by enzyme assay (Hitachi, Tokyo, Japan). The urinary albumin-to-creatinine ratio (ACR) was calculated, and less than 30 μg/mg creatinine was counted as normoalbuminuria and ACR between 30 and 300 μg/mg creatinine as microalbuminuria, based on American Diabetes Association criteria [21]. Microalbuminuria in untimed specimens (30 μg/mg creatinine) corresponds to not less than 30mg albumin in 24 h urine collection and not less than 20 μg/min in timed specimens [22]. Subjects with ACR of more than 300 μg/mg creatinine were excluded from the study due to possible clinical proteinuria.

2.3. Statistical analyses

All analyses were performed using the Statistical Package for the Social Sciences version 10.0J (SPSS Inc., IL, USA). Age, BMI, systolic and diastolic blood pressure, FPG/2 h PG, HbA_{1c}, fasting insulin, insulin resistance, triglycerides, total cholesterol, and HDL-cholesterol were compared among NGT, IFG, isolated IGT, and IGT/FH by general analysis of variance (ANOVA; Table 1). Dunnett's procedure as post hoc analysis was applied to compare with the NGT group. The prevalence of microalbuminuria of each subgroup was evaluated by Chi-square test and was compared with that of the NGT group. In addition, in subjects with IGT, age, BMI, systolic and diastolic blood pressure, FPG, 2 h PG, HbA_{1c}, fasting insulin, insulin resistance, triglycerides, and total and HDL-cholesterol were compared according to the presence of microalbuminuria by unpaired Student's *t*-test (Table 2). In both of these analyses, the value of fasting insulin, insulin resistance (HOMA-IR), triglycelides, total cholesterol, and HDL-cholesterol were log-transformed before analysis so their distribution would be close to normal. Probability (*P*) values less than 0.05 were considered statistically significant. Data are expressed as mean ± S.E.

Multiple logistic regression analyses were performed to investigate the association between microalbuminuria and the various subgroups based on OGTT (Table 3). Microalbuminuria is the dependent variable and the independent variables are (1) glucose tolerance status, (2) glucose tolerance status and age, (3) glucose tolerance status, age, and hypertension, and (4) glucose tolerance status, age, hypertension, and insulin resistance. The Wald test was used to

Table 1
Demographic/metabolic characteristics and prevalence of microalbuminuria of all study subjects

	NGT	IFG	Isolated IGT	IGT/FH	DM	Total
n	71	24	36	23	149	303
Age (years)	45.3 ± 1.2	52.7 ± 1.9***	50.0 ± 1.3*	54.0 ± 1.3***	51.3 ± 0.6***	50.1 ± 0.5
BMI (kg/m^2)	24.1 ± 0.3	23.6 ± 0.6	23.4 ± 0.4	24.3 ± 0.5	24.4 ± 0.2	24.2 ± 0.2
Systolic BP (mmHg)	128 ± 2	132 ± 4	123 ± 3	130 ± 4	130 ± 1	129 ± 1
Diastolic BP (mmHg)	78 ± 2	79 ± 2	75 ± 2	78 ± 3	78 ± 1	77 ± 1
FPG (mmol/l)	5.3 ± 0.1	6.3 ± 0.0***	5.6 ± 0.1	6.5 ± 0.1***	7.8 ± 0.1***	6.7 ± 0.1
2 h glucose (mmol/l)	5.8 ± 0.2	6.2 ± 0.3	8.9 ± 0.2***	9.6 ± 0.2***	15.2 ± 0.3***	11.1 ± 0.3
HbA_{1c} (%)	5.5 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	6.1 ± 0.1*	7.2 ± 0.1***	6.4 ± 0.1
Fasting insulin (pmol/l)[a]	23.8 ± 1.6	26.8 ± 3.1	28.8 ± 2.7	28.9 ± 3.5	27.3 ± 1.2	26.7 ± 0.9
Insulin resistance (mU mmol/l^2)[a,b]	0.9 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.2*	1.6 ± 0.1***	1.3 ± 0.2
Triglycerides (mmol/l)[a]	1.13 ± 0.09	1.01 ± 0.13	1.65 ± 0.17*	1.52 ± 0.20	1.54 ± 0.08***	1.40 ± 0.05
Total cholesterol (mmol/l)[a]	5.04 ± 0.11	5.06 ± 0.18	5.08 ± 0.15	5.18 ± 0.19	5.25 ± 0.08	5.16 ± 0.05
HDL-cholesterol (mmol/l)[a]	1.25 ± 0.04	1.37 ± 0.07	1.02 ± 0.04**	1.15 ± 0.06	1.16 ± 0.03	1.18 ± 0.02
Prevalence of microalbuminuria (%)	9 (6/71)	13 (3/24)	14 (5/36)	26 (6/23)†	26 (39/149)†	19 (59/303)

Data are means ± standard error. Microalbuminuria: urine albumin-to-creatinine ratio ≥30 μg/mg creatinine.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. NGT.
[a] Data are adjusted for age and BMI.
[b] HOMA-IR = fasting insulin × fasting glucose/22.5.
† $P < 0.05$ vs. NGT by Chi-square test.

Table 2
Demographic/metabolic characteristics of IGT subjects in relation to the presence of microalbuminuria

	Microalbuminuria		
	No	Yes	*P*-value
n	48	11	
Age (years)	50.9 ± 1.1	54.6 ± 2.5	0.139
BMI (kg/m^2)	23.7 ± 0.4	24.1 ± 1.0	0.705
Systolic BP (mmHg)	124 ± 3	134 ± 6	0.115
Diastolic BP (mmHg)	75 ± 2	78 ± 4	0.509
FPG (mmol/l)	5.9 ± 0.1	6.1 ± 0.2	0.335
2 h glucose(mmol/l)	9.1 ± 0.1	9.7 ± 0.4	0.082
HbA_{1c} (%)	6.0 ± 0.1	5.9 ± 0.2	0.742
Fasting insulin (pmol/l)	26 ± 2	37 ± 7	0.048
Insulin sensitivity (mU mmol/l^2)[a]	1.2 ± 0.2	1.7 ± 0.3	0.041
Triglycerides (mmol/l)	1.57 ± 0.15	1.57 ± 0.36	1.000
Total cholesterol (mmol/l)	5.15 ± 0.13	5.02 ± 0.27	0.677
HDL-cholesterol (mmol/l)	1.09 ± 0.04	1.06 ± 0.13	0.716

[a] HOMA-IR = fasting insulin × fasting glucose/22.5.

determine the statistical significance of each coefficient. To quantify the proportion of variation due to dependent variables, Nagelkerke R^2-statistics were calculated on each model [23]. For these analyses, age was divided into four groups: <40, 40–50, 50–60, and >60 years. Hypertension was defined as systolic blood pressure more than 140 mmHg or diastolic blood pressure more than 90 mmHg [24]. HOMA-IR was used to measure insulin resistance, and was divided to create quartiles for analysis.

To evaluate the metabolic factors associated with microalbuminuria in IGT subjects (n = 59), linear regression analyses of ACR as the dependent variable and each of the metabolic variables as independent variables were performed (Table 4). A partial standardized regression coefficient (β) was used to show the magnitude of association between ACR and each independent variable. Age was included in all models as an independent variable. For analysis, ACR, HOMA-IR, fasting insulin, triglycerides, total cholesterol, and HDL-cholesterol were transformed to natural logarithms to satisfy statistical assumptions.

Table 4
Standardized partial regression coefficients of urine albumin-to-creatinine ratio and metabolic variables in IGT subjects (n = 59)

	Standardized β	*P*-value
Insulin resistance[a]	0.306	0.012
sBP	0.238	0.060
dBP	0.147	0.250
BMI	0.101	0.420
FPG	0.122	0.352
2 h glucose	0.148	0.235
Fasting insulin[b]	0.255	0.039
HbA_{1c}	−0.109	0.390
Triglycerides[b]	0.132	0.299
Total cholesterol[b]	0.008	0.950
HDL-cholesterol[b]	0.018	0.891

Partial correlation coefficients adjusted for age by linear regression.
[a] HOMA-IR = fasting insulin × fasting glucose/22.5.
[b] Log-transformed.

Table 3
Multiple logistic regression analysis of the presence of microalbuminuria across the subgroups based on OGTT

Adjusted for	Glucose tolerance status								R^2
	IFG		Isolated IGT		IGT/FH		DM		
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	
None	1.55	(0.36–6.74)	1.75	(0.50–6.17)	3.82	(1.09–13.36)*	3.84	(1.54–9.57)**	0.066
Age	1.68	(0.37–7.52)	1.86	(0.52–6.68)	4.39	(1.17–16.40)*	4.40	(1.67–11.58)**	0.072
Age, hypertension	1.62	(0.36–7.31)	1.85	(0.51–6.66)	4.41	(1.17–16.54)*	4.56	(1.73–11.99)**	0.090
Age, hypertension, insulin resistance	1.38	(0.30–6.30)	1.59	(0.43–5.83)	3.45	(0.89–13.41)	3.26	(1.18–9.02)*	0.126

Divided into three groups: <40, 40–50, 50–60 and >60 years. Hypertension: a systolic blood pressure of ≥140 mmHg, or a diastolic blood pressure of ≥90 mmHg. Quartile of HOMA-IR as a measurement of insulin sensitivity.
*$P < 0.05$; **$P < 0.01$ vs. NGT.

3. Results

Demographic and metabolic characteristics of the subjects are shown in Table 1. Three hundred three subjects were enrolled. The average age and BMI was 50.1 ± 0.5 and 24.2 ± 0.2, respectively. Altogether, 19% of the subjects were found to have microalbuminuria (ACR ≥ 0 μg/mg creatinine). There was no significant difference in BMI and blood pressure among the four groups. The IGT/FH group had significantly higher FPG, 2 h PG, and HbA_{1c} than the NGT group, while the isolated IGT group had only significantly higher 2 h PG level. Insulin resistance (HOMA-IR) in the IGT/FH group was significantly higher than in the NGT group ($P = 0.024$), but there was no significant difference between the isolated IGT and the NGT groups. The lipid profile of the isolated IGT group was similar to that of the IGT/FH group, and was deteriorated relative to the NGT group. The prevalence of microalbuminuria in the IGT/FH group (26%, $P = 0.028$ versus NGT by Chi-square test) was higher than in the isolated IGT group (14%, $P = 0.381$ versus NGT by Chi-square test). Mean ACR was 16.2 ± 3.6 μg/mg creatinine in the isolated IGT group and 42.2±10.7 μg/mg creatinine in the IGT/FH group, while the mean ACR in the NGT group was 15.5 ± 2.6 μg/mg creatinine.

The demographic and metabolic characteristics of the IGT subjects according to the presence or absence of microalbuminuria are shown in Table 2. Subjects with microalbuminuria had significantly higher insulin resistance (higher HOMA-IR) and higher fasting plasma insulin than those without microalbuminuria ($P = 0.041$ and 0.048, respectively). The lipid profiles of both groups were similar.

The results of multiple logistic regression analysis with microalbuminuria as a dependent variable and glucose tolerance status as an independent variable are shown in Table 3. With no adjustment, microalbuminuria showed a significant association with IGT/FH ($P = 0.036$). After adjustment for age and hypertension, the odds ratio of microalbuminuria in the IGT/FH group was still significant (OR = 4.41, 95% CI 1.17-16.54; $P = 0.028$). There was no significant association of microalbuminuria with isolated IGT (OR = 1.85, 95% CI 0.51-6.66; $P = 0.348$). The R^2-values suggest that 9% of the variance was due to age, hypertension, and glucose tolerance status. The addition of insulin resistance to the analysis resulted in loss of significant association of microalbuminuria with IGT/FH, but increased the proportion of variation explained (R^2) by 3%.

Table 4 shows the partial correlation coefficients of urine albumin-to-creatinine ratio (ACR; log-transformed) with insulin resistance and other metabolic variables in subjects with IGT. After adjustment for age, there was a significant correlation between ACR and insulin resistance ($P = 0.012$). Fasting insulin also had a significant relation to ACR ($P = 0.039$), while systolic blood pressure had a marginal relation ($P = 0.060$) and there was no significant relation with the other variables. In all measured variables, insulin resistance was the strongest determinant of ACR (standardized $\beta = 0.306$).

4. Discussion

Microalbuminuria has been shown to be a predictor of renal dysfunction and cardiovascular diseases in both non-diabetic and type 2 DM subjects [4,5], and is significantly associated with IGT [8,9]. As previous studies have shown that isolated IGT and IGT/FH have different metabolic profiles [13–15], we compared the prevalence of microalbuminuria in the two subgroups. Our analyses clearly demonstrate that microalbuminuria in these subjects is more strongly associated with IGT/FH than with isolated IGT, which reflects the higher insulin resistance in IGT/FH.

In addition to higher insulin resistance, subjects with IGT/FH exhibit higher age, blood pressure, BMI, plasma glucose, cholesterol, and fasting insulin than those with isolated IGT and NGT, so these factors all might be related to the appearance of microalbuminuria in IGT/FH [10,11,25]. Regarding age and hypertension, the absolute differences between subjects with isolated IGT and IGT/FH are small, and the odds ratio of microalbuminuria in IGT/FH is high even after adjustment for age and hypertension. In addition, linear regression analysis of urine ACR shows insulin resistance to be the stronger determinant of microalbuminuria and fasting insulin the weaker determinant, and no significant correlation with other variables such as BMI, plasma glucose level, or cholesterol level. However, the link between insulin resistance and microalbuminuria is unclear.

Some studies report that hyperglycemia and/or hyperinsulinemia alters permeability of the glomerular membrane and increases intraglomerular pressure [26,27]. Epidemiological studies suggest that hyperglycemia or hyperinsulinemia itself is not the major cause of increased microalbuminuria among IGT subjects [8,10]. However, insulin resistance has been shown to produce changes in the amount and/or the effects of nitric oxide and other chemical mediators by causing endothelial dysfunction [28,29]. Dysfunction at the glomerular capillary wall might well induce increased leakage of albumin at the glomerulus that results in microalbuminuria [30,31]. Another possibility is that endothelial dysfunction causes parallel defects of microalbuminuria and insulin insensitivity, since several studies report that insulin resistance correlates with impaired peripheral vasodilation [32]. However, the role of blood flow in the modulation of insulin-mediated glucose utilization is controversial.

In conclusion, our results show an increased prevalence of microalbuminuria in IGT/FH subjects compared to isolated IGT subjects that is closely associated with increased insulin resistance. Because IGT/FH patients have heightened risk of cardiovascular and other diabetic complications requiring more intensive therapeutic approaches, the results of 75g OGTT should be clinically useful in pre-diabetic patients.

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References

[1] M. Graves, R.G. Malia, K. Goodfellow, et al., EURODIAB IDDM Complication Study Group: Fibrinogen and van Willebrand factor in IDDM: relationships to lipid vascular riskfactors, blood pressure, glycaemic control and urinary albumin excretion rate: the EURODIAB IDDM complications study, Diabetolgia 40 (1997) 698–705.

[2] C.D. Stehouwer, J.J.P. Nauta, G.C. Zeldenrust, W.H.L. Hackeng, A.J.M. Donker, G.J.H. Den Ottolander, Urinary albumin excretion, cardiovascular disease, and endothelial dysfunction in non-insulin-dependent diabetes mellitus, Lancet 340 (1992) 319–323.

[3] B.G.S. Seligman, A. Biolo, C.A. Planczyk, J.L. Gross, N. Clausell, Increased plasma levels of endothelin 1 and van Willebrand factor in patients with Type 2 diabetes and dyslipidemia, Diabetes Care 23 (2000) 1395–1400.

[4] J.S. Yudkin, R.D. Forrest, C.A. Jackson, Microalbuminuria as predictor of vascular disease in non-diabetic subjects: Islington Diabetes Study, Lancet ii (1988) 530–533.

[5] M.B. Mattock, N.J. Morrish, G.C. Viberti, H. Keen, A.P. Fitzgerald, G. Jackson, Prospective study of microalbuminuria as predictor of mortality in NIDDM, Diabetes 41 (1992) 736–741.

[6] M.B. Mattock, D.J. Barnes, B. Vibetrti, et al., Microalbuminuria and coronary heart disease in NIDDM: An Incidence Study, Diabetes 47 (1998) 1786–1792.

[7] S. Savage, R.O. Estacio, B. Jeffers, R.W. Schrier, Urinary albumin excretion as a predictor of diabetic retinopathy, neuropathy, and cardiovascular disease in NIDDM, Diabetes Care 19 (1996) 1243–1248.

[8] R.G. Nelson, C.L. Runzelman, D.J. Pettitt, M.F. Saad, P.H. Bennett, W.C. Knowler, Albuminuria in Type 2 (non-insulin-dependent) diabetes mellitus and impaired glucose tolerance in Pima Indians, Diabetologia 32 (1989) 870–876.

[9] S.M. Haffner, C. Gonzales, R.A. Valdes, et al., Is microalbuminuria part of the prediabetic state? The Mexico City Diabetes Study, Diabetologia 36 (1993) 1002–1006.

[10] L. Mykkanen, D.J. Zaccaro, L.E. Wagenknecht, D.C. Robbins, M. Gaberiel, S.M. Haffner, Microalbuninuria is associated with insulin resistance in nondiabetic subjects: The Insulin Resistance Atherosclerosis Study, Diabetes 47 (1998) 793–800.

[11] K.G. Rowley, D.M. Iser, J.D. Best, K. O'Dea, D. Leonard, R. McDermot, Albuminuria in Australian aboriginal people: prevalence and association with components of the metabolic syndrome, Diabetologia 43 (2000) 1397–1403.

[12] K.G. Alberti, P. Zimmet, Definition, diagnosis and classification of diabetes mellitus and its complications. I. Diagnosis and classification of diabetes mellitus: provisional report of a WHO consultation, Diabet. Med. 15 (1998) 539–553.

[13] D. Tripathy, M. Carlsson, P. Almgren, et al., Insulin secretion and insulin sensitivity in relation to glucose tolerance: lesson from the Botnia Study, Diabetes 49 (2000) 975–980.

[14] H. Suzuki, M. Fukushima, M. Usami, et al., Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia, Diabetes Care 26 (2003) 1211–1215.

[15] D.J. Kim, M.S. Lee, K.W. Kim, M.K. Lee, Insulin secretory dysfunction and insulin resistance in the pathogenesis of Korean type 2 diabetes mellitus, Metabolism 50 (2001) 590–593.

[16] National Diabetes Data Group, Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance, Diabetes 28 (1979) 1039–1057.

[17] A. Taniguchi, M. Fukushima, M. Sakai, et al., Remnant-like particle cholesterol, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients, Diabetes Care 23 (2000) 1766–1769.
[18] D.R. Matthews, J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, R.C. Turner, Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man, Diabetologia 28 (1985) 412–419.
[19] E. Bonora, F. Saggiani, G. Targher, et al., Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity, Diabetes Care 23 (2000) 57–63.
[20] M. Fukushima, A. Taniguchi, M. Sakai, et al., Homeostasis model assessment as a clinical index of insulin resistance. Comparison with the minimal model analysis, Diabetes Care 22 (1999) 1911–1912.
[21] American Diabetes Association: Diabetic Nephropathy (Position Statement), Diabetes Care 25 (Suppl. 1) (2002) S85–S89.
[22] R.L. Chaiken, R. Khowaja, M. Bard M, M. Echkert-Morton, M.A. Banerji, H.E. Lebovitz, Utility of untimed urinary albumin measurement in assessing albuminuria in black NIDDM subjects, Diabetes Care 20 (1997) 709–713.
[23] N.J. Norusis, SPSS Regression Models 10.0, SPSS Inc., Chicago, IL, 1999, pp. 45–47.
[24] Guidelines Subcommittee, 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension, J. Hypertens 17 (1999) 151–183.
[25] F.K. Keane, The role of lipids in renal disease: future challenges, Kidney Int. 57 (Suppl. 75) (2000) S27–S31.
[26] G. Arcaro, A. Cretti, S. Balzano, et al., Insulin causes endothelial dysfuntion in humans: sites and mechanisms, Circulation 15 (2002) 576–582.
[27] E. Ritz, Nephropathy in type 2 diabetes, J. Inter. Med. 245 (1999) 111–126.
[28] M.C. Stuhlinger, F. Abbasi, J.W. Chu, et al., Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor, JAMA 287 (2002) 1420–1426.
[29] G.S. Hotamisligil, P. Arner, J.F. Cargo, R.L. Atkinson, B.M. Spiegelman, Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance, J. Clin. Invest. 95 (1995) 2409–2415.
[30] S.B. Cine, E.S. Pollak, C.A. Buck, et al., Endothelial cell in physiology and in the pathophysiology of vascular disorders, Blood 91 (1998) 3527–3561.
[31] M.S. Goligorsky, J. Chen, S. Brodsky, Endothelial cell dysfunction leading to diabetic nephropathy: focus on nitric oxide, Hypertension 37 (2001) 744–748.
[32] A.D. Baron, M. Tarshoby, G. Hook, et al., Interaction between insulin sensitivity and muscle perfusion on glucose uptake in human skeletal muscle: evidence for capillary recruitment, Diabetes 49 (2000) 768–774.

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Insulin Secretion and Insulin Sensitivity at Different Stages of Glucose Tolerance: A Cross-Sectional Study of Japanese Type 2 Diabetes

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To evaluate the factors causing glucose intolerance in type 2 diabetes in Japan, insulin secretion and insulin sensitivity were compared across the range of glucose tolerance. Subjects were divided into 3 groups: normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and type 2 diabetes (DM) according to the criteria of the World Health Organization (WHO). We examined insulin secretion and insulin sensitivity using fasting blood glucose and insulin levels and 75 g oral glucose tolerance test (OGTT). We used homeostasis model assessment (HOMA) β-cell and insulinogenic index (30 minutes) to estimate insulin secretion and HOMA-insulin resistance (IR) and insulin sensitivity index (ISI) composite for insulin sensitivity. Although insulin resistance plays an important role in the development of diabetes in many ethnic populations, the differences in insulin sensitivity between NGT and IGT and between IGT and DM are small in Japanese patients. On the other hand, as glucose intolerance increases, insulin secretion decreases most remarkably both between NGT and IGT and between IGT and DM in Japanese patients. Decreasing insulin secretion and decreasing insulin sensitivity both occur in developing type 2 diabetes in Japanese patients, but decreased basal and early-phase insulin secretion had more pronounced contribution to glucose tolerance than the indices of insulin sensitivity. Japanese type 2 diabetic patients are characterized by a larger decrease in insulin secretion and show less attribution of insulin resistance.

TYPE 2 DIABETES is characterized by both decreased insulin secretion and decreased insulin sensitivity, but the degree of contribution of these 2 factors in the etiology varies.[1,2] Impaired insulin secretion and impaired insulin sensitivity both occur in the development of type 2 diabetes, but the contribution of these factors differs in certain ethnic populations.[3-6] The prevalence of diabetes is increasing in Japan and is now comparable to other countries. However, there are some differences between Japanese and other ethnic populations. The mean body mass index (BMI) of epidemiologic studies of type 2 diabetes in Japanese is around 24, which is lower than the studies of other ethnic populations.[7-11] In previous studies, we have examined insulin secretion and sensitivity using 75 g oral glucose tolerance test (OGTT) and minimal model analysis.[4,12,13] There were some differences in factors responsible for glucose tolerance of Japanese subjects in comparison to the other studies. We have reported that lower insulin secretory capacity in Japanese subjects would be unlikely to compensate for only a slight decrease in insulin sensitivity.[14] However, to understand the profile of Japanese subjects at various stages of glucose tolerance, a large number of subjects had to be examined.

In the present study, we have investigated insulin secretion and insulin sensitivity of 684 Japanese subjects across the range of glucose tolerance: normal glucose tolerance (NGT) (fasting plasma glucose [FPG] level < 6.1 mmol/L and 2-hour plasma glucose [PG] level < 7.8); impaired glucose tolerance (IGT) (FPG level < 7 and 7.8 < 2-hour PG level < 11.1); and type

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Table 1. Clinical Characteristics of the Subjects With Varing Degrees of Glucose Tolerance

	NGT	IGT	DM
N (M/F)	176 (125/51)	158 (112/46)	350 (248/102)
Age (yr)	49.1 ± 0.9	52.7 ± 0.7*	52.6 ± 0.4*
BMI (kg/m^2)	23.5 ± 0.2	23.9 ± 0.2	24.7 ± 0.2*†
FPG (mmo/L)	5.3 ± 0.03	5.8 ± 0.04*	8.1 ± 0.10*†
2-h PG (mmol/L)	5.9 ± 0.09	9.2 ± 0.08*	15.6 ± 0.23*†
Insulin-0 (μU/mL)	5.1 ± 0.19	5.7 ± 0.23	6.6 ± 0.22*†
Insulin-30	31.3 ± 1.78	24.8 ± 1.32*	15.1 ± 0.61*†
Insulin-60	43.7 ± 2.37	35.5 ± 2.15*	24.2 ± 1.04*†
Insulin-90	45.9 ± 3.76	43.9 ± 3.29	27.4 ± 1.26*†
Insulin-120	32.2 ± 2.03	41.1 ± 2.42*	29.3 ± 1.25†
Triglycerides (mg/dL)	120.4 ± 6.9	190.4 ± 33.5*	191.5 ± 13.5*
Total cholesterol (mg/dL)	205.8 ± 2.9	211.9 ± 3.0	211.4 ± 2.2
HDL-cholesterol (mg/dL)	56.3 ± 1.6	52.3 ± 1.6*	50.8 ± 0.8*

*Significant difference *v* NGT; †significant difference *v* IGT.

2 diabetes (DM) (FPG level ≥ 7 or 2-hour PG level > 11.1).[15] The homeostasis model assessment (HOMA) β-cell and HOMA-insulin resistance (IR) indices calculated by HOMA were used to determine insulin secretion and sensitivity at the fasting state.[16-18] The insulinogenic index (30 minutes) and insulin sensitivity index (ISI) composite were determined by 75 g OGTT.[19-21] We compared these indices across the range of glucose tolerance from normal to type 2 diabetes to evaluate the causative factors.

SUBJECTS AND METHODS

OGTT (75 g) was used to divide 684 Japanese subjects into 3 groups: NGT, IGT, and DM according to the criteria of the World Health Organization (WHO) in 1998.[15] There were 102 isolated IGT subjects (FPG level < 6.1 and 7.8 < 2-hour PG level < 11.1) in 158 IGT subjects. We recruited subjects from Kyoto University Hospital, Ikeda Hospital, Kanai Hospital, Kansai Health Management Center, and Kansai-Denryoku Hospital during 1990 to 2003. The subjects showed no signs of hypertension, hepatic or renal diseases, engaged in no heavy exercise, or took any medications before the study. Blood was drawn in the morning after a 12-hour fast. The plasma glucose was measured by the glucose oxidase method, and serum insulin was measured using 2-site immunoradiometric assay (Insulin Riabead I; Dainabot, 1990-1991 and Insulin Riabead II, Dainabot, 1992-2003, Tokyo, Japan). The assay results of the same samples with these 2 insulin assay methods showed a very high correlation ($r = 0.99$, $P < .0001$) in the usual assay range. The lipid profiles were measured as reported previously.[22]

The indices of basal insulin secretion and sensitivity were evaluated by HOMA and calculated as follows: HOMA-IR = FIRI × FPG/22.5, HOMA β-cell = 20 × FIRI/(FPG-3.5), where FIRI is fasting plasma insulin level(μU/mL) and FPG is fasting plasma glucose levels (mmol/L).[14-16] ISI composite was calculated according to the formula as follows: 10,000/(Glu 0 × Ins 0 × mean Glu 0−120 × mean Ins 0−120)$^{0.5}$.[19] Insulinogenic index (30 minutes) was estimated as follows: (Ins 30 − Ins 0)/(Glu 30 − Glu 0).[20,21]

Statistical Analysis

Statistical analysis was performed with the StatView 5 system (Abacus Concepts, Berkeley, CA). Unpaired student's *t* tests and simple regression analysis were used for the comparisons of clinical parameters. For the analysis of variance, Bonferroni test was used and $P < .05$ was considered significant. We used multiple regression analysis for the comparison of the relationship between area under the curve for glucose (G-AUC) and the indices of insulin secretion and sensitivity. The data are expressed as mean ± SEM.

RESULTS

Table 1 shows the characteristics of the subjects in this study. There was a 3.6-year difference between NGT and IGT, and no significant difference between IGT and DM in age. There was no significant difference between NGT and IGT, and only 0.8 difference between IGT and DM in BMI. The mean values of the HOMA-IR of NGT, IGT, and DM were 1.2, 1.5, and 2.4, respectively, only representing somewhat small differences between each of the groups, as shown in Fig 1A. The mean values of the ISI composite for NGT, IGT, and DM were 8.6, 7.1, and 5.8, respectively, also representing relatively small differences between the 3 groups (Fig 1B). In contrast, there was a dramatic decrease in HOMA β-cell among the 3 groups, as shown in Fig 2A, as there was also in the insulinogenic index, as shown in Fig 2B.

We then examined the relationship between the G-AUC and the indices of insulin secretion and sensitivity. The scattered plots of simple regression analysis between the G-AUC and the 4 indices are presented in Fig 3. There were significant relationships between G-AUC and the 4 indexes. Multiple regression analysis showed that HOMA-IR, HOMA β-cell, ISI composite, and insulinogenic index were independent factors to explain the variability of 60.7% of G-AUC ($P < .0001$). The

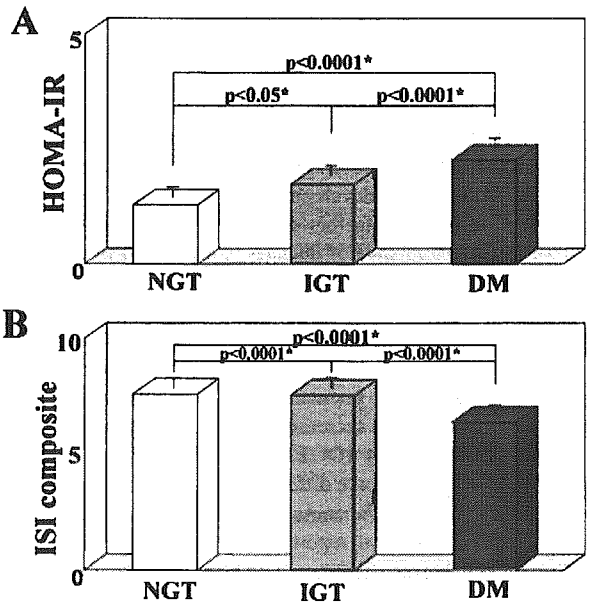

Fig 1. (A) Insulin resistance index at basal state was compared across the range of glucose tolerance. Insulin resistance increases with increasing glucose intolerance, but the differences are relatively small in Japanese subjects. (B) Insulin sensitivity decreases with increasing glucose intolerance according to the ISI composite, and the differences also are relatively small. *Significant differences assessed by analysis of variance.

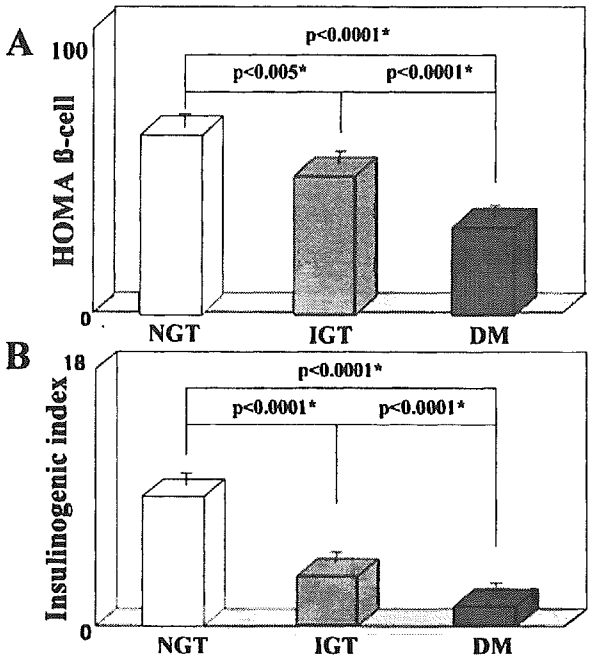

Fig 2. (A) Comparison of insulin secretion index in the basal state across the range of glucose tolerance. Insulin secretion decreases with increasing glucose intolerance. (B) Early-phase insulin secretion decreases remarkably with increasing glucose intolerance. *Significant differences assessed by analysis of variance.

correlation coefficients of these indices with G-AUC in simple regression analysis and β values and P values of multiple regression analysis are shown in Table 2. As estimates of basal insulin secretion and sensitivity, β value of HOMA β-cell was higher than HOMA-IR. As estimates of postchallenge insulin secretion and sensitivity, β value of insulinogenic index was considerably higher than ISI composite.

DISCUSSION

Indices of insulin secretion (HOMA β-cell) and insulin resistance (HOMA-IR) were evaluated from a fasting sample by HOMA.[16-18] These estimations correlated well with the insulin secretion and insulin sensitivity indices of minimal model analysis.[17] Matsuda and DeFronzo[19] have reported a new index of insulin sensitivity as an ISI composite, which has been validated by glucose clamp study. The insulinogenic index (30minutes) is a well-known measure of early-phase insulin secretion during OGTT.[20,21] Comparison of these 4 indexes across the range of glucose tolerance indicates that Japanese type 2 diabetic patients are characterized primarily by a decrease in insulin secretion and show less attribution of insulin resistance. BMI is a strong determinant of insulin resistance, and it is concordant with the evidence that the mean BMIs of representative epidemiologic studies of Japanese diabetic patients are from 23 to 25, lower than the studies of the other ethnic populations.[7-10]

These data indicate that the major factor in glucose intolerance that is characteristic of type 2 diabetes also differs in Japanese patients. Tripathy et al[11] found using OGTT in the Botnia study that the factors responsible for the development of glucose intolerance are decreased insulin secretion and sensitivity. Using HOMA-IR, insulin resistance increased nearly 2-fold from 1.7 as glucose intolerance increased from NGT to IGT and 3.6-fold in DM in that study. Using the same index, insulin resistance of Japanese subjects also increased from 1.2 to 1.5 as glucose intolerance increased from NGT to IGT and from 1.5 to 2.4 as glucose intolerance increased from IGT to DM, remarkably less than in the Botnia study. The difference of HOMA-IR in DM patients between Caucasian and Japanese becomes more than double as a number. Considering even the difference of insulin assay method and the existence of proinsulin, insulin resistance indices of Caucasian are remarkably higher than those of the Japanese. The HOMA-IR of Japanese subjects also is lower compared with that in other ethnic populations of previous studies.[23-27]

On the other hand, the reduction in insulin secretion in Japanese subjects is remarkable. The insulinogenic index (30minutes) of Japanese subjects decreased from 10.0 to 5.3 as glucose intolerance increases from NGT to IGT and from 5.3 to 1.7 as glucose intolerance increases from IGT to DM. In the Botnia study, insulin secretion decreased from 22 by half as glucose intolerance increased from NGT to IGT and by half as glucose intolerance increased from IGT to DM. The insulin secretion in Japanese subjects is considerably lower both than these and those reported in other populations.[28,29] These findings are in accord with those in the Japanese-American population, suggesting a common predisposition of Japanese populations.[30,31]). Multiple regression analysis revealed that HOMA-IR, HOMA β-cell, ISI composite, and insulinogenic index are independently associated with G-AUC. The correlation coefficients of insulinogenic index are considerably higher than the ISI composite (Table 2). In this study, the mean of all the subjects of fasting and 2-hour glucose levels were 6.8 mmol/L and 11.3 mmol/L, respectively, and their glucose intolerance was very mild. Compensately, increase in insulin secretion can make the fasting glucose levels stay near the normal range in these subjects. However, glucose intolerance, expressed as G-AUC during OGTT, appears after the challenge of glucose. Thus, indices using the results not only fasting levels, but after the glucose load, can detect a slight difference of glucose tolerance in subjects with mild glucose intolerance. Matsuda and DeFronzo[19] reported ISI composite is a good surrogate measure of whole body insulin sensitivity in comparison to clamp studies. We also have confirmed the validity of ISI composite using the minimal model analysis.[32]

The factors responsible for the ethnic differences in glucose tolerance are not yet fully clarified. Body fat distribution plays an important role in insulin resistance and glucose tolerance in some studies. We have reported not only visceral, but subcutaneous adiposity contributes to glucose intolerance suggesting the characteristic of Japanese patients.[33] Recently, the contribution of β-cell function to ethnic difference and genetic predisposition was described using precise estimation method of insulin secretion by simultaneous measurement of glucose, insulin, and C-peptide.[34,35] The analysis of body fat distribution and further estimation of insulin secretory capacity will give more explanations for ethnic differences in glucose tolerance.

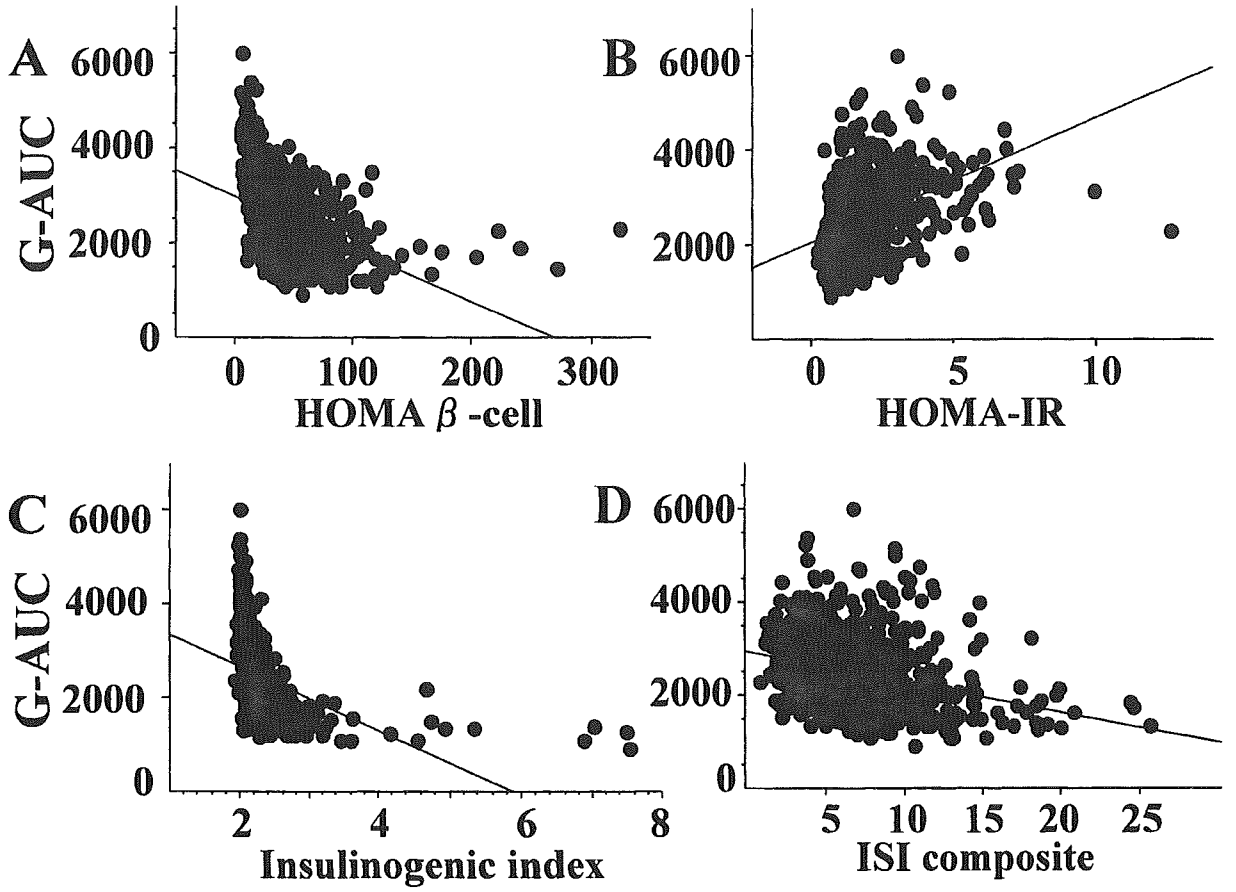

Fig 3. The relationship between G-AUC and the indices of insulin secretion and sensitivity. (A, B) As estimates of basal state, HOMA β-cell and HOMA-IR had significant relationships with G-AUC ($r = -0.45$, $P < .0001$, and $r = 0.41$, $P < .0001$, respectively). (C, D) As estimates of insulin secretion and sensitivy including postchallenge state, there were significant relationships between G-AUC and insulinogenic index ($r = -0.42$, $P < .0001$), and ISI composite ($r = -0.29$, $P < .0001$).

In addition, we compared the indices of insulin secretion and sensitivity between the subgroups of the prediabetic state to elucidate the profile of glucose tolerance (isolated IFG: 6.1 < FPG < 7 and 2-hour PG level < 7.8(n = 44) and isolated IGT: FPG level < 6.1 and 7.8 < 2-hour PG level < 11.1(n = 102)). Isolated IFG is characterized that they cannot keep fasting plasma glucose levels within normal limit at basal steady state, even if they have reserve capacity of insulin secretion after the glucose challenge. In these study subjects, we found HOMA β-cell of isolated IFG was significantly higher than that of isolated IGT (36.5 and 58.1, respectively, $P < .0001$), but there were no significant differences in other indices. It is considered that the difference between IFG and IGT is, at least in part, in the different disrupted balance of insulin secretion and sensitivity at the fasting state. We described the importance of early-phase insulin secretion for the elevation of 2-hour PG levels in Japanese subjects.[36] Further studies are necessary to clarify the different mechanisms of regulation between FPG and 2-hour PG levels.

Table 2. Relationship of the Indices of Insulin Secretion and Sensitivity With G-AUC

	Correlation Coefficients	Standardized β	P Value
HOMA β-cell	−0.45	−0.61	<.0001
HOMA-IR	0.41	0.53	<.0001
Insulinogenic index	−0.42	−0.20	<.0001
ISI composite	−0.29	−0.11	<.001

The incidence of type 2 diabetes has increased recently in Japan and is now comparable to that in other countries, but the causation of the glucose intolerance differs.[3,25,30,31] It is important in terms of prognosis and therapeutic strategy for each diabetic patient to consider the contribution of impaired insulin secretion and insulin resistance to glucose intolerance.[37] The present study clearly shows the clinical relevance of lower basal and impaired early-phase insulin secretion in type 2 diabetes in Japanese patients.

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REFERENCES

1. DeFronzo RA: Lilly lecture 1987. The triumvirate: Beta-cell, muscle, liver. A collusion responsible for NIDDM. Diabetes 37:667-687, 1988
2. Porte D Jr: Banting lecture 1990. Beta-cells in type II diabetes mellitus. Diabetes 40:16-180, 1991
3. Haffner SM, Howard G, Mayer E, et al: Insulin sensitivity and acute insulin response in African-Americans, non-Hispanic whites, and Hispanics with NIDDM: The Insulin Resistance Atherosclerosis Study. Diabetes 46:63-69, 1997
4. Taniguchi A, Nakai Y, Fukushima M, et al: Pathogenic factors responsible for glucose intolerance in patients with NIDDM. Diabetes 41:1540-1546, 1992
5. Fukushima M, Nakai Y, Taniguchi A, et al: Insulin sensitivity, insulin secretion, and glucose effectiveness in anorexia nervosa: A minimal model analysis. Metabolism 42:1164-1168, 1993
6. Taniguchi A, Nakai Y, Doi K, et al: Glucose effectiveness in two subtypes within impaired glucose tolerance. Diabetes 43:1211-1217, 1994
7. Sekikawa A, Tominaga M, Takahashi K, et al: Prevalence of diabetes and impaired glucose tolerance in Funagata area, Japan. Diabetes Care 16:570-574, 1993
8. Ohmura T, Ueda K, Kiyohara Y, et al: The association of the insulin resistance syndrome with impaired glucose tolerance and NIDDM in the Japanese general population: The Hisayama study. Diabetologia 37:897-904, 1994
9. Kosaka K, Kuzuya T, Yoshinaga H, et al: A prospective study of health check examinees for the development of non-insulin-dependent diabetes mellitus: Relationship of the incidence of diabetes with the initial insulinogenic index and degree of obesity. Diabet Med 13:S120-126, 1996
10. Qiao Q, Nakagami T, Tuomilehto J, et al: DECODA Study Group; International Diabetes Epidemiology Group: Comparison of the fasting and the 2-h glucose criteria for diabetes in different Asian cohorts. Diabetologia 43:1470-1475, 2000
11. Tripathy D, Carlsson M, Almgren P, et al: Insulin secretion and insulin sensitivity in relation to glucose tolerance: Lessons from the Botnia Study. Diabetes 49:975-980, 2000
12. Seino Y, Kurahachi H, Goto Y, et al: Comparative insulinogenic effects of glucose, arginine and glucagon in patients with diabetes mellitus, endocrine disorders and liver disease. Acta Diabetol 12:89-99, 1975
13. Taniguchi A, Fukushima M, Sakai M, et al: The role of the body mass index and triglyceride levels in identifying insulin-sensitive and insulin-resistant variants in Japanese non-insulin-dependent diabetic patients. Metabolism 49:1001-1005, 2000
14. Kuroe A, Fukushima M, Usami M, et al: Impaired β-cell function and insulin sensitivity in Japanese subjects with normal glucose tolerance. Diabetes Res Clin Pract 59:71-77, 2003
15. Alberti KG, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539-553, 1998
16. Matthews DR, Hosker JP, Rudenski AS, et al: Homeostasis model assessment: Insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28: 412-419, 1985
17. Fukushima M, Taniguchi A, Sakai M, et al: Homeostasis model assessment as a clinical index of insulin resistance. Comparison with the minimal model analysis. Diabetes Care 22:1911-1912, 1999 (letter)
18. Katsuki A, Sumida Y, Gabazza EC, et al: Homeostasis model assessment is a reliable indicator of insulin resistance during follow-up of patients with type 2 diabetes. Diabetes Care 24:362-365, 2001
19. Matsuda M, DeFronzo RA: Insulin sensitivity indices obtained from oral glucose tolerance testing: Comparison with the euglycemic insulin clamp. Diabetes Care 22:1462-1470, 1999
20. Seltzer HS, Allen EW, Herron AL Jr, et al: Insulin secretion in response to glycemic stimulus: Relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. J Clin Invest 46: 323-335, 1967
21. Seino Y, Ikeda M, Yawata M, et al: The insulinogenic index in secondary diabetes. Horm Metab Res 7:107-115, 1975
22. Taniguchi A, Fukushima M, Sakai M, et al: Remnant-like particle cholesterol, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients. Diabetes Care 23:1766-1769, 2000
23. Taniguchi A, Fukushima M, Sakai M, et al: Effect of bezafibrate on insulin sensitivity and insulin secretion in non-obese Japanese type 2 diabetic patients. Metabolism 50:477-480, 2001
24. Haffner SM, Miettinen H, Stern MP: The homeostasis model in the San Antonio Heart Study. Diabetes Care 20:1087-1092, 1997
25. Yeni-Komshian H, Carantoni M, Abbasi F, et al: Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy nondiabetic volunteers. Diabetes Care 23:171-175, 2000
26. Bonora E, Targher G, Alberiche M, et al: Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity. Diabetes Care 23:57-63, 2000
27. Matsumoto K, Miyake S, Yano M, et al: Glucose tolerance, insulin secretion, and insulin sensitivity in nonobese and obese Japanese subjects. Diabetes Care 20:1562-1568, 1997
28. Haffner SM, Miettinen H, Gaskill SP, et al: Decreased insulin secretion and increased insulin resistance are independently related to the 7-year risk of NIDDM in Mexican-Americans. Diabetes 44:1386-1391, 1995
29. Tripathy D, Carsson AL, Lehto M, et al: Insulin secretion and insulin sensitivity in diabetic subgroup: Studies in the prediabetic and diabetic state. Diabetologia 43:1476-1483, 2000
30. Chen KW, Boyko EJ, Bergstrom RW, et al: Earlier appearance of impaired insulin secretion than of visceral adiposity in the pathogenesis of NIDDM. 5-Year follow-up of initially nondiabetic Japanese-American men. Diabetes Care 18:747-753, 1995
31. Christine CJ, Miriam C, Rebecca L, et al: β-Cell function is a major contributor to oral glucose tolerance in high-risk relatives of four ethnic groups in the U.S. Diabetes 51:2170-2178, 2002
32. Taniguchi A, Nagasaka S, Fukushima M, et al: Assessment of insulin sensitivity and insulin secretion from the oral glucose tolerance test in nonobese Japanese type 2 diabetic patients: Comparison with minimal model approach. Diabetes Care 23:1439-1440, 2000 (letter)
33. Taniguchi A, Nakai Y, Sakai M, et al: Relationship of regional adiposity to insulin resistance and serum triglycerides level in nonobese Japanese type 2 diabetic patients. Metabolism 51:544-548, 2002
34. Chen X, Scholl TO: Ethnic differences in C-peptide/insulin/glucose dynamics in young pregnant women. J Clin Endocrinol Metab 87:4642-4646, 2002
35. Bonadonna RC, Stumvoll M, Fritsche A, et al: Altered homeostatic adaptation of first- and second-phase beta-cell secretion in the offspring of patients with type 2 diabetes: Studies with a minimal model to assess beta-cell function. Diabetes 52:470-480, 2000
36. Suzuki H, Fukushima M, Usami M, et al: Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia. Diabetes Care 26:1211-1215, 2003
37. Wysowski DK, Armstrong G, Governale L: Rapid increase in the use of oral antidiabetic drugs in the United States, 1990-2001. Diabetes Care 26:1852-1855, 2003

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Heparin-binding EGF-like growth factor induces expression of lectin-like oxidized LDL receptor-1 in vascular smooth muscle cells

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Abstract

Receptor-mediated endocytosis of oxidized LDL (Ox-LDL) has been implicated in lipid accumulation and vascular cell dysfunction. Lectin-like Ox-LDL receptor-1 (LOX-1) is highly inducible by proinflammatory cytokines, as well as angiotensin II and Ox-LDL in vitro. LOX-1 is expressed in macrophages and smooth muscle cells accumulated in the intima of advanced atherosclerotic plaques in vivo. Here we show that heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent mitogen for vascular smooth muscle cells, induces LOX-1 expression in cultured bovine aortic smooth muscle cells. HB-EGF (1–100 ng/ml) induced LOX-1 expression, which was peaked between 8 and 16 h after HB-EGF stimulation. HB-EGF-induced expression of LOX-1 was suppressed by ZD1839, an inhibitor of EGF receptor phosphorylation. Both MEK and p38 mitogen-activated protein kinase (MAPK) inhibitors significantly blocked LOX-1 upregulation induced by HB-EGF. Phosphatidylinositol 3-kinase (PI3K) inhibitors also blocked HB-EGF-induced LOX-1 expression. HB-EGF induced phosphorylation of ERK, p38 MAPK and Akt, which were suppressed by ZD1839. Upregulated expression of LOX-1 was associated with enhanced uptake of DiI-labeled Ox-LDL in smooth muscle cells. Taken together, HB-EGF can also act as an inducer of LOX-1 expression and play an integral role in foam cell transformation, cellular dysfunction, and proliferation of smooth muscle cells in atherogenesis.

Keywords: Oxidized LDL; Scavenger receptors; LOX-1; HB-EGF; Vascular smooth muscle cells

1. Introduction

Several lines of evidence have suggested that oxidized LDL (Ox-LDL) appears to play key roles in atherogenesis [1,2]. Receptor-mediated binding and uptake of Ox-LDL may be crucial in activation of vascular cells, as well as lipid accumulation and subsequent foam cell transformation [3].

Several different classes of receptors for atherogenic Ox-LDL have been identified and characterized. Among them, lectin-like Ox-LDL receptor-1 (LOX-1) is a 40–50 kDa type II membrane protein with a C-type lectin-like extracellular domain and a short cytoplasmic tail, which was originally cloned in vascular endothelial cells [4]. LOX-1 can support binding, internalization, and degradation of Ox-LDL [5]. Subsequent studies have revealed that expression of LOX-1 can also be found in macrophages [6,7] and activated vascular smooth muscle cells [8–11] in culture. LOX-1 expression is not constitutive, but dynamically induced by proinflammatory cytokines, such as tumor necrosis factor (TNF) α [12] and transforming growth factor (TGF) β [13], angiotensin II [14,15], Ox-LDL [16,17], fluid shear stress [18] at the level of gene transcription. More importantly, LOX-1 is highly expressed by macrophages and smooth muscle cells accumulated in the intima of advanced atherosclerotic lesions, as well as endothelial cells covering early atherosclerotic lesions in vivo [19,20]. These data suggest that LOX-1 may play important roles in both endothelial activation and foam cell transformation of macrophages and vascular smooth muscle cells in atherogenesis.

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a potent mitogen for vascular smooth muscle cells, which can be expressed by

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macrophages, endothelial cells, as well as vascular smooth muscle cells [21–23]. HB-EGF can induce migration, proliferation, and phenotypic modulation of smooth muscle cells through its interaction with the EGF receptor [21,23]. Expression of HB-EGF can be induced by proinflammatory cytokines [24], angiotensin II [22], a phospholipid component of Ox-LDL [25–27], as well as fluid shear stress [28]. Furthermore, upregulated expression of HB-EGF in atherosclerotic lesions has also been demonstrated [29–31].

In the present study, therefore, we have tested the hypothesis if HB-EGF may induce expression of LOX-1 in vascular smooth muscle cells.

2. Materials and methods

2.1. Materials

HB-EGF was purchased from R&D Systems. PD98059, SB203580, U0126, LY294002, and wortmannin were from Calbiochem. ZD1839 was a generous gift from AstraZeneca. A mouse anti-bovine LOX-1 monoclonal antibody was prepared by immunization with an recombinant bovine LOX-1 extracelluler domain as previously described [4]. A rat anti-human LOX-1 monoclonal antibody that cross-reacts with bovine LOX-1 was prepared by immunization with an recombinant human LOX-1 extracelluler domain generated by BAC TO BAC Baculovirus Expression System (Life Technologies). Antibodies for ERK and phosphorylated ERK were purchased from Cell Signaling, and antibodies for p38 mitogen-activated protein kinase (MAPK), phosphorylated p38 MAPK, Akt and phosphorylated Akt were from Santa Cruz Biotechnology. Irrelevant rat IgG was purchased from Chemicon.

2.2. Cell culture

Bovine aortic smooth muscle cells (BSMCs) were isolated by an explant method, after removing endothelial cells by scraping the inner surface of bovine aortas with a razor blade, and cultured in DMEM containing 10% (v/v) FBS. Cells were used for experiments at the passage levels between 2 and 6.

2.3. Immunoblot analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed in PBS containing 1% Triton X-100. After heated at 95 °C for 5 min, equal protein concentrations of the cell lysates were subjected to SDS–polyacrylamide (12%) gel electrophoresis and transferred onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell) by electroblotting. After preincubation with blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat dry milk) for 2 h at room temperature, blotted membranes were incubated with each primary antibody overnight at 4 °C, followed by washing twice with blocking buffer. Membranes were then incubated with a horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Amersham) for 1 h at room temperature, washed twice in PBS containing 0.04% Tween 20, and visualized by ECL Western blotting detection reagents (Amersham).

2.4. Northern blot analysis

Total cellular RNA was isolated by TRIZOL Reagent (Invitrogen). Total RNA (15 μg) was subjected to electrophoresis through 1% agarose gel containing formaldehyde, and transferred onto nitrocellulose membranes (OPTITRAN, Schleicher & Schuell). Membranes were hybridized with a *Xho*I fragment of bovine LOX-1 cDNA which had been labeled with [α-^{32}P] dCTP (Amersham) using random nonamer primers (Megaprime DNA labelling systems, Amersham).

2.5. Cellular uptake of DiI-labeled Ox-LDL

LDL (density: 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from human plasma. Oxidative modification of LDL was carried out with cupric ion in vitro. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances and electrophoretic mobility of the LDL particles. Our Ox-LDL contained approximately 10 nmol malondialdehyde equivalent/mg protein. Agarose gel electrophoresis showed increased electrophoretic mobility, which is almost equal to that of acetylated LDL, and minimal aggregation of the Ox-LDL particles. Labeling of Ox-LDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) was performed as previously described [32]. To examine cellular uptake of Ox-LDL, confluent monolayers of BSMCs were incubated with DiI-labeled Ox-LDL (5 μg/ml) in DMEM/10% FBS for additional 2 h after treatment with the indicated reagents for 12 h and washed three times with the cell culture medium. Fluorescence microscopy was performed to detect DiI-Ox-LDL accumulated in cytoplasm.

2.6. Statistical analysis

Data are expressed as the mean ± standard deviations (S.D.). Statistical significance of the differences was evaluated by one-factorial ANOVA followed by Fisher's PLSD test, and $P < 0.05$ was considered significant.

3. Results

3.1. LOX-1 expression is upregulated by HB-EGF in cultured BSMCs

After BSMCs were treated with or without HB-EGF (0.01–100 ng/ml) for 16 h, total cell lysates were isolated

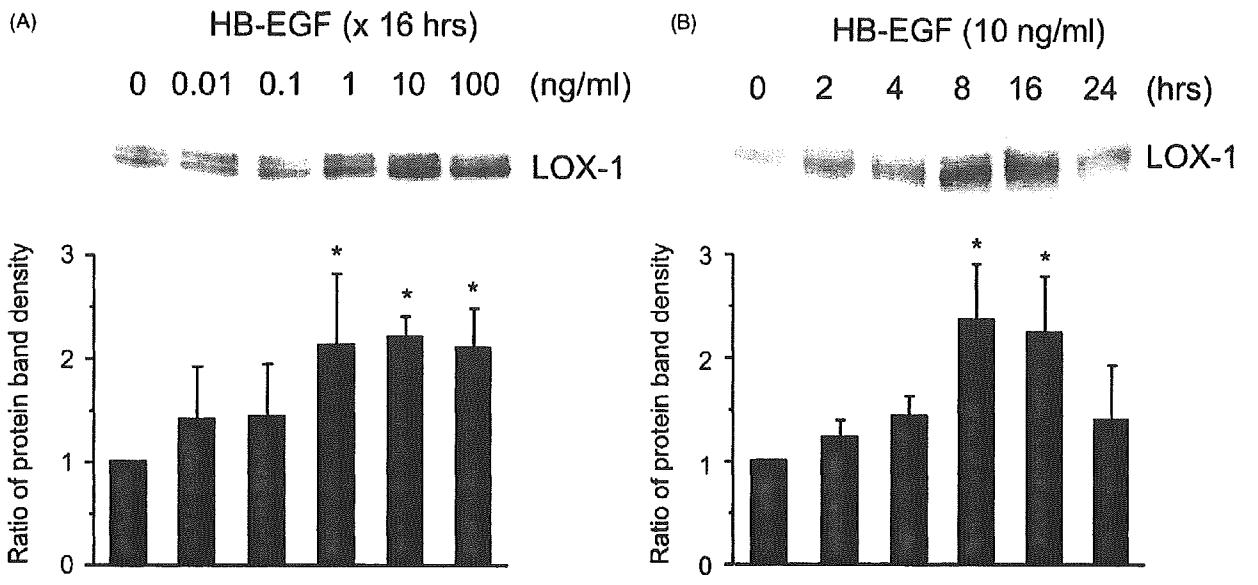

Fig. 1. Effects of HB-EGF on LOX-1 protein induction in BSMCs. After BSMCs were treated with the indicated concentrations of HB-EGF for 16 h (A), or with 10 ng/ml of the reagent for the indicated time periods (B), total cell lysates were subjected to immunoblot analyses with a mouse anti-LOX-1 monoclonal antibody. Data illustrated on the graph bar are the mean ± S.D. of four independent experiments, respectively. $^{*}P < 0.005$ vs. 0 ng/ml of HB-EGF.

and subjected to immunoblot analyses. Fig. 1A demonstrates that LOX-1 protein levels were remarkably induced by HB-EGF in a concentration-dependent manner. Up-regulation of LOX-1 protein expression was observed at concentrations above 1 ng/ml of HB-EGF and peaked at 10 ng/ml of the regent, which resulted in 2.2-fold increase by densitometric analyses. LOX-1 protein expression was time-dependently induced by 10 ng/ml of HB-EGF; increased LOX-1 protein expression was detectable as early as 2 h after the addition of HB-EGF, peaked at 8–16 h (2.4- and 2.3-fold increases, respectively), and declined after 24 h (Fig. 1B).

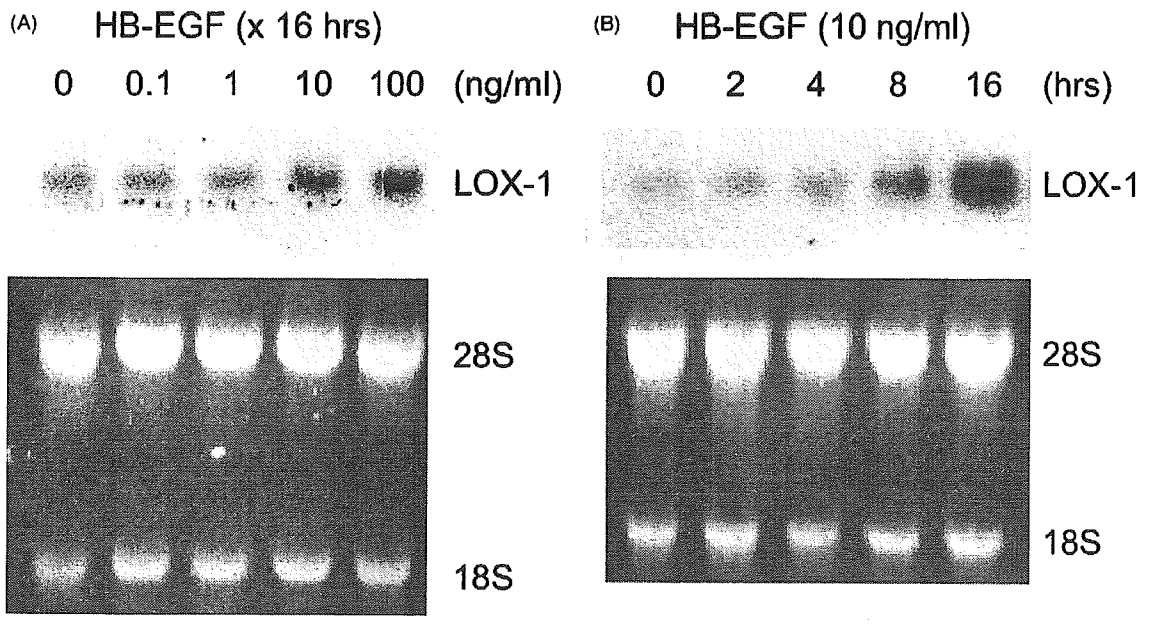

Fig. 2. Effects of HB-EGF on LOX-1 mRNA expression in BSMCs. After BSMCs were treated with the indicated concentrations of HB-EGF for 16 h (A), or with 10 ng/ml of the reagent for the indicated time periods (B), total cellular RNA was subjected to Northern blot analyses (15 μg RNA per lane) with ^{32}P-labeled cDNA probes. Bands for 28S and 18S ribosomal RNA visualized by ethidium bromide staining to control the amount of RNA loaded are also shown.

To determined whether increased expression of LOX-1 protein by HB-EGF depends upon induced expression of LOX-1 mRNA, Northern blot analyses were performed. As shown in Fig. 2A, HB-EGF increased the amount of LOX-1 mRNA concentration-dependently. Time-course experiments showed that increased levels of LOX-1 mRNA were detectable similarly to the protein expression (Fig. 2B).

3.2. HB-EGF increased the uptake of DiI-labeled Ox-LDL

To determine whether upregulated expression of LOX-1 by HB-EGF is correlated with enhanced uptake of Ox-LDL, uptake of DiI-labeled Ox-LDL into BSMCs were measured. After treatment with or without HB-EGF for 12 h, BSMCs were incubated with DiI-labeled Ox-LDL for additional 2 h. As shown in Fig. 3A, HB-EGF increased the internalization of DiI-Ox-LDL into BSMCs. The internalization of Di-Ox-LDL increased by HB-EGF was inhibited by the 100-fold excess amount of unlabeled Ox-LDL. Increased internalization of Di-Ox-LDL by HB-EGF was also inhibited by anti-LOX-1 monoclonal antibody (Fig. 3B). These results demonstrated that increases in LOX-1 expression by HB-EGF were associated with enhanced specific Ox-LDL uptake in BSMCs.

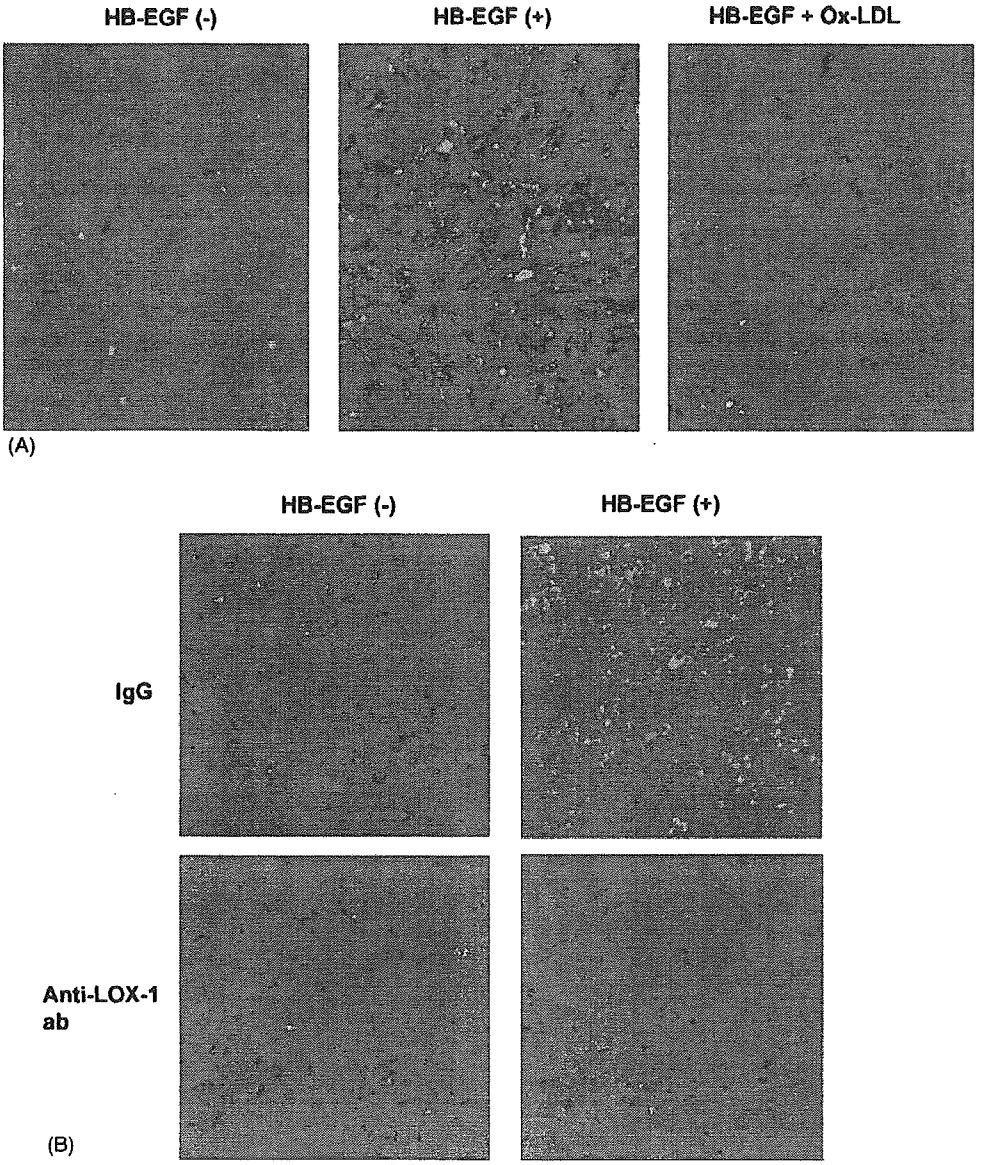

Fig. 3. HB-EGF increases the specific uptake of Ox-LDL in BSMCs. After treatment with or without 10 ng/ml of HB-EGF for 12 h, BSMCs were incubated with 5 μg/ml of DiI-labeled Ox-LDL for additional 2 h, in the presence or absence of 500 μg/ml of unlabeled Ox-LDL (A), and in the presence of a rat anti-LOX-1 monoclonal antibody or an irrelevant rat IgG (B). Representative pictures under fluorescence microscopy are shown.

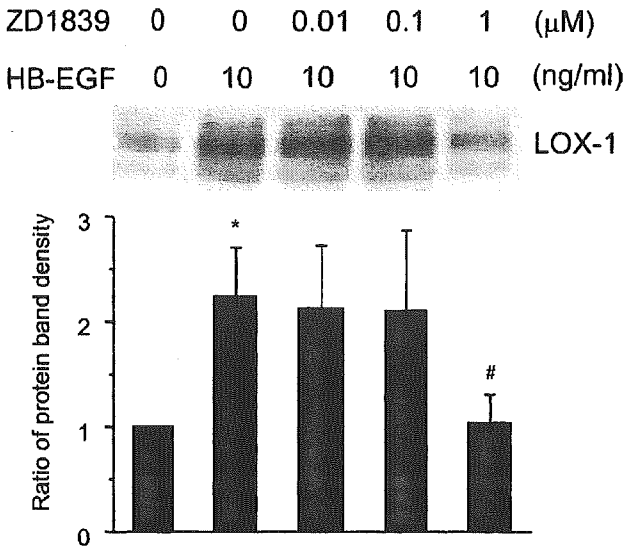

Fig. 4. HB-EGF-induced LOX-1 expression depends upon EGF receptor phosphorylation. After pretreatment with the indicated concentrations of ZD1839 for 1 h, BSMCs were treated with HB-EGF in the presence of ZD1839 for 10 h and then total cell lysates were subjected to Western blotting with a mouse anti-LOX-1 monoclonal antibody. Bar graphs indicate the mean ± S.D. of three independent experiments. $^{*}P < 0.001$, vs. 0 ng/ml of HB-EGF and $^{\#}P < 0.005$ vs. 10 ng/ml of HB-EGF.

3.3. HB-EGF-induced expression of LOX-1 depends upon EGF receptor phosphorylation

Previous studies have shown that EGF receptor phosphorylation mediates HB-EGF-dependent intracellular signal transduction. Therefore, we sought to determine if HB-EGF-induced LOX-1 expression depends upon EGF receptor phosphorylation. As shown in Fig. 4, ZD1839 (1 μM), an EGF receptor tyrosine kinase inhibitor that blocks EGF receptor phosphorylation [33], inhibited LOX-1 expression induced by HB-EGF to the basal level.

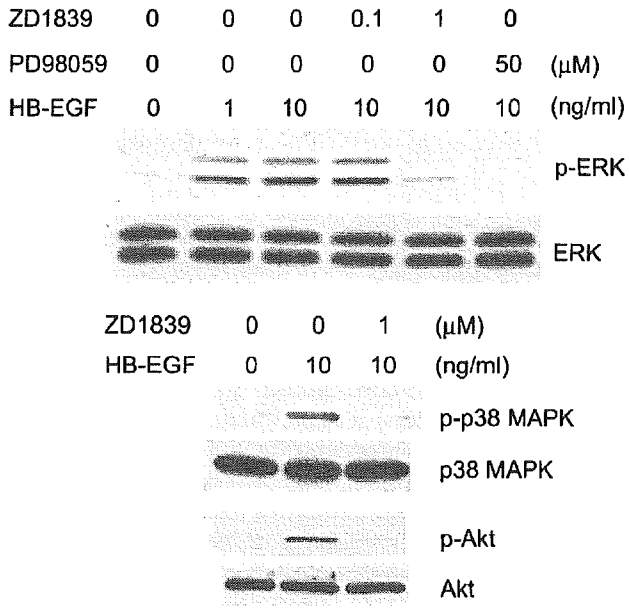

Fig. 5. HB-EGF induces phosphorylation of ERK, p38 MAPK and Akt. After pretreatment with the indicated concentrations of ZD1839 or PD98059 for 1 h, BSMCs were treated with HB-EGF in the presence of each reagent for 10 h and then total cell lysates were subjected to Western blotting with an anti-ERK, anti-phosphorylated ERK, anti-p38 MAPK, anti-phosphorylated p38 MAPK, anti-Akt or anti-phosphorylated Akt polyclonal antibody. A representative result from three independent experiments is shown.

3.4. HB-EGF induces phosphorylation of ERK, p38 MAPK and Akt depending upon EGF receptor phosphorylation

To determine whether HB-EGF, in fact, activates mitogen-activated protein kinases, such as ERK and p38 MAPK, or phosphatidylinositol 3-kinase (PI3K) in BSMCs, phosphorylation of ERK, p38 MAPK and Akt elicited by HB-EGF was measured. HB-EGF dose-dependently activated phosphorylation of ERK, as shown in Fig. 5. HB-EGF-induced

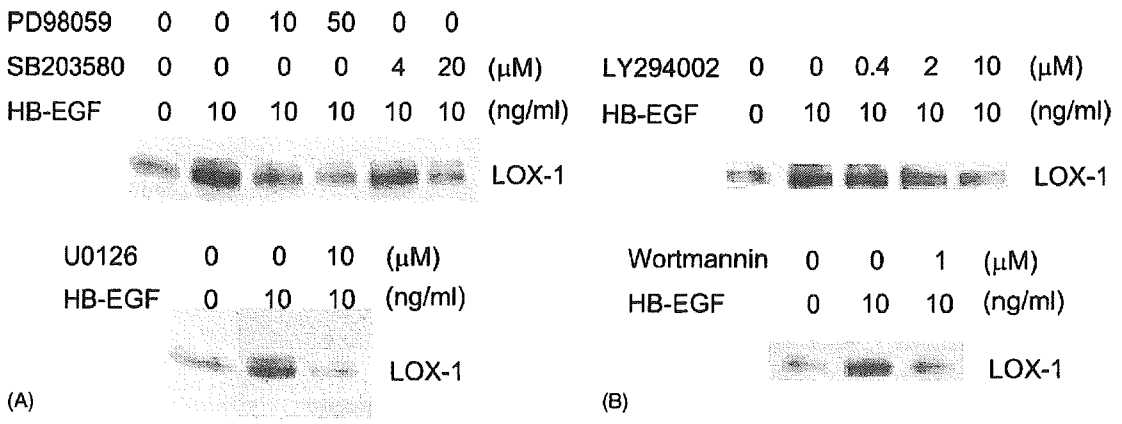

Fig. 6. HB-EGF-induced LOX-1 expression depends upon activation of MAPKs and PI3K. After pretreatment with the indicated concentrations of PD98059, SB203580, or U0126 (A), and LY294002 or wortmannin (B) for 1 h, BSMCs were treated with HB-EGF in the presence of each reagent for 10 h and then total cell lysates were subjected to Western blotting with a mouse anti-LOX-1 monoclonal antibody. A representative result from three independent experiments is shown.

ERK phosphorylation was suppressed by ZD1839, as well as PD98059, a MEK1 inhibitor, indicating the dependency upon EGF receptor phosphorylation. HB-EGF also slightly activated phosphorylation of p38 MAPK and Akt, which were suppressed by the EGF receptor phosphorylation inhibitor.

3.5. HB-EGF-induced expression of LOX-1 depends upon ERK, p38 MAPK and PI3K activation

We have further examined the dependency of HB-EGF-induced LOX-1 expression upon ERK and p38 MAPK as well as PI3K. As shown in Fig. 6A, both PD98059 and SB203580, inhibitors of MEK1 and p38 MAPK, respectively, significantly suppressed LOX-1 expression induced by HB-EGF. U0126, a strong and specific inhibitor of MEK1/2, also suppressed HB-EGF-induced LOX-1 expression. PI3K inhibitors, such as LY294002 and wortmannin, also suppressed HB-EGF-induced LOX-1 expression (Fig. 6B).

4. Discussion

Ox-LDL appears to play key roles in atherosclerotic progression and atherosclerotic plaque rupture. Effects of Ox-LDL on vascular cells appear to be mediated, at least in part, by Ox-LDL receptors, including LOX-1. In fact, LOX-1-mediated Ox-LDL uptake has been shown to induce cellular oxidative stress and activation of the proinflammatory transcription factor NF-κB[34] in vascular endothelial cells. In vascular smooth muscle cells, LOX-1-mediated uptake of Ox-LDL induces apoptosis [10], which may potentially stimulate rupture of atheromatous plaques in concert with other proinflammatory responses [35–37].

On the other hand, HB-EGF is a potent mitogen for smooth muscle cells, which is produced by vascular endothelial cells, smooth muscle cells, macrophages, and T lymphocytes [21–23]. HB-EGF has been shown to modulate smooth muscle phenotype, including induction of a macrophage colony-stimulating factor receptor (c-fms) [38]. Expression of HB-EGF can be induced by a variety of biological stimuli, including proinflammatory cytokines [24] and a phospholipid component in Ox-LDL [25–27]. Upregulated expression of HB-EGF and EGF receptors has been demonstrated in human atherosclerotic lesions [29–31], as well as animal models of vascular injury [39], thus suggesting a pivotal role in atherogenesis.

In the present study, we have shown that LOX-1, a receptor for atherogenic Ox-LDL, can be induced by HB-EGF in cultured vascular smooth muscle cells. Enhanced expression of LOX-1 in intimal smooth muscle cells, as well as endothelial cells, in atherosclerotic lesions has also been demonstrated previously [19,20]. Therefore, HB-EGF produced in the intima of atherosclerotic lesions may induce expression of LOX-1, and thereby enhance Ox-LDL-induced smooth muscle cell apoptosis, if abundant Ox-LDL is present, and thus may destabilize the atherosclerotic plaque and make it prone to rupture, although HB-EGF itself can also directly stimulate smooth muscle cell proliferation and inhibit apoptosis. This mechanism may represent one of the links between smooth muscle proliferation and lipid accumulation in atherogenesis, in addition to induced expression of HB-EGF by a lipid component of Ox-LDL [25–27].

Effects of HB-EGF on LOX-1 expression appear to be mediated by EGF receptor, because ZD1938, which blocks EGF receptor phosphorylation through inhibition of the receptor tyrosine kinase, suppressed HB-EGF-induced LOX-1 expression. In addition, both ERK and p38 MAPK, which are shown to be phosphorylated by HB-EGF, are involved in HB-EGF-induced LOX-1 expression, since PD98059, U0126, and SB203580 inhibited HB-EGF-induced LOX-1 expression. Furthermore, ERK and p38 MAPK phosphorylation by HB-EGF was suppressed by ZD1839, an inhibitor of EGF receptor tyrosine kinase. Moreover, PI3K is also involved in this process, because LY294002 and wortmannin inhibited HB-EGF-induced LOX-1 expression and HB-EGF, in fact, stimulated Akt phosphorylation. These results are consistent with previous reports showing that HB-EGF induces phosphorylation of EGF receptor and activates MAPK, and that HB-EGF-induced DNA synthesis is suppressed by PD98059 and LY294002 [40]. Although transcriptional regulatory mechanisms of LOX-1 gene have not been fully understood, oxidative stress has been implicated in LOX-1 gene induction [41,42]. Therefore, reactive oxygen species or redox-sensitive transcription factors may be upstream or downstream of MAPKs.

HB-EGF is a potent stimulus for smooth muscle cell migration and proliferation, and thus may modify atherosclerotic plaques into smooth muscle-rich stable ones. On the other hand, HB-EGF also induces LOX-1 which is a receptor for Ox-LDL and involved in Ox-LDL-induced apoptosis. Therefore, Ox-LDL might be a key factor to determine the stability of atherosclerotic plaques by modulating the function of smooth muscle cells, in addition to its actions on foam cell transformation and the production of matrix metalloproteinases.

In summary, the present report provides evidence, for the first time, that LOX-1 expression can be induced by HB-EGF in vascular smooth muscle cells. Further studies would elucidate the pathophysiological relevance of HB-EGF-induced smooth muscle LOX-1 expression in atherogenesis in vivo.

Acknowledgements

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References

[1] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. J Clin Invest 1991;88:1785–92.
[2] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–9.
[3] Steinberg D, Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 1997;95:1062–71.
[4] Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low-density lipoprotein. Nature 1997;386:73–7.
[5] Moriwaki H, Kume N, Sawamura T, et al. Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. Arterioscler Thromb Vasc Biol 1998;18:1541–7.
[6] Moriwaki H, Kume N, Kataoka H, et al. Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by TNF-alpha. FEBS Lett 1998;440:29–32.
[7] Yoshida H, Kondratenko N, Green S, Steinberg D, Quehenberger O. Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor. Biochem J 1998;334(Pt1):9–13.
[8] Draude G, Hrboticky N, Lorenz RL. The expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) on human vascular smooth muscle cells and monocytes and its down-regulation by lovastatin. Biochem Pharmacol 1999;57:383–6.
[9] Aoyama T, Chen M, Fujiwara H, Masaki T, Sawamura T. LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells. FEBS Lett 2000;467:217–20.
[10] Kataoka H, Kume N, Miyamoto S, et al. Oxidized LDL modulates Bax/Bcl-2 through the lectinlike Ox-LDL receptor-1 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2001;21:955–60.
[11] Kume N, Kita T. Apoptosis of vascular cells by oxidized LDL: involvement of caspases and LOX-1 and its implication in atherosclerotic plaque rupture. Circ Res 2004;94:269–70.
[12] Kume N, Murase T, Moriwaki H, et al. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. Circ Res 1998;83:322–37.
[13] Minami M, Kume N, Kataoka H, et al. Transforming growth factor-beta(1) increases the expression of lectin-like oxidized low-density lipoprotein receptor-1. Biochem Biophys Res Commun 2000;272:357–61.
[14] Morawietz H, Rueckschloss U, Niemann B, et al. Angiotensin II induces LOX-1, the human endothelial receptor for oxidized low-density lipoprotein. Circulation 1999;100:899–902.
[15] Li DY, Zhang YC, Philips MI, Sawamura T, Mehta JL. Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. Circ Res 1999;84:1043–9.
[16] Aoyama T, Fujiwara H, Masaki T, Sawamura T. Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. J Mol Cell Cardiol 1999;31:2101–14.
[17] Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. Arterioscler Thromb Vasc Biol 2000;20:1116–22.
[18] Murase T, Kume N, Korenaga R, et al. Fluid shear stress transcriptionally induces lectin-like oxidized LDL receptor-1 in vascular endothelial cells. Circ Res 1998;83:328–33.
[19] Kataoka H, Kume N, Miyamoto S, et al. Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. Circulation 1999;99:3110–7.
[20] Chen M, Kakutani M, Minami M, et al. Increased expression of lectin-like oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesion of Watanabe heritable hyperlipidemic rabbits. Arterioscler Thromb Vasc Biol 2000;20:1107–15.
[21] Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 1991;251:936–9.
[22] Temizer DH, Yoshizumi M, Perrella MA, Susanni EE, Quertermous T, Lee ME. Induction of heparin-binding epidermal growth factor-like growth factor mRNA by phorbol ester and angiotensin II in rat aortic smooth muscle cells. J Biol Chem 1992;267:24892–6.
[23] Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor. Biochim Biophys Acta 1997;1333:F179–99.
[24] Yoshizumi M, Kourembanas S, Temizer DH, Cambria RP, Quertermous T, Lee ME. Tumor necrosis factor increases transcription of the heparin-binding epidermal growth factor-like growth factor gene in vascular endothelial cells. J Biol Chem 1992;267:9467–9.
[25] Kume N, Gimbrone Jr MA. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J Clin Invest 1994;93:907–11.
[26] Nakano T, Raines EW, Abraham JA, Klagsbrun M, Ross R. Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. Proc Natl Acad Sci USA 1994;91:1069–73.
[27] Nishi E, Kume N, Ochi H, et al. Lysophosphatidylcholine increases expression of heparin-binding epidermal growth factor-like growth factor in human T lymphocytes. Circ Res 1997;80:638–44.
[28] Morita T, Yoshizumi M, Kurihara H, Maemura K, Nagai R, Yazaki Y. Shear stress increases heparin-binding epidermal growth factor-like growth factor mRNA levels in human vascular endothelial cells. Biochem Biophys Res Commun 1993;197:256–62.
[29] Miyagawa J, Higashiyama S, Kawata S, et al. Localization of heparin-binding EGF-like growth factor in the smooth muscle cells and macrophages of human atherosclerotic plaques. J Clin Invest 1995;95:404–11.
[30] Nakata A, Miyagawa J, Yamashita S, et al. Localization of heparin-binding epidermal growth factor-like growth factor in human coronary arteries. Possible roles of HB-EGF in the formation of coronary atherosclerosis. Circulation 1996;94:2778–86.
[31] Reape TJ, Wilson VJ, Kanczler JM, Ward JP, Burnand KG, Thomas CR. Detection and cellular localization of heparin-binding epidermal growth factor-like growth factor mRNA and protein in human atherosclerotic tissue. J Mol Cell Cardiol 1997;29:1639–48.
[32] Stephan ZF, Yurachek EC. Rapid fluorometric assay of LDL receptor activity by Dil-labeled LDL. J Lipid Res 1993;34:325–30.
[33] Ranson M, Mansoor W, Jay son G. ZD1839 (IRES S A): a selective EGFR-TK inhibitor. Expert Rev Anticancer Ther 2002;2:161–8.
[34] Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem 2000;275:12633–8.
[35] Bjorkerud B, Bjorkerud S. Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. Arterioscler Thromb Vasc Biol 1996;16:416–24.
[36] Jovinge S, Crisby M, Thyberg J, Nilsson J. DNA fragmentation and ultrastructural changes of degenerating cells in atherosclerotic

lesions and smooth muscle cells exposed to oxidized LDL in vitro. Arterioscler Thromb Vasc Biol 1997;17:2225–31.
[37] Bennett MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest 1995;95:2266–74.
[38] Inaba T, Ishibashi S, Harada K, et al. Induction of macrophage colony-stimulating factor receptor (c-fms) expression in vascular medial smooth muscle cells treated with heparin binding epidermal growth factor-like growth factor. J Biol Chem 1996;271:24413–7.
[39] Igura T, Kawata S, Miyagawa J, et al. Expression of heparin-binding epidermal growth factor-like growth factor in neointimal cells induced by balloon injury in rat carotid arteries. Arterioscler Thromb Vasc Biol 1996;16:1524–31.
[40] Reynolds CM, Eguchi S, Frank GD, Motley ED. Signaling mechanisms of heparin-binding epidermal growth factor-like growth factor in vascular smooth muscle cells. Hypertension 2002;39:525–9.
[41] Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox-sensitive regulation of lox-1 gene expression in vascular endothelium. Biochem Biophys Res Commun 2001;281:720–5.
[42] Halvorsen B, Staff AC, Henriksen T, Sawamura T, Ranheim T. 8-*iso*-Prostaglandin F(2alpha) increases expression of LOX-1 in JAR cells. Hypertension 2001;37:1184–90.

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EXPERIMENTAL STUDY

Gastric inhibitory polypeptide is the major insulinotropic factor in K_{ATP} null mice

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Abstract

Objective: ATP-sensitive K^+ (K_{ATP}) channels in pancreatic β-cells are crucial in the regulation of glucose-induced insulin secretion. Recently, K_{ATP} channel-deficient mice were generated by genetic disruption of Kir6.2, the pore-forming component of K_{ATP} channels, but the mice still showed a significant insulin response after oral glucose loading *in vivo*. Gastric inhibitory polypeptide (GIP) is a physiological incretin that stimulates insulin release upon ingestion of nutrients. To determine if GIP is the insulinotropic factor in insulin secretion in K_{ATP} channel-deficient mice, we generated double-knockout Kir6.2 and GIP receptor null mice and compared them with Kir6.2 knockout mice.

Methods: Double-knockout mice were generated by intercrossing Kir6.2-knockout mice with GIP receptor-knockout mice. An oral glucose tolerance test, insulin tolerance test and batch incubation study of pancreatic islets were performed on double-knockout mice and Kir6.2-knockout mice.

Results: Fasting glucose and insulin levels were similar in both groups. After oral glucose loading, blood glucose levels of double-knockout mice became elevated compared with Kir6.2-knockout mice, especially at 15 min (345±10 mg/dl vs 294±20 mg/dl, $P < 0.05$) and 30 min (453±20 mg/dl vs 381±26 mg/dl, $P < 0.05$). The insulin response was almost completely lost in double-knockout mice, although insulin secretion from isolated islets was stimulated by another incretin, glucagon-like peptide-1 in the double-knockout mice. Double-knockout mice and Kir6.2-knockout mice were similarly insulin sensitive as assessed by the insulin tolerance test.

Conclusion: GIP is the major insulinotropic factor in the secretion of insulin in response to glucose load in K_{ATP} channel-deficient mice.

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Introduction

The regulation of glucose-induced insulin secretion depends critically on glucose metabolism in pancreatic β-cells, and electrical activity controlled by plasma membrane ion channels is especially important (1). The ATP-sensitive K^+ (K_{ATP}) channel links glucose metabolism to membrane potentials (2–4). ATP closes the K_{ATP} channels, which activates voltage-dependent calcium channels, initiating insulin release.

Recently, K_{ATP} channel-deficient mice were generated by genetic disruption of Kir6.2, which forms the K^+ ion-selective pore of the channel (5). While there is no increment in insulin secretion in response to high glucose concentration *in vitro* in Kir6.2-knockout ($Kir6.2^{-/-}$) mice, as assessed by perifusion and batch incubation of isolated pancreatic islets, the mice still show the insulin response after oral glucose loading *in vivo*.

Insulinotropic hormones secreted from the gut upon nutritional ingestion play an important role in glucose-induced insulin secretion (6, 7). Gastric inhibitory polypeptide (GIP) is released from duodenal and upper small intestinal endocrine K-cells upon absorption of glucose or fat (8), and potentiates insulin secretion from pancreatic β-cells by binding to its specific receptor, the GIP receptor (GIPR). This incretin stimulates adenylyl cyclase, raises cyclic AMP (cAMP), and activates protein kinase A or type-II isoform of cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF II) (9). We previously generated mice with a targeted disruption of the GIPR gene (10). Islets from GIPR-knockout

($GIPR^{-/-}$) mice respond to glucose as well as wild-type mice, but $GIPR^{-/-}$ mice exhibit higher blood glucose levels and an impaired initial insulin response after oral glucose loading.

In the present study, we have generated double-knockout Kir6.2 and GIPR null ($Kir6.2^{-/-}GIPR^{-/-}$) mice, and have found that $Kir6.2^{-/-}GIPR^{-/-}$ mice lack the insulin response to oral glucose load seen in $Kir6.2^{-/-}$ mice, clearly indicating that GIP is the major insulinotropic factor in the insulin secretory activity in these mice.

Design and methods

Generation of animal models

$Kir6.2^{-/-}$ mice were provided by T Miki and S Seino, Chiba University Graduate School of Medicine, Japan. $GIPR^{-/-}$ mice were generated as described previously (10). $Kir6.2^{+/-}GIPR^{+/-}$ mice were generated by intercrossing $Kir6.2^{-/-}$ mice with $GIPR^{-/-}$ mice. Cross-breeding of $Kir6.2^{+/-}GIPR^{+/-}$ mice yielded $Kir6.2^{-/-}GIPR^{-/-}$ mice. All animals received care in accordance with the principles of laboratory animal care adopted by Kyoto University and the Declaration of Helsinki.

Batch incubation study

Pancreatic islets were isolated from 18- to 19-week-old male mice by collagenase digestion, and batch incubation was performed as described previously (11), with slight modification. Briefly, pancreatic islets (10 in each tube) were preincubated at 37 °C for 60 min in Hepes-Krebs buffer containing 118.4 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 2.4 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 20 mmol/l $NaHCO_3$, 2.8 mmol/l glucose, and 10 mmol/l Hepes, supplemented with 0.2% (wt/vol) BSA. The islets were incubated for 30 min in 400 μl buffer containing various stimuli of insulin secretion. GIP and glucagon-like peptide-1 (GLP-1) were obtained from Peptide Institute, Inc., Osaka, Japan. Tolbutamide was from Nacalai Tesque Inc., Kyoto, Japan.

Immunohistochemical analysis

The pancreata of 18-week-old male mice were removed and fixed in Bouin's solution. Pancreatic specimens were embedded in paraffin and sectioned at 3.5 μm. The avidin–biotin complex method with alkaline phosphatase was used as previously described (12), with a slight modification. After deparaffinization, normal goat serum (diluted to 1:75) (DAKO, Kyoto, Japan) for inhibition of nonspecific binding of primary antibody, rabbit anti-insulin antibody (diluted to 1:350) (DAKO) or rabbit anti-glucagon antibody (OAL-123, kindly provided by Otsuka Assay Laboratory, Tokushima, Japan) (13) in a final dilution of 1:500, the biotin-labeled goat anti-rabbit IgG antibody (diluted to 1:300) (DAKO), and avidin-biotin-alkaline phosphatase complex (diluted to 1:100) (Vector Laboratories, Burlingame, CA, USA) were sequentially applied on sections, followed by hematoxylin nuclear counterstaining. Staining was visualized in red and black by alkaline phosphatase substrate (Vector Laboratories) for insulin and glucagon respectively. The sections were analyzed using the NIH Image software (http://rsb.info.nih.gov/nih-image/), and the area containing insulin-positive β-cells was calculated.

Measurements of blood glucose and insulin levels

An oral glucose tolerance test (OGTT) was performed on age-matched male mice at 10 to 11 weeks of age. After a 16-h fast, plasma insulin and blood glucose levels were measured and glucose (2 g/kg body weight) was loaded orally. In the insulin tolerance test, 0.1 unit/kg body weight of human insulin (Eli Lilly, Indianapolis, IN, USA) was injected subcutaneously in male mice (17 weeks of age) after a 16-h fast. Blood samples were taken from the tail vein at the times indicated. Blood glucose levels were measured by an enzyme-electrode method in whole blood. Plasma insulin levels were determined by a high-sensitive ELISA kit (Shibayagi, Gunma, Japan).

Glucose uptake in jejunum in vitro

Incorporation of D-glucose into everted jejunal rings was determined as described previously (14), with slight modifications. Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg s.c.) and a segment of jejunum was quickly excised, rinsed with saline solution (140 mmol/l NaCl, 10 mmol/l $KHCO_3$, 0.4 mmol/l KH_2PO_4, 2.4 mmol/l K_2HPO_4, 1.2 mmol/l $CaCl_2$ and 1.2 mmol/l $MgCl_2$, pH 7.4), everted, and cut into ~30 mg pieces. Groups of 4 intestinal rings were incubated for 15 min at 37 °C and continuously gassed with O_2 in buffer containing 1.0 mmol/l D-glucose and 0.1 μCi/10 ml D-[^{14}C]glucose (specific activity 50–60 mCi/mmol; Amersham Radiochemical Center, London, UK) in the absence and presence of 1 mmol/l phlorizin (phloretin 2′-glucoside), a potent inhibitor of Na^+/glucose cotransporter (SGLT). At the end of the incubation period, the tissues were washed in ice-cold saline solution twice, blotted carefully to remove excess moisture, weighed, and extracted by shaking for 24 h in 1 ml 100 mmol/l HNO_3 (at 4 °C). An aliquot was taken and radioactivity was determined by liquid scintillation counting on a Liquid Scintillation Analyzer (model TRI-CARB 1900CA, Packard Instrument Company Inc., Downers Grove, IL, USA). SGLT-dependent glucose uptake was determined as the glucose uptake