OBSERVATIONS

Postprandial Hyperglycemia Is a Better Predictor of the Progression of Diabetic Retinopathy Than HbA_{1c} in Japanese Type 2 Diabetic Patients

It is well known that postchallenge and postprandial hyperglycemia are related to the progression of diabetic macroangiopathy (1–6). However, there is little information regarding the association between diabetic microangiopathy and postprandial hyperglycemia in human subjects. In this study, we performed a follow-up study to elucidate the relationship between diabetic retinopathy and postprandial glycemia or insulinemia.

We recruited 151 Japanese patients with type 2 diabetes (74 men, aged 58.1 ± 10.2 years, and 77 women, aged 57.9 ± 9.2 years) who were admitted to Osaka Prefectural General Hospital between 1 January 1995 and 31 December 1999. The mean duration of diabetes of these patients was 7.4 ± 6.7 years. The mean BMI and HbA_{1c} (A1C) were 25.7 ± 4.4 kg/m^2 and 8.15 ± 1.51%, respectively. Eighty-three patients were treated with diet alone and 65 with oral hypoglycemic agents. Two to 4 days before admission, patients were given an oral glucose load of 75 g and postchallenge plasma glucose and insulin levels were determined 2 h later. Within 2–4 days after admission, postprandial plasma glucose and insulin levels were determined 2 h after the intake of an isocaloric mixed breakfast (10 kcal/kg body wt; 57% carbohydrate, 15% fat, and 28% protein), representative of a standard Japanese breakfast. Within a week after admission, retinopathy was assessed through dilated pupils by ophthalmologists. One hundred twenty-one subjects showed no evidence of diabetic retinopathy, 23 simple diabetic retinopathy, and 7 preproliferative retinopathy or proliferative retinopathy. After discharge from the hospital, subjects were followed prospectively for 5.0 ± 1.5 years.

During the follow-up periods, diabetic retinopathy worsened in 34 patients. Since A1C, postprandial plasma glucose and insulin, postchallenge plasma glucose and insulin, fasting plasma glucose and insulin, and duration of diabetes are closely linked, we performed multiple logistic model analyses, including sex, smoking, blood pressure, and serum lipid profile, to identify the independent and important predictors of the progression of diabetic retinopathy. Also, it is noted that in multiple regression analyses, the significance of several factors can be lost when there is a very close correlation among these several factors in addition to another factor having a stronger correlation. Postprandial plasma glucose levels (odds ratio 1.008, $P = 0.016$) correlated with the progression of diabetic retinopathy, and the significance in correlation between A1C levels and the progression of retinopathy was lost in our multiple regression analyses. These results indicate that postprandial hyperglycemia is a stronger predictor of the progression of diabetic retinopathy than A1C in Japanese type 2 diabetic patients. In addition, postprandial plasma insulin levels independently correlated with the progression of diabetic retinopathy (0.918, $P < 0.0001$) (Fig. 1). Thus, we assume that the control of excessive glucose excursions, especially in the postprandial state, may provide clinical benefit on not only carotid atherosclerosis but also diabetic retinopathy (7).

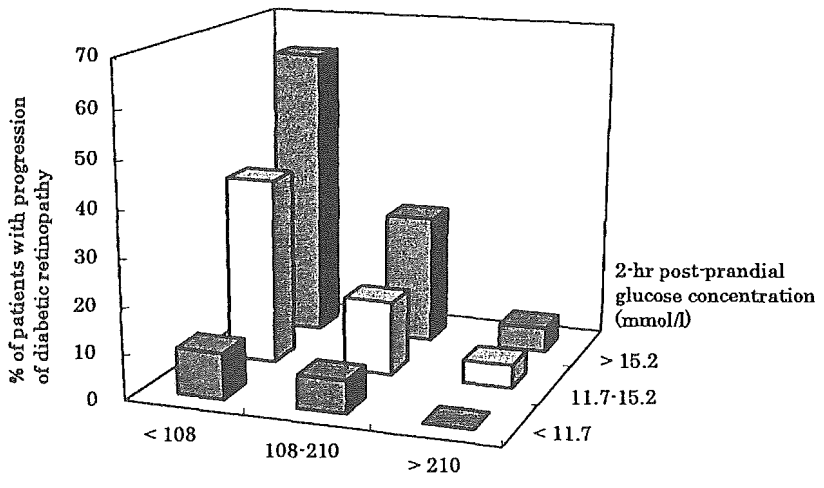

Figure 1—*Effects of 2-h postprandial insulin and glucose concentrations on the progression of diabetic retinopathy during a 5-year follow-up period according to tertiles of 2-h postprandial insulin and glucose concentrations.*

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Post-prandial hyperglycemia is an important predictor of the incidence of diabetic microangiopathy in Japanese type 2 diabetic patients ☆

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Abstract

Diabetic microangiopathy is often observed in diabetic patients, but there is little evidence regarding the relationship between post-prandial glycemia or insulinemia and the incidence of diabetic microangiopathy. In this study, to elucidate the relationship between post-prandial glycemia (or insulinemia) and diabetic microangiopathy, we performed a cross-sectional study of 232 subjects with type 2 diabetes mellitus who were not being treated with insulin injections. A multiple regression analysis showed that post-prandial hyperglycemia independently correlated with the incidence of diabetic retinopathy and neuropathy. Post-prandial hyperglycemia also correlated, although not independently, with the incidence of diabetic nephropathy. In addition, interestingly, post-prandial hypoinsulinemia independently correlated with the incidence of diabetic retinopathy, although not correlated with diabetic neuropathy or nephropathy. In conclusion, post-prandial hyperglycemia, rather than fasting glycemia or hemoglobin A1c levels, is an important predictor of the incidence of diabetic microangiopathy in Japanese type 2 diabetic patients.

Keywords: Diabetes; Post-prandial hyperglycemia; Post-prandial hypoinsulinemia; Diabetic microangiopathy; Retinopathy; Nephropathy; Neuropathy

It is well known that post-challenge hyperglycemia, which refers to high blood glucose levels following a 75 g oral glucose load, is related to the progression of diabetic macroangiopathy [1] and to mortality rate in subjects with impaired glucose tolerance and type 2 diabetes mellitus [2–4]. In addition to post-challenge hyperglycemia, the importance of post-prandial hyperglycemia as a risk factor of diabetic macroangiopathy is suggested from the results of various epidemiological studies [5–7]. However, there have only been a few reports regarding the association between post-prandial hyperglycemia and diabetic microangiopathy in human subjects. In this study, we examined whether there is a relationship between post-prandial glucose (or insulin) levels and the incidence of diabetic microangiopathy in Japanese type 2 diabetic patients, and found that post-prandial hyperglycemia, rather than fasting glycemia or hemoglobin A1c levels, is an important predictor of the incidence

☆ *Abbreviations:* PPG, post-prandial plasma glucose; PPI, post-prandial plasma insulin; PCG, post-challenge plasma glucose; PCI, post-challenge plasma insulin; WHO, World Health Organization; FPG, fasting plasma glucose; FPI, fasting plasma insulin; ECG, electric cardiograms; CVRR, coefficient variation of ECG R-R interval; AER, albumin excretion rate; DCCT, diabetes control and complications trial; 1,5-AG, 1,5-anhydro-D-glucitol; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; ET-1, endothelin-1; NDR, non-diabetic retinopathy; SDR, simple diabetic retinopathy; PDR, proliferative diabetic retinopathy.

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of diabetic microangiopathy such as retinopathy, neuropathy, and nephropathy.

Methods

Study cohort. We recruited 232 Japanese patients with type 2 (non-insulin-dependent) diabetes mellitus aged between 24 and 82 years, who were admitted to Osaka Prefectural General Hospital for the education of diabetes. The assessment of type 2 diabetes was based on World Health Organization (WHO) criteria. Patients were recruited for the study when they met the following criteria: (1) no episode of ketoacidosis, (2) no treatment with insulin injections, (3) absence of overt diabetic nephropathy (daily urinary protein excretion levels: less than 1 g/24 h) or other renal diseases such as glomerulonephritis, nephrotic syndrome, and renal failure, and (4) absence of acute coronary heart disease, cerebral vascular disease, or peripheral artery disease after careful evaluation of clinical records.

During the first week after admission, we did not change the patients' prescription for the treatment of diabetes, hyperlipidemia, and hypertension, and treated them in the same way as they had been just before admission. One hundred and thirty patients were treated with diet alone and 102 patients were treated with oral hypoglycemic agents (95 received a sulfonylurea, 38 received an α-glucosidase inhibitor, 16 received biguanide, and 7 received thiazolidinedione). Antihypertensive drugs were given to 75 patients (11 received diuretics, 12 received β-blockers, 2 received α-blockers, 47 received calcium channel blockers, 23 received angiotensin-converting enzyme inhibitors, and 4 received angiotensin-2 receptor blockers). Antihyperlipidemic drugs were administered to 41 patients (5 received clofibrates, 2 received probucol, and 34 received 3-hydroxyl-3-methylglutaryl-CoA reductase inhibitors).

Two to four days before admission, patients were given an oral load of 75 g glucose. Blood was withdrawn using standard laboratory techniques before, and 60 and 120 min after the glucose load for the determination of plasma glucose and insulin concentrations. Within 2–4 days after admission, blood samples were taken for analyses of fasting plasma glucose (FPG), fasting plasma insulin (FPI), hemoglobin A_{1C} (HbA_{1C}), serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and serum triglyceride levels. Two hours after the intake of an isocaloric mixed breakfast (10 kcal/kg of body weight, 57% carbohydrate, 15% fat, and 28% protein), representative of a standard Japanese breakfast, blood was withdrawn to measure post-prandial plasma glucose (PPG) and post-prandial plasma insulin (PPI) levels. To reflect daily glycemic excursion, strenuous exercise was prohibited by providing a written direction to each patient before post-prandial blood withdrawal; the patients were permitted only mild exercise, which is a level equal to daily exercise of each subject. Systolic and diastolic blood pressure was measured using a mercury sphygmomanometer. Exposure to smoking was estimated as the mean number of cigarettes smoked daily. Laboratory methods were kept constant throughout the study period.

Within a week after admission, retinopathy was assessed through dilated pupils by ophthalmologists, and classified as no evidence of diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), pre-proliferative retinopathy (pre-PDR), or proliferative retinopathy (PDR). If the eyes were asymmetric, we used data from the eye that was graded as having the worse retinopathy. Electro cardiograms (ECGs) were taken to calculate the coefficient variation of ECG R-R interval (CVRR), as an index of cardiac autonomic nervous function. Albumin excretion rate (AER) was measured using a fresh 24-h urine collection sample, as an index of diabetic nephropathy. Upon completion of each collection, a midstream specimen of urine was examined by microscopy or by culturing to exclude urinary tract infection and hematuria. Albumin concentrations were determined by radioimmunoassay (RIA).

Statistical analyses. Data are presented as means ± SD. The laboratory data were compared by the unpaired *t* test or Mann–Whitney's *U* test. Stepwise multivariate regression analyses, including sex and smoking, were performed to evaluate the relationship between possible risk factors and diabetic nephropathy and cardiac autonomic nerve dysfunction in diabetic patients. Multiple logistic model analysis was performed to evaluate the relationship between possible risk factors and diabetic retinopathy where NDR was set at 0, and SDR, pre-PDR, and PDR were set at 1. All analyses were conducted using the SPSS statistical package (SPSS, IL, USA).

Results

Post-prandial plasma glucose levels are significantly higher in type 2 diabetic patients with diabetic microangiopathy

The patients' baseline characteristics are shown in Table 1. The mean age of the 232 enrolled subjects with type 2 diabetes was 57.3 ± 11.5 years. The duration of diabetes was 7.2 ± 7.5 years. To examine the association of plasma glucose and insulin levels with the incidence of diabetic microangiopathy, we compared post-prandial and post-challenge plasma glucose and insulin concentrations between patients with and without diabetic retinopathy, neuropathy, and nephropathy. As shown in Table 2 and Fig. 1, post-prandial and post-challenge plasma glucose concentrations were significantly higher in subjects with diabetic retinopathy than in subjects without retinopathy. On the other hand, post-prandial and post-challenge plasma insulin concentrations were significantly lower in subjects with diabetic retinopathy than in subjects without retinopathy. Post-prandial and post-challenge plasma glucose concentrations were significantly higher in subjects with a

Table 1
Baseline characteristics of the patients[a]

Characteristic	Sulfonylurea treatment	Diet alone
Total number of patients	130	102
Sex (F/M)	67/63	53/49
Age (years)	58.8 ± 10.1	55.4 ± 12.9*
Duration of diabetes (years)	8.9 ± 8.2	5.1 ± 5.9*
Body-mass index (kg/m^2)[b]	24.2 ± 4.31	25.9 ± 4.39*
Glycosylated hemoglobin (%)	8.40 ± 1.33	7.89 ± 1.45*
Blood pressure		
Systolic (mmHg)	122.9 ± 17.6	121.9 ± 15.2
Diastolic (mmHg)	77.2 ± 11.4	76.7 ± 10.2
Cholesterol		
Total (mmol/L)	5.1 ± 0.9	5.3 ± 1.1
Low-density lipoprotein (mmol/L)	3.2 ± 0.9	3.2 ± 1.0
High-density lipoprotein (mmol/L)	1.3 ± 0.6	1.3 ± 0.9
Triglyceride (mmol/L)	1.5 ± 0.8	1.6 ± 1.0
Coefficient variation of ECG R-R interval (%)	2.45 ± 1.12	2.49 ± 1.07
Retinopathy		
NDR (no. of subjects)	93	87
SDR (no. of subjects)	27	10*
Pre-PDR, PDR (no. of subjects)	10	5*
Urine albumin excretion rate (mg/g creatinine)	39.5 ± 64.2	27.2 ± 49.7

Abbreviations: NDR, non-diabetic retinopathy; SDR, simple diabetic retinopathy; PDR, proliferative diabetic retinopathy.

[a] Values expressed with plus/minus signs are means ± SD.

[b] The body-mass index is the weight in kilograms divided by the square of the height in meters.

* $p < 0.05$ for the comparison of sulfonylurea treatment with diet therapy alone.

Table 2
Comparison of post-prandial and post-challenge plasma glucose and insulin concentrations between patients with and without diabetic microangiopathy

	Retinopathy		Neuropathy		Nephropathy	
	NDR	SDR, pre-PDR, PDR	CVRR ≧ 2.00 (%)	CVRR < 2.00 (%)	AER < 20 (μg/min)	AER ≧ 20 (μg/min)
PCG (mmol/L)	18.6 ± 5.6	20.6 ± 5.3*	18.5 ± 5.3	20.3 ± 6.2†	18.6 ± 5.7	20.1 ± 5.4‡
PPG (mmol/L)	12.4 ± 3.9	15.4 ± 4.3***	12.4 ± 3.9	14.4 ± 4.3 ††	12.5 ± 4.1	14.1 ± 4.1‡‡
PCI (pmol/L)	220.4 ± 164.9	133.2 ± 82.3**	207.9 ± 155.2	193.2 ± 159.5	206.7 ± 150.6	195.4 ± 169.8
PPI (pmol/L)	213.5 ± 120.2	124.6 ± 61.9***	209.1 ± 128.0	176.9 ± 90.8	207.6 ± 123.6	173.4 ± 97.7

Values are means ± SD.
Abbreviations: PPG, postprandial plasma glucose; PPI, postprandial plasma insulin; NDR, non-diabetic retinopathy; SDR, simple diabetic retinopathy; PDR, proliferative diabetic retinopathy; CVRR, coefficient variation of ECG R-R interval.
* $p = 0.02$ vs NDR.
** $p = 0.002$ vs NDR.
*** $p < 0.0005$ vs NDR.
† $p = 0.01$ vs CVRR ≧ 2.00 (%).
†† $p < 0.0005$ vs CVRR ≧ 2.00 (%).
‡ $p = 0.05$ vs AER < 20 (μg/min).
‡‡ $p = 0.005$ vs AER < 20 (μg/min).

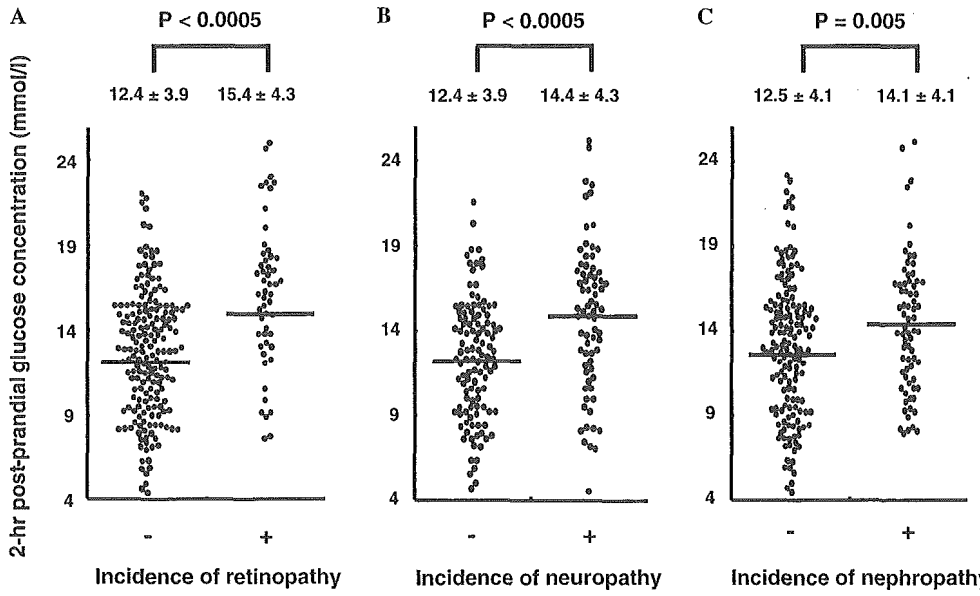

Fig. 1. Comparison of 2-h post-prandial plasma glucose concentrations between subjects with and without the incidence of diabetic retinopathy (A), neuropathy (B), and nephropathy (C).

CVRR of less than 2.00%, or with an AER of more than 20 μg/min, although there was no difference in post-prandial and post-challenge plasma insulin concentrations between patients with and without diabetic neuropathy or nephropathy. Taken together, post-prandial as well as post-challenge plasma glucose levels were significantly higher in Japanese type 2 diabetic patients with diabetic microangiopathy such as retinopathy, neuropathy, and nephropathy.

Post-prandial hyperglycemia and hypoinsulinemia are important predictors of the incidence of diabetic retinopathy

Since HbA1c, post-prandial glucose and insulin levels, post-challenge glucose and insulin levels, fasting plasma glucose and insulin levels, and duration of diabetes are closely linked, we performed multiple logistic model analyses including sex, smoking, blood pressure, and serum lipid profile, to identify the independent and important predictors of the incidence of diabetic retinopathy and possible risk factors (Table 3). Also, it is noted that, in multiple regression analyses, the significance of several factors can be lost when there is a very close correlation among these several factors and also another factor has a stronger correlation. Systolic blood pressure (odds ratio 1.05, 95% confidence interval 1.026–1.075, $p < 0.0001$), post-prandial plasma glucose concentration (odds ratio 1.01, 95% confidence interval 1.005–1.016, $p = 0.0002$), post-prandial plasma insulin concentration (odds ratio 0.964, 95% confidence interval 0.939–0.989, $p = 0.0049$), and duration of diabetes (odds ratio 1.067, 95% confidence interval 1.018–1.117, $p = 0.0063$) independently correlated with the incidence of diabetic retinopathy. In this analysis, significance in cor-

Table 3
Data from multivariate analyses evaluating association between the microangiopathy and various clinical parameters

Parameter	Retinopathy[a]				CVRR[b]				Log AER[b]			
	75 g OGTT		Standard meal		75 g OGTT		Standard meal		75 g OGTT		Standard meal	
	Odds ratio (95% CI)	*p* value	Odds ratio (95%CI)	*p* value	Partial correlation coefficient	*p* value	Partial correlation coefficient	*p* value	Partial correlation coefficient	*p* value	Partial correlation coefficient	*p* value
Age (years)	NS	NS	NS	NS	−0.2978	0.0007	−0.3446	<0.0001	NS	NS	NS	NS
Duration (years)	1.114 (1.053–1.178)	0.0002	1.067 (1.018–1.117)	0.0063	NS	NS	NS	NS	NS	NS	NS	NS
BMI (kg/m^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FPG (mmol/L)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.2010	0.0023
FPI (pmpl/L)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PCG (mmol/L)	NS	NS	ND	ND	NS	NS	ND	ND	NS	NS	ND	ND
PPG (mmol/L)	ND	ND	1.010 (1.005–1.016)	0.0002	ND	ND	−0.1887	0.0080	ND	ND	NS	NS
PCI (pmol/L)	0.963 (0.938–0.988)	0.0044	ND	ND	NS	NS	ND	ND	NS	NS	ND	ND
PPI (pmol/L)	ND	ND	0.964 (0.939–0.989)	0.0049	ND	ND	NS	NS	ND	ND	NS	NS
HbA1C (%)	NS	NS	NS	NS	NS	NS	NS	NS	0.1784	0.0098	NS	NS
sBP (mmHg)	1.068 (1.037–1.101)	<0.0001	1.050 (1.026–1.075)	<0.0001	NS	NS	NS	NS	0.1535	0.0261	0.2334	0.0004
dBP (mmHg)	0.951 (0.911–0.994)	0.0248	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
T-CHO (mmol/L)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TG (mmol/L)	NS	NS	NS	NS	−0.1855	0.0094	NS	NS	0.1387	0.0445	NS	NS
R^2					0.1504		0.1957		0.2831		0.1173	

Abbreviation: CVRR, coefficient variation of ECG R-R interval; OGTT, oral glucose tolerance test; BMI, body-mass index; FPG, fasting plasma glucose; FPI, fasting plasma insulin; PCG, postchallenge glucose; PPG, postprandial plasma glucose; PCI, postchallenge insulin; PPI, postprandial plasma insulin; sBP, systolic blood pressure; dBP, diastolic blood pressure; T-CHO, total-cholesterol; TG, triglyceride; ND, not determined; NS, not significant.

[a] Evaluated by multiple logistic regression model analysis was performed.

[b] Evaluated by stepwise multivariate regression analysis was performed.

relation between HbA1c levels and the incidence of retinopathy was lost because post-prandial glucose levels had a stronger correlation with the incidence of diabetic neuropathy rather than HbA1c levels. Furthermore, multiple logistic model analyses including post-challenge glucose and insulin concentrations showed that systolic blood pressure (odds ratio = 1.068, $p < 0.0001$), duration of diabetes (odds ratio = 1.114, $p = 0.0002$), 2-h post-challenge plasma insulin concentration (odds ratio = 0.963, $p = 0.0044$), and diastolic blood pressure (odds ratio = 0.951, $p = 0.0248$) independently correlated with the incidence of diabetic retinopathy. In addition to this, post-prandial insulin levels also independently correlated with the incidence of diabetic retinopathy. Fig. 2 shows the association between 2-h post-prandial insulin concentration, 2-h post-prandial glucose concentration, and the incidence of diabetic retinopathy. The highest incidence of diabetic retinopathy was found at both the high tertile of 2-h post-prandial glucose concentration and the low tertile of 2-h post-prandial insulin concentration. On the other hand, the lowest incidence of diabetic retinopathy was found at both the low tertile of 2-h post-prandial glucose concentration and the high tertile of 2-h post-prandial insulin concentration. Taken together, post-prandial hyperglycemia and hypoinsulinemia are independent and important predictors of the incidence of diabetic retinopathy.

Post-prandial hyperglycemia is an important predictor of the incidence of diabetic neuropathy and nephropathy

In addition, to evaluate the correlation between cardiac autonomic neuropathy and risk factors, we performed stepwise multivariate regression analysis of the coefficient variation of ECG R-R intervals (CVRR), an index of cardiac autonomic neuropathy. As shown in Table 3, this analysis showed that age ($p < 0.0001$), 2-h post-prandial plasma glucose levels ($p = 0.0080$), independently correlate with cardiac autonomic neuropathy. In this analysis, significance of HbA1c levels was lost because post-prandial glucose levels had a stronger correlation with the incidence of diabetic neuropathy rather than HbA1c. On the other hand, multiple regression analysis of post-challenge plasma glucose and insulin concentrations showed that age ($p = 0.0007$), and triglyceride levels ($p = 0.0094$), but not post-challenge plasma glucose and insulin concentrations, independently correlated with cardiac autonomic neuropathy. Taken together, post-prandial hyperglycemia is an independent and important predictor of the incidence of diabetic neuropathy.

Similarly, to evaluate the correlation of diabetic nephropathy and risk factors, we performed stepwise multivariate regression analysis including the urinary excretion rate of albumin, an index of diabetic nephropathy. As

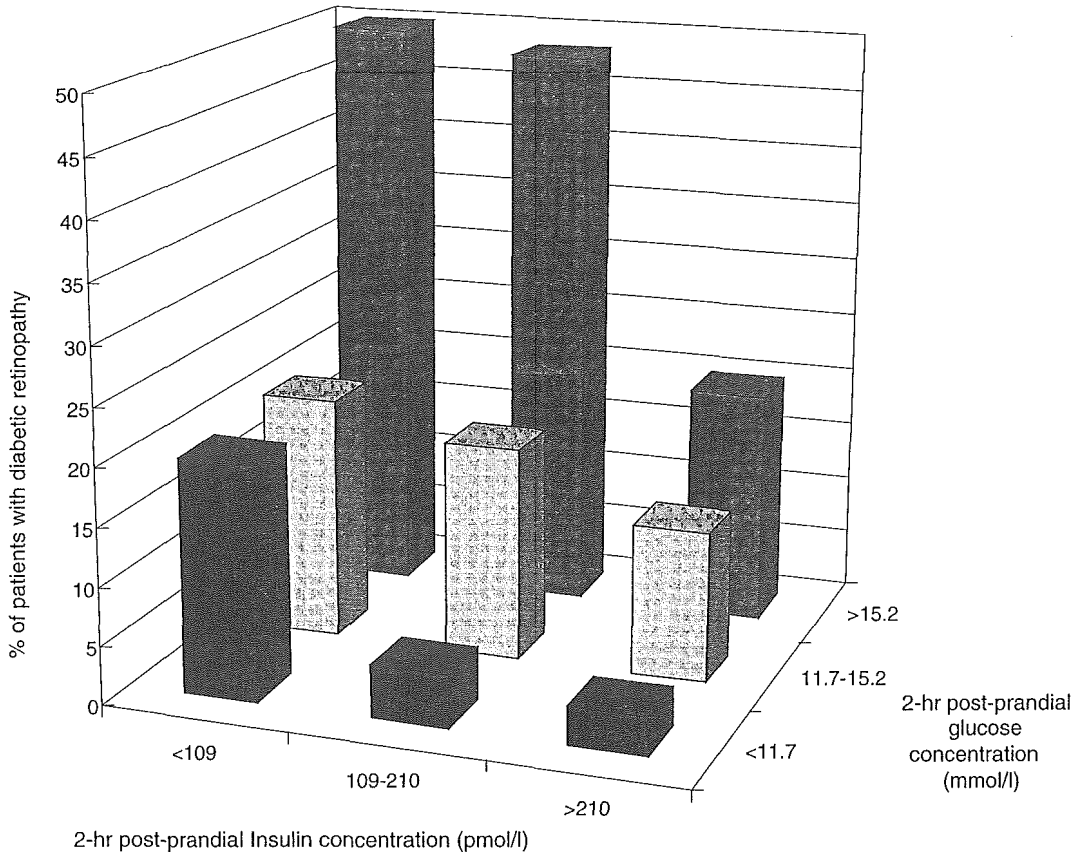

Fig. 2. Incidence of diabetic retinopathy analyzed by tertiles of 2-h post-prandial insulin concentrations and 2-h post-prandial glucose concentrations.

shown in Table 3, this analysis showed that systolic blood pressure ($p = 0.0004$), fasting plasma glucose ($p = 0.0023$) independently correlate with diabetic nephropathy. This analysis also suggests that post-prandial hyperglycemia is not an independent predictor of the incidence of diabetic nephropathy. As shown in Table 2, however, post-prandial hyperglycemia could be an important predictor of the incidence of diabetic nephropathy. Thus, we assume we should conclude that post-prandial hyperglycemia is an important, although not independent, predictor of the incidence of diabetic nephropathy.

Discussion

The importance of post-prandial hyperglycemia as a risk factor of diabetic macroangiopathy has been suggested from the results of various epidemiological studies [5–7]. However, there have only been a few reports on the relationship between diabetic microangiopathy and post-prandial hyperglycemia in human subjects. An analysis of the diabetes control and complications trial (DCCT) showed that none of the 3 different post-prandial blood glucose levels may predict the incidence of diabetic retinopathy in type 1 diabetic patients [8]. In this study, we first demonstrated that post-prandial hyperglycemia, rather than fasting plasma glucose levels and HbA_{1C} levels, is a better indicator of daily glycemic excursion, which is responsible for the diabetic retinopathy and cardiac autonomic neuropathy in type 2 diabetic patients.

Epidemiological and intervention studies have clearly indicated the importance of HbA_{1C} levels in predicting long-term diabetic complications. In a further analysis of the DCCT results [9–11], at similar mean HbA_{1C} values, the worsening of diabetic retinopathy in type 1 diabetic patients on conventional treatment was greater than in patients on intensified insulin treatment. In addition, we previously described that subjects with type 2 diabetes treated by conventional insulin treatment possessed a significantly lower value of 1,5-anhydro-D-glucitol (1,5-AG) which indicates higher glycemic excursion than those treated by multiple insulin injections [12,13]. Because these data imply that diabetic subjects treated by conventional insulin therapy show profound post-prandial hyperglycemia compared with those treated by multiple insulin injections, we assume that diabetic retinopathy results from the extent of post-prandial glucose excursions. In addition, it has been reported that hyperglycemia increases the blood concentration of some growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which lead to vascular construction and neovascularization [14]. In addition, it is known that hyperglycemia increases apoptosis in retinal pericytes and endothelial cells [15,16]. Thus, although not examined in this study, it is plausible that post-prandial hyperglycemia contributes to the progression of diabetic retinopathy at least in part through the increased expression of several growth factors and an induction of apoptosis in retinal cells.

The multiple regression analyses pointed out that post-prandial hypoinsulinemia independently correlates with diabetic retinopathy and cardiac autonomic neuropathy (Table 3). This observation has not been reported previously, probably because of the insufficient number of post-prandial insulin determinations. It has been reported that physiological concentrations of insulin rescue cultured optic nerve oligodendrocytes from apoptosis and are necessary for the survival of retinal ganglion cells in culture [17]. Thus, although not examined in this study, we assume that post-prandial hypoinsulinemia contributes to the incidence of diabetic retinopathy at least in part through the increased expression of several growth factors and an induction of apoptosis in retinal cells.

In conclusion, post-prandial hyperglycemia, rather than fasting glycemia or HbA1c levels, is a stronger predictor of the incidence of each diabetic microangiopathy in Japanese type 2 diabetic patients.

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The Endoplasmic Reticulum Chaperone Improves Insulin Resistance in Type 2 Diabetes

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To determine the role of the endoplasmic reticulum (ER) in diabetes, Akita mice, a mouse model of type 2 diabetes, were mated with either heterozygous knockout mice or two types of transgenic mice of 150-kDa oxygen-regulated protein (ORP150), a molecular chaperone located in the ER. Systemic expression of ORP150 in Akita mice improves insulin intolerance, whereas the exclusive overexpression of ORP150 in pancreatic β-cells of Akita mice did not change their glucose tolerance. Both an insulin tolerance test and hyperinsulinemic-euglycemic clamp revealed that ORP150 enhanced glucose uptake, accompanied by suppression of oxidized protein. Furthermore, ORP150 enhanced the insulin sensitivity of myoblast cells treated with hydrogen peroxide. These data suggest that ORP150 plays an important role in insulin sensitivity and is a potential target for the treatment of diabetes. *Diabetes* 54:657–663, 2005

Hyperglycemia occurs with the progressive failure of pancreatic β-cells to secrete sufficient amounts of insulin to compensate for insulin resistance (1). Mice lacking PKR-like endoplasmic reticulum kinase (PERK) or eukaryotic initiation factor (eIF)-2α exhibited β-cell overload in pancreatic β-cells (2,3), which is observed during conditions such as hyperglycemia and obesity. Furthermore, nitric oxide induces apoptosis by endoplasmic reticulum (ER) stress via the induction of C/EBP homologous protein (Chop), and pancreatic islets from Chop knockout mice exhibit resistance to nitric oxide (4). The Akita mouse, which carries a conformation-altering missense mutation (Cys96Tyr) in Insulin 2, displays hyperglycemia without obesity (5,6). During the development of diabetes in Akita mice, both the transcriptional factor Chop and the molecular chaperone GRP78 in the ER were induced in the pancreas, and targeted disruption of the Chop gene improved the glucose intolerance of heterozygous Akita mice (7). These reports show that ER plays an important role in insulin secretion in β-cells of the pancreas.

ORP150 (150-kDa oxygen-regulated protein) is a molecular chaperone in the ER that has been identified in cultured astrocytes exposed to hypoxia (8). The expression of ORP150 is essential for the maintenance of cellular viability under hypoxia (9), and neurons overexpressing ORP150 resist acute ischemic damage (10). ORP150 also plays an important role in the secretion of vascular endothelial growth factor (VEGF) as a molecular chaperone (11,12), and it is induced via the unfolded protein pathway (13). Kobayashi et al. (14) showed that the strong expression of ORP150 protein by islets of pancreas tissue is reduced by fasting, suggesting that ORP150 is involved in the secretion of insulin. In contrast, polymorphism analysis revealed that some single nucleotide polymorphisms (SNPs) in the ORP150 genome of Pima Indians are associated with insulin sensitivity and not the secretion of insulin (15).

In this report, we show that the systemic overexpression of ORP150 delayed the onset of disease in heterozygous Akita mice and improved insulin sensitivity, whereas heterozygous disruption of the ORP150 gene facilitated the progress of diabetes and caused insulin resistance. Furthermore, the overexpression of ORP150 reduced oxidative stress and augmented insulin signaling in the liver and skeletal muscle of Akita mice and in rat skeletal myoblast cell lines, suggesting that ORP150 improves the insulin sensitivity impaired by oxidative stress.

RESEARCH DESIGN AND METHODS

For Western blotting, anti-human ORP150 (1 μg/ml) (9), anti–insulin receptor substrate (IRS)-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine (Calbiochem-Novabiochem, San Diego, CA), and anti–β-actin Ab IgG (Sigma Chemical, St. Louis, MO) were used. We used anti-human ORP150 IgG (5 μg/ml) (16) and anti-insulin IgG for immunohistochemical analysis. The construct of the rat insulin promoter was a kind gift from Dr. Richard D.

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ALA, α-lipoic acid; CAG, chicken β-actin; Chop, C/EBP homologous protein; eIF, eukaryotic initiation factor; GRP, glucose-regulated protein; ER, endoplasmic reticulum; IPGTT, intraperitoneal glucose tolerance test; IRS, insulin receptor substrate; ITT, insulin tolerance test; ORP150, 150-kDa oxygen-regulated protein; PERK, PKR-like ER kinase; TM, tunicamycin.

Palmiter at the University of Washington. L6 cells were purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank (no. IFO50364; Osaka, Japan).

All procedures involving animals were approved by the Animal Care and Use Committee of Kanazawa University. ORP150 transgenic mice using a cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter (16) were a kind gift from the HSP institute (17). ORP150 heterozygous knockout mice have been generated previously (18). Akita mice established from C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Transgenic mice using insulin promoter were generated at the Genome Information Research Center (Osaka University, Osaka, Japan) (10). The genotype of the mutant mice was determined by Southern blot analysis and PCR. These mice were crossed into the C57BL/6 background.

The levels of HbA_{1c} were measured from tail-vein blood using a DCA2000 analyzer (Bayer Medical, Tokyo, Japan) (19). The concentration of insulin was measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions (Shibayagi, Shibukawa, Japan).

Glucose metabolism of mutant mice. Intraperitoneal glucose tolerance tests (IPGTTs) and insulin tolerance tests (ITTs) were performed as described previously (20). Hyperinsulinemic-euglycemic clamp was performed as described previously (21,22). The rates of glucose appearance (R_a) and disappearance (R_d) were calculated according to Steele's non–steady-state equations. Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived R_a and exogenous infusion rates of glucose (GIRs) and tracer.

Assessment of insulin signaling. Animals that fasted overnight were anesthetized and injected with either saline or 5 IU human insulin via the inferior vena cava. The liver was obtained after 2 min and the skeletal muscle after 5 min. L6 cells were harvested after 5 min treatment with hydrogen peroxide. Protein samples from the liver, skeletal muscle, and L6 cells underwent immunoprecipitation for IRS-1 followed by Western blotting with an anti–IRS-1 antibody and anti-phosphorylated IRS-1 antibody.

Treatment with α-lipoic acid. At 6 weeks of age, C57BL/6 and Akita mice received an intraperitoneal injection of either vehicle or 30 mg/kg body wt α-lipoic acid over 5 days (23).

Protein oxidation detection. The formation of protein carbonyl groups was assessed by a OxyBlot protein oxidation detection kit (Integen) used in accordance with the manufacturer's protocol.

Adenovirus infection. Adenovirus coding for ORP150 in the sense (Ad/S-ORP150) or antisense (Ad/AS-ORP150) orientation and coding LacZ (AxCALacZ) have been generated previously (10). L6 cells were infected with an adenovirus at 100 multiplicities of infection (MOI) for 24 h as described previously.

Data analysis. Statistical analysis was performed by either an unpaired *t* test or an ANOVA followed by multiple comparison analysis using Newman-Kuehl's equation. Where indicated, the data were analyzed by a two-way ANOVA followed by multiple contrast analysis. For nonparametric data, either a Kruskal-Wallis analysis or χ^2 analysis was applied.

RESULTS

Targeted disruption of ORP150 heterozygously accelerated the onset of diabetes in Akita mice. ORP150 heterozygous mice ($ORP150^{-/+}$) were mated with heterozygous Akita mice ($Ins2^{WT/C96Y}$). Consistent with a previous report (16), ORP150 levels of $ORP150^{-/+}$ mice were reduced by ~50% compared with those of their wild-type littermates (Fig. 1*A*), and similar results were obtained between $ORP150^{-/+}Ins2^{WT/C96Y}$ and $Ins2^{WT/C96Y}$ mice (data not shown). There was no significant difference in the body weights of $ORP150^{-/+}Ins2^{WT/C96Y}$ and $Ins2^{WT/C96Y}$ mice (Fig. 1*B*). The IPGTT showed there was no significant difference between $ORP150^{-/+}$ and their wild-type littermates (data not shown), whereas at 6 weeks the serum glucose levels of $ORP150^{-/+}Ins2^{WT/C96Y}$ were significantly higher than those of $Ins2^{WT/C96Y}$ mice (Fig. 1*C*). The measurement of HbA_{1c} showed a similar trend as IPGTT at 6 and 9 weeks; however, at 12 weeks there was no significant difference in HbA_{1c} levels between $Ins2^{WT/C96Y}$ and $ORP150^{-/+}Ins2^{WT/C96Y}$ mice (Fig. 1*D*). Contrary to our prediction, there was no significant difference in the insulin content of the pancreas of $ORP150^{-/+}Ins2^{WT/C96Y}$ and $Ins2^{WT/C96Y}$ mice (Fig. 1*E*).

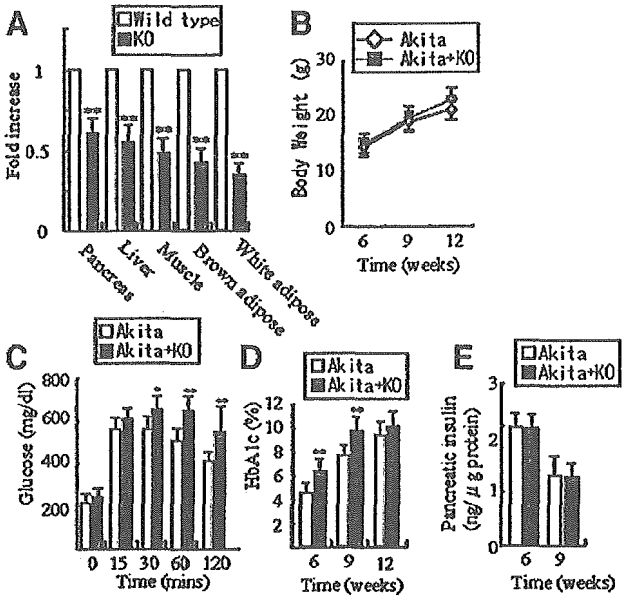

FIG. 1. Glucose and insulin homeostasis in Akita mice heterozygous for ORP150 deficiency. *A*: Indicated tissues of $ORP150^{-/+}$ and wild-type littermates underwent immunoblotting, and semiquantitative analysis was performed (n = 4). *B*: Growth curve of $Ins2^{WT/CY}$ and $ORP150^{-/+}Ins2^{WT/CY}$ mice (n = 8). *C*: The glucose tolerance test was performed on male $Ins2^{WT/CY}$ and $ORP150^{-/+}Ins2^{WT/CY}$ mice (n = 5). *D*: Blood HbA_{1c} levels measured in $Ins2^{WT/CY}$ and $ORP150^{-/+}Ins2^{WT/CY}$ mice (n = 6). *E*: Pancreatic insulin content of $Ins2^{WT/CY}$ and $ORP150^{-/+}Ins2^{WT/CY}$ mice (n = 5). Data are means ± SD. *P < 0.05 and **P < 0.01 by multiple comparison analysis compared with the control.

Glucose metabolism of Akita mice overexpressing ORP150. ORP150 transgenic mice generated using the CAG promoter ($ORP150^{CAG}$) were mated with $Ins2^{WT/C96Y}$ mice. The levels of ORP150 protein were significantly greater in $ORP150^{CAG}$ compared with their wild-type littermates (Fig. 2*A*), and similar results were obtained between $ORP150^{CAG}Ins2^{WT/C96Y}$ and $Ins2^{WT/C96Y}$ (data not shown). Consistent with a previous report (17), $ORP150^{CAG}Ins2^{WT/C96Y}$ had significantly lower body weights than $Ins2^{WT/C96Y}$ mice (Fig. 2*B*). IPGTT showed that at 6 weeks, the glucose tolerance of $ORP150^{CAG}Ins2^{WT/C96Y}$ was greater than that of $Ins2^{WT/C96Y}$ (Fig. 2*C*). HbA_{1c} showed a similar trend as IPGTT at 6 and 9 weeks; however, at 12 weeks there was no significant difference (Fig. 2*D*). There was no significant difference in the insulin content of the pancreas (Fig. 2*E*).

To determine whether overexpression of ORP150 in β-cells of the islets improves the glucose intolerance of $Ins2^{WT/C96Y}$, ORP150 transgenic mice were generated using the rat insulin promoter ($ORP150^{Ins}$), as described in RESEARCH DESIGN AND METHODS. Western blot analysis revealed that ORP150 levels in the pancreas of $ORP150^{Ins}$ mice lines were significantly higher than those of their nontransgenic littermates. In comparison, there was no significant difference in the levels of ORP150 in the liver, skeletal muscle, white fat tissue, and brown fat tissue between the three lines of $ORP150^{Ins}$ and their wild-type littermates (Fig. 3*A*). Furthermore, immunohistochemical analysis showed that ORP150 expression in the pancreas of $ORP150^{Ins}$ was limited to the islets of Langerhans and overlapped with that of insulin (Fig. 3*B* and *C*), suggesting that, as pre-

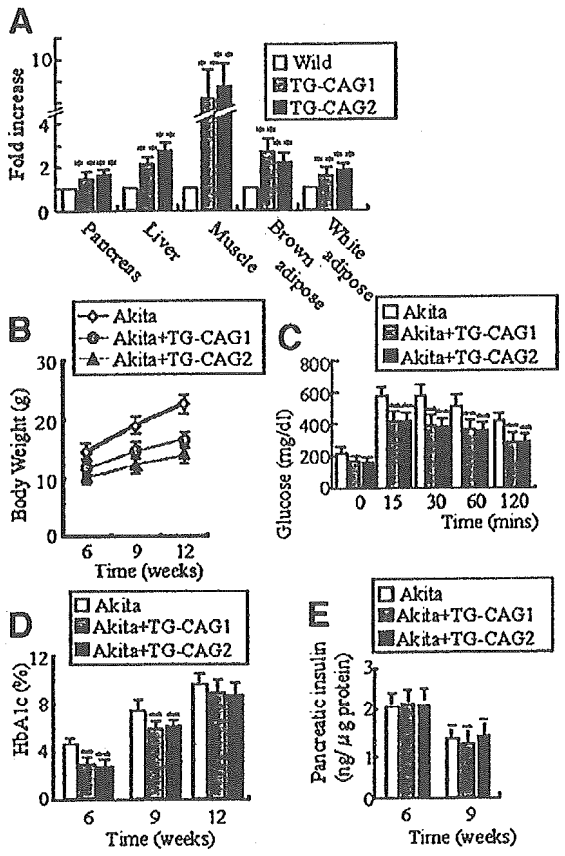

FIG. 2. Glucose and insulin homeostasis in Akita mice overexpressing ORP150 using CAG promoter. *A*: Indicated tissues of two lines of ORP150^{CAG}(TG-cag1 and 2) and their wild-type littermates (wild) underwent immunoblotting, and semiquantitative analysis was performed (n = 4). *B*: The growth curve of Ins2$^{WT/CY}$ (Akita) and ORP150^{CAG}Ins2$^{WT/CY}$ (Akita+TG-CAG1 and -2) (n = 8). *C*: Male Ins2$^{WT/CY}$ and ORP150^{CAG}Ins2$^{WT/CY}$ underwent the glucose tolerance test (n = 5). *D*: Blood HbA_{1c} levels measured in Ins2$^{WT/CY}$ and ORP150^{CAG}Ins2$^{WT/CY}$ (n = 6). *E*: Pancreatic insulin content of Ins2$^{WT/CY}$ and ORP150^{CAG}Ins2$^{WT/CY}$ mice (n = 5). Data are means ± SD. $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

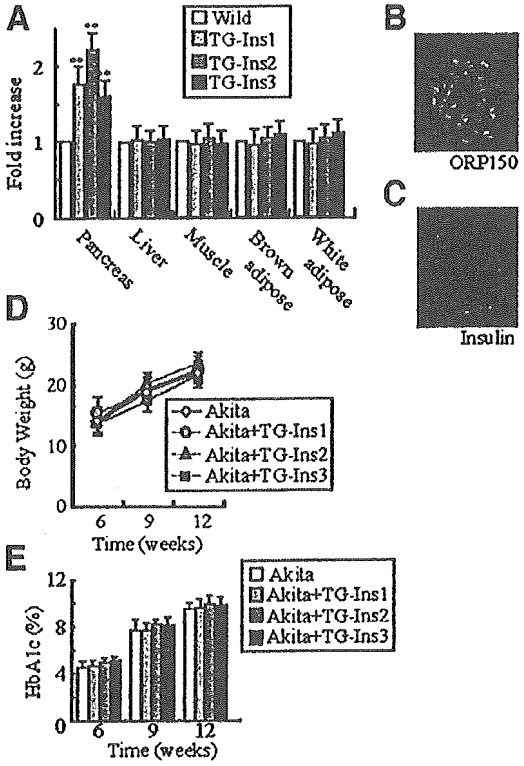

FIG. 3. Glucose and insulin homeostasis in Akita mice overexpressing ORP150 using insulin promoter. *A*: Indicated tissues of two lines of three lines of ORP150^{Ins} (TG-ins1, -2, and -3) and their wild-type littermates (wild) underwent immunoblotting, and semiquantitative analysis was performed (n = 4). *B* and *C*: Pancreatic sections of ORP150^{Ins} mice that underwent immunohistochemical analysis using an anti-ORP150 (*B*) and anti-insulin antibody (*C*). *D*: The growth curve of Ins2$^{WT/CY}$ (Akita) and ORP150^{Ins}Ins2$^{WT/CY}$ (Akita+ TG-ins1, -2, and -3) (n = 8). *E*: Blood HbA_{1c} levels measured in Ins2$^{WT/CY}$ and ORP150^{Ins}Ins2$^{WT/CY}$ mice (n = 6). Data are means ± SD (n = 4). $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

dicted, the transgene was expressed in β-cells. There was no significant difference between the growth curves of Ins2$^{WT/C96Y}$ and ORP150^{Ins}Ins2$^{WT/C96Y}$ mice (Fig. 3*D*). Both glucose tolerance assessed by IPGTT (data not shown) and measurement of HbA_{1c} (Fig. 3*E*) were not significantly different between Ins2$^{WT/C96Y}$ and ORP150^{Ins} Ins2$^{WT/C96Y}$ mice. In addition, there was no significant difference in the pancreatic levels of insulin in Ins2$^{WT/C96Y}$ and ORP150^{Ins}Ins2$^{WT/C96Y}$ mice (data not shown). The results shown in Figs. 1 and 2 suggest that ORP150 improves the glucose intolerance of Akita mice but not the secretion of insulin.

ORP150 is involved in insulin sensitivity. To determine whether ORP150 expression was lessened by fasting (14), the pancreas (Fig. 4*A*), liver (Fig. 4*B*), and skeletal muscle (Fig. 4*C*) of C57BL/6 starved for indicated times were used for Western blotting with an anti-ORP150 antibody. In contrast to previous findings (14), there was no significant difference in the levels of ORP150 in the pancreatic tissue of control and starved mice, whereas starvation for 48 h significantly reduced the levels of ORP150 in the liver and skeletal muscle.

We assessed the levels of ORP150 in Akita mice. The levels of ORP150 transcript in the pancreas of Ins2$^{WT/C96Y}$ were significantly higher than those of C57BL/6 (Fig. 4*D* and 4e). However, there was no significant difference in the levels of ORP150 protein between Ins2$^{WT/C96Y}$ and C57BL/6 mice (Fig. 4*F* and *G*). Immunoblotting also revealed that the levels of ORP150 in the liver (Fig. 4*H* and *I*) and skeletal muscle (Fig. 4*J* and *K*) of Ins2$^{WT/C96Y}$ were significantly greater compared with that of C57BL/6 mice at 6 weeks, whereas those of Ins2$^{WT/C96Y}$ were significantly lower compared with those of C57BL/6 mice at 12 weeks.

To determine whether ORP150 is involved in the insulin sensitivity of Ins2$^{WT/C96Y}$, an ITT was performed. The ITT revealed that overexpression of ORP150 increased the insulin sensitivity of Ins2$^{WT/C96Y}$ (Fig. 5*A*), whereas heterozygous disruption of ORP150 reduced insulin sensitivity (Fig. 5*B*). To further investigate these findings, ORP150^{CAG} and ORP150$^{-/+}$ mice underwent hyperinsulinemic-euglycemic clamp. As described previously (17), ORP150^{CAG} mice displayed the phenotype of myocardial degeneration and died of heart failure during the clamp test. The average GIR of 90–120 min in ORP150$^{-/+}$ was significantly lower than that of their littermates (Fig. 5*C*).

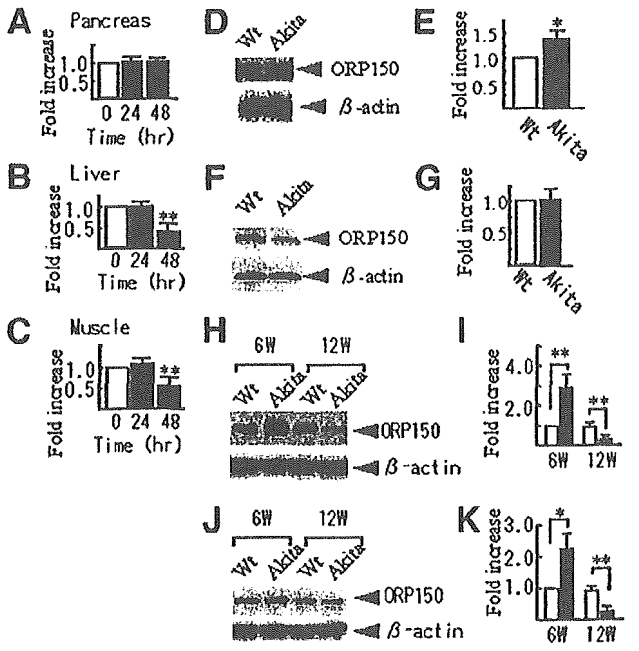

FIG. 4. Expression of ORP150 in starved and diabetic mice. *A–C*: Wild-type mice (C57BL/6) were starved 0–48 h and the indicated tissues harvested for Western blot analysis using an anti-ORP150 and anti–β-actin antibody; semiquantitative analysis is shown. The intensity of ORP150 is represented as the percentage increase over the control. *D* and *E*: Total RNA from the pancreas of male wild-type (C57BL/6) and 6-week-old Akita mice used in Northern blot analysis. A typical example of the blot is shown in *D*. Semiquantitative analysis is shown in *E*, where the intensity of ORP150 is represented as the percentage increase over the control. *F–K*: Protein samples from the pancreas (*F* and *G*), liver (*H* and *I*), and skeletal muscle (*J* and *K*) of male C57BL/6 and Akita mice ($Ins2^{WT/CY}$) used in Western blot analysis. A typical example of the blot is shown in *F*, *H*, and *J*. Semiquantitative analysis is shown in *G*, *I*, and *K*, where the intensity of ORP150 is represented as the percentage increase over the control. Data are shown as means ± SD (n = 4). *P < 0.05 and **P < 0.01 by multiple comparison analysis compared with the control.

EGP showed there was no significant difference between $ORP150^{-/+}$ and their wild-type littermates (data not shown). We assessed insulin signaling in $Ins2^{WT/C96Y}$, $ORP150^{CAG}Ins2^{WT/C96Y}$, and $ORP150^{-/+}Ins2^{WT/C96Y}$ mice. After overnight starvation and treatment with either saline or insulin, protein was extracted from the liver (Fig. 5*D* and *E*) and skeletal muscle (Fig. 4*F* and *G*) for immunoprecipitation with an anti–IRS-1 IgG, followed by Western blot analysis with an anti-phosphorylated IRS-1 IgG and anti–IRS-1. The levels of phosphorylated IRS-1 in $ORP150^{CAG}Ins2^{WT/C96Y}$ were increased compared with $Ins2^{WT/C96Y}$ but were reduced in $ORP150^{-/+}Ins2^{WT/C96Y}$ mice (Fig. 4*E* and *G*). These results indicate that ORP150 might play a role in the insulin sensitivity of the liver and skeletal muscle but not in the secretion of insulin.

ORP150 decreased oxidative stress in Akita mice. We speculated that oxidative stress caused by hyperglycemia might induce ORP150 expression. To determine whether there was a relationship between oxidative stress and the induction of ORP150 in diabetes, Akita mice at 5 weeks were treated with α-lipoic acid (ALA), an antioxidant drug that improves insulin resistance (23). Northern blot analysis showed that ALA reduced the levels of ORP150 transcript in the liver (Fig. 6*A* and *B*) and skeletal muscle (Fig. 6*C* and *D*) of Akita mice. In addition, 10–40 μmol/l hydrogen peroxide induced ORP150 and GRP78 transcripts in L6 skeletal myoblast cells (Fig. 7*A* and *B*). In contrast, Chop transcripts were only induced by 100 μmol/l hydrogen peroxide (Fig. 7*A* and *C*). ORP150 and GRP78 transcript expression peaked 6–12 h after the treatment with hydrogen peroxide (data not shown). Immunoblot analysis showed the similar results as Northern blot analysis (data not shown). These data suggest that oxidative stress could induce ORP150 and GRP78. To further investigate the role of hydrogen peroxide in ER, L6 cells were treated with hydrogen peroxide for 24 h and then L6 cells were treated with 20 μmol/l hydrogen peroxide and tunicamycin (TM). TM induced ORP150 (Fig. 7*D* and *E*) and GRP78 (Fig. 7*D* and *F*), and hydrogen peroxide decreased their induction (Fig. 7*D–F*). These data are consistent with the in vivo data that levels of ORP150 in liver and muscle of Akita mice were decreased compared with those of wild-type mice at 12 weeks. Taken together, these data suggest that oxidative stress could induce ER chaperone; however, prolonged oxidative stress attenuates the induction of ER chaperone.

Furthermore, Western blotting using an anti-DNP (1-3 dinitrophenyl hydrazone) antibody revealed that overexpression of ORP150 reduced the levels of carbonylated protein in Akita mice (Fig. 8*A*) but that carbonylated protein was increased with heterozygous disruption of ORP150 (Fig. 8*B*). To further determine a possible role for ORP150, the levels of ORP150 were modulated in L6 myoblast cells using adenoviruses coding for ORP150 in the sense (Ad/S-ORP150) or antisense (Ad/AS-ORP150) orientation. Treatment of L6 cells with Ad/S-ORP150 significantly increased the levels of ORP150, whereas treatment with Ad/AS-ORP150 suppressed ORP150 levels (Fig. 8*C*). L6 cells infected with Ad/S-ORP150, Ad/AS-ORP150, or an adenovirus encoding LacZ (AxCALacZ) were treated with 20 μmol/l hydrogen peroxide and then used in Western blot analysis in conjunction with an anti-DNP antibody. The levels of oxidative protein in L6 cells treated with hydrogen peroxide were suppressed Ad/S-ORP150 but increased by Ad/AS-ORP150 (Fig. 8*D*). Using immunoprecipitation, insulin signals were further assessed in L6 cells treated with hydrogen peroxide. Ad/S-ORP150 consistently enhanced phosphorylation of IRS-1, but phosphorylation was suppressed by Ad/AS-ORP150 (Fig. 8*E* and *F*), suggesting that the ER chaperone might protect insulin signaling from oxidative stress in myoblast cells.

DISCUSSION

In this report, we demonstrated that ORP150 expression could be induced by hydrogen peroxide in myoblast cells and that overexpression of ORP150 using an adenovirus reduced oxidized protein. However, in a previous study, we showed that ORP150 had no influence on hydrogen peroxide–induced cell death (9,10). This discrepancy might arise from the different concentrations of hydrogen peroxide added to the cells. In previous reports, 100 μmol/l to 10 mmol/l hydrogen peroxide has been added to either 293 cells or primary neurons (9,10), whereas in this study, 10–40 μmol/l hydrogen peroxide induced ORP150 but produced no significant difference in cell growth and death (data not shown).

$ORP150^{CAG}Ins2^{WT/C96Y}$ showed lower body weight com-

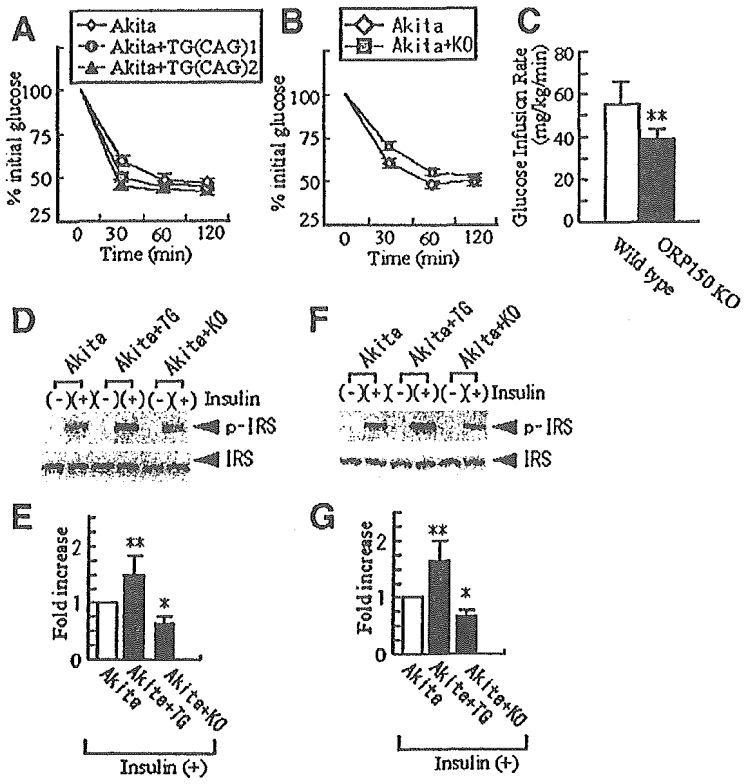

FIG. 5. Assessment of insulin sensitivity of mutant mice. *A* and *B*: ITT of $Ins2^{WT/CY}$, $ORP150^{CAG}Ins2^{WT/CY}$, and $ORP150^{-/+}Ins2^{WT/CY}$ mice ($n = 5$). *C*: GIR of wild-type littermates and $ORP150^{-/+}$ mice (seven littermates and five $ORP150^{-/+}$). *D–G*: Phosphorylation of IRS-1 in $Ins2^{WT/CY}$, $ORP150^{CAG}Ins2^{WT/CY}$, and $ORP150^{-/+}Ins2^{WT/CY}$ mice were assessed. A typical example of the blot is shown in *D* and *F*. Semiquantitative analysis is shown in *E* and *G* ($n = 4$). Data are means ± SD ($n = 4$). $^*P < 0.05$ and $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

pared with the nontransgenic Akita ($Ins2^{WT/C96Y}$). It is possible that these differences in body weight might account for the differences in insulin sensitivity. To eliminate this possibility, we performed IPGTT and ITT using $ORP150^{CAG}$ and weight-matched control. IPGTT showed no significant difference between $ORP150^{CAG}$ and weight-matched control (see online appendix at http://diabetes.diabetesjournals.org). However, ITT showed that overexpression of ORP150 improved insulin sensitivity compared with weight-matched control (online appendix).

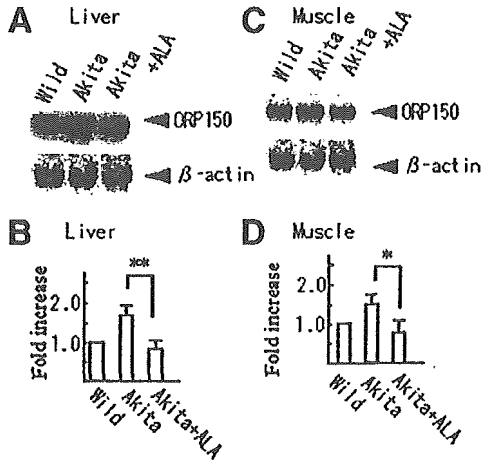

FIG. 6. Antioxidant drug decreased the expression of ORP150. C57BL/6 and Akita mice were treated with either saline or ALA, and the indicated tissues underwent Northern blot analysis for ORP150 and β-actin. A typical example of the blot is shown in *A* and *C*. Semiquantitative analysis is shown in *B* and *D*, where the intensity of ORP150 is represented as the percentage increase over the wild-type mice treated with saline and normalized to the levels of β-actin. Data are means ± SD ($n = 4$). $^*P < 0.05$ and $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

Using immunoblotting, we have shown that fasting reduces the levels of ORP150 in the liver and skeletal muscle (Fig. 4*B* and *C*). These data are consistent with a previous report showing that the expression of some ER chaperones, including ORP150, was lower in the liver of mice fed energy-restricted food compared with that of freely fed mice (24). In addition, we have shown that the levels of ORP150 in the liver and skeletal muscle of Akita mice were significantly greater compared with that of wild-type mice (Fig. 4*H–K*). Given that mutation of the insulin two gene is responsible for the phenotype of Akita mice, we expected hyperglycemia might secondarily induce ORP150 expression in the liver and skeletal muscle. This idea is consistent with our pilot data showing increased ORP150 expression in the liver and skeletal muscle of *db/db* diabetic mice or C57BL/6 mice treated with streptozotocin.

Although there is no significant difference in glucose levels during IPGTT between $ORP150^{-/+}$ and wild-type littermates (data not shown), hyperinsulinemic-euglycemic clamp revealed that insulin sensitivity of $ORP150^{-/+}$ was greater than that of wild-type littermates (Fig. 5*C*). As described in RESEARCH DESIGN AND METHODS, excess insulin was infused to maintain levels of glucose during clamp test, and insulin accelerates the process of mRNA translation (25) and results in increasing protein synthesis and ER stress. These reports led us to the idea that more ORP150 is required during clamp test than during IPGTT, and this is consistent with our pilot study that ORP150

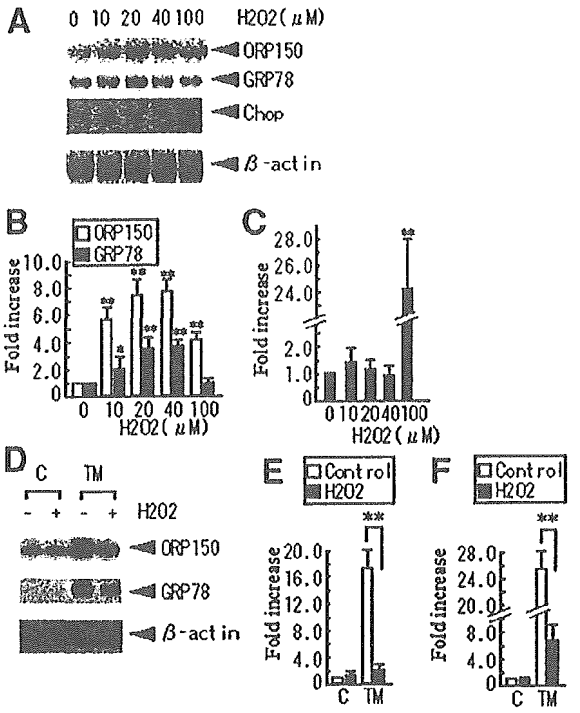

FIG. 7. The relationship between ORP150 and oxidative stress. *A–C*: L6 myoblast cells treated with hydrogen peroxide underwent Northern blot analysis for ORP150, GRP78, Chop, and β-actin. A typical example of the blot is shown in *A*. Semiquantitative analysis is shown in *B* and *C*, where the intensity of ORP150 (*B*), GRP78 (*B*), and Chop (*C*) is represented as the percentage increase over the control (0 h) normalized to the levels of β-actin. Data are means ± SD (n = 4). *D–F*: L6 myoblast cells, treated with hydrogen peroxide for 24 h, were subjected to TM and hydrogen peroxide for 6 h and then underwent Northern blot analysis for ORP150, GRP78, and β-actin. A typical example of the blot is shown in *D*. Semiquantitative analysis is shown in *E* and *F*, where the intensity of ORP150 (*E*) and GRP78 (*F*) is represented as the percentage increase over the control (0 h) normalized to the levels of β-actin (n = 4). Data are means ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

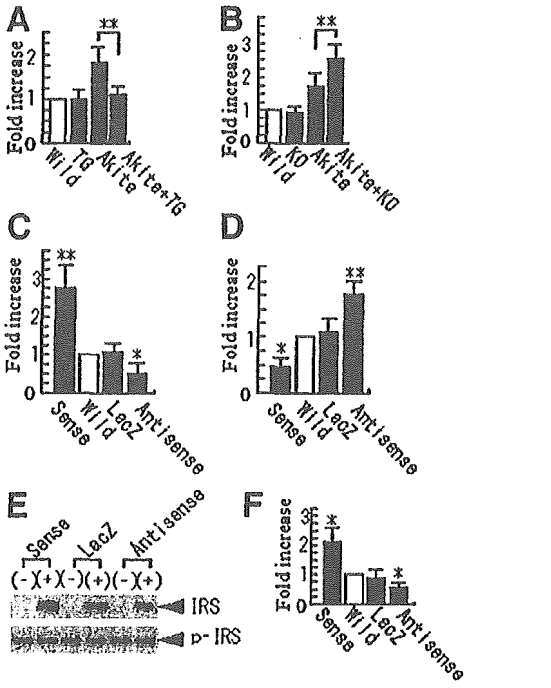

FIG. 8. ORP150 enhanced insulin signaling. *A* and *B*: The liver and skeletal muscle of wild-type (wild), ORP150$^{-/+}$ (KO), ORP150^{CAG} (TG), Ins2$^{WT/CY}$ (Akita), ORP150$^{-/+}$Ins2$^{WT/CY}$ (Akita+KO), and ORP150^{CAG} Ins2$^{WT/CY}$ (Akita+TG) mice that underwent Western blot analysis using an anti-DNP antibody and semiquantitative analysis (n = 4). *C* and *D*: L6 cells infected by Ad/S-ORP150, Ad/AS-ORP150, or AxCALacZ and treated with hydrogen peroxide underwent Western blot analysis using either an anti-ORP150 antibody or anti-DNP antibody. Semiquantitative analysis is shown, where the intensity of ORP150 (*C*) and anti-DNP antibody (*D*) is represented as the percentage increase over the control (not infected with adenovirus) normalized to the levels of β-actin (n = 4). *E* and *F*: L6 cells infected by Ad/S-ORP150, Ad/AS-ORP150, or AxCALacZ and treated with hydrogen peroxide were stimulated with either saline (−) or 100 nmol/l of human insulin (+) underwent immunoprecipitation of IRS-1 followed by Western blotting with an anti-phosphotyrosine antibody and anti–IRS-1 antibody. A semiquantitative analysis was performed (n = 4). Data are means ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ by multiple comparison analysis compared with the control.

levels in liver and muscle of *db/db* mice were greater than that of Akita mice (data not shown).

We have reported that ORP150 enhances the secretion of VEGF in wound healing and tumor formation (11,12), and, therefore, we expected that ORP150 might enhance the secretion of ACRP30/adiponectin, the expression of which correlates with insulin sensitivity (26), resulting in improved insulin resistance of Akita mice. To determine whether ORP150 increases the secretion of ACRP30, we assessed the levels of ACRP30 in serum and white/brown adipose tissues of Ins2$^{WT/C96Y}$, ORP150$^{-/+}$Ins2$^{WT/C96Y}$, and ORP150^{CAG}Ins2$^{WT/C96Y}$ by ELISA; however, no significant differences were shown between Ins2$^{WT/C96Y}$ and ORP150$^{-/+}$ Ins2$^{WT/C96Y}$ or between Ins2$^{WT/C96Y}$ and ORP150^{CAG} Ins2$^{WT/C96Y}$(online appendix).

In contrast, the ER of proteins consumes oxidizing equivalents during the process of disulphide-bond formation (27,28), and ER stress in PERK$^{-/-}$ or ATF4$^{-/-}$ cells leads to the acute production of reactive oxygen species (ROS) through the accumulation of proteins oxidized by protein disulfide isomerase (29). These findings suggest that ER can reduce ROS in the liver and skeletal muscle, as well as improve insulin sensitivity in type 2 diabetes. As a molecular chaperone, ORP150 increases folding capacity and enhances protein secretion during ER stress (10,11,12,30). Consequently, ORP150 could enhance the role of the ER by reducing excess oxidizing equivalents and improving insulin sensitivity in type 2 diabetes.

Taken together, these data demonstrate that the ER chaperone ORP150 could remit insulin resistance caused by hyperglycemia by reducing oxidative stress and that it might be a novel therapeutic target to reduce the insulin resistance characteristic of type 2 diabetes.

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Association of soluble epoxide hydrolase gene polymorphism with insulin resistance in type 2 diabetic patients

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Abstract

The insulin resistance found in diabetes is influenced by vascular tone and local blood flow. Endothelial-derived hyperpolarizing factor (EDHF) functions as a potent vasodilator to regulate vascular tone, and its production is regulated by soluble epoxide hydrolase (sEH). In this study, we examined the genotype distribution and allele frequency of sEH gene G860A (Arg287Gln) polymorphism in Japanese subjects ($n = 499$) (non-diabetic subjects, $n = 205$; type 2 diabetic patients, $n = 294$). Also, to accurately evaluate insulin resistance, we performed the euglycemic hyperinsulinemic clamp test for each type 2 diabetic patient ($n = 86$) from whom agreement was obtained, and then examined a possible association of sEH gene G860A polymorphism with insulin resistance status. There was no significant difference in genotype distribution and allele frequency between non-diabetic subjects and type 2 diabetic patients. Interestingly, however, there was close association of sEH gene G860A (Arg287Gln) polymorphism with insulin resistance in type 2 diabetic patients, which was not observed in non-diabetic subjects. These results suggest that sEH and EDHF play some important role in the pathogenesis of insulin resistance found in type 2 diabetes.

Keywords: Diabetes; Insulin resistance; Euglycemic hyperinsulinemic clamp; Soluble epoxide hydrolase; Polymorphism

Type 2 diabetes is the most prevalent and serious metabolic disease affecting people around the world. The hallmark of the disease is insulin resistance which is influenced by vascular tone and local blood flow. Glucose uptake into various tissues has been reported to be enhanced by insulin-mediated vasodilation and the decrease in local blood flow to lead to the development of insulin resistance [1,2]. An endothelial-derived hyperpolarizing factor (EDHF) that functions as a potent vasodilator is thought to be epoxyeicosatrienoic acids (EETs), lipid metabolites of arachidonic acid which are synthesized by the cytochrome P450 system [3–5]. EETs function as regulators of the vascular tone as well as an anti-inflammatory molecules [6–14]. Their production is regulated by soluble epoxide hydrolase (sEH), a ubiquitous enzyme that catalyzes the degradation of EETs. It has been reported that a common polymorphism in exon 8 of the sEH gene, which results in an amino acid substitution from arginine to glutamine at codon 287 (G860A), reduces its enzymatic activity and decreases its protein stability [15,16]. Very recently, the coronary artery risk development in young adults (CARDIA) study demonstrated that the sEH Arg287Gln polymorphism is associated with coronary artery calcification, suggesting a role of sEH in the pathogenesis of atherosclerosis [17]. In this study, we examined whether sEH Arg287Gln polymorphism is associated with insulin resistance in type 2 diabetes. This is the first report showing the association of sEH and insulin resistance.

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Methods

Study subjects. Japanese type 2 diabetic patients ($n = 294$, aged 30–76 years, duration of diabetes: 12.1 ± 9.2 years) undergoing periodic follow-up examinations at the Diabetes Clinic in Osaka University Hospital were enrolled in this study. The determination of type 2 diabetes was based on World Health Organization criteria. Patients were recruited for the study if they met the following inclusion criteria: (1) no episodes of ketoacidosis, (2) absence of overt diabetic nephropathy, and (3) absence of cardiovascular, cerebral vascular, and peripheral artery disease. Of the 294 diabetic patients, 72 patients were controlled with diet only, 114 with oral agents, and 108 with insulin injection. As control subjects, we enrolled healthy non-diabetic subjects ($n = 205$, aged 21–66 years) without cardiovascular, cerebral vascular or peripheral artery disease. Written informed consent was obtained from all subjects enrolled in this study.

Euglycemic hyperinsulinemic clamp test. For non-diabetic subjects ($n = 205$), insulin sensitivity was determined using fasting insulin and glucose concentrations by homeostasis model assessment of insulin resistance (HOMA). Plasma insulin concentrations were measured by radioimmunoassay (SRL, Tokyo, Japan). For type 2 diabetic patients, insulin sensitivity was determined by the euglycemic hyperinsulinemic clamp test. Of type 2 diabetic patients ($n = 294$), 86, from whom agreement was obtained, were subjected to determination of insulin-mediated glucose uptake by the euglycemic hyperinsulinemic clamp technique using an artificial pancreas (STG22; Nikkiso, Tokyo, Japan). Before the clamp study, the patients were hospitalized at Osaka University Hospital for at least 2 weeks and were confirmed to be free of glucose toxicity. Briefly, regular insulin (Humalin-R Eli Lilly, Indianapolis, IN, USA) was infused in a primed continuous manner at a rate of 8.7 pmol/kg/min for 2 h. Normoglycemia was maintained by adjusting the rate of a 10% D-glucose infusion based on plasma glucose measurements performed at 1-min intervals. Glucose infusion rate (GIR) was calculated by averaging the glucose infusion rates achieved over the last 30 min of the clamp as endogenous glucose production is completely suppressed at the elevated concentrations achieved.

Table 1
Clinical characteristics of non-diabetic and type 2 diabetic subjects

Variables	Non-diabetic subjects	Type 2 diabetic patients
n	205	294
Male/female	132/73	210/84
Age (year)	44.1 ± 0.8	57.4 ± 0.6
Duration (years)	—	12 ± 0.5
BMI (kg/m^2)	22.3 ± 0.2	23.7 ± 0.2
Systolic blood pressure (mmHg)	122 ± 0.9	133 ± 1.0
Diastolic blood pressure (mmHg)	75 ± 0.7	76 ± 0.5
HbA1c (%)	4.9 ± 0.02	7.7 ± 0.1
Total cholesterol (mg/dl)	193 ± 2.0	203 ± 2.1
Triglycerides (mg/dl)	95 ± 3.0	133 ± 4.2
HDL cholesterol (mg/dl)	61 ± 1.0	52 ± 0.9

Data are shown as means ± SE.

Genotype determination. Whole blood was first collected in a Vacutainer CPT tube (Becton–Dickinson, Franklin Lakes, NJ, USA). After the tube was centrifuged in a horizontal, swing-out rotor (20 min, 1500*g*, 24 °C), the leukocyte-enriched fraction was collected. This was then added to lysing buffer and DNA was extracted using the QIAamp Blood Kit (Qiagen, Chatsworth, CA, USA). Genotyping for the G860A (Arg287Gln) polymorphism was performed using the TaqMan assay kit (Applied Biosystems) as described previously [17]. A 64-bp product was amplified by polymerase chain reaction from 15-ng DNA using 0.9 μmol/L each of forward primer (AGA TCC CTG CTC TGG CCC) and reverse primer(TCT CCA TAG CCT TTC ATG TCC A). The sequence-specific probes (FAM-TAG GAC CcG GTA ACC and VIC-CTA GGA CCt GGT AAC C) were used in the allele discrimination assay, and allele detection and genotype calling were performed using the ABI7700 instrument and Sequence Detection System software.

Statistical analysis. The difference in quantitative variables was examined by one-way ANOVA and differences in allele frequency were examined by χ^2 analysis. Data are shown as means ± SEM.

Results

Genotype distribution and allele frequency of soluble epoxide hydrolase gene G860A (Arg287Gln) polymorphism in Japanese non-diabetic and diabetic subjects

We first examined the genotype distribution and allele frequency of sEH gene G860A (Arg287Gln) polymorphism in Japanese subjects ($n = 499$). Table 1 shows clinical characteristics of non-diabetic ($n = 205$) and type 2 diabetic subjects ($n = 294$), and Table 2 shows genotype distribution and allele frequency in non-diabetic subjects and type 2 diabetic patients. Genotype distribution and allele frequency of sEH G860A polymorphism in non-diabetic subjects and type 2 diabetic patients were in accordance with the Hardy–Weinberg equilibrium. Also, as shown in Table 2, there was no significant difference in genotype distribution (χ^2 analysis; genotype: $\chi^2 = 0.081$, $p = 0.97$) and allele frequency ($\chi^2 = 0.074$, $p = 0.85$) between Japanese non-diabetic subjects ($n = 205$) and type 2 diabetic patients ($n = 294$).

Association of soluble epoxide hydrolase gene polymorphism with insulin resistance in type 2 diabetic patients

We evaluated a possible association of sEH gene G860A polymorphism with insulin resistance status in non-diabetic subjects and type 2 diabetic patients. The

Table 2
Genotype distribution and allele frequency of sEH gene G860A polymorphism in non-diabetic subjects and type 2 diabetic patients

	n	Genotype			Allele frequency	
		GG	GA	AA	G	A
Non-diabetic subjects	205	126 (61.5%)	71 (34.6%)	8 (3.9%)	0.788	0.212
Type 2 diabetic patients	294	177 (60.2%)	105 (35.7%)	12 (4.1%)	0.781	0.219

number of homozygous mutants was so small that we combined the data of homozygous (AA) and heterozygous mutants (GA) in the following analysis. To evaluate insulin resistance in non-diabetic subjects, we calculated their HOMA values. We did not perform the euglycemic hyperinsulinemic clamp test in non-diabetic subjects from the ethical point of view. As shown in Table 3, there was no significant difference in age, BMI, HbA1c, blood pressure, and cholesterol levels between with (GA + AA) and without the 860A allele (GG) in both non-diabetic ($n = 205$) and diabetic subjects ($n = 294$). Also, there was no difference in HOMA values between with (GA + AA) and without the 860A allele (GG) in non-diabetic subjects. Next, to accurately evaluate insulin resistance in type 2 diabetic patients from whom agreement was obtained ($n = 86$), we performed the euglycemic hyperinsulinemic clamp test and calculated their glucose infusion rate which indicates insulin sensitivity in the whole body. As shown in Table 4, there was no significant difference in age, BMI, HbA1c levels between with (GA + AA) and without the 860A allele (GG). Interestingly, however, the glucose infusion rate in type 2 diabetic patients with the sEH gene 860A allele (GA + AA) was significantly lower than those without the 860A allele (GG) (5.06 ± 0.26 vs. 6.01 ± 0.28 mg/kg/min, $p = 0.038$). It is noted that glucose infusion rate indicates insulin sensitivity in the whole body. Thus, insulin sensitivity in type 2 diabetic patients with the sEH gene 860A allele (GA + AA) was significantly lower than those without the 860A allele (GG). These results indicate that the presence of the 860A allele in the sEH gene is associated with the insulin resistance status found in type 2 diabetic patients.

Table 4
Association of sEH gene G860A polymorphism with insulin resistance in type 2 diabetic patients

	Genotype		
	GG	GA or AA	
n	51	35	
Gender (male/female)	39/17	24/11	n.s.
Age (years)	50.1 ± 1.8	52.4 ± 2.2	n.s.
BMI (kg/m^2)	25.2 ± 0.8	25.9 ± 0.8	n.s.
HbA1c (%)	7.5 ± 0.3	7 ± 0.3	n.s.
Glucose infusion rate (mg/kg/min)	6.01 ± 0.28	5.05 ± 0.26	$p = 0.038$

Data are shown as means ± SE.

Discussion

In this study, we examined the genotype distribution and allele frequency of sEH gene G860A (Arg287Gln) polymorphism in Japanese subjects ($n = 499$) (non-diabetic subjects, $n = 205$; type 2 diabetic patients, $n = 294$) and a possible association of sEH gene G860A polymorphism with insulin resistance status which was accurately evaluated by the euglycemic hyperinsulinemic clamp technique using artificial pancreas. The main finding in this

Table 3
Clinical characteristics according to G860A polymorphism of sEH gene

	Non-diabetic subjects			Type 2 diabetic patients		
	GG	GA or AA	p	GG	GA or AA	p
Gender (male/female)	81/45	51/28		130/47	80/37	
Age (years)	44.1 ± 0.9	41.2 ± 1.2	n.s.	57.8 ± 0.8	57.2 ± 1.0	n.s.
BMI (kg/m^2)	22.3 ± 0.3	22.8 ± 0.4	n.s.	23.8 ± 0.3	23.6 ± 0.3	n.s.
Systolic BP (mmHg)	122 ± 1.2	122 ± 1.3	n.s.	132 ± 1.2	133 ± 1.6	n.s.
Diastolic BP (mmHg)	75 ± 0.1	76 ± 0.1	n.s.	76 ± 0.7	76 ± 0.9	n.s.
HbA1c (%)	4.9 ± 0.03	4.8 ± 0.03	n.s.	7.9 ± 0.1	7.6 ± 0.1	n.s.
Total cholesterol (mmol/L)	194 ± 2.5	190 ± 3.5	n.s.	203 ± 2.6	203 ± 3.7	n.s.
Triglycerides (mmol/L)	92 ± 3.7	100 ± 5.2	n.s.	130 ± 5.0	137 ± 7.1	n.s.
HDL cholesterol (mmol/L)	61 ± 1.3	62 ± 1.5	n.s.	51 ± 1.1	52 ± 1.4	n.s.
Insulin (pmol/L)	7.2 ± 0.3	7.1 ± 0.3	n.s.	—	—	
HOMA-R	1.63 ± 0.06	1.64 ± 0.08	n.s.	—	—	
HT-Risk[a]	17	11	n.s.	41	25	n.s.
HL-Risk[a]	31	8	n.s.	48	30	n.s.
SM-Risk[a]	29	5	n.s.	50	28	n.s.
Nephropathy[a]	—	—		155/22	99/18	n.s.
Retinopathy[a]	—	—		149/28	96/21	n.s.
Treatment of diabetes[a]	—	—		45/68/64	23/49/45	n.s.

Data are shown as means ± SE. HT-Risk, number of subjects with either systolic >160, diastolic pressure >95 mmHg, or taking anti-hypertensive drugs; HL-Risk, number of subjects with either total cholesterol >240 mg/dl, triglyceride >150 mg/dl, HDL-cholesterol <40 mg/dl or taking hypolipidemic drugs; SM-Risk, number of subjects who smoke more than one pack of cigarettes per day for 20 years; nephropathy, normoalbuminuria + albuminuria/proteinuria with normal serum creatinine; retinopathy, no diabetic retinopathy + background diabetic retinopathy/preproliferative diabetic retinopathy + proliferative diabetic retinopathy; treatment of diabetes, diet therapy/oral agents/insulin injection.

[a] χ^2 test.

study is that sEH gene G860A (Arg287Gln) polymorphism is closely associated with insulin resistance in type 2 diabetic patients. These results suggest that sEH and EDHF play some important role in the pathogenesis of insulin resistance found in type 2 diabetes.

In contrast to type 2 diabetic patients, there was no association between sEH gene G860A polymorphism and insulin sensitivity in non-diabetic subjects. Since endothelial cell dysfunction is often observed in type 2 diabetic patients, but not in non-diabetic subjects, we assume that the presence of the 860A allele in the sEH gene, together with endothelial cell dysfunction, leads to the substantial progression of insulin resistance. It is noted that although the development of obesity and/or poor glycemic control are well known to induce insulin resistance, there was no difference in BMI and HbA1c levels between the two groups. Thus, we assume that the sEH gene G860A polymorphism directly affects the insulin resistance status in type 2 diabetic patients by altering vascular tone. Although we could not measure the plasma EET levels in the patients due to the very short half-life of EETs, we assume that plasma EET levels are lower in type 2 diabetic patients with sEH G860A, which leads to a disturbed vascular tone and to an increase of insulin resistance.

In conclusion, although no significant difference was observed in the genotype distribution and allele frequency of sEH gene G860A (Arg287Gln) polymorphism between Japanese non-diabetic subjects and type 2 diabetic patients, the glucose infusion rate in type 2 diabetic patients with the sEH gene 860A allele (GA + AA) was significantly lower than those without the 860A allele (GG). This is the first report showing the association of sEH and insulin resistance, implying that sEH and EDHF play some important role in the pathogenesis of insulin resistance found in type 2 diabetes.

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