Metabolism
Clinical and Experimental

VOL 51, NO 5 MAY 2002

Ultrasonographically Assessed Carotid Atherosclerosis in Japanese Type 2 Diabetic Patients: Role of Nonesterified Fatty Acids

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The aim of the present study was to evaluate the association of carotid atherosclerosis (intimal-medial thickness [IMT] in plaque-free segments and carotid stenosis in plaque segments) with serum nonesterified fatty acids (NEFA) in diabetic and nondiabetic patients. Fifty-one nonobese nonhypertensive Japanese type 2 diabetic patients aged 38 to 83 years (60.0 ± 1.5 years, mean ± SEM) and 23 age-matched (60.4 ± 2.2 years, $P = .439$; range, 36 to 74 years) and sex-matched nondiabetic subjects were examined. The duration of diabetes was 9.6 ± 1.0 years. Body mass index (BMI), blood pressure (systolic pressure, diastolic pressure), glycosylated hemoglobin (HbA_{1c}), and fasting concentrations of plasma glucose, serum lipids (triglycerides, total, and high-density lipoprotein [HDL] cholesterol, low-density lipoprotein [LDL] cholesterol) and serum NEFA were measured. Using high-resolution B-mode ultrasound scan, we measured IMT in plaque-free segments of bilateral common carotid arteries, and the mean of IMT in 2 vessels was used for the analysis. Furthermore, we calculated the degree of stenosis in plaque segments of bilateral common carotid arteries. The degree of carotid stenosis was expressed as a percentage ratio between the area of plaque and that of the lumen using the formula (Lumen Area − Residual Lumen) × 100. Both the areas were automatically measured by the system on a frozen transverse scanning plane at the site of maximal narrowing. When 2 or more plaques were present in the vessel, only that causing the greatest degree of stenosis was considered for analysis. Univariate regression analyses showed that mean IMT in plaque-free segments was positively correlated with age ($r = .498$, $P = .0004$) and NEFA ($r = .354$, $P = .0188$) in type 2 diabetic patients. The degree of stenosis was positively correlated to age ($r = .422$, $P = .0028$), duration of diabetes ($r = .313$, $P = .0268$) and NEFA ($r = .540$, $P = .0003$) in diabetic patients. Other variables, including BMI and lipid profile, were not associated both with mean IMT in plaque-free segments and the degree of stenosis in plaque segments in our diabetic patients. Multiple regression analyses showed that mean IMT in plaque-free segments was independently associated with age ($P = .0003$, $F = 15.2$), which explained 26.1% of the variability of IMT in our diabetic patients. The degree of stenosis was independently predicted by NEFA ($P = .0047$, $F = 8.9$), which explained 17.2% of the variability of the carotid stenosis in our diabetic patients. In contrast, mean IMT in plaque-free segments was positively correlated to age in nondiabetic subjects ($r = .450$, $P = .0347$). There was, however, no relationship between the degree of stenosis and the variables, including age and NEFA, in nondiabetic subjects. These results indicate that the factors contributing to IMT in plaque-free segments and the degree of carotid stenosis in plaque segments are different in nonobese nonhypertensive Japanese type 2 diabetic patients. IMT in plaque-free segments was independently associated with age both in nondiabetic and diabetic subjects, whereas the serum NEFA level independently predicted the degree of stenosis in plaque segments in our diabetic patients, while not in nondiabetic subjects. Thus, NEFA is considered to be one of the new risk factors responsible for the progression of carotid atherosclerosis in nonobese nonhypertensive Japanese type 2 diabetic patients.

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Submitted October 9, 2000; accepted November 19, 2001.

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0026-0495/02/5105-0002$35.00/0
doi:10.1053/meta.2002.31970

DIABETES IS ASSOCIATED with a greater risk of morbidity and mortality from cardiovascular disease. Coronary heart disease (CHD) is the leading cause of death among people with diabetes. Mortality from CHD and the incidence of nonfatal CHD events are 2 to 4 times higher in patients with type 2 diabetes than in age-matched, nondiabetic subjects.[1,2] Therefore, an effort to reduce the risk of CHD through evaluation of risk factors is a primary focus when caring for diabetic patients. Several factors have been found to be associated with an increased risk of major manifestations of CHD. One of the major factors is abnormality in lipids and lipoproteins. Many previous studies have shown that total cholesterol, especially low-density lipoprotein (LDL) cholesterol, is associated with atherosclerosis. Kugiyama et al[3] recently demonstrated that fasting remnant lipoprotein levels predict coronary events in patients with CHD.

Fatty acids seem to participate in the development and progression of atherosclerosis. Hoak et al[4] found thrombosis to be associated with mobilization of fatty acids. Botti et al[5] found that long-chain saturated fatty acids promote clotting. Connor et al[6] reported the induction of fatal occlusive thrombi within minutes of infusing fatty acids. Although type 2 diabetes is frequently associated with atherosclerosis and high serum nonesterified fatty acids (NEFA),[7] there is little in the literature dealing with the relationship between the degree of atherosclerosis and fatty acids in type 2 diabetic patients.

The degree of atherosclerosis can be evaluated by high-resolution B-mode ultrasound scan. This is a reliable noninvasive method for the assessment of carotid atherosclerosis.[8] Carotid atherosclerosis is important in view of its relationship to cerebrovascular ischemic diseases and to coronary atherosclerosis.[9] It is recognized that the degree of overweight, hypertension, or hyperglycemia per se is associated with atherosclerosis. To accomplish this, we recruited nonobese, nonhypertensive, well-controlled unique Japanese type 2 diabetic patients who had no evidence of cardiovascular disease or ischemic stroke and investigated the relationships between NEFA and the degree of carotid atherosclerosis using high-resolution B-mode ultrasound scan. Age- and sex-matched nondiabetic subjects served as controls.

MATERIALS AND METHODS

A total of 51 type 2 diabetic patients without hypertension who visited Kansai-Denryoku Hospital were enrolled in the present study. Twenty-three age- and sex-matched nondiabetic subjects served as controls. They all were nonobese Japanese subjects.[10] Type 2 diabetes mellitus was diagnosed based on the criteria of the World Health Organization (WHO).[11] Twenty-six of the 51 diabetic patients were taking sulfonylureas (gliclazide), and the rest were treated with diet alone. None of these diabetic 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. They did not receive any medications affecting lipid metabolism. They did not consume alcohol or perform heavy exercise for at least 1 week before the study.

The blood was drawn in the morning after a 12-hour fast. Plasma glucose was measured with the glucose oxidase method. The triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol were also measured. The range of triglyceride level was 34 to 346 mg/dL in our present study. The LDL cholesterol was calculated using the Friedewald formula.[12] Serum NEFAs were measured in duplicate using an enzymatic method (NEFA HR kit; Wako Chemicals, Osaka, Japan), and the mean of the 2 values was used.[13] The coefficient of variation (CV) for NEFA was 2%. Blood pressure was measured twice in the sitting position, and the mean of the 2 values was used.

A carotid sonography was performed with high-resolution B-mode scanning equipment (Logic 500 GE; Yokogawa, Milwaukee, WI) with a 7.5-MHz sector scanner probe. The common carotid arteries of both sides were examined with longitudinal and transverse scans, because we could not analyze the internal and external carotid arteries fully in all patients. The CV for interobserver variability was found to be 8.5%, and the CV for intraobserver variability was 6.0%. The intimal plus medial thickness (IMT) of the common carotid artery was measured in plaque-free segments as the distance from the leading edge of the first ehogenic line, corresponding to the lumen-intimal interface, to that of the second echogenic line, corresponding to the collagen-contained upper layer of tunic adventitia.[14] The mean of IMT in plaque-free segments of bilateral common carotid arteries was used for the analysis. The degree of stenosis was also measured in the plaque segments of bilateral common carotid arteries. It was calculated as a percentage ratio between the area of the plaque and that of the lumen using the formula (Lumen Area − Residual Lumen) × 100.[15] Both the areas were automatically measured by the system on a frozen transverse scanning plane at the site of maximal narrowing. When 2 or more plaques were present in the vessel, only that causing the greatest degree of stenosis was considered for analysis.

Statistical Analysis

The statistical analyses were conducted using the StatView 5 system (Statview, Berkeley, CA). Simple (Spearman's rank) correlation coefficients between the degree of carotid atherosclerosis (IMT, carotid stenosis) and measures of variables were calculated, and a stepwise multiple regression analysis was then used to evaluate the independent association of these variables with the degree of carotid atherosclerosis. Data were presented as means ± SEM unless otherwise stated. $P < .05$ was considered significant. In multivariate analysis, F value ≥ was considered significant.

RESULTS

The clinical characteristics of the 51 patients (38 men and 13 women) and 23 nondiabetic subjects (13 men and 10 women) are listed in Table 1. There was no significant difference in sex, age, BMI, and systolic and diastolic blood pressure between the

Table 1. Clinical Profile of Diabetic and Nondiabetic Subjects

Clinical Characteristics	Diabetic	Nondiabetic	*P*
No.	51	23	–
Sex (M/F)	38/13	13/10	.063
Age (yr)	60.0 ± 1.5	60.4 ± 2.2	.439
BMI (kg/m^2)	22.6 ± 0.3	22.6 ± 0.5	.499
Systolic blood pressure (mm Hg)	124 ± 2	122 ± 3	.274
Diastolic blood pressure (mm Hg)	72 ± 1	71 ± 2	.362
Fasting glucose (mg/dL)	154 ± 5	93 ± 2	<.001
HbA_{1c} (%)	7.1 ± 0.2	5.2 ± 0.1	<.001
Triglycerides (mg/dL)	121 ± 8	114 ± 3	.342
Total cholesterol (mg/dL)	190 ± 4	199 ± 4	.080
HDL cholesterol (mg/dL)	51 ± 2	56 ± 2	.082
LDL cholesterol (mg/dL)	115 ± 3	121 ± 4	.128
NEFA (mEq/L)	0.63 ± 0.03	0.52 ± 0.05	.037
Mean IMT (mm)	0.71 ± 0.02	0.70 ± 0.02	.379
Degree of carotid stenosis	8.5 ± 2.2	2.0 ± 1.2	.028

Table 2. Correlation of IMT in Plaque-Free Segments to Measures of Variables in Diabetic and Nondiabetic Patients

	Diabetic			Nondiabetic	
	Univariate		Multivariate	Univariate	
	r	*P*	*F*	*r*	*P*
Age	.498	.0004	15.2	.450	.0347
NEFA	.354	.0188	3.1	.226	.3119
Sex	.215	.1284		.187	.3813
Diabetes duration	.111	.4342		–	–
BMI	−.103	.4660		−.070	.7444
FBS	.063	.6537		−.288	.1771
HbA_{1c}	.083	.5559		−.228	.2859
Triglycerides	−.182	.1998		.009	.9652
Total cholesterol	−.040	.7793		−.041	.8471
HDL cholesterol	.131	.3530		.095	.6553
LDL cholesterol	.090	.5268		−.201	.3451
Systolic blood pressure	.091	.5257		−.105	.6220
Diastolic blood pressure	−.031	.8287		−.307	.1499

2 groups. Fasting glucose and glycosylated hemoglobin (HbA_{1c}) levels were significantly higher in diabetic patients as compared with nondiabetic subjects. Serum triglyceride level was higher in diabetic patients than in nondiabetic patients, but it was not statistically significant. Total, HDL, and LDL cholesterol concentrations were similar between the 2 groups. On the other hand, serum NEFA concentration was significantly higher in diabetic patients than in nondiabetic subjects (0.63 ± 0.03 *v* 0.52 ± 0.05 mEq/L, $P = .037$). Mean IMT in plaque-free segments was higher in diabetic patients than in nondiabetic subjects, but was not statistically significant (0.71 ± 0.02 *v* 0.70 ± 0.02 mm, $P = .379$). In contrast, the degree of carotid stenosis (% stenosis) in diabetic patients was significantly higher than in nondiabetic subjects ($8.5\% \pm 2.2\%$ *v* $2.0\% \pm 1.2\%$, $P = .028$).

Spearman's rank correlations of mean IMT in plaque-free segments or the degree of carotid stenosis with measures of variables were next calculated for diabetic and nondiabetic subjects. IMT in plaque-free segments in diabetic patients was positively correlated with age and NEFA (Table 2). The degree of stenosis was positively correlated to age, duration of diabetes, and NEFA in diabetic patients (Table 3). Whereas IMT in plaque-free segments was positively correlated to age, the degree of stenosis was not associated with any variables, including age and NEFA, in nondiabetic subjects studied (Tables 2 and 3).

Multiple regression analyses were performed using the stepwise procedure in diabetic patients (Tables 2 and 3). The analysis included IMT or the degree of stenosis as dependent variable and candidate risk factors (age, NEFA) as independent variables. IMT in plaque-free segments was independently predicted by age ($P = .0003$, $F = 15.2$), which explained 26.1% of the variability of IMT in our diabetic patients. In contrast, the degree of stenosis was independently associated with NEFA ($P = .0047$, $F = 8.9$), which explained 17.2% of variability of the carotid stenosis in our diabetic patients. Other variables, including BMI and lipid profile, were not associated with both IMT in plaque-free segments and the degree of carotid stenosis in our nonobese, nonhypertensive Japanese type 2 diabetic patients.

DISCUSSION

Our main observations in the present study were that although age was independently associated with IMT in plaque-free segments of carotid artery both in diabetic and nondiabetic patients, NEFA affected the degree of carotid atherosclerotic plaque in diabetic patients, while not in nondiabetic subjects. This is the first description that NEFA is associated with the development and progression of atherosclerosis in type 2 diabetic patients.

Regarding the risk factors responsible for the evolution of atherosclerosis in diabetic patients, Bierman[16] previously estimated that typical risk factors including smoking, cholesterol, and blood pressure can account for no more than 25% to 30% of the excess cardiovascular risk factors in diabetic patients. This suggests that other factors might play a key role in the progression of atherosclerosis in diabetes. One of the candidates is the disturbance of lipid mobilization and metabolism. Atherothrombotic changes and high serum NEFA frequently accompany type 2 diabetic patients.[7] We confirmed in the present study that both the degree of carotid stenosis and serum NEFA were significantly higher in diabetic patients than in nondiabetic subjects.

There are some data in the literature suggesting that fatty acids are associated with atherosclerosis. Hoak et al[4] found thrombosis to be associated with mobilization of fatty acids. Botti et al[5] found that long-chain saturated fatty acids promote clotting. Connor et al[6] reported the induction of fatal occlusive thrombi within minutes of infusing fatty acids. To the best of our knowledge, however, the relationship between atherosclerosis and serum NEFA has not yet been examined in diabetic patients. In the present study, we selected nonobese, nonhypertensive, well-controlled unique type 2 diabetic patients (mean HbA_{1c} 7.1%) who had no evidence of cardiovascular disease or

Table 3. Correlation of the Degree of Stenosis to Measures of Variables in Diabetic and Nondiabetic Patients

	Diabetic			Nondiabetic	
	Univariate		Multivariate	Univariate	
	r	*P*	*F*	*r*	P
NEFA	.540	.0003	8.9	−.040	.8596
Age	.422	.0028	2.8	−.001	.9953
Diabetes duration	.313	.0268	0.1	–	–
Sex	.117	.4078		−.339	.1119
BMI	−.247	.0804		−.269	.2078
FBS	.160	.2578		.143	.5026
HbA_{1c}	.211	.1366		.260	.2228
Triglycerides	−.029	.8351		.001	.9968
Total cholesterol	.053	.7101		−.087	.6831
HDL cholesterol	.110	.4380		.258	.2265
LDL cholesterol	.086	.5430		−.129	.5445
Systolic blood pressure	.075	.6001		.003	.9905
Diastolic blood pressure	−.075	.5975		−.025	.9053

ischemic stroke, taking into account any conditions affecting atherosclerosis and serum NEFA. The degree of overweight, hypertension, and hyperglycemia per se is known to affect atherosclerosis and serum NEFA level in man. As an index of atherosclerosis, we evaluated IMT in the plaque-free segments and carotid stenosis (% stenosis) in the segments of plaque using high-resolution B-mode ultrasound scan. We also investigated common carotid artery, because we could not fully observe the internal and external carotid artery.

Our present study disclosed that age was independently associated with IMT in plaque-free segments both in diabetic and nondiabetic patients. However, the effect of NEFA on the degree of carotid stenosis in plaque segments was different between diabetic and nondiabetic patients. NEFA was independently associated with carotid atherosclerotic plaque in type 2 diabetic patients, while not in nondiabetic subjects.

The mechanism by which NEFA affects the degree of carotid atherosclerotic plaque in our diabetic patients is not known at present. One possible explanation is that fatty acids act as energy sources of macrophage in atherosclerotic plaque. Macrophage is able to use not only glucose and glutamine, but also utilize fatty acids as energy sources. However, complete oxidation of glucose and glutamine is limited in macrophage.[17] Thus, fatty acids may constitute the crucial fuel for activated macrophage energy expenditure in the energy-limited environment of the atherosclerotic plaque. Another possible explanation is that fatty acids are associated with increased coagulation and/or decreased fibrinolytic abnormalities. Didisheim et al[18] previously reported that saturated long-chain fatty acids activate Hageman factor (factor XII). Hageman factor initiates the cascade sequence of enzymatic reactions, culminating in the production of thrombin and the conversion of fibrinogen to fibrin. Thrombin also induces an increase in fibrinogen biosynthesis. Pilgeram and Pickart[19] have shown that free fatty acids, unbound by protein, stimulate the rate of biosynthesis of fibrinogen in vitro. Schneider et al[20] later demonstrated that free fatty acids had synergistic effects on insulin-stimulated increase in plasminogen activator inhibitor type I (PAI-I) in blood of type 2 diabetic patients. Alternatively, another factor might work the mechanism. Kwok et al[21] showed that linoleic acid and oleic acid increased the endothelin-1 binding and action in cultured rat aortic smooth muscle cells. Hennig et al[22] disclosed that linoleic acid and other omega-6 fatty acids appear to be the most proinflammatory and atherogenic fatty acids.

The reason why NEFA was associated with carotid atherosclerosis in diabetic patients, but not in nondiabetic subjects, is still unknown at present. Serum NEFA level was significantly higher in diabetic patients than in nondiabetic subjects. Thus, higher NEFA level might be required to develop or promote carotid atherosclerosis in man.

Finally, we could not find an association between carotid atherosclerosis and conventional risk factors, including LDL cholesterol, in our unique populations. The reason is unclear, but it may mean that the diabetic state per se is such a powerful factor on carotid atherosclerosis that the effect of other risk factors is masked.[23] Alternatively, it could be due to the clinical characteristics studied. Our patients were well-controlled unique type 2 diabetic patients (mean HbA_{1c} 7.1%) who had no evidence of obesity, hypertension, cardiovascular disease, or ischemic stroke.[24] This idea is supported from the recent study shown by Mohan et al[23] that diabetes and age, but not conventional risk factors, are the most important risk factors associated with increased IMT in South Indian diabetic patients with a BMI of 24.5 kg/m^2.

In summary, we first demonstrated that serum NEFA is associated with carotid atherosclerosis in nonobese, nonhypertensive, well-controlled unique Japanese type 2 diabetic patients, while not in nondiabetic subjects. The mechanism by which NEFA is associated with carotid atherosclerosis is not clarified, but our present study might imply that NEFA predicts the degree of carotid atherosclerotic plaque in nonobese, nonhypertensive, Japanese type 2 diabetic patients. Further study should be undertaken to confirm the validity of our findings.

ACKNOWLEDGMENT

We are very grateful to Chiaki Taniguchi and Nina Hayasaki for their help in our research.

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