method. For the synthesis of cDNA, 1 μg of total RNA was reverse-transcribed with random hexamers using Super Script II (Gibco-BRL, Life Technologies). The transcribed cDNA was amplified by PCR with specific primers for rabbit LOX-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Two specific primer pairs corresponding to published sequences were used to amplify LOX-1 (5′-TATGCACAGGTGCTGAAGG-3′ and 5′-CAAGAGGCTCTGAAGAGAATGG-3′) [3], and GAPDH (5′-CAGGAATTCGGTGAAGGTCGGAGTCAAGGG-3′ and 5′-AGTGGATCCGGTCATGAGTCCTCCCAGGAT-3′). The PCR amplification included 35 cycles of denaturing, annealing, and elongation with Ex *Taq* polymerase (Takara Shuzo). Equal amounts of PCR product were subjected to electrophoresis through 1.5% agarose gels and visualized with ethidium bromide. Densitometric analysis was performed to quantify the PCR products using NIH Image software. GAPDH expression was used as a reference for quantification of LOX-1 mRNA.

In situ hybridization for LOX-1 mRNA. In situ hybridization was carried out with thymine–thymine (T–T) dimerized synthetic oligonucleotides complementary to a rabbit LOX-1 mRNA as a probe as reported previously [9]. A 45-base sequence (italic type) complementary to rabbit LOX-1 mRNA was chosen. A computer-assisted search (GenBank) of antisense sequences, as well as the sense sequences, revealed no significant homology with any known sequences other than that of LOX-1. For the haptenization of oligo-DNAs with T–T dimers, 2 or 3 TTA repeats were added to the 5′- and 3′-ends of the native sequences as follows: antisense probe, 5′-TTATTA*CAAGAGGCTCTGAAGAGAATGGACAACTTTTCAGGTCCTTGTCCC*ATTATTATT-3′; sense probe, 5′-TTATTA*GTTCTCCGAGACTTCTCTTACCTGTTGAAAAGTCCAGGAACAGGG*ATTATTATT-3′.

Cell culture. Rabbit SMC were isolated from the media of rabbit aorta and were cultured as previously described [9]. We confirmed that these cells were 100% positive for smooth muscle α-actin expression. Seventh passage of SMC was seeded into 48-well microplates and incubated with Medium 199 (M199; Gibco-BRL, Life Technologies) containing 10% fetal bovine serum (FBS). To make the cell growth arresting quiescent, they were incubated with M199 containing 0.1% FBS for 24 h.

Antisense phosphorothioate oligonucleotides and sense phosphorothioate oligonucleotides directed to 5′-coding sequence of the human LOX-1 mRNA were designed and manufactured by Sigma Genosys. The antisense oligonucleotides (antisense LOX-1) were synthesized at 16-mer targeted at 5′-CAGTTAAATGAGGCCG-3′part of the LOX-1 cDNA sequence. The sense oligonucleotides (sense LOX-1) were 16-mer targeted at 5′-ACCTACGTGACTACGT-3′ [12,13]. In order to examine effects of ox-LDL–LOX-1 interactions on SMC proliferation, SMC were preincubated with antisense LOX-1 or sense LOX-1 (0.5 μmol/L) containing 0.1% FBS for 48 h and then incubated with ox-LDL (10 μg/mL) containing 0.1% FBS for 24 h. We counted the cell numbers to determine the SMC proliferation. To analyze the expression of LOX-1 and proliferating cell nuclear antigen (PCNA), SMC were cultivated on chamber slides in the presence of antisense or sense LOX-1 (0.5 μmol/L) for 48 h and incubated with ox-LDL for additional 24 h. Then, immunofluorescence staining was performed using a monoclonal antibody against rabbit LOX-1 or a polyclonal antibody against PCNA. Ox-LDL was purchased from Biogenesis. All the experiments were performed in triplicate, and each experiment was repeated three times.

Human atherectomy specimens. To investigate the expression of LOX-1 in restenotic lesions from humans, we performed immunohistochemical analysis of atherectomy specimens after angioplasty with an anti-LOX-1 antibody as reported previously [9]. In order to further assess relationships between LOX-1 expression and the presence of ox-LDL in SMC of restenotic lesions, we performed immunohistochemical staining with a monoclonal antibody raised against ox-LDL. In addition, to address the role of LOX-1 in restenosis after PCI, colocalization of LOX-1 and PCNA was analyzed by double immunofluorescence staining, using a polyclonal antibody raised against PCNA (Santa Cruz Biotechnology).

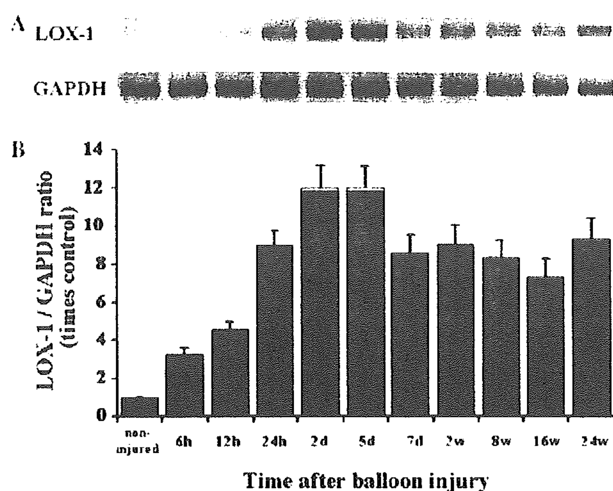

Fig. 1. Time course of LOX-1 mRNA expression after balloon injury in the rabbit aorta. Total RNA was isolated from the non-injured aorta, and the injured aorta obtained at 6 hours (6h), 12 hours (12h), 24 hours (24h), 2 days (2d), 5 days (5d), 7 days (7d), 2 weeks (2w), 8 weeks (8w), 16 weeks (16w), and 24 weeks (24w) after balloon injury. (A) A representative time course of LOX-1 mRNA expression after balloon injury by RT-PCR. (B) Quantification of LOX-1 mRNA in three independent experiments. Intensities of the bands for LOX-1 were normalized in comparison with those for GAPDH. Bars indicate fold increases relative to non-injured aorta (mean ± SD).

Statistical analysis. All calculated data are presented as means ± SD and analyzed by ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

Results

Vascular injury model of the rabbit aorta

We used a balloon-injury model of rabbit aorta in which LOX-1 mRNA and protein expression levels were analyzed at 6, 12, and 24 h, 2, 5, and 7 days, and 2, 4, 8, 16, and 24 weeks after the balloon injury. Neointima appeared 5 days after balloon injury and the neointimal area increased until 8 weeks (data not shown). We have previously shown that not only media, but also the neointima, are composed predominantly of SMC based on immunohistochemical staining for muscle actin [9].

Time course of LOX-1 mRNA expression

Based on RT-PCR analysis for the time-dependent changes in LOX-1 mRNA expression after balloon injury, LOX-1 mRNA expression was minimal in the non-injured aorta, showed a peak level 2 days after the injury (a 12-fold increase compared with non-injured aorta), declined slightly from the peak value after 7 days, and remained elevated until 24 weeks after injury (Fig. 1).

Expression of LOX-1 protein

To analyze the localization of LOX-1 protein in the balloon-injured rabbit aorta, we performed immunohistochemical staining with a monoclonal antibody raised against rabbit LOX-1 (Fig. 2A). The LOX-1 protein was

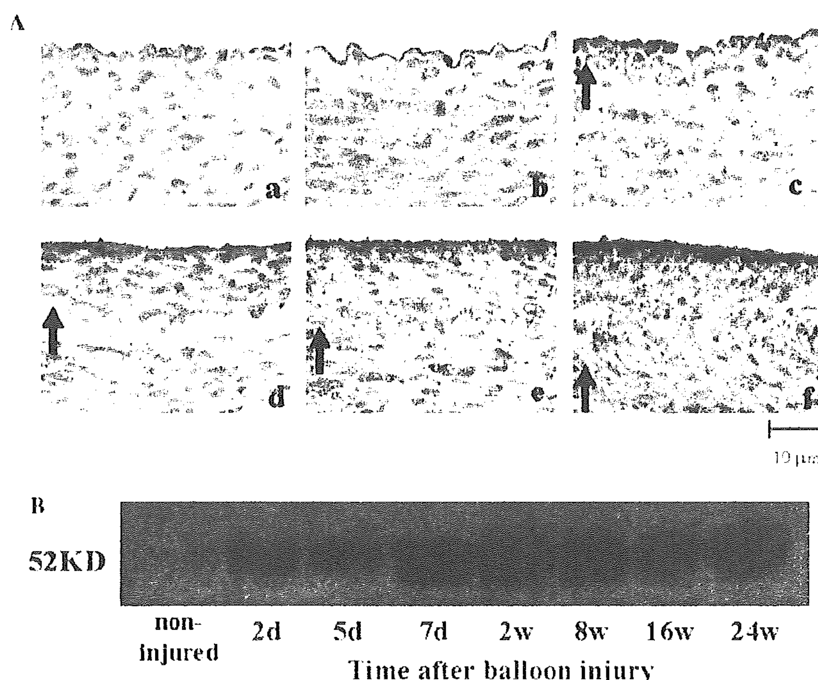

Fig. 2. Immunohistochemical staining for LOX-1 in the balloon-injured rabbit aorta. (A) Representative pictures of immunohistochemical staining. LOX-1 protein was not detected in the media of the non-injured aorta (a), but was expressed in SMC of the media at 2 days (b) after balloon injury, as well as in the SMC of the media and neointima at 5 days (c), 2 weeks (d), 8 weeks (e), and 24 weeks (f) after balloon injury. Arrows indicate the internal elastic lamina. (B) Time course of LOX-1 protein expression after balloon injury in the rabbit aorta by Western blot analysis.

not detectable in the media of non-injured aorta, but was expressed in the medial SMC 2 days after vascular injury. Five days after injury, neointimal formation was observed and LOX-1 protein was expressed in both medial and neointimal SMC. LOX-1 protein expression persisted in the medial and neointimal SMC until 24 weeks after injury.

Furthermore, in order to quantify the expression levels of LOX-1 protein after vascular injury, Western blot analysis of LOX-1 was performed. The time course of LOX-1 protein expression is shown in Fig. 2B. The expression of LOX-1 was undetectable in the non-injured aorta, but appeared at 2 days after vascular injury, gradually increased, and sustained until 24 weeks after injury.

In situ hybridization of LOX-1 mRNA

We performed *in situ* hybridization to assess whether medial and neointimal SMC produced mRNA encoding LOX-1. LOX-1 mRNA was not detected in the medial SMC of non-injured aortas, whereas LOX-1 mRNA was expressed in the medial and neointimal SMC at both 2 and 24 weeks after injury (Fig. 3), as well as in the medial SMC 2 days after injury (data not shown).

Effect of LOX-1 on SMC proliferation

In order to clarify the role of LOX-1 in neointimal formation after vascular injury, we performed cell culture experiments using cultured rabbit SMC, ox-LDL, and antisense or sense LOX-1. Using immunofluorescence staining, we analyzed the expression of LOX-1 and PCNA. Immunofluorescence staining demonstrated that ox-LDL (10 μg/mL) upregulated the expression of both LOX-1 and PCNA (Figs. 4B and F). In addition, antisense LOX-1 decreased the ox-LDL-induced expression of LOX-1 and PCNA (Figs. 4C and G); however, sense

Fig. 3. In situ hybridization of LOX-1 in the balloon-injured rabbit aorta. Using an antisense probe, LOX-1 mRNA was not detected in the media of the non-injured aorta (A), but was observed in SMC of the media and neointima at 2 (C) and 24 (E) weeks after balloon injury. Using a sense probe, no signal was detected in the non-injured aorta (B), or the aorta at 2 (D) or 24 (F) weeks after balloon injury. Arrows indicate the internal elastic lamina.

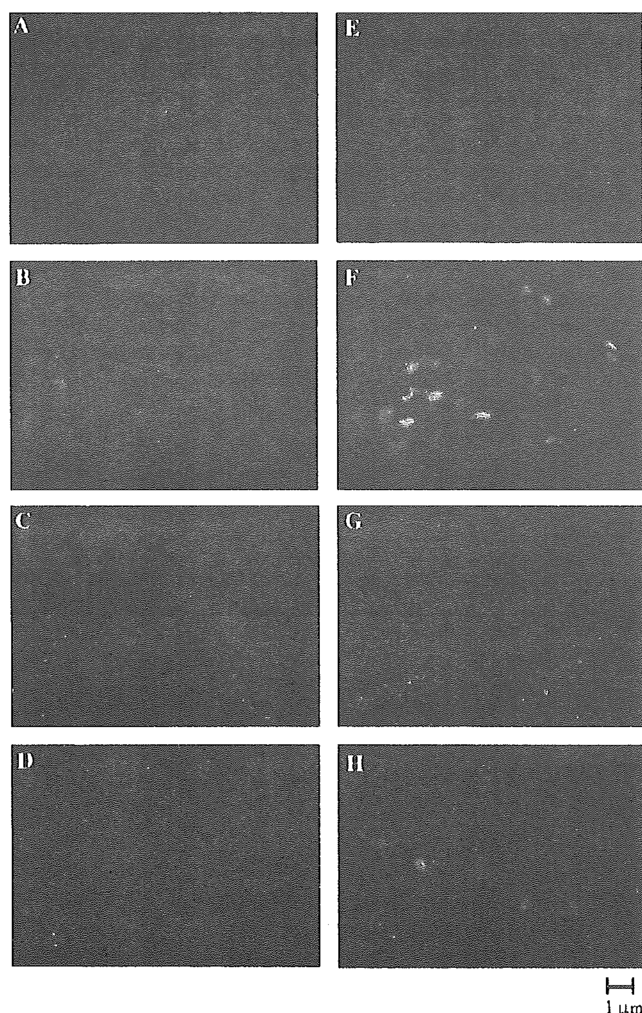

Fig. 4. Immunofluorescence staining for LOX-1 (A–D) and PCNA (E–H) in cultured SMC. (A,E) 0.1% FBS; (B,F) 0.1% FBS + 10 μg/mL ox-LDL; (C,G) 0.1% FBS + 10 μg/mL ox-LDL + antisense LOX-1; (D,H) 0.1% FBS + 10 μg/mL ox-LDL + sense LOX-1. Ox-LDL upregulated the expression of LOX-1 (B) and PCNA (F). Antisense LOX-1 decreased the ox-LDL-induced expression of LOX-1 (C) and PCNA (G); however, sense LOX-1 did not affect the ox-LDL-stimulated expression of LOX-1 (D) or PCNA (H).

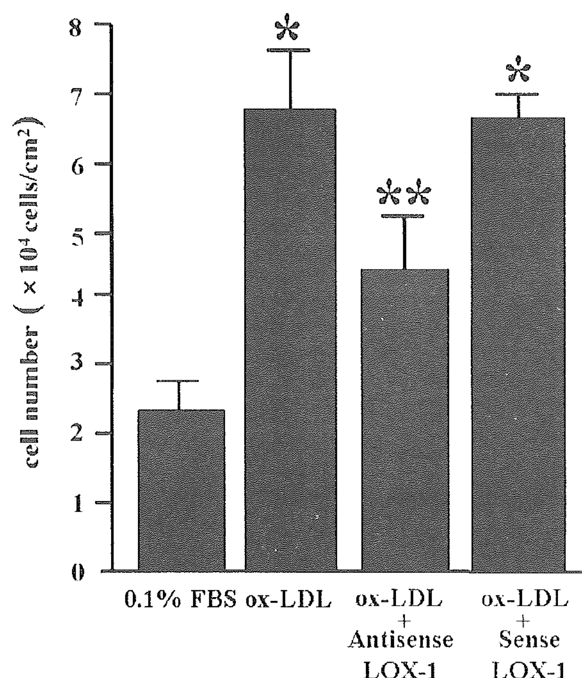

Fig. 5. Effect of antisense LOX-1 on SMC proliferation. Ox-LDL (10 μg/mL) significantly increased the number of SMC. Preincubation of SMC with antisense LOX-1, but not sense LOX-1, significantly decreased the cell number. $^*P < 0.01$ vs. 0.1% FBS. $^{**}P < 0.01$ vs. ox-LDL or ox-LDL + sense LOX-1.

LOX-1 did not affect the ox-LDL-induced LOX-1 or PCNA expression (Figs. 4D and H). Furthermore, we analyzed proliferation of SMC by counting the cell numbers of cultured SMC 3 days after stimulation by ox-LDL (Fig. 5), and found that ox-LDL (10 μg/mL) significantly increased the cell number. Preincubation of SMC with antisense LOX-1, but not sense LOX-1, significantly suppressed the ox-LDL-induced increase in the cell number.

Expression of LOX-1 in human restenotic lesion

To determine if LOX-1 was expressed in human restenotic lesions after balloon-angioplasty, we performed immunohistochemical analysis, using an anti-rabbit LOX-1 monoclonal antibody in human atherectomy specimens obtained from human coronary restenotic lesions. LOX-1 was strongly expressed in human restenotic lesions after balloon-angioplasty (Figs. 6A and B). Oxidized LDL was also detected in the intimal SMC of human restenotic lesions, suggesting colocalization of LOX-1 and ox-LDL in human coronary restenotic lesions (Figs. 6C and D). Furthermore, double immunofluorescence staining revealed a colocalization of LOX-1 and PCNA in restenotic lesion after PCI (Figs. 6E and F). It is suggested that LOX-1 appears to be involved in SMC proliferation in restenosis after PCI.

Discussion

In this study, we demonstrated that LOX-1 mRNA and protein are induced and persistently expressed in neointimal and medial SMC of the rabbit aorta for 24 weeks after balloon injury. LOX-1 protein is similarly expressed in restenotic lesions after balloon-angioplasty in humans. In cell culture experiments, the chemical inhibitor of LOX-1 reduced the SMC proliferation stimulated by relatively low concentrations of ox-LDL which was hard to induce apoptosis, although previous studies have shown that higher concentrations induce LOX-1-dependent apoptosis of SMC [14,15]. These results suggest that LOX-1 may play a role in restenosis after PCI and that LOX-1 might be a molecular target for preventing restenosis after PCI.

LOX-1 has been reported to be upregulated by proinflammatory stimuli, such as tumor necrosis factor-alpha (TNF-α) [16,17], transforming growth factor-beta (TGF-β) [18], and

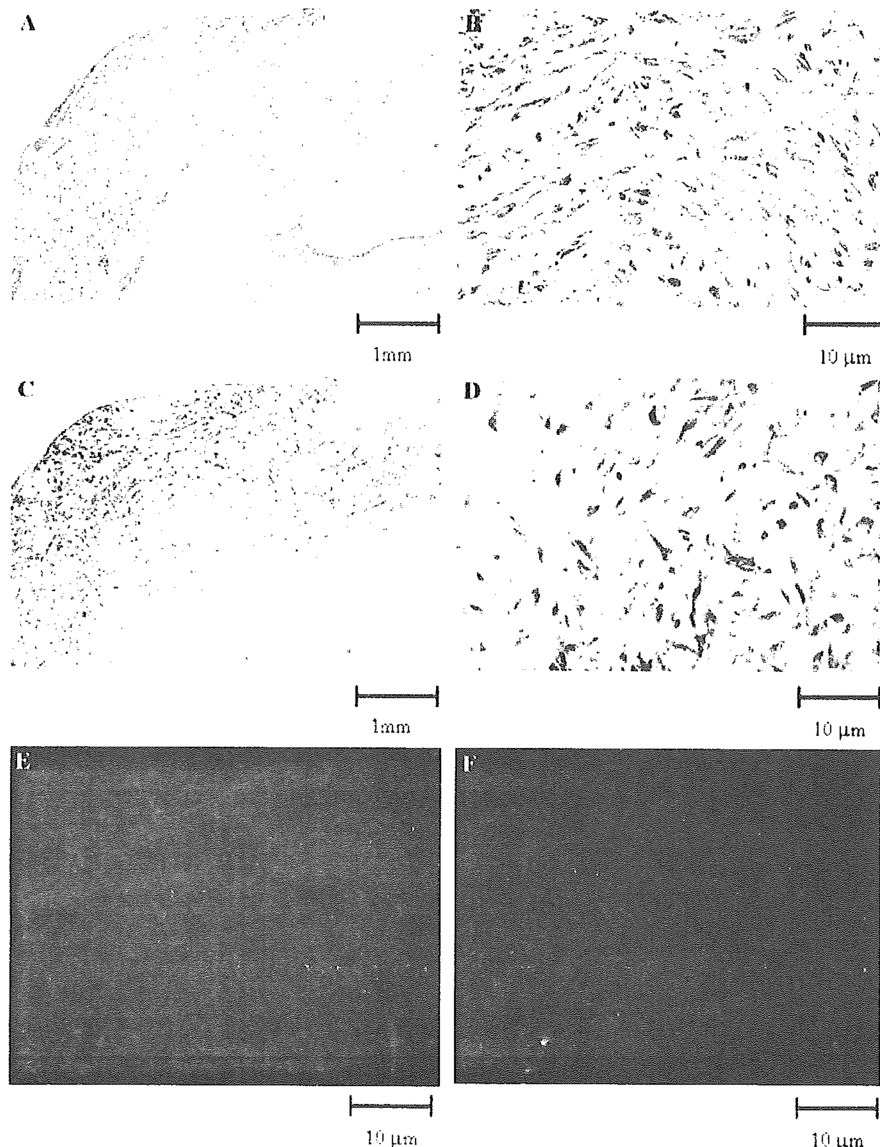

Fig. 6. Expression of LOX-1 (A,B) and oxidized LDL (C,D) in human restenotic lesions by immunohistochemistry. (A,C) Low power magnification; (B,D) high power magnification. Double immunofluorescense staining showed a colocalization of LOX-1 and PCNA in restenotic lesion after PCI (E, LOX-1; F, PCNA).

bacterial endotoxin [17], as well as by phorbol esters [16], angiotensin II [20,21], oxidized LDL [13,22], fluid shear stress [19,23], and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [24]. Specifically, angiotensin II, TGF-β, and HB-EGF are known to play roles in restenosis after balloon injury or atherogenesis of human coronary arteries. Furthermore, Mehta et al. [25,26] reported that statins and aspirine inhibit ox-LDL-mediated LOX-1 expression in endothelial cells.

In the present study, we also demonstrated that LOX-1 colocalizes with ox-LDL in human restenotic lesions after balloon-angioplasty. This suggests that the interaction between ox-LDL and LOX-1 appears to occur in human coronary restenotic lesions and that ox-LDL may be one of the stimuli that induce LOX-1 expression and smooth muscle cell proliferation after balloon injury. LOX-1 also is reported to be the receptor for apoptotic cells, in addition to ox-LDL [13].

Significant numbers of apoptotic cells are present in restenotic lesions after balloon injury [27]. Higher concentrations of ox-LDL can induce smooth muscle cell apoptosis by the interactions with LOX-1 [14,15]. Moreover, LOX-1 can act as a phagocytic receptor for apoptotic cells [28]. Therefore, LOX-1 may also play an important role in inducing and removing apoptotic cells from restenotic lesions after PCI [28].

Recently, Muscali et al. [29] reported that LOX-1 was expressed in neointima of rat common carotid artery until 2 weeks after balloon injury. Here, we demonstrated that LOX-1 was induced in neointima and media of rabbit aorta

in a time-dependent manner and its expression was sustained until 24 weeks after balloon injury. Muscali et al. did not show the direct evidence that LOX-1 is associated with SMC proliferation. Therefore, we performed the cell culture experiments in order to address the role of LOX-1 in restenotic lesion after PCI. Relatively low concentrations of ox-LDL stimulated the SMC proliferation, which was inhibited by antisense LOX-1. In addition, for the first time, we demonstrated the expression of LOX-1 and its colocalization with PCNA in human restenotic lesion by double immunofluorescense staining. Our results thus suggest that LOX-1 appears to play significant roles in proliferation of SMC.

LOX-1 has been reported to be converted into soluble molecules by proteolytic cleavage at the membrane-proximal sites of the extracellular domain [30]. Among the scavenger receptors, LOX-1 is the first molecule shown to be cleaved from the cell surface and released as a soluble molecule. Recently, soluble LOX-1 was reported to be a useful marker for early diagnosis of acute coronary syndrome [31]. We speculate that the concentration of soluble LOX-1 in the circulating plasma might also be elevated in patients with restenosis after PCI, and thus might possibly be a predictor or indicator of restenosis after PCI.

In conclusion, the present study revealed that LOX-1 is expressed by SMC in the media and neointima after balloon injury in humans and animal models in a time-dependent fashion. Furthermore, immunohistostaining and cell culture experiments demonstrated that LOX-1 appears to be involved in ox-LDL-induced SMC proliferation. LOX-1 thus may play a key role in neointimal formation and restenosis after balloon-angioplasty and may be one of the target molecules for the diagnosis and prevention of restenosis after PCI.

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Angiotensin converting enzyme inhibitor attenuates oxidative stress-induced endothelial cell apoptosis via p38 MAP kinase inhibition

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Abstract

Background: The effects of angiotensin converting enzyme (ACE) inhibitors on oxidative stress-induced apoptosis of endothelial cells and the intracellular signaling were investigated.

Methods: Cultured endothelial cells derived from a bovine carotid artery were treated with H_2O_2 or TNF-α to induce apoptosis. Apoptosis was evaluated by DNA fragmentation and cell viability, p38 MAP kinase activity by Western blotting, and oxidative stress by formation of 8-isoprostane. The effects of ACE inhibitors were examined by adding them into the medium throughout the experiments.

Results: Apoptosis was attenuated by ACE inhibitors, temocapril and captopril, in a dose-dependent manner (1–100 μmol/l). H_2O_2 (0.2 mmol/l for 1.5 h) or TNF-α (10 ng/ml for 72 h) treatment stimulated the activities of p38 MAP kinase. Temocapril and captopril decreased the activity of p38 MAP kinase as well as 8-isoprostane formation induced by H_2O_2. A p38 MAP kinase inhibitor, SB203580, partially inhibited the effect of temocapril on apoptosis.

Conclusions: These results suggest that ACE inhibitors protect endothelial cells from oxidative stress-induced apoptosis, and that p38 MAP kinase plays a critical role in the process.
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Keywords: Apoptosis; ACE inhibitor; Endothelial cell; p38 MAP kinase

1. Introduction

Stress-induced injury of vascular endothelial cells (ECs) is considered to be an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species is upregulated in vascular lesions [4,5], and that lesion formation such as endothelial dysfunction is accelerated by superoxide anion [6] and, in contrast, is attenuated by free radical scavengers including vitamin E [7] and superoxide dismutase [8].

Angiotensin converting enzyme (ACE) inhibitors effectively interfere with the renin angiotensin system and exert various beneficial actions on vascular structure and function beyond their blood pressure-lowering effects [9,10]. ACE inhibitors attenuate neointimal formation after vascular injury in animals [11] and endothelial dysfunction in humans [12]. It has been demonstrated that ACE activation induces oxidative stress [13]. However, it has not been elucidated whether ACE inhibitors could attenuate oxidative stress-induced EC apoptosis, an initial and important process in atherosclerosis [14,15].

In this study, we examined the effects of ACE inhibitors, temocapril and captopril, on H_2O_2- and TNF-α-induced EC apoptosis and the pro-apoptotic intracellular signaling, p38 mitogen-activated protein (MAP) kinase, to clarify the underlying mechanism.

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