Endothelial cells actively regulate vascular tone and permeability, the balance between coagulation and fibrolysis and interaction with platelets (Davignon & Ganz, 2004). Vascular endothelium is resistant to interaction with circulating platelets under normal circumstances. An adhesive glycoprotein, von Willebrand factor (VWF), is synthesized by endothelial cells and stored in intracellular granules. When endothelial cells are injured, this molecule mediates platelet adhesion to the inflamed endothelial cells and participates in thrombus formation to arrest hemorrhage at the sites of vascular injury (Ruggeri & Mendolicchio, 2007). Serotonin is a monoamine neurotransmitter mainly synthesized in the enterochromaffin cells of the gastrointestinal mucosa and is released into the portal blood. This molecule is either rapidly stored in platelets for use in vasoconstriction to stop bleeding or metabolized by the liver and kidney to 5-hydroxyindole acetic acid (5-HIAA) (Tyce, 1990). Associations of these endothelial factors with peripheral circulation in lower-leg arteries among diabetic patients with normal ABI are not fully understood.

In the present study, we attempted to clarify whether circulating levels of VWF or 5-HIAA are associated with blood flow in lower-leg arteries in type 2 diabetic patients with normal ABI using gated 2D-cine-PC MRI.

2. Patients and methods

2.1. Patients

One hundred twenty-three type 2 diabetic patients and 30 nondiabetic subjects ranging in age from 45 to 75 years consecutively admitted to our hospital between May 2006 and March 2009 were recruited for the study. All diabetic patients were admitted for strict glycemic control or assessment of diabetic complications including eye, renal, neurological, and circulatory disorders. Diabetic patients taking antiplatelet agents for the primary prevention of cardiovascular disease and diabetic patients with clinical history of cerebrovascular disease, coronary artery disease, or PAD were excluded from the study. Patients who had abused alcohol or had foot edema caused by heart failure, liver cirrhosis, severe nephropathy (serum creatinine > 177 μmol/l), malignant neoplasm, autoimmune disorder, acute illness, or urinary tract infections were excluded from the study. Hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) were used for the treatment of hyperlipidemia (LDL cholesterol ≥ 3.35 mmol/l) in diabetic patients. All diabetic patients with hypertension (>140/90 mm Hg) received renin–angiotensin system (RAS) inhibitors such as angiotensin-converting enzyme inhibitor (ACEI) or angiotensin II receptor blocker (ARB) for the management of high blood pressure. A 75-g glucose tolerance test was performed in our outpatient clinic for the diagnosis of patients with normal glucose regulation, impaired glucose tolerance, and diabetes mellitus (Alberti & Zimmet, 1998). Individuals with normal glucose tolerance were used as nondiabetic subjects in this study. The study was approved by the ethics committee of our institution, and informed consent was obtained from all patients before the examinations done during their stay in our hospital.

2.2. Clinical methods

Blood samples were drawn before breakfast in the morning after a 12-hour overnight fast. The HbA1c (%) is estimated as an NGSP equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS; %) + 0.4%, considering the relational expression of HbA1c (JDS; %) measured by the previous Japanese standard materials and measurement methods and HbA1c (NGSP) (NGSP [%] = 1.019 × JDS [%] + 0.30) and the coefficient of variance of 2%–3% in the measurement of HbA1c (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus, 2010). Blood pressure was measured by a sphygmomanometer with the patients in the sitting position after 5 min of rest. Three readings separated by 2 min were taken, and the average was used for analysis. An automatic device (BP-203RPE; Colin, Komaki, Japan) was used to measure both ABI and brachial-ankle pulse-wave velocity (baPWV) as an index of arterial stiffness. A trained ophthalmologist carried out fundus ophthalmoscopies and classified diabetic patients as without retinopathy or as having simple, preproliferative, or proliferative retinopathy. Diabetic patients were classified by the measurement of urinary albumin excretion in 24-h urine collection as having normo-, micro-, or macroalbuminuria when at least two of three specimens were at diagnostic threshold of less than 30, 30–300, or greater than 300 mg/24 h, respectively. Estimated glomerular filtration rate (eGFR) was calculated by the modification of diet in renal disease formula with Japanese ethnic factor of 0.881 as follows: eGFR (ml/min per 1.73 m^2) $= 0.881 \times 186.3 \times \mathrm{Age}^{-0.203} \times \mathrm{SCr}^{-1.154}$ (if female × 0.742), where SCr is serum creatinine (mg/dl) (Imai et al., 2007). Diabetic patients were screened for distal symmetric polyneuropathy using a 128-Hz tuning fork applied to the bony prominence at the dorsal surface of both great toes, just proximal to the nail bed. If the patient feels vibration for more than 10 s, vibration perception was regarded as a normal response (Boulton et al., 2005). Each subject was also classified based on smoking habits as being a current smoker or nonsmoker. Nonsmokers were defined as not having consumed tobacco for at least the previous 3 years. Plasma soluble intercellular adhesion molecule-1 (sICAM-1) and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured by enzyme-linked immunosorbent assay kit (Human sICAM/CD54 or Human MCP-1 Quantikine ELISA kit; R&D Systems, Minneapolis, MN). Serum high-sensitivity C-reactive protein (hsCRP) levels were measured by a microparticle-enhanced immunonephelometric assay (CardioPhase hsCRP; Dade Behring, Newark, DE). Plasma von Willebrand factor ristocetin cofactor activity (VWF) was tested using reagents (BC von Willebrand Reagent; Dade Behring, Marburg, Germany). Plasma 5-HIAA concentrations were measured by the high-performance liquid chromatography system using a Model L-7100 pump (Hitachi, Tokyo, Japan) and a Model ECD-300 electrochemical detector (Eicom, Kyoto, Japan). An MRI scanner operating at 1.5-Tesla (Signa Horizon-LX; GE Medical Systems, Milwaukee, WI) was used for the following experimental protocols as previously described (Suzuki et al., 2001). All patients were at rest in the supine position during examinations, which were done in a temperature-controlled room at 25°C. A single slice at the popliteal artery was oriented perpendicular to the flow direction, and flow data were obtained using two-dimensional cine-mode phase-contrast magnetic resonance imaging with 80-cm/s velocity encoding triggered by peripheral gating. The accuracy and reproducibility of this methodology to measure flow volume for triphasic waveforms created from a pulsatile pump have been reported (McCauley et al., 1995). Flow data were analyzed on an Advantage Windows version 4.2 workstation (GE Medical Systems). The instantaneous flow volume at 20 equally spaced time points through the cardiac cycle was calculated from the individual velocity images by integrating the velocity across the area of the vessel. A resistive index, which is associated with arterial resistance to blood flow, has been defined as $A - B)/A$, where A is the systolic peak velocity and B is the end-diastolic velocity (Halpern et al., 1998).

2.3. Statistical analysis

Statistical evaluation was done on SPSS software version 11.0 for Windows (SPSS Inc., Chicago, IL). Normality of distribution of each variable was assessed with the Kolmogorov–Smirnov test. Comparison between the two groups was performed using the unpaired Student's t-test. A multiple comparison of significant differences

among the three groups was carried out by one-way ANOVA followed by Scheffe's F test. The χ^2 test for 2-by-2 contingency table or Bonferroni test for 2-by-3 contingency table was used to compare frequencies between two or among three groups. Simple linear regression analyses were performed to assess the relation between normally distributed variables. Stepwise multiple regression analyses were performed to evaluate the association of blood flow with possible risk factors. The F value was set at 4.0 at each step. Values were expressed as the means ± SD. P values <.05 were considered statistically significant.

3. Results

3.1. Clinical characteristics in all subjects

Clinical characteristics in all subjects are shown in Table 1. There were no significant differences between the groups for prevalence of male gender, age, body mass index (BMI), LDL cholesterol (LDL-C), triglycerides (TGs), diastolic blood pressure (dBP), prevalence of smokers, and eGFR. However, diabetic patients had higher fasting plasma glucose (FPG) (P<.0001), hemoglobin A1c (HbA1c) (P<.0001), systolic blood pressure (sBP) (P=.0069), and frequency of micro- or macroalbuminuria (P<.0001) and neuropathy (P<.0001) and lower HDL cholesterol (HDL-C) (P=.0175) than nondiabetic subjects. Although ABI, heart rate, and early diastolic flow reversal were similar between the groups, diabetic patients had higher baPWV (P<.0001) and resistive index (P<.0001) and lower total (P<.0001), systolic (P=.0013), and late diastolic (P<.0001) flow volumes than nondiabetic subjects. Circulating levels of sICAM-1 (P<.0001), MCP-1 (P=.0224), log hsCRP (P<.0001), VWF (P<.0001), and 5-HIAA (P=.0015) in diabetic patients were higher than nondiabetic subjects.

Table 1
Clinical characteristics in diabetic patients with normal ABI and nondiabetic subjects.

	Nondiabetic subjects	Diabetic patients	P value
Number	30	123	–
Male gender (%)	15 (50.0)	78 (63.4)	.2534
Age (years)	60.3 ± 4.8	62.9 ± 7.8	.0828
BMI (kg/m^2)	22.8 ± 2.4	24.3 ± 4.2	.0594
Duration of diabetes (years)	–	10.8 ± 8.7	–
Treatment (diet/OHA/insulin)	–	5/67/51	–
FPG (mmol/l)	5.48 ± 0.59	7.85 ± 1.98	<.0001
HbA1c (%)	5.7 ± 0.4	8.9 ± 1.9	<.0001
LDL-C (mmol/l)	3.34 ± 0.75	3.07 ± 0.82	.0976
HDL-C (mmol/l)	1.54 ± 0.46	1.36 ± 0.36	.0175
TGs (mmol/l)	1.41 ± 0.58	1.60 ± 0.89	.2840
Statins (%)	–	26 (21.1)	–
Blood pressure (mm Hg)			
Systolic	125 ± 12	133 ± 15	.0069
Diastolic	79 ± 10	75 ± 10	.0698
ACEI or ARB (%)	–	41 (33.3)	–
Smokers (%)	8 (26.7)	35 (28.5)	.9999
Retinopathy (%)	–	48 (39.0)	–
Micro- or macroalbuminuria (%)	0 (0)	54 (43.9)	<.0001
eGFR (ml/min per 1.73 m^2)	71.5 ± 7.4	68.3 ± 15.9	.2906
Neuropathy (%)	0 (0)	80 (65.0)	<.0001
ABI	1.14 ± 0.07	1.13 ± 0.08	.6418
Brachial-ankle PWV (cm/s)	1407 ± 187	1673 ± 274	<.0001
Heart rate (bpm)	68 ± 10	68 ± 10	.8597
Flow volume (ml/min)			
Total	99.4 ± 19.2	69.6 ± 24.5	<.0001
Systolic	96.4 ± 20.9	81.9 ± 21.9	.0013
Early diastolic	−18.3 ± 14.0	−18.0 ± 10.6	.9193
Late diastolic	21.2 ± 8.5	5.7 ± 11.4	<.0001
Resistive index	0.999 ± 0.030	1.045 ± 0.048	<.0001
sICAM-1 (ng/ml)	150 ± 42	209 ± 73	<.0001
MCP-1 (pg/ml)	248 ± 60	281 ± 72	.0224
Log hsCRP	2.21 ± 0.38	2.69 ± 0.52	<.0001
VWF (%)	36.5 ± 16.0	91.9 ± 56.5	<.0001
5-HIAA (ng/ml)	4.1 ± 1.1	5.4 ± 2.2	.0015

Data are expressed as n (%) or means ± SD. OHA, oral hypoglycemic agent.

3.2. Associations of circulating molecules with vascular parameters

To clarify the associations of proinflammatory molecules (sICAM-1, MCP-1 and hsCRP) and endothelial factors (VWF and 5-HIAA) with vascular parameters (total flow volume, resistive index, and baPWV) in diabetic patients, simple linear regression analyses were performed as shown in Table 2. VWF (P=.0019) and 5-HIAA (P=.0011), but not sICAM-1, MCP-1, or log hsCRP, negatively correlated with total flow volume. Only 5-HIAA positively correlated with resistive index (P=.0199) and baPWV (P=.0003). To clarify the influence of 5-HIAA on blood flow, diabetic patients were classified into tertiles according to their 5-HIAA levels. Arterial waveforms recorded at the popliteal artery in each subgroup are shown in Fig. 1. The lowest group had a normal triphasic waveform, which could clearly be separated into systolic, early diastolic flow reversal, and late diastolic forward flow (Fig. 1A). The highest group showed reduced forward flow and new flow reversal in late diastole (Fig. 1C). Clinical characteristics and quantitative assessments of peripheral circulation in each subgroup are shown in Table 3. There were no significant differences among the groups for frequency of male gender, FPG, HbA1c, LDL-C, HDL-C, TGs, sBP, dBP, prevalence of patients taking statins or RAS inhibitors, frequency of smokers or neuropathy, sICAM-1, MCP-1, and log hsCRP. However, the highest group had the highest age (P=.0117), duration of diabetes (P=.0239), frequency of retinopathy (P<.01), and micro- or macroalbuminuria (P<.01) and VWF (P=.0297) and the lowest BMI (P=.0113) and eGFR (P=.0008) among the groups. There were no significant differences among the groups for ABI, heart rate, and systolic, early, and late diastolic flow volumes, while the highest group had the highest baPWV (P=.0453) and resistive index (P=.0383) and the lowest total flow volume (P=.0150) among the groups.

3.3. Variables associated with impaired blood flow

Stepwise multiple linear regression analyses were performed to examine the associations of blood flow with 10 possible risk factors for atherosclerosis (age, duration of diabetes, FBS, HbA1c, sBP, dBP, LDL-C, HDL-C, TGs, and smoking habit), three for microangiopathy (retinopathy, micro- or macroalbuminuria and eGFR), and two for medications (statins and RAS inhibitors) as well as two for endothelial factors (VWF and 5-HIAA). The significant independent determinants of blood flow were sBP (β=−0.287, F=4.447), VWF (β=−0.084, F=5.228), 5-HIAA (β=−2.382, F=6.047), and use of RAS inhibitors (β=12.538, F=7.640) (r^2=0.198, P<.0001) in diabetic patients.

4. Discussion

Our multivariate analysis demonstrates that the significant independent determinants of insufficient blood flow caused by

Table 2
Simple linear regression analyses of proinflammatory molecules (sICAM-1, MCP-1, and hsCRP) and endothelial factors (VWF and 5-HIAA) with vascular parameters (total flow volume and resistive index at the popliteal artery and baPWV) in diabetic patients with normal ABI.

	Total flow volume	Resistive index	baPWV
sICAM-1	n.s.	n.s.	n.s.
MCP-1	n.s.	n.s.	r=0.180, P=.0459
Log hsCRP	n.s.	n.s.	r=0.197, P=.0289
VWF	r=−0.277, P=.0019	r=0.238, P=.0081	n.s.
5-HIAA	r=−0.291, P=.0011	r=0.210, P=.0199	r=0.320, P=.0003

n.s., not significant.

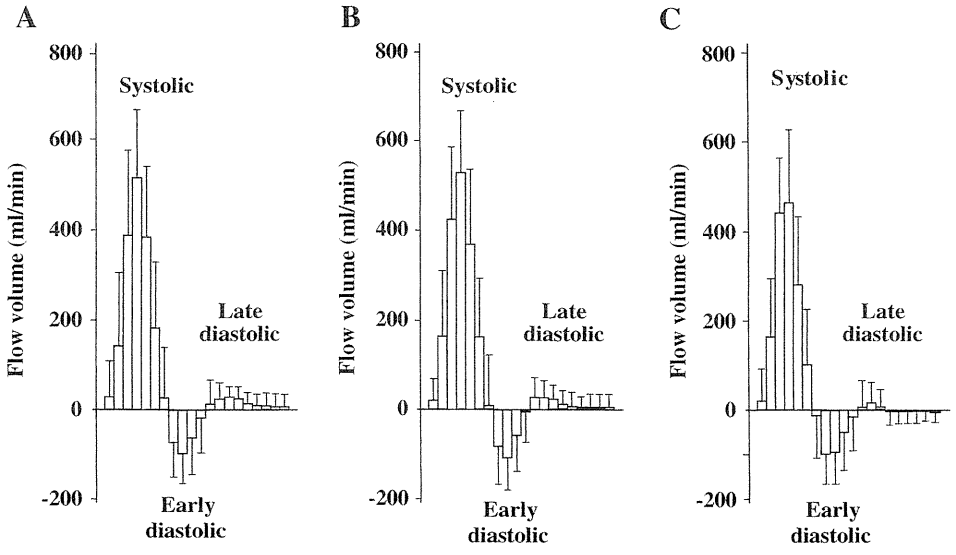

Fig. 1. Arterial waveforms recorded at the popliteal artery in diabetic patients with normal ABI grouped into tertiles according to their plasma 5-hydroxyindole acetic acid concentrations. (A) The lowest group. (B) The intermediate group. (C) The highest group. The instantaneous flow volume at 20 equally spaced time points through the cardiac cycle was reconstructed. Data are expressed as means ± SD.

higher arterial rigidity and greater peripheral vascular resistance in lower-leg arteries in diabetic patients with normal ABI are plasma levels of VWF and 5-HIAA, hypertension, and use of RAS inhibitors. An increase in plasma VWF concentration reflects endothelial damage and loss of adequate endothelial function. Plasma VWF concentration is elevated in diabetic patients with retinopathy (Morise et al., 1995) or nephropathy (Stehouwer et al., 1991). The release of serotonin from activated platelets is enhanced and intraplatelet serotonin content is diminished, resulting in elevated plasma serotonin concentrations in diabetic patients (Barradas et al., 1988; Malyszko et al., 1994). Plasma 5-HIAA concentrations are elevated and associated with arterial stiffness in diabetic patients (Fukui et al., 2007). Vasoconstrictor effects of 5-HIAA is extremely low compared to those of serotonin in normotensive rats (Thompson & Webb, 1987). Thus, the elevated plasma 5-HIAA concentrations found in this study might well contribute to higher arterial stiffness, greater vascular resistance, and lower blood flow in lower-leg arteries through vasoconstrictive actions of serotonin in diabetic patients with normal ABI.

Waveform analysis at the popliteal artery using gated 2D-cine-PC MRI is useful to assess peripheral circulation in both normal and diseased arteries (Suzuki et al., 2001). Normal subjects show a typically triphasic waveform, which can be clearly separated into systolic and early and late diastolic phases of the cardiac cycle. In late diastole, a positive waveform smaller than during systole occurs as the distended arterial reservoirs force blood antegrade through the arterioles into the venous circulation (Caputo & Higgings, 1992). When diabetic patients in this study were classified into tertiles based on their levels of 5-HIAA, those in the highest range demonstrate that waveform analysis at the popliteal artery is characterized not only by reduced forward flow but also a flow reversal in late diastole. Elastic arteries and muscular arteries have different vascular functions that are frequently impaired in diabetic patients (Henry et al. 2003; Kimoto et al., 2003). Large arteries, including the aorta and its major branches, exhibit elastic properties of the vessel wall and act as carrying vessels and blood supply reservoirs (London & Guerin, 1999). In the case of decreased arterial elasticity, less blood can be stored in these arteries, resulting in reduced late diastolic forward flow. The small-caliber arteries and arterioles act as resistance vessels that regulate blood flow to the capillaries (London & Guerin, 1999). Endothelial dysfunction and reduced limen diameter (Rizzoni et al., 2001a, 2001b) in small vessels are major determinants of peripheral vascular resistance. We have reported that diabetic patients with stiffer arteries show an abnormal vasculature in calf and foot arteries on magnetic resonance angiography (Suzuki et al., 2001), suggesting the presence of lumen narrowing or vessel wall distensibility even when ABI is normal. Angiographic evaluation in diabetic patients with foot ulcers reveals that stenosis involving ≥50% of the vessel lumen is found in 99% of the subjects and is detected in 16% of the subjects with normal ABI (Faglia et al., 1998). Vasodilatory capacity in common femoral artery is reduced in diabetic patients, with a more marked alteration in diabetic patients with microalbuminuria (Zenere et al., 1995). Capillary blood volume expansion during exercise assessed by near-infrared spectroscopy is impaired in the lower-extremity skeletal muscle of diabetic patients with normal ABI (Mohler et al., 2006). These findings support the notion that the presence of lumen narrowing or vessel wall distensibility and coexisting endothelial dysfunction in resistance vessels can contribute to hemodynamic change in diabetic patients even though they have a normal ABI.

Endothelium-dependent vascular relaxation is impaired in patients with essential hypertension (Panza et al., 1990). Nitric oxide is a potent vasodilator released by endothelial cells, and endothelial dysfunction attenuates vasodilation due to reduced nitric oxide bioavailability in diabetic patients (Williams et al., 1996). The use of RAS inhibitors ameliorates endothelial function in diabetic patients (Cheetham et al., 2000).

Among the limitations of this study, we used a cross-sectional study design in diabetic patients with normal ABI. Further prospective study is required to clarify the efficacy of oral administration of 5-HT2A receptor antagonist on plasma 5-HIAA concentrations and insufficient blood flow in lower-leg arteries in diabetic patients with normal ABI. In addition, our data were obtained in a Japanese population, and therefore, it remains to be established that these results can be generalized to other ethnicities.

In conclusion, we have revealed that plasma VWF and 5-HIAA concentrations are associated with blood flow, and these endothelial factors are involved in the pathogenesis of impaired peripheral

Table 3
Clinical characteristics of diabetic patients with normal ABI classified into tertiles based on the levels of plasma concentrations of 5-HIAA.

Group and range of 5-HIAA (ng/ml)	Lowest 1.7–4.3 (3.4 ± 0.6)	Intermediate 4.4–5.9 (5.0 ± 0.5)	Highest 6.0–13.6 (7.9 ± 1.9)
Number	41	41	41
Male gender (%)	24 (58.5)	28 (68.3)	26 (63.4)
Age (years)	60.4 ± 8.7	62.9 ± 7.4	65.5 ± 6.5[a]
BMI (kg/m^2)	25.6 ± 4.3	24.4 ± 4.3	22.8 ± 3.7[a]
Duration of diabetes (years)	7.6 ± 7.1	11.9 ± 8.2	12.8 ± 9.9[a]
Treatment (diet/OHA/insulin)	2/24/15	2/21/18	1/22/18
FPG (mmol/l)	7.69 ± 1.73	8.03 ± 2.07	7.82 ± 2.17
HbA1c (%)	8.9 ± 2.0	8.9 ± 2.0	8.7 ± 1.8
LDL-C (mmol/l)	3.22 ± 0.87	3.09 ± 0.77	2.90 ± 0.81
HDL-C (mmol/l)	1.29 ± 0.31	1.39 ± 0.40	1.39 ± 0.36
TGs (mmol/l)	1.63 ± 0.96	1.58 ± 0.87	1.58 ± 0.87
Statins (%)	7 (17.1)	10 (24.4)	9 (22.0)
Blood pressure (mm Hg)			
Systolic	130 ± 16	131 ± 14	138 ± 15[a]
Diastolic	76 ± 11	75 ± 9	74 ± 12
ACEI or ARB (%)	12 (29.3)	14 (34.1)	15 (36.6)
Smokers (%)	11 (26.8)	12 (29.3)	12 (29.3)
Retinopathy (%)	9 (22.0)	15 (36.6)	24 (58.5)[b]
Micro- or macroalbuminuria (%)	12 (29.3)	15 (36.6)	27 (65.9)[b,d]
eGFR (ml/min per 1.73 m^2)	73.1 ± 13.4	71.5 ± 16.1	60.3 ± 15.3[c,e]
Neuropathy (%)	23 (56.1)	29 (70.7)	28 (68.3)
ABI	1.13 ± 0.07	1.13 ± 0.09	1.14 ± 0.08
Brachial-ankle PWV (cm/s)	1604 ± 223	1660 ± 294	1754 ± 285[a]
Heart rate (bpm)	70 ± 11	67 ± 11	68 ± 8
Flow volume (ml/min)			
Total	73.9 ± 23.5	76.2 ± 23.3	58.6 ± 23.5[a,e]
Systolic	85.3 ± 23.4	85.6 ± 19.3	74.8 ± 21.6
Early diastolic	−18.5 ± 8.8	−17.2 ± 10.1	−18.4 ± 12.7
Late diastolic	7.1 ± 11.3	7.7 ± 11.2	2.2 ± 11.2
Resistive index	1.040 ± 0.048	1.035 ± 0.046	1.062 ± 0.046[e]
sICAM-1 (ng/ml)	203 ± 71	205 ± 78	217 ± 72
MCP-1 (pg/ml)	270 ± 64	283 ± 76	291 ± 77
Log hsCRP	2.66 ± 0.47	2.76 ± 0.60	2.66 ± 0.49
VWF (%)	76.1 ± 51.7	90.5 ± 54.3	109.0 ± 59.7[a]

Data are expressed as n (%) or means ± SD. OHA, oral hypoglycemic agent.
[a] $P<.05$ vs. the lowest group.
[b] $P<.01$ vs. the lowest group.
[c] $P<.001$ vs. the lowest group.
[d] $P<.05$ vs. the intermediate group.
[e] $P<.01$ vs. the intermediate group.

circulation in lower-leg arteries in diabetic patients with normal ABI. These findings contribute to understanding the mechanism of insufficient arterial blood flow to the lower limbs in diabetes.

References

Alberti, K. G., & Zimmet, P. Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabetic Medicine*, *15*(7), 539–553.

American Diabetes Association. (2003). Peripheral arterial disease in people with diabetes. *Diabetes Care*, *26*(12), 3333–3341.

Barradas, M. A., Gill, D. S., et al. (1988). Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease. *European Journal of Clinical Investigation*, *18*(4), 399–404.

Boulton, A. J., Vinik, A. I., et al. (2005). Diabetic neuropathies: a statement by the American Diabetes Association. *Diabetes Care*, *28*(4), 956–962.

Caputo, G. R., & Higgings, C. B. (1992). Magnetic resonance angiography and measurement of blood flow in the peripheral vessels. *Investigative Radiology*, *27*(Suppl 2), S97–S102.

Cheetham, C., Collis, J., et al. (2000). Losartan, an angiotensin type 1 receptor antagonist, improves endothelial function in non-insulin-dependent diabetes. *Journal of the American College of Cardiology*, *36*(5), 1461–1466.

Davignon, J., & Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation*, *109*(23 Suppl 1), III27–III32.

Faglia, E., Favales, F., et al. (1998). Angiographic evaluation of peripheral arterial occlusive disease and its role as a prognostic determinant for major amputation in diabetic subjects with foot ulcers. *Diabetes Care*, *21*(4), 625–630.

Faglia, E., Clerici, G., et al. (2009). Long-term prognosis of diabetic patients with critical limb ischemia: a population-based cohort study. *Diabetes Care*, *32*(5), 822–827.

Fukui, M., Ose, H., et al. (2007). Association between urinary albumin excretion and plasma 5-hydroxyindole-3-acetic acid concentration in men with type 2 diabetes. *Diabetes Care*, *30*(10), 2649–2651.

Gorogawa, S., Iida, O., et al. (2006). Characteristics of peripheral arterial disease in Japanese type 2 diabetic patients. *Diabetologia*, *49*(6), 1449–1451.

Halpern, E. J., Merton, D. A., et al. (1998). Effect of distal resistance on Doppler US flow patterns. *Radiology*, *206*(3), 761–766.

Henry, R. M., Kostense, P. J., et al. (2003). Arterial stiffness increases with deteriorating glucose tolerance status: the Hoorn Study. *Circulation*, *107*(16), 2089–2095.

Imai, E., Horio, M., et al. (2007). Estimation of glomerular filtration rate by the MDRD study equation modified for Japanese patients with chronic kidney disease. *Clinical and Experimental Nephrology*, *11*(1), 41–50.

Kimoto, E., Shoji, T., et al. (2003). Preferential stiffening of central over peripheral arteries in type 2 diabetes. *Diabetes*, *52*(2), 448–452.

London, G. M., & Guerin, A. P. (1999). Influence of arterial pulse and reflected waves on blood pressure and cardiac function. *American Heart Journal*, *138*(3 Pt 2), 220–224.

Maeda, Y., Inoguchi, T., et al. (2008). High prevalence of peripheral arterial disease diagnosed by low ankle-brachial index in Japanese patients with diabetes: The Kyushu Prevention Study for Atherosclerosis. *Diabetes Research and Clinical Practice*, *82*(3), 378–382.

Malyszko, J., Urano, T., et al. (1994). Daily variations of platelet aggregation in relation to blood and plasma serotonin in diabetes. *Thrombosis Research*, *75*(5), 569–576.

McCauley, T. R., Peña, C. S., et al. (1995). Validation of volume flow measurements with cine phase-contrast MR imaging for peripheral arterial waveforms. *Journal of Magnetic Resonance Imaging*, *5*(6), 663–668.

Mohler, E. R., III, Lech, G., et al. (2006). Impaired exercise-induced blood volume in type 2 diabetes with or without peripheral arterial disease measured by continuous-wave near-infrared spectroscopy. *Diabetes Care*, *29*(8), 1856–1859.

Morise, T., Takeuchi, Y., et al. (1995). Increased plasma levels of immunoreactive endothelin and von Willebrand factor in NIDDM patients. *Diabetes Care*, *18*(1), 87–89.

Panza, J. A., Quyyumi, A. A., et al. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *New England Journal of Medicine*, *323*(1), 22–27.

Rizzoni, D., Porteri, E., et al. (2001). Endothelial dysfunction in small resistance arteries of patients with non-insulin-dependent diabetes mellitus. *Journal of Hypertension*, *19*(5), 913–919.

Rizzoni, D., Porteri, E., et al. (2001). Structural alterations in subcutaneous small arteries of normotensive and hypertensive patients with non-insulin-dependent diabetes mellitus. *Circulation*, *103*(9), 1238–1244.

Ruggeri, Z. M., & Mendolicchio, G. L. (2007). Adhesion mechanisms in platelet function. *Circulation Research*, *100*(12), 1673–1685.

Stehouwer, C. D., Stroes, E. S., et al. (1991). von Willebrand factor and development of diabetic nephropathy in IDDM. *Diabetes*, *40*(8), 971–976.

Suzuki, E., Egawa, K., et al. (2003). Prevalence and major risk factors of reduced flow volume in lower extremities with normal ankle-brachial index in Japanese patients with type 2 diabetes. *Diabetes Care*, *26*(6), 1764–1769.

Suzuki, E., Kashiwagi, A., et al. (2001). Increased arterial wall stiffness limits flow volume in the lower extremities in type 2 diabetic patients. *Diabetes Care*, *24*(12), 2107–2114.

The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. (2010). Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *Journal of Diabetes Investigation*, *1*(5), 212–228.

Thompson, L. P., & Webb, R. C. (1987). Vascular responsiveness to serotonin metabolites in mineral corticoid hypertension. *Hypertension*, *9*(3), 277–281.

Tyce, G. M. (1990). Origin and metabolism of serotonin. *Journal of Cardiovascular Pharmacology*, *16*(Suppl 3), S1–S7.

Williams, S. B., Cusco, J. A., et al. (1996). Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. *Journal of the American College of Cardiology*, *27*(3), 567–574.

Zenere, B. M., Arcaro, G., et al. (1995). Noninvasive detection of functional alterations of the arterial wall in IDDM patients with and without microalbuminuria. *Diabetes Care*, *18*(7), 975–982.

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CASE REPORT

Effectiveness of the glucagon test in estimating islet function for liraglutide treatment in a lean diabetic patient with impaired insulin response to glucose

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Abstract A 52-year-old lean woman was admitted to hospital for a medical examination in July 2010. A 75 g oral glucose test (75 g OGTT) revealed postprandial hyperglycemia (above 200 mg/dl at 120 min) and she was diagnosed with diabetes mellitus. She was negative for diabetes-related autoantibodies. The 75 g OGTT also revealed a very low insulinogenic index (I.I.) of 0.024 μU/ml/mg/dl; however, her plasma C-peptide immunoreactivity (CPR) response to glucagon was preserved (0.94 and 5.56 at 0 and 6 min, respectively). At the same time, she also suffered from Fe-deficiency anemia due to endometriosis, for which treatment with leuprorelin was initiated after hospitalization in August 2010. Meanwhile, her postprandial plasma glucose level continued to increase. Subsequently, on November 30, 2010, alogliptin therapy at a dose of 6.25 mg was initiated, which was increased to 25 mg on December 28, 2010. Leuprorelin therapy was discontinued on January 14, 2011; however, her plasma glucose level remained high despite the alogliptin therapy. Administration of 0.3 mg liraglutide was initiated on March 15, 2011, and the dose was increased to 0.6 mg on June 7, 2011. The plasma glucose level, glycosylated hemoglobin (HbA1c) level, and 1.5 anhydroglucitol level gradually improved, as did her I.I. Moreover, fasting plasma glucagon levels were suppressed by liraglutide. Her fasting serum CPR to plasma glucose ratio and homeostasis model assessment (HOMA-R) were low both before and after liraglutide administration. These results suggest that the fasting CPR to glucose ratio may underestimate residual islet function in lean patients with high insulin sensitivity. Thus, a glucagon test may be useful to estimate residual islet function when administering liraglutide treatment in lean diabetes patients.

Keywords Liraglutide · Glucagon-like peptide · Glucagon test · Glucose responsiveness

Introduction

Type 2 diabetes mellitus (T2DM) is emerging as a major social and economic problem across the world. This form of diabetes is characterized by a combination of insulin resistance and β-cell dysfunction [1]. Unlike Caucasian T2DM, Japanese T2DM is characterized by impaired insulin response to glucose rather than insulin resistance [2]. To overcome impaired glucose-stimulated insulin secretion, several medications such as glinides, biguanides, α-glucosidase inhibitors, thiazolidine, and rapid-acting insulin are widely used. However, these drugs have severe side effects, including nausea, abdominal bloating, indigestion, hypoglycemia, and weight gain. Recently, glucagon-like peptide-1 (GLP-1) analogs have emerged as promising drugs with significantly less severe side effects [3].

GLP-1 and glucose-dependent insulinotropic peptide (GIP) are gastrointestinal hormones that comprise the incretins [4]. GLP-1 and GIP are secreted in the small intestine from L cells and K cells, respectively [4]. GLP-1

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is responsible for potentiating insulin secretion, delaying gastric emptying, lowering appetite and reducing body weight, and suppressing glucagon secretion [4]. In contrast, GIP lacks all of these actions except for the potentiation of insulin secretion [4]. GLP-1 analogs are therefore commonly used to maintain glucose homeostasis without the side effects of body weight gain and hypoglycemia [3–5].

In T2DM patients generally, the impaired insulin response to glucose is caused by abnormal glucose sensing, a decreased number of pancreatic islets, and/or abnormal accumulation of toxic molecules such as amylin [6–8]. Some groups have reported that pancreatic islet volumes are much lower in T2DM patients than in healthy subjects [6].

Thus, evaluation of residual β-cell function is important to determine whether insulin treatment is warranted [9–12]. Fasting serum C-peptide immunoreactivity (CPR) to plasma glucose ratio and glucagon tests are often used to estimate pancreatic islet function in order to determine the requirement for insulin therapy [11, 12]. However, clinical estimation of the amount of residual islet function, which is required for the successful introduction of a GLP-1 analog, remains elusive.

We report the case of a 52-year-old lean diabetic patient whose islet function was estimated using the fasting CPR to plasma glucose ratio and a glucagon test to determine the feasibility of liraglutide treatment. The results show that the glucagon test can be beneficial for the introduction of liraglutide therapy in a lean diabetic patient with impaired insulin response to glucose.

Case report

A 52-year-old lean woman was admitted to hospital for a medical examination in July 2010. On admission, her plasma glucose level was 203 mg/dl. A 75 g oral glucose tolerance test (75 g OGTT) was performed on July 12, 2010. Plasma glucose levels at 0, 30, 60, and 120 min were 100, 236, 228, and 238 mg/dl, respectively. On the basis of these findings, the patient was diagnosed with diabetes mellitus. Her insulinogenic index (I.I.), which reflects insulin response to glucose, was as low as 0.024 and she was hospitalized to undergo treatment for diabetes mellitus on August 9, 2010. She reported a family history of diabetes (two brothers) and an unremarkable past medical history except for hypermenorrhea and endometriosis.

Physical data on admission

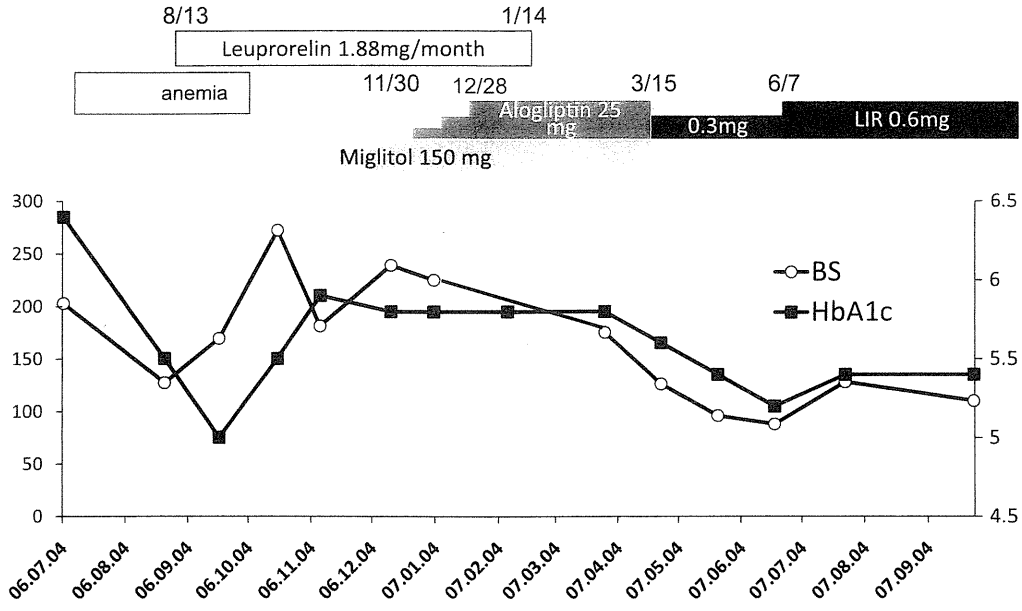
The patient's height and weight were 148.8 cm and 40.1 kg, respectively, with a body mass index of 18.11 kg/m^2. Her resting blood pressure was 92/63 mmHg. There were no abnormal findings in the thoracoabdominal region and no abnormal neurogenic signs were detected.

Laboratory findings

The laboratory data are presented in Table S1 of the Electronic supplementary material (ESM). Urinalysis revealed no abnormalities. Hemoglobin, iron, and ferritin levels were decreased, whereas unbound iron binding capacity was increased. She was diagnosed with iron-deficiency anemia caused by hypermenorrhea and endometriosis. Levels of transaminases, creatinine, electrolytes, blood urea nitrogen, and uric acid were within normal limits. The percentage value of glycosylated hemoglobin (HbA1c) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value calculated by the formula HbA1c (%) = HbA1c (Japan Diabetes Society, JDS) (%) + 0.4%, considering the relational expression of HbA1c (JDS)(%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [13]. The HbA1c level was as high as 6.3% (NGSP equivalent value) [13]. Results for diabetes-related autoantibodies, including anti-glutamic acid decarboxylase antibody, anti-insulin antibody, and anti-IA2 antibody, were all negative. Unlike the insulin response to glucose in 75 g OGTT, the patient's plasma CPR response to glucagon on August 11, 2010 was preserved (0.94 and 5.56 ng/ml at 0 and 6 min, respectively). Her plasma insulin response to glucagon was similarly preserved (1.5 and 47.4 μU/ml). Her plasma glucose levels were measured at the same time (99 and 137 mg/dl at 0 and 6 min, respectively). Her CPR index was low (0.83 and 0.95 on August 10 and 11, 2010, respectively) (Fig. 1; Table S1).

Clinical course

Diet therapy (1200 kcal/day) and 150 mg miglitol were initiated after hospitalization on August 9, 2010. On August 10, 2010, the patient's plasma glucose levels at 0800, 1000, 1200, 1400, 1800, 2000, 2400, 0300, and 0800 hours were 98, 206, 144, 198, 109, 197, 112, 103, and 98 mg/dl, respectively. The corresponding insulin levels at 0800 and 1000 hours were 0.81 and 4.36 ng/ml, respectively. Administration of leuprorelin (1.88 mg/month) for endometriosis was initiated on August 13, 2010. During the course of leuprorelin treatment, the patient's plasma glucose level (1400 hours) gradually increased. Her HbA1c levels increased after an initial decrease because of the restoration of anemia. To improve postprandial hyperglycemia, she was administered 6.25 mg of alogliptin + 150 mg of miglitol on November 30, 2010. The dose of



	2010.8.10	2011.2.9	2011.5.25	2011.9.7
FPG (mg/dl)	98	111	106	103
CPR (ng/ml)	0.81	0.98	0.74	0.68
CPR index	0.83	0.88	0.7	0.66
proinsulin (pmol/l)	<2	<2	4.5	2.3
Insulinogenic Index	ND	0.034	0.08	0.044
glucagon (pg/ml)	67	51	22	29
HOMA-R	0.267	0.6	0.55	0.25
1,5-AG (μg/ml)	ND	15	17.1	17.5

Fig. 1 Clinical course of the patient. Miglitol at a dose of 150 mg was initiated on August 9, 2011. After the administration of leuprorelin (1.88 mg/month) was initiated, her plasma glucose level increased immediately. On November 30, 2010, 6.25 mg of alogliptin was initiated; this dose was increased to 25 mg on December 25, 2010. On March 15, 2011, administration of 0.3 mg liraglutide was initiated; this dose was increased to 0.6 mg on June 7, 2011, and continued thereafter. After the administration of liraglutide, plasma glucose levels (1400 hours) (*open circles*) and HbA1c (*filled squares*) gradually decreased. Fasting plasma glucose, C-peptide, proinsulin, glucagon, and 1,5-anhydroglucitol (1,5-AG) levels were measured on August 10, 2010, February 9, 2011, May 25, 2011, and September 7, 2011. CPR index was calculated by the formula CPR index = CPR (ng/ml)/glucose (mg/dl) × 100. Insulinogenic index (I.I.) was calculated by the formula I.I. = (insulin at 30 min − insulin at 0 min)/(glucose at 30 min − glucose at 0 min). HOMA-R was calculated by the formula HOMA-R = fasting glucose (mg/dl) × insulin (μU/ml)/405

alogliptin was increased to 25 mg on December 28, 2010. On January 14, 2011, the course of leuprorelin therapy was completed. To evaluate the effect of alogliptin on glucose tolerance, we performed a 75 g OGTT on February 9, 2011. Plasma glucose levels at 30, 60, and 120 min were much higher than those measured on July 12, 2010, although her insulin levels were also higher (Fig. 2). This indicated worsening glycemic control. To protect the pancreatic β cells from hyperglycemia, we recommended intensive insulin therapy with a low dose of rapid-acting insulin three times per day. However, the patient rejected our proposal because she was afraid of hypoglycemia. As an alternative, we recommended GLP-1 analog therapy, which she accepted. Administration of 0.3 mg liraglutide began on March 15, 2011. To evaluate the effect of liraglutide on blood glucose control, we performed a 75 g OGTT on May 25, 2011. Plasma glucose levels at 30, 60, and 120 min were improved compared with those measured on July 12, 2010 and February 9, 2011. The dose of liraglutide was increased to 0.6 mg on June 7, 2011. After the introduction of liraglutide, her plasma glucose and HbA1c levels immediately improved (Fig. 1). A 75 g OGTT performed on September 7, 2011 revealed that her plasma glucose levels at 30, 60, and 120 min were below 200 mg/dl (Fig. 2). When the dose of liraglutide was increased to 0.6 mg, she experienced soft stools and indigestion; nevertheless, her body weight remained unchanged throughout the course of liraglutide treatment.

To evaluate the effect of treatment with the various medications used, we performed 75 g OGTT on July 12, 2010 (no medication); February 9, 2011 (25 mg alogliptin + 150 mg miglitol); May 25, 2011 (0.3 mg liraglutide); and September 7, 2011 (0.6 mg liraglutide). The areas under the curve of glucose (AUCglucose) in the 75 g

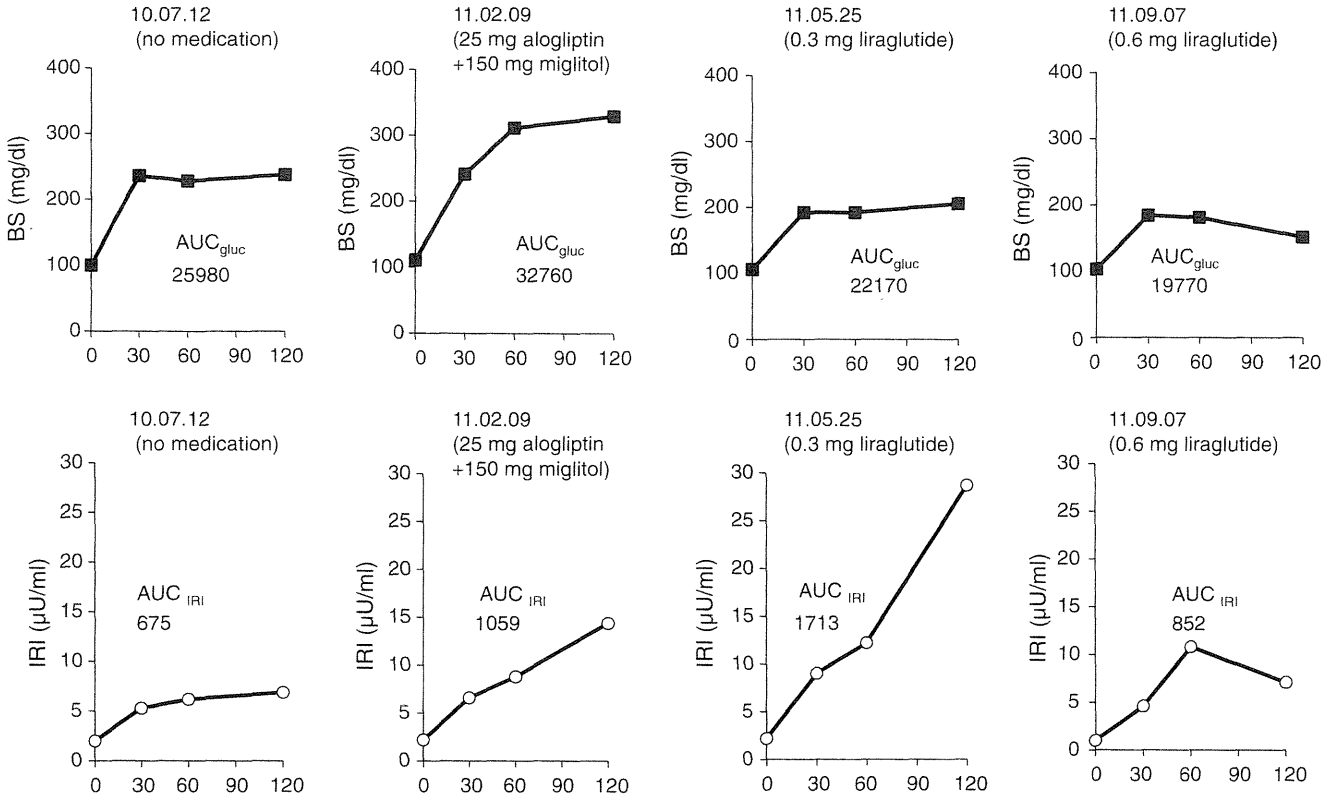


Fig. 2 The effects of plasma glucose and insulin levels on the 75 g oral glucose tolerance test for the various medications used in this patient. We performed a 75 g oral glucose tolerance test (75 g OGTT) on July 12, 2010, February 9, 2011, May 25, 2011, and September 7, 2011. Plasma glucose (*filled squares*) and plasma insulin (*open circles*) levels were measured at 0, 30, 60, and 120 min

OGTTs conducted on the abovementioned dates were 25980, 32760, 22170, and 19770 mg/dl min, respectively. Consistent with this result, HbA1c (6.4, 5.8, 5.4, and 5.4%, respectively) and 1.5-anhydroglucitol (1,5-AG) levels (15, 17.1, and 17.5 μg/ml in February, May, and September 2011, respectively) also showed an improving trend, particularly after liraglutide administration. The area under the curve of insulin (AUCinsulin) improved from 675 μU/ml min and 1059 μU/ml min in July 2010 and February 2011, respectively, to 1713 and 852 μU/ml min in May and September 2011 (after liraglutide administration), respectively. Similarly, the I.I. improved from 0.024 and 0.034 μU/ml/mg/dl in July 2010 and February 2011, respectively, to 0.080 and 0.044 μU/ml/mg/dl in May and September 2011 (after liraglutide administration), respectively (Fig. 2). Plasma proinsulin levels improved from <2.0 and <2.0 pmol/l in July 2010 and February 2011, respectively, to 4.5 and 2.3 pmol/l in May and September 2011 (after liraglutide administration), respectively (Fig. 1), whereas fasting plasma glucagon levels decreased from 67 and 51 pg/ml in July 2010 and February 2011, respectively, to 22 and 29 pg/ml in May and September 2011 (after liraglutide administration), respectively (Fig. 1).

Discussion

We report that the glucagon test was beneficial for assessing islet function when administering liraglutide treatment in a lean diabetic patient with impaired insulin response to glucose. Liraglutide improved glucose tolerance by delaying gastric emptying, suppressing the glucagon level, and improving insulin sensitivity. Thus, in cases where the insulin response to glucagon, but not to glucose, is preserved and insulin resistance is moderate, liraglutide may be an effective alternative for the treatment of postprandial hyperglycemia.

Glucagon, GLP-1, and GIP potentiate glucose-stimulated insulin secretion and insulin synthesis by increasing the intracellular cyclic adenosine monophosphate (cAMP) level [4]. In the present case, evidence of intact effects of cAMP on islet function were as follows: (1) insulin response to glucose was preserved, (2) postprandial serum CPR (4.36 ng/ml) and proinsulin levels (23.2 pmol/l) were much higher than fasting CPR (0.81 ng/ml) and proinsulin (<2 pmol/l) levels, and (3) I.I., which reflects first-phase insulin release, was not improved by liraglutide, which potentiated only second-phase insulin secretion. These results suggest that the effect of cAMP on insulin granule

movement and insulin synthesis is comparatively preserved [14].

In contrast, the insulin response to glucose and first-phase insulin secretion were remarkably disordered. The first phase required a rapid and marked elevation of $[Ca^{2+}]_i$ and corresponded to the release of insulin granules from a limited pool [15]. This suggests that glucose stimulation did not increase the intracellular calcium level in pancreatic β cells of this patient. We therefore suspected that glucose metabolism in pancreatic β cells was disordered. However, fasting plasma pyruvate and lactate levels in this patient were within normal limits (data not shown). Further investigation is required to clarify the mechanism of glucose unresponsiveness seen in this patient.

Liraglutide improved glucose tolerance at doses of 0.3 and 0.6 mg. Plasma glucagon levels decreased dramatically with doses of 0.3 and 0.6 mg, whereas the insulin response to glucose on May 25, 2011 (0.3 mg liraglutide) became much higher than that on September 7, 2011 (0.6 mg liraglutide). After the administration of 0.6 mg liraglutide, the patient sometimes experienced gastrointestinal symptoms (soft stool and indigestion). Moreover, the HOMA-R level was lower with 0.6 mg liraglutide than with 0.3 mg liraglutide. These results suggest that the improvement in glucose tolerance with 0.6 mg liraglutide was achieved by delayed gastric emptying, suppression of glucagon secretion, and improvement of insulin sensitivity rather than by augmentation of insulin secretion.

In this patient, postprandial glucose levels were remarkably high, although HbA1c was within normal limits. Consistently, the plasma 1,5-AG level was at the lower end of the normal spectrum, and was improved with liraglutide administration. Thus, in cases of fasting normoglycemia, HbA1c may be readily underestimated. Moreover, postprandial hyperglycemia poses a potential risk for the development of atherosclerosis [16, 17]. In this patient, as the pancreatic β cells would be protected from glucotoxicity because the insulin secretion capacity was very low, normalization of postprandial hyperglycemia and protection against pancreatic β-cell failure and diabetic complications caused by glucose toxicity were crucial.

There are recent reports that fasting CPR, CPR index, and I.I. are useful parameters for predicting the efficacy of liraglutide in patients with type 2 diabetes [18]. In contrast, both the fasting plasma glucose levels and the insulin levels were low in this patient because hepatic insulin sensitivity was preserved. Furthermore, the insulin response to glucagon and the postprandial serum CPR to plasma glucose ratio, rather than the fasting serum CPR to plasma glucose ratio, better reflected islet function in this patient. Consistently, it was reported that the serum CPR to plasma glucose ratio after oral glucose ingestion better predicts β-cell area than fasting measures such as the HOMA-β index [11, 19]. Thus, the insulin response to glucagon and the postprandial serum CPR to plasma glucose ratio are useful tools for estimating pancreatic β-cell function, particularly in lean diabetic patients. However, we should keep in mind that the plasma CPR levels are affected by renal function [20, 21], and that the CPR response to glucagon is affected by the plasma blood glucose levels and may overestimate β-cell function in poorly controlled NIDDM patients with elevated blood glucose levels [22, 23]. Furthermore, in patients with a long-term history of diabetes mellitus, the residual β-cell function and CPR response to glucagon can be expected to be decreased [9]. For these reasons, any estimation of residual β-cell function using a glucagon test is limited to patients with short-duration diabetes mellitus.

Leuprorelin used for the treatment of endometriosis often worsens insulin resistance, subsequently causing postprandial hyperglycemia [24]. In the present case, leuprorelin increased the postprandial glucose level, while alogliptin did not improve glucose tolerance. Similarly, glucocorticoids induce insulin resistance and postprandial hyperglycemia [25], whereas liraglutide improves postprandial hyperglycemia and insulin resistance induced by glucocorticoids. This is due to delayed gastric emptying and suppressed glucagon secretion rather than the potentiation of insulin secretion [26]. Thus, liraglutide may be an effective agent to treat leuprorelin-induced diabetes mellitus.

In conclusion, we found that liraglutide is effective for glycemic control in a lean diabetic patient with an impaired insulin response to glucose. Thus, in patients with a low fasting CPR to glucose ratio, the glucagon test may be appropriate for estimating pancreatic β-cell function when liraglutide is administered.

References

1. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1998;37:1595–607.
2. Fukushima M, Suzuki H, Seino Y. Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. Diabetes Res Clin Pract. 2004;66:S37–43.
3. Drucker DJ, Sherman SI, Bergenstal RM, Buse JB, Faber OK, Binder C. The safety of incretin-based therapies—review of the scientific evidence. J Clin Endocrinol Metab. 2011;96:2027–31.
4. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007;87:1409–39.
5. Iizuka K, Tomita R, Suwa T, Horikawa Y, Takeda J. A case report: normalization of fasting hyperglycemia is beneficial for successful introduction of small amount of the GLP-1 analog liraglutide in an obese patient with type 2 diabetes mellitus. Diabetol Int. 2011 (in press). doi:10.1007/s13340-011-0052-9.
6. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes. 2003;52:102–10.

7. Ward WK, Bolgiano DC, McKnight B, Halter JB, Porte D Jr. Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. J Clin Invest. 1984;74:1318–28.
8. Clark A, Charge SB, Badman MK, MacArthur DA, de Koning EJ. Islet amyloid polypeptide: actions and role in the pathogenesis of diabetes. Biochem Soc Trans. 1996;24:594–9.
9. Faber OK, Binder C. C-peptide response to glucagon. A test for the residual beta-cell function in diabetes mellitus. Diabetes. 1977;26:605–10.
10. Hendriksen C, Faber OK, Drejer J, Binder C. Prevalence of residual B-cell function in insulin-treated diabetics evaluated by the plasma C-peptide response to intravenous glucagon. Diabetologia. 1977;13:615–9.
11. Meier JJ, Menge BA, Breuer TG, Müller CA, Tannapfel A, Uhl W, Schmidt WE, Schrader H. Functional assessment of pancreatic beta-cell area in humans. Diabetes. 2009;58:1595–603.
12. Goto A, Takaichi M, Kishimoto M, Takahashi Y, Kajio H, Shimbo T, Noda M. Body mass index, fasting plasma glucose levels, and C-peptide levels as predictors of the future insulin use in Japanese type 2 diabetic patients. Endocr J. 2010;57:237–44.
13. Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, Araki E, Ito C, Inagaki N, Iwamoto Y, Kasuga M, Hanafusa T, Haneda M, Ueki K. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Diabetol Int. 2010;1:2–20.
14. Seino S, Takahashi H, Fujimoto W, Shibasaki T. Roles of cAMP signaling in insulin granule exocytosis. Diabetes Obes Metab. 2009;11(Suppl 4):180–8.
15. Henquin JC, Ishiyama N, Nenquin M, Ravier MA, Jonas JC. Signals and pools underlying biphasic insulin secretion. Diabetes. 2002;51(Suppl 1):S60–7.
16. Milicevic Z, Raz I, Beattie SD, Campaigne BN, Sarwat S, Gromniak E, Kowalska I, Galic E, Tan M, Hanefeld M. Natural history of cardiovascular disease in patients with diabetes: role of hyperglycemia. Diabetes Care. 2008;31(Suppl 2):S155–60.
17. Ceriello A. Postprandial hyperglycemia and diabetes complications: is it time to treat? Diabetes. 2005;54:1–7.
18. Kozawa J, Inoue K, Iwamoto R, Kurashiki Y, Okauchi Y, Kashine S, Kitamura T, Maeda N, Okita K, Iwahashi H, Funahashi T, Imagawa A, Shimomura I. Liraglutide is effective in type 2 diabetic patients with sustained endogenous insulin-secreting capacity. J Diabetes Invest. 2011 (in press). doi: 10.1111/j.2040-1124.2011.00168.x.
19. Saisho Y, Kou K, Tanaka K, Abe T, Kurosawa H, Shimada A, Meguro S, Kawai T, Itoh H. Postprandial serum C-peptide to plasma glucose ratio as a predictor of subsequent insulin treatment in patients with type 2 diabetes. Endocr J. 2011;58:315–22.
20. Ramirez G, O'Neill WM Jr, Bloomer HA, Jubiz W. Serum C-peptide in renal failure patients following stimulation of pancreatic secretion. Clin Nephrol. 1979;11:297–301.
21. Kajinuma H, Kanazawa Y, Sando H, Hayashi M, Kawazu S, Kosaka K. Human plasma C-peptide immunoreactivity: its correlation with immunoreactive insulin in diabetes, and chronic liver and renal diseases. Endocrinol Jpn. 1979;26:65–73.
22. Nosari I, Lepore G, Maglio ML, Cortinovis F, Pagani G. The effect of various blood glucose levels on post-glucagon C-peptide secretion in type 2 (non insulin-dependent) diabetes. J Endocrinol Invest. 1992;15:143–6.
23. Park SW, Ihm SH, Yoo HJ, Park JY, Lee KU. Differential effects of ambient blood glucose level and degree of obesity on basal serum C-peptide level and the C-peptide response to glucose and glucagon in non-insulin-dependent diabetes mellitus. Diabetes Res Clin Pract. 1997;37:165–71.
24. Coddington CC, Hassiakos DK, Harrison HC, Brzyski RG, Jones GS. Effect of a gonadotropin-releasing hormone analogue on the glucose metabolism in a diabetic patient. Gynecol Obstet Invest. 1990;30:246–8.
25. Rizza RA, Mandarino LJ, Gerich JE. Cortisol-induced insulin resistance in man: impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action. J Clin Endocrinol Metab. 1982;54:131–8.
26. van Raalte DH, van Genugten RE, Linssen MM, Ouwens DM, Diamant M. Glucagon-like peptide-1 receptor agonist treatment prevents glucocorticoid-induced glucose intolerance and islet-cell dysfunction in humans. Diabetes Care. 2011;34:412–7.

The Evaluation of Risk Factors Associated with Adverse Drug Reactions by Metformin in Type 2 Diabetes Mellitus

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Metformin is a drug to improve glycemic control by reducing insulin resistance and is currently considered to be one of the first-choice drugs for type 2 diabetes mellitus (T2DM). However, during metformin use, adverse drug reactions (ADRs) including gastrointestinal adverse events were frequently observed. Thus, in the present study, we investigated the incidence of ADRs induced by metformin and further analyzed risk factors for ADRs in Japanese patients with type 2 diabetes mellitus who initially administered metformin (500–750 mg). One hundred and one hospitalized patients receiving metformin during September 1, 2009 and August 31, 2010 were studied. The incidence of ADRs and changes in laboratory data including hemoglobin A1c (HbA1c) were monitored retrospectively. The anti-glycemic effect of metformin was successfully observed as indicated by decreased HbA1c. Among ADRs, diarrhea was most frequently occurred during metformin use (26.7% of patients) although the symptom of diarrhea was mild in most cases and disappeared within 3 d after the initial use. A logistic regression analysis showed the existence of six risk factors, including initial dose (750 mg), female, age (≦65), body mass index (≧25), aspartate aminotransferase (≧30 IU/L) and alkaline phosphatase (≧270 IU/L). The incidence of diarrhea increased linearly as the number of risk factors increased. In conclusion, in order to avoid ADRs, especially diarrhea, subsequently improving the quality of life during metformin use, the optimization of the dose of metformin by considering risk factors would be beneficial for patients with T2DM.

Key words metformin; type 2 diabetes mellitus; diarrhea; risk factor; dose optimization

Metformin improves insulin resistance by inhibiting gluconeogenesis and enhancing peripheral glucose uptake through stimulation of AMP-activated kinase.[1] *meta*-Analysis has shown its effectiveness in type 2 diabetes mellitus (T2DM).[2] Metformin has been recommended as a first-choice drug in new-onset type 2 diabetes in a consensus algorithm from the American Diabetes Association and the European Association for the Study of Diabetes.[3] Moreover, recent reports suggest that metformin would be effective 1) in preventing cardiovascular events in type 2 diabetes,[4] 2) in preventing the development of T2DM in nondiabetic subjects,[3,5] 3), in improving impaired glucose tolerance in patients with metabolic syndrome,[6] and 4) limiting weight-gain induced by atypical antipsychotics.[7]

Metformin is generally well tolerated. However, it is well known that metformin frequently causes adverse drug reactions (ADRs) including gastrointestinal adverse events, especially diarrhea.[3] These ADRs occasionally leads to withdrawal of metformin treatment.[8,9] In the present study, we initially clarified the incidence and profiles of ADRs caused by initial treatment of metformin in patients hospitalized with T2DM patients. Secondly, we aimed to identify risk-factors related to ADR(s) to encourage the appropriate use of metformin.

MATERIALS AND METHODS

Patients Our study was carried out in accordance with the guidelines for the care for human study adopted by the ethics committee of the Gifu Graduate School of Medicine and notified by the Japanese government (approved No. 22-174 of the institutional review board). One hundred and one patients admitted to our hospital and started on metformin during September 1, 2009 and August 31, 2010 were enrolled. The enrolled patients possessed normal renal function (serum creatinine <1.2 mg/dL) since in our hospital patients with reduced renal function (serum creatinine ≧1.2 mg/dL) were not given metformin to avoid the risk of metformin-induced lactic acidosis although this complication is rare.[10] Moreover, it should be noted that patients who have any diseases that frequently cause diarrhea (ex. infectious disease, gastrointestinal disease *etc.*)[11–13] or who take any drugs that frequently cause diarrhea (ex. antibiotics, anticancer drugs, non-steroidal anti-inflammatory drugs *etc.*)[14] were eliminated.

Survey of ADRs Associated with Metformin Metformin (Melbin® 250 mg tablet, Dainippon-Sumitomo Pharma Co., Ltd., Osaka, Japan) was administered at a daily dose of 500 mg or 750 mg. Data were retrospectively obtained from the patients' electronic medical records and pharmaceutical records. Briefly, ADRs, assessed by pharmacists, such as diarrhea, nausea, and vomiting during the hospitalization (*i.e.* up to 7 d after starting metformin use) were obtained from pharmaceutical records. The severity of ADRs was assessed by the Common Terminology Criteria for Adverse Events (CTCAE version 4.0).

Monitoring the Laboratory Data and the Patients' Characteristics The laboratory data for alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin, serum creatinine (Cr) and hemoglobin A1c (HbA1c), as well as the patient's characteristics including age, gender, body mass index (BMI) and prescribed drugs before metformin use were obtained from the medical records. Creatinine clearance (CCr) was obtained

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by the Cockcroft–Gault formula[15] from data on age, sex, body weight and Cr.

Statistical Analyses Data analysis was with the Statistics Program for Social Science for Windows (SPSS II, ver. 11, IBM Co., Somers, NY, U.S.A.). Non-parametric data were analyzed using Fisher's exact probability test or Mann–Whitney *U*-test, while parametric data were analyzed by *t*-test. Risk analyses were carried out by univariate logistic regression analysis.

RESULTS

Patient's Demographics Characteristics of patients were shown in Table 1. Metformin was administered at a dose of 500 mg and 750 mg in 52 and 49 patients, respectively. Before metformin treatment, insulin was administered in 49 patients (48.5%), sulfonylurea in 6 patients (5.9%), α-glucosidase inhibitors in 31 patients (30.7%), and thiazolidines in 12 patients (11.9%).

Hypoglycemic Effect of Metformin As shown in Table 2, HbA1c gradually decreased after treatment with metformin in either group. Significant reduction in HbA1c was observed at 12 weeks in both groups. The extent of the reduction in HbA1c was significantly ($p<0.05$) greater in 750 mg-treated group than in 500 mg-treated group.

Metformin-Induced ADRs As shown in Table 3, the most-frequently observed ADR was diarrhea that occurred in 27 patients of 101 patients (27%). Another ADR was a mild anorexia (*i.e.* G1) which appeared in 3 patients (3%) without diarrhea. Nausea, vomiting, hepatic and renal dysfunctions were not observed. Although it is well known that one of serious ADRs caused by metformin is lactic acidosis,[10] no ADRs related to lactic acidosis (ex. blood pH *etc.*) were observed in the present study (data not shown).

Profiles of Metformin-Induced Diarrhea Profiles of diarrhea were compared between the two initial doses 500 mg and 750 mg. As shown in Table 4, the incidence was significantly higher in 750 mg-treated group than in 500 mg-treated group (36.7% *vs.* 17.3%, $p<0.05$). The onset of diarrhea was in all cases within a day of metformin treatment. The symptom

Table 1. Patients' Demographics

Number of patients (male/female)	101 (62/39)
Age (range)	60.7 (14–86)
HbA1c (%)	9.4±2.2
Initial daily dose	
500 mg	52 patients (51.5%)
750 mg	49 patients (48.5%)
Dose regimen	
Before meal	12 patients (11.9%)
After meal	89 patients (88.1%)
Alanine aminotransferase (ALT, U/L)	30.7±24.2
Aspartate aminotransferase (AST, U/L)	25.2±13.3
Alkaline phosphatase (ALP, IU/L)	233.3±71.4
Total bilirubin (mg/dL)	0.9±0.4
Creatinine clearance (mL/min)	94.7±28.5
Co-administered drugs	
Insulin	49 patients (48.5%)
Sulfonylurea	6 patients (5.9%)
α-Glucosidase inhibitors	31 patients (30.7%)
Thiazolidines	12 patients (11.9%)

Table 2. Effect of Initial Doses of Metformin on the Levels of HbA1c in Patients with T2DM

	Initial daily dose of metformin		Statistical significance[b]
	500 mg (N=52)	750 mg (N=49)	
Hypoglycemic effect (HbA1c, %)			
Baseline	9.0±2.1	9.8±2.2	0.056
12w	7.1±0.9 (N=16[a])*	6.6±0.9 (N=12[a])**	0.137
ΔHbA1c	−1.4±0.4 (N=16[a])	−3.3±0.7 (N=12[a])	0.02

*,** $p<0.05$ and $p<0.01$ *vs.* corresponding baseline. *a*) Patients whose HbA1c was not examined or recorded were excluded from the data. *b*) Unpaired *t*-test.

Table 3. The Incidence of ADRs of Metformin (N=101)

ADRs	Number of patients (%)
Diarrhea[a]	27 (26.7%)
Anorexia[a,b]	3 (3.0%)
Nausea [a]	0 (0%)
Vomiting [a]	0 (0%)
Hepatic dysfunctions[c,d]	0 (0%)
Renal dysfunctions[e]	0 (0%)

ADRs were monitored during the hospitalization (up to 7 d after metformin treatment). *a*) We counted the number of patients who developed diarrhea, anorexia, nausea and vomiting as more than G0. *b*) No patients with anorexia occurred diarrhea during metformin use. *c*) Values of AST and ALT 3 times above ULN (*i.e.* G1) considered being hepatic dysfunction after metformin use. *d*) One patient who had high ALT (G1) before metformin use together with unchanged ALT after metformin use was excluded from the analysis. This patient caused diarrhea after metformin use for 3 d. *e*) Values of serum creatinine 1.5 times above ULN (*i.e.* G1) considered being renal dysfunction after metformin use.

Table 4. Effect of Initial Doses of Metformin on the Profiles of Metformin-Induced Diarrhea in Patients with T2DM

	Initial daily dose of metformin		Statistical significance[e]
	500 mg (N=52)	750 mg (N=49)	
Incidence (n=27)[a]	9 (17.3%)	18 (36.7%)	0.042
Gender			
Male	3	9	
Female	6	9	0.681
Severity[b]			
G1 (n=21)	6	15	
G2 (n=6)	3	3	0.623
Duration[b]			
Within 3 d (n=18)	6	12	
Over 3 d (n=6)[c]	1	5	0.629
Tolerability[b]			
Treatment continued (n=24)	7	17	
Treatment discontinued (n=3)[d]	2	1	0.795

Onset, gender, grade, duration and tolerability of diarrhea were monitored during the hospitalization (up to 7 d after metformin treatment). *a*) Diarrhea appeared in all cases within a day of metformin treatment. *b*) There are no statistical differences of gender and age (≦65) between groups in severity, duration and tolerability. *c*) Symptom was G1 at the end of the observation in 5 patients. *d*) All were G2 but the symptom recovered immediately after stopping medication. *e*) Fisher's exact probability test.

Table 5. Comparison of the Characteristics of Patients between Those with and without Diarrhea

	With diarrhea (N=27)	Without diarrhea (N=74)	Statistical significance
Age	56.1±15.2	62.4±13.8	0.028[b]
Gender			
Male	12 (44.4%)	50 (67.6%)	
Female	15 (55.6%)	24 (32.4%)	0.040[a]
Body mass index	26.6±5.6	24.0±3.7	0.036[c]
HbA1c (%)	9.3±2.1	9.4±2.2	0.918[c]
Initial daily dose (mg)	667±120	605±124	0.028[c]
Dose			
500 mg	6 (40.0%)	18 (75.0%)	
750 mg	9 (60.0%)	6 (25.0%)	0.065[a]
Dose regimen			
Before meal	2 (7.4%)	10 (13.5%)	
After meal	25 (92.6%)	64 (86.5%)	0.507[a]
ALT (U/L)	41.9±35.2	25.6±17.3	0.038[c]
AST (U/L)	28.4±15.2	24.0±12.5	0.140[c]
ALP (IU/L)	270.6±98.6	219.1±52.1	0.015[c]
Total bilirubin (mg/dL)	0.94±0.54	0.88±0.41	0.567[c]
Creatinine clearance (mL/min)	103.4±26.5	91.6±28.7	0.066[c]
Co-administered drugs			
Sulfonylurea	1 (3.7%)	5 (6.8%)	1.000[a]
α-Glucosidase inhibitors	8 (29.6%)	23 (31.1%)	1.000[a]
Thiazolidines	1 (3.7%)	11 (14.9%)	0.125[a]
Insulin	12 (44.4%)	37 (50%)	0.659[a]
Laxatives	3 (11.1%)	11 (14.9%)	0.754[a]

a) Fisher's exact probability test. *b*) Mann–Whitney *U*-test. *c*) Student's *t*-test.

of metformin-induced diarrhea was mild (*i.e.* G1) in many cases in either group and most patients recovered from diarrhea within a few days. Three of 27 patients with diarrhea discontinued metformin therapy due to G2 diarrhea, where the symptom disappeared immediately after the cessation of metformin use. There are no significant differences of the severity, duration and tolerability of diarrhea between the initial doses of metformin.

Comparison of the Characteristics between Patients with and without Diarrhea Table 5 shows a comparison of patient characteristics between the two groups with and without diarrhea. Six risk factors identified were age (p=0.028), sex (p=0.04), initial daily dose of metformin (p=0.028), BMI (p=0.036), ALT (p=0.038) and ALP (p=0.015). The odds ratio in a univariate analysis was 2.774 (95% CI, 1.101–6.988, p=0.030) for initial daily dose of 750 mg, 2.604 (1.057–6.416, p=0.037) for female, 3.694 (1.338–10.198, p=0.012) for age ≦65, 2.532 (1.028–6.239, p=0.043) for BMI ≧25, 2.90 (1.13–7.42, p=0.026) for ALT ≧30 IU/L and 3.380 (1.126–9.071, p=0.016) for ALP ≧270 IU/L (Fig. 1).

The Relationship between Risk Factors and the Incidence of Diarrhea It was notable that the incidence of diarrhea was elevated linearly as the number of risk factors increased, when the number of risks exceeded 3 (Fig. 2).

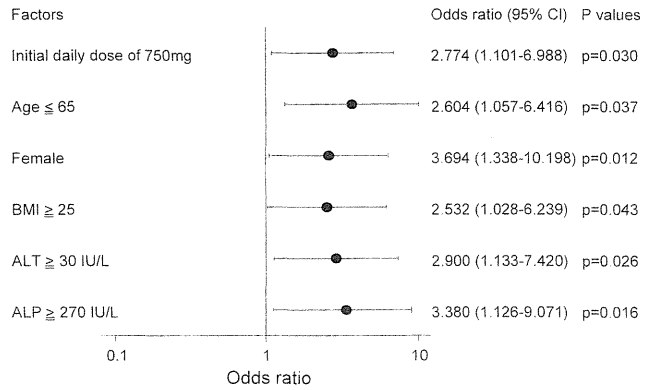
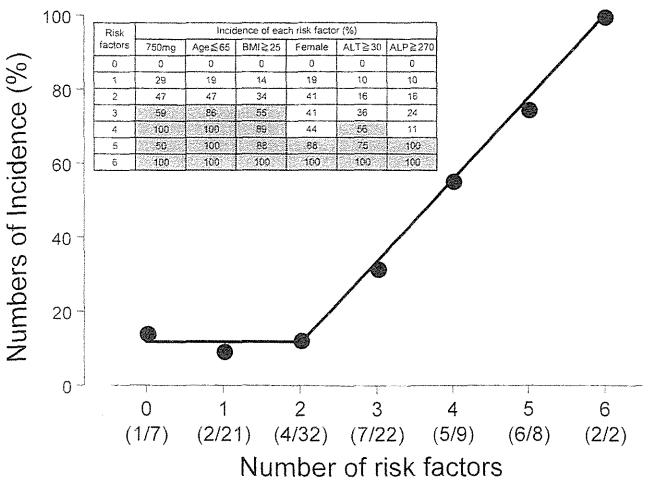


Fig. 1. Risk Factor Analysis for Metformin-Induced Diarrhea
Data were analyzed by univariate logistic regression analyses.

DISCUSSION

The current study revealed that diarrhea is most-frequently occurred ADR during metformin use at an initial dose of 500 mg or 750 mg as shown previously.[3,16] The overall incidence of diarrhea observed in the present study was 26.7% (27 of 101 patients), which was consistent with the data (23.7%) reported in a large clinical trial such as A Diabetes Outcome Progression Trial (ADOPT),[17] although the incident rate was varied among studies.[16] The differences of initial daily doses of metformin failed to affect the severity, duration and tolerability of diarrhea although further large scale study would be needed to analyze the profiles of metformin-induced diarrhea in detail.

In the present study, we determined the risks of metformin-induced diarrhea. An univariate logistic regression analysis indicated that six risk factors for metformin-induced diarrhea was found, including an initial dose of 750 mg, female, age ≦65, BMI ≧25, AST ≧30 IU/L and ALP ≧270 IU/L. It was notable that the incidence of diarrhea was proportionally elevated as the number of these risk factors increased, in which the incidence was dramatically increased when the number of risks exceeded 3. Thus, the number of possessed risk factors



Risk factors	Incidence of each risk factor (%)					
	750mg	Age≦65	BMI≧25	Female	ALT≧30	ALP≧270
0	0	0	0	0	0	0
1	29	19	14	19	10	10
2	47	47	34	41	16	16
3	59	86	55	41	36	24
4	100	100	89	44	56	11
5	50	100	88	88	75	100
6	100	100	100	100	100	100

Fig. 2. Relationship between the Number of Risks and the Incidence of Diarrhea Associated with Metformin Administration

Risk factors for metformin-induced diarrhea included age ≦65, female, body mass index ≧25, initial daily dose of 750 mg, ALT ≧30 IU/L and ALP ≧270 IU/L. Numbers in each parenthesis represent the number of patients with diarrhea per total number of patients. Table in the figure showed the incidence of each risk factor. Shaded column indicated the value over 50%.

would become a good predictor for metformin-induced diarrhea.

Consistent with previous studies,[8,16] we found the importance of dose of metformin since the incidence of diarrhea was significantly higher in patients who received metformin at an initial dose of 750 mg than in those who took at 500 mg (17.3% *vs.* 36.7%). Thus, in order to use a maximal effective dose of metformin without the ADRs as well as the discontinuation, the assessment of risk factors together with appropriate supports such as prophylactic use of antidiarrhoics would be expected to reduce the number of patients who should decrease the dose of metformin due to diarrhea.

The elevation of ALT and ALP also became risk factors. So far, it has been reported that metformin itself induces hepatic dysfunction, including cholestatic damage, as shown by an increase in alkaline phosphatase.[18] Moreover, liver dysfunction by cholestasis decreases the expression of organic cation transporter (OCT) 1 and 3, both of which play a crucial role in the uptake of metformin into liver.[19] Taken together, it is possible that liver damage including cholestasis might modify the incidence of diarrhea.

Several hypotheses underlying metformin-induced gastrointestinal side effects have been postulated although some of them were controversial.[1,20] Although, so far, no patients had a sign of lactic acidosis such as altered blood pH level, it is difficult to exclude out the involvement of lactic acidosis in metformin-induced diarrhea since diarrhea is the most frequent initial symptom of lactic acidosis.[1] The involvement of malabsorption of bile acid from the ilium by metformin in metformin-induced gastrointestinal side effects has also been suggested,[21] although the evidence of the malabsorption of bile salts by metformin was controversial.[21,22] Similarly, the involvement of serotonin and ghrelin in the gastrointestinal side effects of metformin was also conflicting.[20] Other possible mechanism to induce gastrointestinal side effects is the involvement of glucagon-like peptide 1 (GLP-1) since metformin elevated GLP-1 in plasma by inhibiting the degradation of GLP-1[23] and GLP-1 analogs and dipeptidyl peptidase-IV (DPP-IV) inhibitors frequently cause the gastrointestinal side effects.[24,25]

In the present study, age ≦65 became a risk factor although it is well accepted that reduced functions of kidney, liver and other organs in elderly subjects might strengthen the effect of drugs. Furthermore, we also found that female and BMI also became risk factors. It has been reported that genders and body weight failed to affect the pharmacokinetics of metformin.[26] Thus, the further investigation would be needed to understand these data.

Finally, we introduced our preliminary trial that pharmacists recommended three choices to the physicians who underwent the treatment of T2DM by metformin in patients with the risk factors exceeded 3; 1) the use of 500 mg of metformin instead of 750 mg, 2) the prophylactic use of antidiarrhoics, or 3) the discontinuation of currently-used laxatives. Results were likely to be promising since the rate of diarrhea was reduced to 12.0% (3 out of 25 patients) together with the use of 500 mg of metformin in most patients (24 out of 25 patients).

In conclusion, we found six risk factors to cause metformin-induced diarrhea: age ≦65, female, BMI ≧25, initial daily dose of 750 mg, ALT ≧30 IU/L and ALP ≧270 IU/L. The incidence of diarrhea was linearly elevated as the number of these risks increased. Thus, checking the risk factors for metformin-induced diarrhea may aid medical staffs to pre-caution the possible occurrence of metformin-induced diarrhea to the patients together with the use of antidiarrhoics, subsequently expecting to improve the adherence of metformin by reducing the prevalence of metformin-induced diarrhea.

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REFERENCES

1) Scarpello JH, Howlett HC. Metformin therapy and clinical uses. *Diab. Vasc. Dis. Res.*, **5**, 157–167 (2008).
2) Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M, Moher D. Metformin monotherapy for type 2 diabetes mellitus. *Cochrane Database Syst. Rev.*, CD002966 (2005).
3) Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B, American Diabetes Association European Association for Study of Diabetes. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care*, **32**, 193–203 (2009).
4) Schramm TK, Gislason GH, Vaag A, Rasmussen JN, Folke F, Hansen ML, Fosbøl EL, Køber L, Norgaard ML, Madsen M, Hansen PR, Torp-Pedersen C. Mortality and cardiovascular risk associated with different insulin secretagogues compared with metformin in type 2 diabetes, with or without a previous myocardial infarction: a nationwide study. *Eur. Heart J.*, **32**, 1900–1908 (2011).
5) Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM, Diabetes Prevention Program Research Group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N. Engl. J. Med.*, **346**, 393–403 (2002).
6) Orchard TJ, Temprosa M, Goldberg R, Haffner S, Ratner R, Marcovina S, Fowler S, Diabetes Prevention Program Research Group. The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the Diabetes Prevention Program randomized

trial. *Ann. Intern. Med.*, **142**, 611–619 (2005).
7) Lee YJ, Jeong JH. A systematic review of metformin to limit weight-gain with atypical antipsychotics. *J. Clin. Pharm. Ther.*, **36**, 537–545 (2011).
8) Garber AJ, Duncan TG, Goodman AM, Mills DJ, Rohlf JL. Efficacy of metformin in type II diabetes: results of a double-blind, placebo-controlled, dose-response trial. *Am. J. Med.*, **103**, 491–497 (1997).
9) Belcher G, Lambert C, Edwards G, Urquhart R, Matthews DR. Safety and tolerability of pioglitazone, metformin, and gliclazide in the treatment of type 2 diabetes. *Diabetes Res. Clin. Pract.*, **70**, 53–62 (2005).
10) Salpeter SR, Greyber E, Pasternak GA, Salpeter EE. Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus. *Cochrane Database Syst. Rev.*, CD002967 (2010).
11) Juckett G, Trivedi R. Evaluation of chronic diarrhea. *Am. Fam. Physician*, **84**, 1119–1126 (2011).
12) Manatsathit S, Dupont HL, Farthing M, Kositchaiwat C, Leelakusolvong S, Ramakrishna BS, Sabra A, Speelman P, Surangsrirat S, Working Party of the Program Committ of the Bangkok World Congress of Gastroenterology 2002. Guideline for the management of acute diarrhea in adults. *J. Gastroenterol. Hepatol.*, **17** (Suppl.), S54–S71 (2002).
13) Thomas PD, Forbes A, Green J, Howdle P, Long R, Playford R, Sheridan M, Stevens R, Valori R, Walters J, Addison GM, Hill P, Brydon G. Guidelines for the investigation of chronic diarrhoea, 2nd edition. *Gut*, **52** (Suppl. 5), v1–v15 (2003).
14) Chassany O, Michaux A, Bergmann JF. Drug-induced diarrhoea. *Drug Saf.*, **22**, 53–72 (2000).
15) Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron*, **16**, 31–41 (1976).
16) Bennett WL, Wilson LM, Bolen S, Maruthur N, Singh S, Chatterjee R, Marinopoulos SS, Puhan MA, Ranasinghe P, Nicholson WK, Block L, Odelola O, Dalal DS, Ogbeche GE, Chandrasekhar A, Hutfless S, Bass EB, Segal JB. "Oral Diabetes Medications for Adults With Type 2 Diabetes: An Update.": ‹http://www.ncbi.nlm.nih.gov/books/NBK55754›, Rockville MD, Agency for Healthcare Research and Quality, U.S., March, 2011.
17) Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G, ADOPT Study Group. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N. Engl. J. Med.*, **355**, 2427–2443 (2006).
18) Nammour FE, Fayad NF, Peikin SR. Metformin-induced cholestatic hepatitis. *Endocr. Pract.*, **9**, 307–309 (2003).
19) Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, Zanger UM, Keppler D, Schwab M, Schaeffeler E. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology*, **50**, 1227–1240 (2009).
20) Bouchoucha M, Uzzan B, Cohen R. Metformin and digestive disorders. *Diabetes Metab.*, **37**, 90–96 (2011).
21) Caspary WF, Zavada I, Reimold W, Deuticke U, Emrich D, Willms B. Alteration of bile acid metabolism and vitamin-B12-absorption in diabetics on biguanides. *Diabetologia*, **13**, 187–193 (1977).
22) Scarpello JH, Hodgson E, Howlett HC. Effect of metformin on bile salt circulation and intestinal motility in type 2 diabetes mellitus. *Diabet. Med.*, **15**, 651–656 (1998).
23) Cuthbertson J, Patterson S, O'Harte FP, Bell PM. Addition of metformin to exogenous glucagon-like peptide-1 results in increased serum glucagon-like peptide-1 concentrations and greater glucose lowering in type 2 diabetes mellitus. *Metabolism*, **60**, 52–56 (2011).
24) Bergenstal RM, Wysham C, Macconell L, Malloy J, Walsh B, Yan P, Wilhelm K, Malone J, Porter LE, DURATION-2 Study Group. Efficacy and safety of exenatide once weekly *versus* sitagliptin or pioglitazone as an adjunct to metformin for treatment of type 2 diabetes (DURATION-2): a randomised trial. *Lancet*, **376**, 431–439 (2010).
25) Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*, **368**, 1696–1705 (2006).
26) Sambol NC, Chiang J, O'Conner M, Liu CY, Lin ET, Goodman AM, Benet LZ, Karam JH. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. *J. Clin. Pharmacol.*, **36**, 1012–1021 (1996).

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Hepatic FoxO1 Integrates Glucose Utilization and Lipid Synthesis through Regulation of Chrebp O-Glycosylation

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Abstract

In liver, glucose utilization and lipid synthesis are inextricably intertwined. When glucose availability exceeds its utilization, lipogenesis increases, leading to increased intrahepatic lipid content and lipoprotein secretion. Although the fate of three-carbon metabolites is largely determined by flux rate through the relevant enzymes, insulin plays a permissive role in this process. But the mechanism integrating insulin receptor signaling to glucose utilization with lipogenesis is unknown. Forkhead box O1 (FoxO1), a downstream effector of insulin signaling, plays a central role in hepatic glucose metabolism through the regulation of hepatic glucose production. In this study, we investigated the mechanism by which FoxO1 integrates hepatic glucose utilization with lipid synthesis. We show that FoxO1 overexpression in hepatocytes reduces activity of carbohydrate response element binding protein (Chrebp), a key regulator of lipogenesis, by suppressing O-linked glycosylation and reducing the protein stability. FoxO1 inhibits high glucose- or O-GlcNAc transferase (OGT)-induced liver-pyruvate kinase (L-PK) promoter activity by decreasing Chrebp recruitment to the L-PK promoter. Conversely, FoxO1 ablation in liver leads to the enhanced O-glycosylation and increased protein level of Chrebp owing to decreased its ubiquitination. We propose that FoxO1 regulation of Chrebp O-glycosylation is a mechanism linking hepatic glucose utilization with lipid synthesis.

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Introduction

The liver plays a central role in integrating glucose and lipid metabolism, effectively exchanging carbons from one energy source to the other for storage and utilization [1]. This process requires both hormone signaling and feedback control by substrate flux. Examples of the latter are the diversion of three-carbon precursors from glycolysis to esterification of FFA to generate triglycerides, and the shunting of citrate from glycolysis to fatty acid synthesis by way of acetyl-CoA carboxylase and malonyl-CoA [2]. Examples of the former are the effects of insulin on expression of genes that rate-control glucose utilization and its conversion into lipids, like glucokinase, glucose-6-phosphatase, pyruvate kinase, and pyruvate dehydrogenase kinase [1]. In physiological situations, the two control mechanisms cohabit peacefully. But in the metabolic syndrome, there is an apparent discrepancy between the inability of insulin to suppress glucose production, and its preserved ability to promote de novo lipogenesis. Various theories have been advanced, but none of them is entirely satisfactory [3].

Key transcriptional mediators of insulin signaling and glucose signaling are FoxO1 and Chrebp. FoxO1 is an Akt substrate and regulates glucose production and bile acid synthesis [4–5]. Chrebp mediates glucose action on glycolysis and lipid synthesis [6]. Among its targets are liver-pyruvate kinase (*Lpk*), one of the rate-limiting enzymes of glycolysis [7], and lipogenic genes, such as acetyl-CoA carboxylase (*Acaca*) and fatty acid synthase (*Fasn*) [8]. Chrebp is activated via protein phosphatase 2A-dependent dephosphorylation in response to xylulose-5-phosphate (Xu-5-P) generated by the pentose monophosphate shunt [9]. Dephosphorylated Chrebp translocates to the nucleus and activates target gene transcription [10].

Impetus for the present studies came from prior observations that genetic ablation of FoxO1 in liver increases systemic insulin sensitivity, and results in lower hepatic glucose production, increased glycogen storage, and increased lipogenesis [11–12]. We reasoned that this model could be deconstructed for the purpose of identifying the physiological mechanism linking glucose with lipid metabolism. We identify a genetic, biochemical, and molecular pathway linking FoxO1 with Chrebp, and propose that it represents the connection between altered glucose and lipid metabolism in type 2 diabetes.

Methods

Antibodies

We purchased antibodies against Chrebp from Novus Biologicals, O-GlcNAc from Covance, OGT (DM-17) from Sigma, FoxO1 (9462) from Cell Signaling, FoxO1 (H-128), Ubiquitin (P4D1), Tubulin (B-7) from Santa-Cruz, HA (12CA5) from Roche. We used these antibodies for immunoprecipitation or immunoblotting according to manufacturer's protocol.

Expression vectors and Adenoviral vectors

We have previously described expression vectors encoding Flag-tagged FoxO1-ADA and His-HA-Ubiquitin [13], and adenoviral vectors encoding HA-tagged wild type and FoxO1-ADA [14]. pcDNA3-HA-OGT and pCMV4-Flag-Chrebp are gifts from Mark Montminy (Salk Institute, La Jolla, CA) and Howard Towle (Univ of Minnesota, Minneapolis, MN), respectively. We generated a synthetic L-PK luciferase vector containing 3x charbohydrate response element in the L-PK promoter (pGL3-3xL-PK-ChoRE).

Cell culture, siRNA transfection, and viral transduction

We purchased primary culture of mouse hepatocyte from Primary Cell Co., Ltd (Sapporo, Japan) and cultured the cells in DMEM supplemented with 10% FCS. The FoxO1-specific siRNA sequence is 5′-ACGGAGGATTGAACCAGTATA-3′. The OGT specific siRNA sequence is 5′-CGACATGCCTTGCGGCTGA -3′. siRNA was transfected using DharmaFECT Duo (Dharmacon). In some experiments, we infected primary hepatocytes with adenovirus at MOI of 10 or 30, 5 hrs before treatments with high glucose. All experiments were repeated at least three times.

Luciferase assays and Chromatin immunoprecipitation assays

We performed luciferase assays as previously described [15] using pGL3-3xL-PK-ChoRE. We performed ChIP assays in mouse primary hepatocytes or mouse liver extracts with primers; 5′-GATTTGAGCCTTTGATCCAGGCTC-3′ and 5′-AAGTTCCCTCCATCTATACAGTGC-3′ according to the previously described methods [13]. All experiments were repeated at least three times.

Immunoprecipitation and Western blotting

We lysed cultured cells in RIPA buffer containing protease inhibitors (Roche). After centrifugation, cell extracts were diluted with Co-IP buffer (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 5 mM $MgCl_2$), immunoprecipitated and analyzed by immunoblotting.

mRNA isolation and real-time PCR

We isolated mRNA from primary hepatocytes or mouse liver extracts using the Micro Fast Track 2.0 kit (Invitrogen). We performed real-time RT-PCR using ImProm-II™ Reverse Transcription System (Promega) and LightCycler System (Roche). Primer sequences used for real-time PCR are as follows, for Chrebp; 5′- CTG GGG ACC TAA ACA GGA GC -3′ and 5′-GAA GCC ACC CTA TAG CTC CC −3′, for L-PK; 5′- GGG CCG CAT CTA CAT TGA C −3′ and 5′- GTC CCT CTG GGC CAA TTT T-3′. We carried out each reaction in triplicate, using a standard curve with the relevant cDNA for each primer set.

O-GlcNAc enzymatic labeling

We performed metabolic labeling of Chrebp with tetraacetylated azide-modified N-acetylglucosamine (GlcNAz) in mouse primary hepatocytes. After immunoprecipitation with anti-Chrebp antibody, we detected O-glycosylation modification using biothin-avidin system.

Animal generation and analyses

We generated liver specific FoxO1 knockout mice using FoxO1 flox/flox mice [16] and Albumin-cre transgenic mice (a kind gift from Akihiro Harada, Osaka University). The wild-type, null and $Foxo1^{flox}$ alleles were detected using PCR with primers 5′-GCT TAG AGC AGA GAT GTT CTC ACA TT-3′, 5′-CCA GAG TCT TTG TAT CAG GCA AAT AA-3′ and 5′-CAA GTC CAT TAA TTC AGC ACA TTG A-3′. Individually caged mice were housed in a temperature-controlled facility. All animal care and experimental procedures were approved by the Institutional Animal Care and Experimentation Committee at Gunma University. H-E staining was performed using 4- μm-thick paraffin sections following the standard methods. Hepatic triglyceride (TG) contents were measured as described previously [17].

Results

FoxO1 inhibits Chrebp transcriptional activity by suppressing O-glycosylation and reducing protein stability of Chrebp

Although insulin as well as glucose flux regulate hepatic glucose utilization and lipid synthesis, the underlying molecular mechanisms have not been fully understood. Because FoxO1 is a downstream effector of insulin signaling and Chrebp is a key transcriptional regulator of glycolysis and lipogenesis, we tested whether FoxO1 is involved in the regulation of Chrebp. When we overexpressed constitutively active form of FoxO1, FoxO1-ADA (a mutant FoxO1 with the following amino acid substitutions: T24A, S253D, and S316A) [18], in primary hepatocytes, mRNA level of *Lpk*, a target of Chrebp, was significantly decreased, despite unchanged levels of *Chrebp* mRNA (Fig. 1A). Therefore, we next investigated Chrebp protein levels in these samples. As shown in Figure 1B, FoxO1-ADA expression significantly decreased Chrebp protein level (Fig. 1B, second panel from the top). Because it has been reported that Chrebp protein is stabilized by modification of O-glycosylation [19], we investigated it and found that FoxO1-ADA expression decreased Chrebp O-glycosylation (Fig. 1B, top panel and the bottom graph). Consistent with the previous report [19], O-glycosylation and protein level of Chrebp were increased by high glucose (25mM) in primary hepatocytes (Fig. 1B). Conversely, when we knocked down FoxO1 using an adenovirus expressing FoxO1-specific siRNA in primary hepatocytes, Chrebp O-glycosylation was increased independent of glucose concentrations in the medium (Fig. 1C, top panel and the bottom graph).

Because protein level, but not mRNA level of Chrebp was decreased by FoxO1-ADA, we next checked whether FoxO1-ADA expression affects protein degradation of Chrebp by evaluating its ubiquitination. As shown in Fig. 1D, FoxO1-ADA expression enhanced poly-ubiquitination of both endogenous (left two lanes) and exogenously expressed Chrebp (right two lanes), indicating that changes in Chrebp ubiquitination might explain the decreased protein levels observed in our experiments. Indeed, chromatin immunoprecipitation revealed that overexpression of FoxO1-ADA suppressed high-glucose-induced recruitment of Chrebp to the *Lpk* promoter (Fig. 1E). Furthermore, results of luciferase assays using the *Lpk* promoter indicated that FoxO1-ADA inhibited high-glucose-induced or OGT-expression-induced

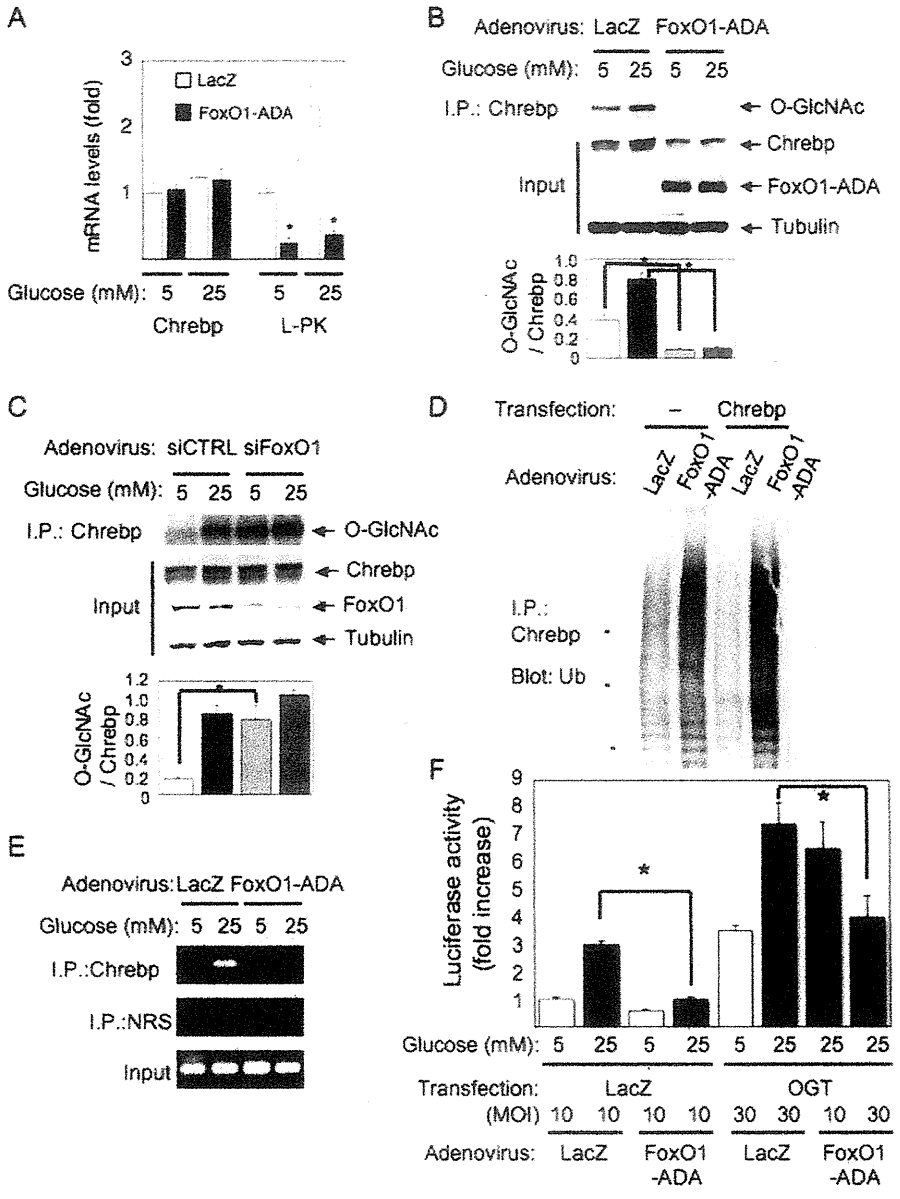

Figure 1. FoxO1-ADA expression and the knockdown of FoxO1 affect Chrebp O-glycosylation, protein stability and transcriptional activity in primary hepatocytes. (A, B, D and E) Mouse primary hepatocytes infected with adenovirus expressing FoxO1-ADA or LacZ were cultured with 5 mM or 25 mM glucose. The cell lysates were subjected to real-time RT-PCR for Chrebp or L-PK (A), immunoprecipitation with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc antibody (B) or anti-ubiquitin antibody (D). The cell lysates were also subjected to chromatin-immunoprecipitation assay using anti-Chrebp antibody and the primers for L-PK promoter (E). (C) Mouse primary hepatocytes infected with adenovirus expressing specific siRNA for FoxO1 or control siRNA were cultured with 5 mM or 25 mM glucose and the cell lysates were immunoprecipitated with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc antibody. Input represents expression levels of Chrebp, FoxO1-ADA, endogenous FoxO1 and Tubulin. Quantitative analyses were performed by assessment of O-glycosylation level compared with the protein level of Chrebp using densitometry, showing as a bar graph below the results of blotting (B and C). (F) Mouse primary hepatocytes co-transfected with pGL3-3XI-PK-ChoRE and OGT or LacZ were infected with adenovirus expressing FoxO1-ADA or LacZ at indicated MOI and cultured with 5 mM or 25 mM glucose for 24 hr. The cell lysates were used for luciferase assays. Experiments were repeated at least three times. Data represent mean ± SEM. *P<0.05.
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Chrebp transcriptional activity (Fig. 1F). Taken together, these results show that FoxO1 inhibits Chrebp transcriptional activity by suppressing O-glycosylation and reducing protein stability of Chrebp.

Chrebp O-glycosylation is regulated by OGT

We used western blotting with anti-GlcNAc antibody to show that Chrebp is O-glycosylated in the presence of elevated glucose concentrations (Fig. 1B). To confirm the modification of Chrebp

O-glycosylation, we also used enzymatic labeling with GlcNAz in primary hepatocytes. As shown in Figure 2A, we detected GlcNAz incorporation into Chrebp protein only in the presence of GlcNAz, indicating that Chrebp is directly modified by O-glycosylation. We next investigated whether high glucose-induced Chrebp O-glycosylation is mediated by OGT. When we transfected primary hepatocytes with OGT, Chrebp O-glycosylation was significantly increased even in low glucose condition (Fig. 2B, top panel and bottom graph). It is notable that OGT was coimmunoprecipitated with Chrebp, and that this interaction was enhanced by high glucose (Fig. 2B, second panel from the top). Conversely, when OGT was depleted by specific siRNA for OGT in primary hepatocytes, both high glucose- and glucosamine-induced O-glycosylation of Chrebp were decreased (Fig. 2C, top panel and bottom graph). Thus, Chrebp interacts with OGT in high glucose condition, leading to Chrebp O-glycosylation.

Increased Chrebp O-glycosylation, protein stability, and recruitment to the Lpk promoter in FoxO1 knockout liver

To assess the effects of FoxO1 ablation in liver on Chrebp protein, we generated liver-specific FoxO1 knockout mice (L-FoxO1-KO) by crossing Albumin-Cre mice with FoxO1 flox mice [16]. We isolated livers from L-FoxO1-KO and control mice following a 24-hr fast or 3-hr re-feeding after 24-hr fast, and performed western blotting or ChIP assays using liver lysates. In the liver of L-FoxO1-KO mice, Chrebp O-glycosylation and protein levels were significantly increased, and–more interestingly–OGT interaction with Chrebp was enhanced in both fasted and refed conditions compared with the liver of control mice (Fig. 3A, top two panels). Consistent with the increase in Chrebp protein level, Chrebp poly-ubiquitination were significantly reduced in the liver of L-FoxO1-KO mice in both fasted and refed conditions (Fig. 3B). We also found that Chrebp recruitment to the *Lpk*

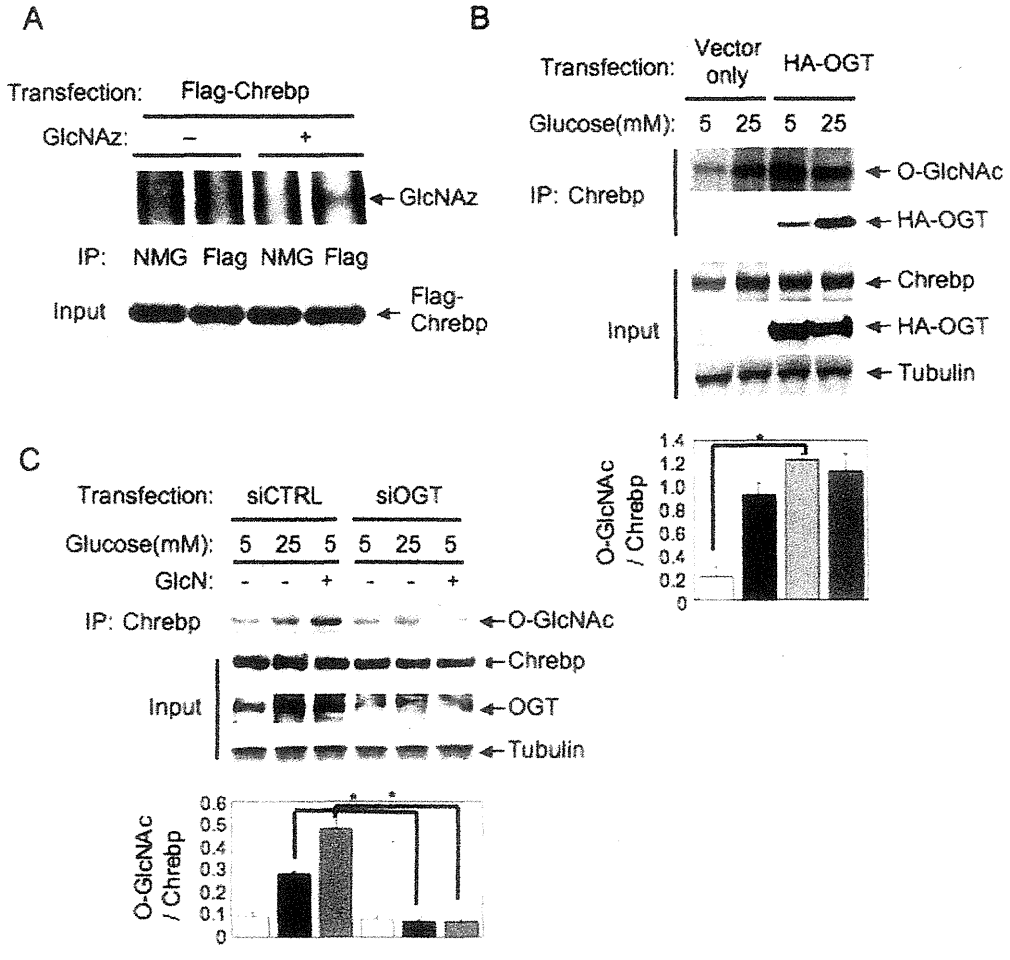

Figure 2. Chrebp O-glycosylation is regulated by OGT. (A) Mouse primary hepatocytes were transfected with Flag-Chrebp and labeled with tetraacetylated azide-modified N-acetylglucosamine (GlcNAz). The cell lysates were immunopricipitated with anti-Flag antibody or normal mouse globlin (NMG) and subjected to detection of O-glycosylation by biothin-avidin system as described in Materials and Methods. Input indicates the expression level of Flag-Chrebp in each lane. (B and C) Mouse primary hepatocytes were transfected with HA-OGT (B) or siRNA for OGT (C) along with empty vector or control siRNA, respectively and cultured with 5 mM or 25 mM glucose for 24 hr. In some experiments, 10 mM glucosamine (GlcN) was added in the medium. The cell lysates were immunoprecipitated with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc or anti-HA antibody. Input indicates the expression level of Chrebp, HA-OGT, endogenous OGT or tubulin. Quantitative analyses were performed by assessment of O-glycosylation level compared with the protein level of Chrebp using densitometry, showing as a bar graph below the results of blotting (B and C). Experiments were repeated at least three times. Data represent mean ± SEM. *P<0.05.
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