titration to 100 mg/day is acceptable. All other medications including statin and antihypertensive agents were not changed or newly prescribed during the study. If the A1C level did not reach 6.9% by treatment with 100 mg/day sitagliptin, the dosage of SUs was increased at physician discretion. Subjects visited the clinic every 4 weeks, and laboratory data including A1C, physical findings and all documented medications were collected every 4 weeks in the first 24 weeks and every 8 weeks in the last 28 weeks. On the day of the final visit, glucagon loading tests were performed under conditions that all prescribed AHAs had been withheld for 72 h.

Measurements

The primary endpoint was change in A1C in 52-week. The secondary endpoints were change in insulin secretion capacity (0-min C-peptide reactin (CPR), 6-min CPR, delta CPR) in 52-week, change in A1C in glimepiride and gliclazide subjects in 52-week, change in A1C in dosage of SUs-reduced and -unreduced subjects in 52-week, unresponsive rate in 12-week, ineffectiveness rate in 52-week, change in BMI, hypoglycaemia, change in blood pressure level, change in urinary albumin excretion and the correlation between change in A1C and insulin secretion capacity or CPR index (CPI) or the secretory unit of islet in transplantation (SUIT) index or BMI. CPI was calculated by the formula: [100 × fasting CPR (ng/ml)]/[18 × FPG (mM)] (14). SUIT index was calculated by the formula: [250 × fasting CPR (ng/ml)]/[(FPG-3.43) (mM)] (15). The day before glucagon loading test, subjects did not eat any food except water and tea without sugar after 9:00 pm. Glucagon loading test was performed in the morning, and blood glucose and C-peptide level were measured before (0 min) and 6 min after intravenous administration of 1 mg glucagon. If A1C reduction was less than 0.5% in 52-week, add-on of sitagliptin to SUs was considered to be ineffective. If A1C level in 12-week was not changed or increased, the combination therapy was judged unresponsive.

Statistical analysis

Sample size was estimated to be 64 to detect a 0.5% change in A1C in 52-week with a power 95%, alpha 0.05 two-tailed, beta 0.20, standardised effect size 0.5. To take the dropout rate of 20% into account, the aim was to include 80 subjects. Dependent samples Student's *t*-test was used to compare the means of A1C level, insulin secretion capacity, BMI and blood pressure between baseline and 52-week. Wilcoxon signed-rank test was used to compare the means of urinary albumin excretion between baseline and 52-week. Person's product-moment correlation test was used to evaluate the relationship between change in A1C and insulin secretion capacity or CPI or SUIT or BMI. p values < 0.05 were considered as statistically significant.

Results

Subjects

We screened 106 patients of whom 87 were provisionally registered. Of these, 82 were eligible and were enrolled consecutively in the study (Figure 1). Five patients declined to participate because of the visiting schedule. Average age of the subjects was 70.1 ± 8.6 years and 43.5% were female; duration of diabetes was 10.2 ± 4.7 years and A1C was 7.90 ± 0.51% (Table 1). Forty-six subjects were treated with glimepiride and 36 were treated with gliclazide. Twenty-five subjects were treated with metformin (average dose 640 ± 127 mg/day) (14

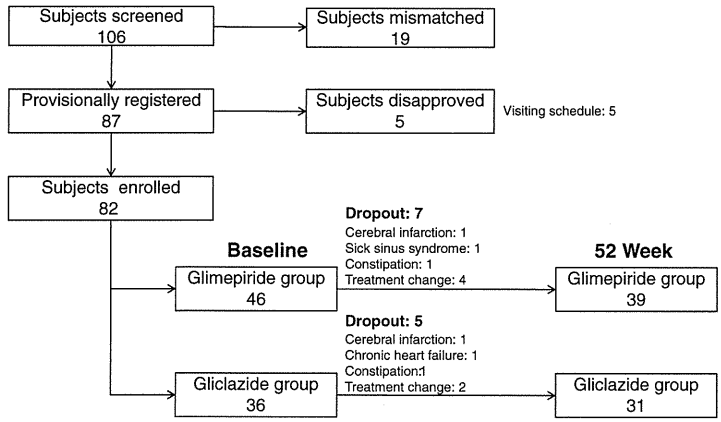

Figure 1 Progress of subjects through the study

Table 1 Background of the subjects

Subjects (*n*)	Age (years)	Female (%)	Diabetes duration (year)	A1C (%)	Nephropathy	Retinopathy	Neuropathy	Cardio vascular diseases	SU (%)	Medication Metformin (%)	αGI (%)
All 82	70.1 ± 8.6	43.5	10.2 ± 4.7	7.90 ± 0.51	36.6%	34.1%	26.8%	24.3%	100	30.4	12.2
Glimepiride 46	68.7 ± 7.5	41.3	9.9 ± 4.8	7.93 ± 0.55	34.7%	32.6%	26.1%	23.9%	56.1	17.1	7.3
Gliclazide 36	72.8 ± 10.0	42.2	10.9 ± 4.7	7.85 ± 0.49	38.9%	36.1%	27.8%	25.0%	43.9	13.4	4.9

glimepiride- and 11 gliclazide-treated subjects). α-glucosidase inhibitors were discontinued in 10 subjects (6 glimepride- and 4 gliclazide-treated subjects). There was no significant difference in the background of glimepiride and gliclizide subjects (Table 1). Twelve subjects were dropped because of hospitalisation for cerebral infarction (one glimepiride- and one gliclazide-treated subject), sick sinus syndrome (one glimepiride-treated subject), chronic heart failure (one gliclazide-treated subject), constipation by sitagliptin (one glimepiride- and one gliclazide-treated subject) or treatment changes from sitagliptin to liralgutide (four glimepiride- and two gliclazide-treated subjects) (Fig. 1). The dropout rate was 14.6% in all subjects, 15.2% in glimepiride subjects and 13.8% in gliclazide subjects (Table 2). The final number of subjects who completed the study was 70: 39 glimepiride subjects and 31 gliclazide subjects (Figure 1).

A1C findings

The A1C level in 52-week in all subjects was significantly decreased from 7.96 ± 0.53% to 7.16 ± 0.53% ($p < 0.001$). Change in A1C in 52-week was −0.80% (95% CI −0.90 to −0.68) ($p < 0.001$) (Table 2). Final dosage of sitagliptin was 69.0 ± 24.5 mg/day. In glimepiride subjects, the A1C level in 52-week was significantly decreased from 8.00 ± 0.57% to 7.19 ± 0.58% ($p < 0.001$) (Table 2). Change in A1C at 52-week from baseline was −0.81 (95% CI −0.96 to −0.62) ($p < 0.001$). The final dosages of glimepiride and sitagliptin were 1.44 ± 0.90 mg/day and 73.1 ± 25.4 mg/day respectively (Table 2). The initial and final dosages of glimepiride were significantly decreased compared with the original dosages of glimepiride ($p < 0.001$). In gliclazide subjects, A1C level in 52-week was significantly decreased from 7.92 ± 0.48% to 7.13 ± 0.49% ($p < 0.001$) (Table 2). Change in A1C at 52-week from baseline was −0.79 (95% CI −0.90 to −0.64) ($p < 0.001$). The initial dosage of gliclazide was slightly but significantly decreased from the original dosage of gliclazide ($p < 0.05$), but the final dosage of gliclazide did not differ from the original dosage. The final dosage of gliclazide and sitagliptin were 34.5 ± 15.3 mg/day and 64.6 ± 23.2 mg/day respectively (Table 2).

Differences in A1C findings in dosage of SUs-unreduced and -reduced subjects

The starting dosage of SUs was reduced to equal to or less than 2.0 mg/day glimepiride or 40 mg/day gliclazide if the two SUs were administrated at more than the suggested dosage. In addition, the dosage of SUs was reduced at physician discretion if subjects had previously experienced hypoglycaemia induced by SUs. In SU-reduced subjects, the initial dosage of glimepiride was decreased from 1.79 ± 0.97 to 1.10 ± 0.51 mg/day in 16 of the 39 glimepiride subjects and that of gliclazide was decreased from 55.0 ± 17.6 to 33.3 ± 10.3 mg/day in 12 of the 31 gliclazide subjects. The dosages for SUs-unreduced subjects of glimepiride and gliclazide were 1.15 ± 0.55 mg/day and 30.0 ± 10.3 mg/day respectively. Thus, the A1C level was almost equally decreased in SUs-unreduced and SU-reduced subjects (Table 3). The change in A1C was −0.80% (95% CI −1.02 to −0.59) ($p < 0.001$) in SUs-unreduced subjects, and −0.81% (95% CI −1.04 to −0.57) ($p < 0.001$) in SUs-reduced subjects. In glimepiride subjects, the change in A1C level was −0.88% (95% CI −1.16 to −0.59) ($p < 0.001$) in SU-unreduced subjects, and −0.70% (95% CI −0.98 to −0.42) ($p < 0.001$) in SU-reduced subjects (Table 3). In gliclazide subjects, the change in A1C was −0.71% (95% CI −1.03 to −0.39%) ($p < 0.001$) in SU-unreduced subjects, and −0.96% (95% CI −1.32 to −0.60%) ($p < 0.001$) in SU-reduced subjects (Table 3).

Hypoglycaemia

During the study no severe hypoglycaemia was documented. Mild hypoglycaemia was observed in three subjects (two glimepiride- and one gliclazide-treated subject) (Table 2). All subjects reported a sense of hunger that diminished when the dosage of glimepiride or glyclazide was reduced.

Table 2 Change in A1C and dosages of SUs and sitagliptin

Final subjects (*n*)	Dropout rate (%)	A1C level baseline	A1C level 52-week	Change in A1C (95% CI)	Original dosage of SUs (mg)	Initial dosage of SUs (mg)	Final dosage of SUs (mg)	Final dosage of sitagliptin (mg)	Hypoglycaemia (*n*)
All 70	14.6	7.96 ± 0.53	7.16 ± 0.53*	−0.80% * (−0.90, −0.68%)	–	–	–	69.0 ± 24.5	3
Glimepiride 39	13.8	8.00 ± 0.57	7.19 ± 0.58*	−0.81% * (−0.96, −0.62%)	1.75 ± 0.96	1.19 ± 0.51*	1.44 ± 0.90*	73.1 ± 25.4	2
Gliclazide 31	15.2	7.92 ± 0.48	7.13 ± 0.49*	−0.79% * (−0.90, −0.64%)	36.7 ± 18.4	30.0 ± 12.0***	34.5 ± 15.3	64.6 ± 23.2	1

*$p < 0.001$; ***$p < 0.05$.

Insulin secretion capacity

Insulin secretion capacity was measured by glucagon loading test, which was performed at baseline in the absence of any AHAs for 24 h and at 52-week in the absence of any AHAs for 72 h. At baseline, 0-min plasma glucose (PG) and CPR level were 7.6 ± 1.5 mM and 1.61 ± 0.59 ng/ml, respectively, and 6-min plasma glucose and C-peptide level were 8.7 ± 1.6 mM and 3.46 ± 1.41 ng/ml respectively (Table 4). At 52-week, 0-min PG and CPR level were 7.7 ± 1.1 mM and 1.71 ± 0.67 ng/ml, respectively, and 6-min PG and CPR level were 8.8 ± 1.1 mM and 3.45 ± 1.13 ng/ml respectively. These results indicate that insulin secretion capacity was not significantly increased, but was maintained for at least 52 weeks after 1-year administration of sitagliptin.

In the glimepiride group, 0-min PG and CPR level at baseline were 8.1 ± 1.6 mM and 1.67 ± 0.52 ng/ml, respectively, and 6-min PG and CPR level were 9.1 ± 1.6 mM and 3.48 ± 1.21 ng/ml respectively (Table 4). At 52-week, 0-min PG and CPR level were 7.9 ± 0.9 mM and 1.59 ± 0.79 ng/ml, respectively, and 6-min PG and CPR level were 8.9 ± 1.0 mg/dl and 3.12 ± 0.93 ng/ml respectively. In the gliclazide group, fasting PG and CPR level at baseline were 6.9 ± 1.3 mM and 1.61 ± 0.61 ng/ml, respectively, and 6-min PG and CPR level were 8.1 ± 1.6 mM and 3.48 ± 1.62 ng/ml respectively (Table 4). At 52-week, 0-min PG and CPR level were 7.6 ± 1.2 mM and 1.84 ± 0.70 ng/ml, respectively, and 6-min PG and CPR level were 8.7 ± 1.2 mM and 3.66 ± 1.23 ng/ml respectively. There was no statistical difference in insulin secretion capacity between the glimepiride group and the gliclazide group at baseline and 52-week. Insulin secretion capacity was preserved for 52 weeks in both the glimepiride and gliclazide groups.

CPI and SUIT index

CPI and SUIT index were evaluated as an indication of treatment outcome in all subjects and glimepiride and glilcazide subjects. If CPI or SUIT index is more than 1.2 or 50, respectively, the patient could be treated with AHAs or diet and exercise. If CPI or SUIT index is less than 0.8 or 20, respectively, patients usually require insulin therapy (14,15). CPI at baseline was 1.22 ± 0.47 in all subjects, 1.17 ± 0.45 in glimepiride subjects and 1.26 ± 0.50 in gliclizide subjects (Table 5). CPI at 52-week was 1.25 ± 0.53 in all subjects, 1.10 ± 0.40 in glimepiride subjects and 1.41 ± 0.61 in gliclizide subjects. SUIT index at baseline was 37.1 ± 18.2 in all subjects, 33.6 ± 16.9 in glimepiride subjects and 40.9 ± 19.0 in gliclizide subjects (Table 5). SUIT index at 52-week was 36.2 ± 17.7 in all subjects, 30.2 ± 11.3 in

Table 3 Change in A1C in dosage of SUs-reduced or -unreduced subjects

Un-reduced subjects(*n*)	Original and Initial dosage of SUs (mg)	Final dosage of SUs (mg)	Final dosage of sitagliptin (mg)	A1C level baseline	A1C level 52-week	Change in A1C (95% CI)
All 42			63.2 ± 22.6	7.98 ± 0.48	7.10 ± 0.55*	−0.80%* (−1.02, −0.59%)
Glimepiride 23	1.15 ± 0.55	1.36 ± 0.95	66.7 ± 24.6	8.05 ± 0.42	7.19 ± 0.60*	−0.88%* (−1.16, −0.59%)
Gliclazide 19	30.0 ± 10.3	35.0 ± 17.1	57.1 ± 18.9	7.91 ± 0.52	7.05 ± 0.53*	−0.71%* (−1.03, −0.39%)

Reduced subjects (*n*)	Original dosage of SUs(mg)	Initial dosage of SUs (mg)	Final dosage of SUs (mg)	Final dosage of sitagliptin (mg)	A1C level baseline	A1C level 52-week	Change in A1C (95% CI)
All 28				72.6 ± 25.4	7.94 ± 0.42	7.17 ± 0.56*	−0.81%* (−1.04, −0.57%)
Glimepiride 16	1.79 ± 0.97	1.10 ± 0.51*	1.53 ± 0.92*	78.6 ± 25.7	7.94 ± 0.43	7.23 ± 0.64*	−0.70%* (−0.98 −0.42%)
Gliclazide 12	55.0 ± 17.6	33.3 ± 10.3**	31.4 ± 10.7**	67.6 ± 24.6	7.93 ± 0.42	7.06 ± 0.43*	−0.96%* (−1.32, −0.60%)

*p < 0.001; **p < 0.01.

Table 4 Glucagon loading test

	Glucagon loading test							
	0-week				52-week			
	0-min		6-min		0-min		6-min	
Final subjects	PG (mm)	CPR (ng/ml)	PG (mm)	CPR (ng/ml)	PG (mm)	CPR (ng/ml)	PG (mm)	CPR (ng/ml)
All 70	7.6 ± 1.5	1.61 ± 0.59	8.7 ± 1.6	3.46 ± 1.41	7.7 ± 1.1	1.71 ± 0.67	8.8 ± 1.1	3.45 ± 1.13
Glimepiride 39	8.1 ± 1.6	1.67 ± 0.52	9.1 ± 1.6	3.48 ± 1.21	7.9 ± 0.9	1.59 ± 0.79	8.9 ± 1.0	3.12 ± 0.93
Gliclazide 31	6.9 ± 1.3	1.61 ± 0.61	8.1 ± 1.6	3.48 ± 1.62	7.6 ± 1.2	1.84 ± 0.70	8.7 ± 1.2	3.66 ± 1.23

glimepiride subjects and 42.2 ± 21.2 in gliclizide subjects. There were no significant differences in CPI and SUIT index between baseline and 52-week or among the three groups. This evidence indicates that combination therapy with any of the SUs and sitagliptin did not worsen the therapeutic selectivity in 52-week.

Correlation between efficacy of sitagliptin on glycaemic control and insulin secretion capacity, CPI, SUIT and BMI.

We then evaluated insulin secretion capacity, CPI, SUIT and BMI at baseline to predict the efficacy of combination therapy with sitagliptin and SUs on glycaemic control in all subjects. There was no correlation between 0-min C-peptide or 6-min C-peptide or delta C-peptide assessed by glucagon loading at baseline test and the efficacy of the combination therapy (Figure 2A, B, C). Other patient background, such as age and disease duration was not correlated with the efficacy of the combination therapy on glycaemic control (Data not shown). In addition, there was no relationship between CPI or SUIT index at baseline and change in A1C in 52-week (Figure 2D, E). BMI at baseline also did not correlate with the change in A1C in 52-week (Figure 2F). In the glimepiride and gliclazide groups, change in A1C in 52-week and insulin secretion capacity, CPI, SUIT index or BMI at baseline was not correlated (data not shown).

BMI, blood pressure and urinary albumin excretion changes

BMI in all subjects at baseline and 52-week were 24.1 ± 3.2 kg/m^2 and 23.7 ± 3.0 kg/m^2 respectively. Change in BMI in 52-week was −0.38 kg/m^2 (95% CI −0.72 to −0.04) ($p < 0.05$) (Table 6). BMI in glimepiride subjects at baseline and 52-week were 23.4 ± 3.0 kg/m^2 and 23.0 ± 2.5 kg/m^2 respectively. Change in BMI in 52-week was −0.43 kg/m^2 (95% CI −0.71 to −0.13) ($p < 0.01$) in glimepiride subjects. BMI in gliclazide subjects at baseline and 52-week were 24.6 ± 3.3 kg/m^2 and 24.4 ± 3.9 kg/m^2 respectively. Change in BMI in 52-week was −0.20 kg/m^2 (95% CI −0.51 to + 0.11) ($p > 0.05$).

Blood pressure at baseline was 129 ± 12.5/73.6 ± 9.6 mmHg in all subjects, 128 ± 12.1/73.2

Table 5 CPI and SUIT in the final, effective and ineffective subjects

Final subjects	CPI baseline	SUIT baseline	CPI 52-week	SUIT 52-week	Effective Subjects	CPI baseline	SUIT baseline	CPI 52-week	SUIT 52-week	Ineffective Subjects	CPI baseline	SUIT baseline	CPI 52-week	SUIT 52-week
All 70	1.22 ± 0.47	37.1 ± 18.2	1.25 ± 0.53	36.2 ± 17.7	All 58	1.23 ± 0.50	37.9 ± 19.5	1.27 ± 0.57	36.9 ± 20.0	All 12	1.12 ± 0.33	31.7 ± 9.8	1.14 ± 0.12	28.9 ± 9.4
Glimepiride 39	1.17 ± 0.45	33.6 ± 16.9	1.10 ± 0.40	30.2 ± 11.3	Glimepiride 33	1.20 ± 0.45	34.0 ± 17.3	1.12 ± 0.45	30.8 ± 12.3	Glimepiride 6	1.01 ± 0.29	31.4 ± 14.7	0.99 ± 0.13	26.9 ± 5.8
Gliclazide 31	1.26 ± 0.50	40.9 ± 19.0	1.41 ± 0.61	42.2 ± 21.2	Gliclazide 25	1.27 ± 0.53	43.0 ± 22.4	1.44 ± 0.73	44.5 ± 23.2	Gliclazide 6	1.22 ± 0.38	32.0 ± 4.8	1.28 ± 0.11	30.8 ± 12.9

± 8.5 mmHg in glimepiride subject and 132 ± 13.2/74.2 ± 11.3 mmHg gliclazide subjects. Blood pressure at 52-week was 122 ± 15.4/70.0 ± 8.8 mmHg in all subjects, 124 ± 10.4/68.9 ± 7.6 mmHg in glimepiride subjects and 124 ± 9.7/71.0 ± 10.3 mmHg in gliclazide subjects. Change in systolic and diastolic blood pressure was −6.7 mmHg (95% CI −10.0 to −3.4) ($p < 0.001$) and −3.6 mmHg (95% CI −4.8 to −2.4) ($p < 0.001$) in all subjects, respectively, −4.4 mmHg (95% CI −7.1 to −1.7) ($p < 0.05$) and −4.3 mmHg (95% CI −5.6 to −3.0 mmHg) ($p < 0.001$) in glimepiride subjects and −7.9 mmHg (95% CI −11.3 to −4.4 mmHg) ($p < 0.001$) and −3.2 mmHg (95% CI −4.7, −1.6 mmHg) ($p < 0.001$) in gliclazide subjects, respectively.

Urinary albumin excretion was decreased from 76.2 ± 95.6 to 33.0 ± 48.1 mg/gCr in all subjects: from 56.9 ± 62.0 to 23.9 ± 13.8 mg/gCr in glimepiride subjects and from 107 ± 129 to 47.4 ± 74.4 mg/gCr in gliclazide subjects. Change in urinary albumin excretion was −43.2 mg/gCr (95% CI −65.7 to −20.8) ($p < 0.001$) in all subjects, −33.0 mg/gCr (95% CI −50.5 to −15.6) ($p < 0.001$) in glimepiride subjects and −59.6 (95% CI −91.2 to −22.5) ($p < 0.01$) in gliclazide subjects respectively.

Ineffective and effective add-on treatment in 52-week

The A1C level of 12 of the 70 subjects was not improved more than 0.5% in 52-week. The ineffectiveness rate was 17.1%. A1C level at baseline and 52-week in ineffective subjects was 7.26 ± 0.43%, and 6.98 ± 0.39% respectively (Table 7). Change in A1C was −0.28% (95% CI −0.34 to −0.21) ($p < 0.001$). On the other hand, A1C level at baseline and 52-week in effective subjects was 7.51 ± 0.56% and 6.58 ± 0.45% respectively. The change in A1C was −0.93% (95% CI −1.06 to −0.81) ($p < 0.001$). The change in A1C between ineffective and effective subjects was significantly different ($p < 0.001$). Although CPI, SUIT index and 0-min CPR in ineffective subjects were relatively lower than those in effective subjects, the differences were not significant (Figure 3, Table 5). Six-minutes CPR and delta CPR evaluated by glucagon loading test also were not significantly different between the two groups of subjects (Data not shown).

Responsive and unresponsive subjects in 12-week

The combination therapy with SUs and sitagliptin was changed to liragrutide in 5 of 82 (6.1%) subjects in 12-week because of worsening in glycaemic control in 12-week. A1C level at baseline and 12-week in

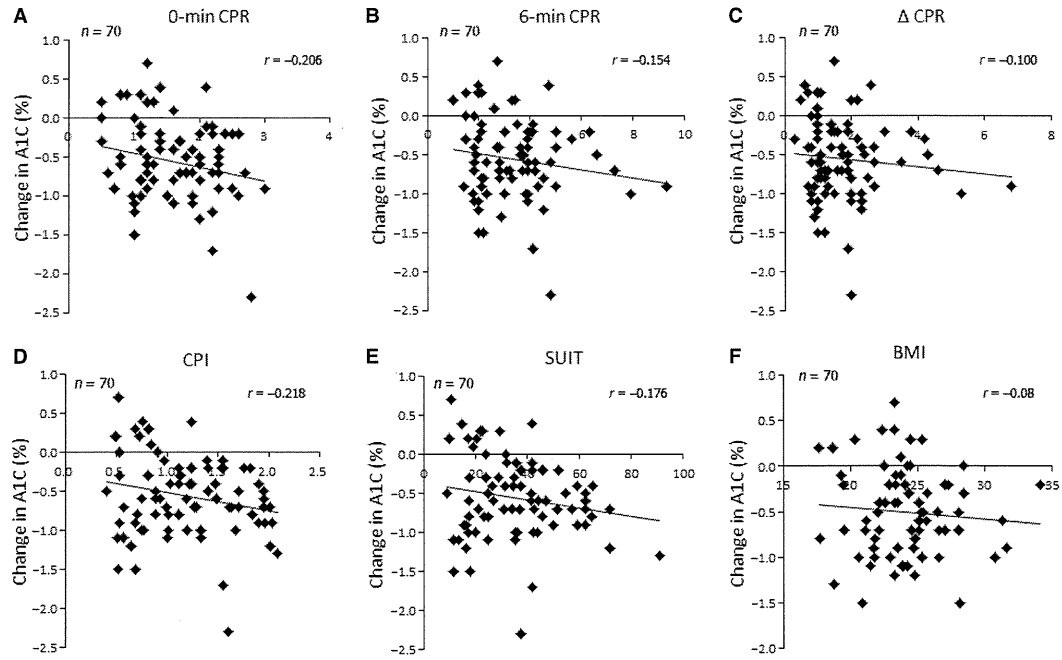

Figure 2 Relationship between change in A1C in 52-week and results of glucagon loading test, CPI, SUIT and BMI at baseline. Change in A1C in 52-week and 0-min CPR (A), 6-min CPR (B), delta CPR (C), CPI (D), SUIT index (E) and BMI (F) at baseline. CPR, C-peptide reaction; CPI, C-peptide index; SUIT, the secretory unit of islet in transplantation; BMI, body mass index

the unresponsive subjects was 7.43 ± 0.26% and 7.70 ± 0.55% respectively (Table 7). Change in A1C was + 0.27% (95% CI −0.05 to + 0.59) ($p > 0.05$). On the other hand, A1C level at baseline and 12-week in responsive subjects were 7.50 ± 0.54% and 6.66 ± 0.48% respectively. Change in A1C was −0.84% (95% CI −0.96 to −0.72) ($p < 0.001$). The change in A1C in unresponsive and responsive subjects was significantly different ($p < 0.001$). However, there was no difference between 0-min CPR and CPI at baseline in unresponsive and responsive subjects (Figure 3). SUIT index, 6-min CPR and delta-CPR at baseline also were similar in responsive and unresponsive subjects (data not shown).

Discussion

Sitagliptin was the first available DPP-4 inhibitor and is broadly administered at any step in the therapeutic algorithm in Japan. The compound is well tolerated and decreases A1C level by 1.05% compared with placebo in Japanese type 2 diabetes (4). Sitagliptin is effective for glycaemic control and is well tolerated in Caucasian type diabetes at least up to 2 years in duration (16,17). However, when combined with SUs, severe hypoglycaemia has occurred in Japanese patients with type 2 diabetes (8,9). The characteristics of these patients are aged, with mild renal dysfunction and treatment with a relatively higher dosage of SUs. In the clinical trials for combination therapy with sitagliptin and glimepiride (1–6 mg/day), such severe hypoglycaemia was never observed (5). The average dosage of glimepiride was about 2.5 mg/day in both the sitagliptin add-on group and placebo (glimepiride alone) group, and the dosage was maintained during the entire 52-week period. The average age was 61.0 years in placebo group and 60.5 years old in sitagliptin group, and subjects with moderate renal dysfunction (serum creatinine > 1.5 mg/dl in men or > 1.3 mg/dl in women) were excluded. The incidence of hypoglycaemia in 12-week was 5.6% and 0.0% in sitagliptin group and placebo group respectively. All hypoglycaemia events were considered either mild or moderate; severe hypoglycaemia was unexpected and was not observed. Japanese type 2 diabetes is characterised mainly by impaired insulin secretion, so that an insulin secretagogue is most commonly used (2). Thus, determination of the adequate dosage of SUs in combination of DPP-4 inhibitors that ensures safety and efficacy is extremely important in Japan. We find here that less than or equal to 40 mg/day of glyclazide and 2 mg/day of glimepride in combination with sitagliptin is sufficient for glycaemic control and does not cause severe hypoglycaemia.

Table 6 Changes in BMI, blood pressure and urinary albumin excretion

Final subjects	BMI Baseline (kg/m^2)	BMI 52-week (kg/m^2)	Change in BMI (kg/m^2) (95% CI)	Blood pressure Baseline (mmHg)	Blood pressure 52-week (mmHg)	Change in blood pressure Systolic/diastolic (mmHg)(95% CI)	Urinary albumin excretion Baseline (mg/gCr)	Urinary albumin excretion 52-week (mg/gCr)	Change in Urinary albumin excretion (mg/gCr)
All 70	24.1 ± 3.2	23.7 ± 3.0	−038*** (−0.72, −0.04)	129 ± 12.5/ 73.6 ± 9.6	122 ± 15.4*/ 70.0 ± 8.8*	−6.7* (−10.0, −3.4) −3.6* (−4.8, −2.4)	76.2 ± 95.6	33. 0 ± 48.1**	−43.2* (−65.7, −20.8)
Glimepiride 39	23.4 ± 3.0	23.0 ± 2.5	−0.43** (−0.71, −0.13)	128 ± 12.1/ 73.2 ± 8.5	124 ± 10.4***/ 68.9 ± 7.6**	−4.4*** (−7.1, −1.7) −4.3* (−5.6, −3.0)	56.9 ± 62.0	23. 9 ± 13.8**	−33.0* (−50.5, −15.6)
Gliclazide 31	24.6 ± 3.3	24.4 ± 3.9	−0.20 (−0.51, 0.11)	132 ± 13.2/ 74.2 ± 11.3	124 ± 9.7**/ 71.0 ± 10.3	−7.9* (−11.3, −4.4) −3.2* (−4.7. −1.6)	107 ± 129	41.4 ± 54.4***	−59.6** (−91.2, −22.5)

*p < 0.001; **p < 0.01; ***p < 0.05.

Table 7 Change in A1C in ineffective and effective subjects

Subjects	A1C Baseline	A1C 52-week	Change in A1C (95% CI)
Ineffective 12	7.26 ± 0.43	6.98 ± 0.39*	−0.28%* (−0.34, −0.21%)
Effective 58	7.51 ± 0.56	6.58 ± 0.45*	−0.93%* (−1.06, −0.81%)
Unresponsive 5	7.43 ± 0.26	7.70 ± 0.55	0.27% (−0.05, 0.59%)
Responsive 77	7.50 ± 0.54	6.66 ± 0.48*	−0.84%* (−0.96, −0.72%)

*p < 0.001.

In the present study, the A1C level in all subjects was decreased from 7.90 ± 0.53% to 7.10 ± 0.53% in 52-week. Change in A1C in 52-week was −0.80% (95% CI −0.90 to −0.68) (p < 0.001). Dosages of glimepiride and gliclazide were decreased from 1.75 ± 0.96 mg/day to 1.19 ± 0.96 mg/day and from 35.3 ± 18.4 mg/day to 30.0 ± 12.0 mg/day, respectively, at the initiation of sitagliptin. In addition, the final dosages of glimepiride and gliclazide were 1.44 ± 0.90 mg/day and 34.5 ± 15.3 mg/day, respectively, which were not significantly increased during the study. The average final dosage of sitagliptinn was 73.1 ± 25.4 mg/day in the glimepiride group and 64.6 ± 23.2 mg/day in the gliclazide group. These results show that when combined with sitagliptin, low dosages of SUs are sufficient for glycaemic control in Japanese type 2 diabetes. On the other hand, in Caucasian type 2 diabetes, change in A1C in 24-week has been found to be −0.74% (95% CI −0.90 to −0.57) by treatment with 100 mg/day sitagliptin and equivalent to 4 mg/day or more glimepiride (6). Thus, Japanese type 2 diabetes requires a lower dosage of sitagliptin and SUs for glycaemic control than Caucasian type 2 diabetes. In addition, the change in A1C in 52-week did not differ between dosages of SUs-reduced and -unreduced subjects: −0.88% (95% CI −1.16 to −0.59) in glimepiride-unreduced subjects and −0.70% (95% CI −0.98 to −0.42) in glimepiride-reduced subjects and −0.71% (95% CI −1.03 to −0.39) in gliclazide-unreduced subjects and −0.96% (95% CI −1.32 to −0.60) in gliclazide-reduced subjects. The observation that the efficacy was similar in SU-reduced and -unreduced subjects is consistent with prior observations that maximal SU efficacy is typically attained at doses that are less than the maximum prescribed dose (18). The average dosage of SUs also did not differ in both glimepiride and gliclazide subjects: the dosage of

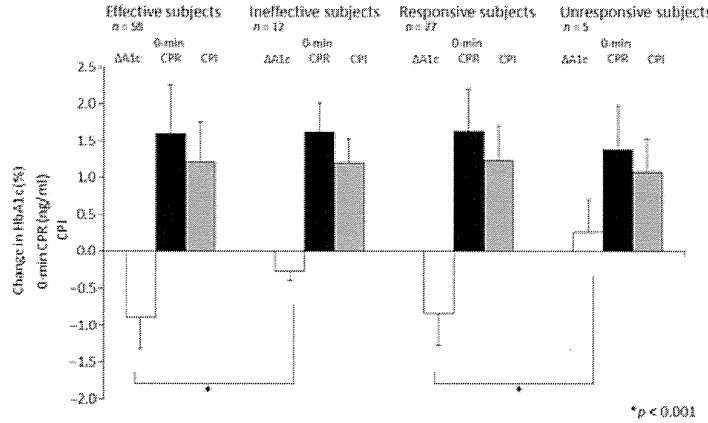

Figure 3 Differences in change in A1C in 52-week, 0-min CPR and CPI in effective and ineffective subjects in 52-week or responsive and unresponsive subjects in 12-week. CPR, C-peptide reaction; CPI, C-peptide index **p < 0.001

glimepiride was 1.53 ± 0.92 mg/day in reduced subjects and 1.36 ± 0.95 mg/day in unreduced subjects; the dosage of gliclazide was 31.4 ± 10.7 mg/day in reduced subjects and 35.0 ± 17.1 mg/day in unreduced subjects. This result indicates that the dosage of SUs can be reduced to less than 2 mg/day of glimepiride and 40 mg/day of gliclazide at initiation of combination therapy with sitagliptin.

The ineffective rate of the combination therapy with SUs and sitagliptin in 52-week was 17.1% in this study. CPI and SUIT index in ineffective subjects were relatively lower than those in effective subjects, but there was no significant difference between the two groups. About 40% of the ineffective subjects achieved a reduction of 0.5% or greater during the course of the study. Diet failure was a cause of an increase in A1C in those cases, further suggesting that sitagliptin might be effective for glycaemic control in these patients. The causes of ineffectiveness of sitagliptin in the remaining ineffective subjects remain unknown. However, the baseline A1C in the ineffective subjects was lower than that in the effective subject group (7.26% vs. 7.51%). Thus, the designation of ineffective for those subjects may simply reflect the lower baseline value and not a lack of responsiveness to sitagliptin. In addition, sulphonylurea efficacy diminishes over time, an effect called secondary failure of sulphonylureas. Thus, the failure to achieve a reduction of 0.5% at 52 weeks might also reflect a reduction in the contribution of the sulphonylurea rather than unresponsiveness to sitagliptin.

Hypoglycaemia was observed in only three cases: two in the glimepiride group and one in the gliclazide group. All patients had a sense of hunger that diminished when the dosage of the SUs was reduced. Therefore, at the beginning of therapy with sitagliptin with SUs, it is safe for patients, especially those who have had experiences of hypoglycaemia, to receive a smaller dosage of SUs.

In the present study, we examined whether or not insulin secretion capacity predicted the efficacy of the combination therapy on glycaemic control. Insulin secretion capacity evaluated by glucagon loading test did not predict the efficacy of sitagliptin, although a tendency to correlation was observed in 0-min CPR and CPI (Figure 2). There were also no differences in insulin secretion capacity, CPI and SUIT index between ineffective and effective subjects or unresponsive and responsive subjects (Figure 3). Thus, insulin secretion capacity does not predict the efficacy of combination therapy with sitagliptin and SUs. Recently, Kim et al. reported that sitagliptin responders had lower body mass index and were younger compared with non-responders in Korean type 2 diabetes (19). Nomiyama et al. reported that treatment with sitagliptin 50 mg/day for 24 weeks was especially effective in patients with higher baseline A1C, lower BMI and duration of diabetes (20). However, in our study, BMI did not correlate with the efficacy of combination therapy. Other patient background, such as age, disease duration, and gender, also did not predict the efficacy of the combination therapy in Japanese type 2 diabetes (data not shown). Thus, no suggestive marker to predict the efficacy of sitagliptin with SUs on glycaemic control has been identified.

BMI was decreased only in glimepiride subjects and not in gliclazide-treated subjects. The dosage of glimepiride was decreased from 1.75 ± 0.96 mg/day to 1.19 ± 0.51 mg/day at baseline, and was not significantly increased during the study. However, the dosage of gliclazide was not significantly decreased during the study. In the clinical trial of the combination therapy with sitagliptin and glimepiride, body weight was significantly increased by 0.50 kg in the

sitagliptin and glimepiride groups compared with that by 0.03 kg in the glimepiride alone group (6). However, the average dose of SUs was higher (2.46 mg) compared with that in our study (1.19 mg). The decrease in dosage of SUs might thus be a cause of the BMI reduction in glimepiride subjects.

Blood pressure and urinary albumin excretion in 52-week also were significantly decreased compared with those at baseline without any change of other medications including statin and angiotensin receptor blocker. Systolic and diastolic blood pressures were decreased by 6.7 mmHg (95% CI −10.0 to −3.4) and 3.6 mmHg (95% CI −4.8 to −3.4) respectively. Urinary albumin excretion was reduced by 43.2 mg/gCr (95% CI −65.7 to −20.8). Ogawa et al. reported that systolic blood pressure was dropped from 130.0 ± 37.2 to 119.7 ± 9.4 mmHg by the treatment of sitagliptin in Japanese hypertensive patients with type 2 diabetes (21). Another study reported that systolic blood pressure was decreased from 133.0 ± 0.9 to 130.0 ± 0.7 mmHg, and diastolic blood pressure from 75.2 ± 0.7 to 73.4 ± 0.7 mmHg (19). Hattori reported that urinary albumin excretion was decreased from 11.6 ± 8.4 to 4.5 ± 5.0 mg/gCr in 6-month (22). GLP-1 enhances sodium excretion and reduces hyperfiltration in obese men (23). Liraglutide, a GLP-1 receptor agonist, decreased systolic blood pressure (24,25). Exenatide, another GLP-1 receptor agonist, also reduced systolic blood pressure (26). For these reasons, the increased active GLP-1 level by sitagliptin might have decreased the blood pressure level and urinary albumin excretion in our study.

Insulin secretion capacity evaluated by glucagon loading test at 52-week was not significantly changed compared with that at baseline. However, all AHAs including sitagliptin were unmedicated for 72 h before the glucagon loading test at 52-week. Under this condition, 0-min C-peptide, 6-min C-peptide, CPI and SUIT index at 52-week were almost the same as those at baseline. These results indicate that insulin secretion capacity was preserved at least for 1 year by the treatment with SUs and sitagliptin. For vildaglipitin, another DPP-4 inhibitor, it has been reported that beta-cell function was preserved by 2-year treatment under condition of 4-week washout (12). Sitagliptin also may improve beta cell function with longer term usage. Therefore, a glucagon loading test is scheduled 2 years after the initiation of combination therapy with sitagliptin and low dosage of SUs.

In summary, combination therapy with sitagliptin and low dosage of SUs is effective for glycaemic control in Japanese type 2 diabetes. The combination therapy is weight neutral, and lowered both the blood pressure level and urinary albumin excretion. As a result of this decrease in the dosage of SUs, hypoglycaemia seldom occurs, and there is no severe hypoglycaemia. When sitagliptin is added on to SUs for the first time, the initial dosage of glimepiride or gliclazide should be less than 2 mg/day and 40 mg/day, respectively, according to the committee's recommendation regarding use of incretin-based therapy.

Author contributions

The study conception and protocol were by Shin-ichi Harashima and Nobuya Inagaki. Patient examinations were by Shin-ichi Harashima, Toru Fukushima, Tadashi Koizumi and Mitsuru Aono. Collection of data was by Yu Wang, Yuko Murata and Mika Seike. The statistical analysis was by Daisuke Tanaka, Shunsuke Yamane and Masahito Ogura. The manuscript development was by Shin-ichi Harashima and Nobuya Inagaki. All authors reviewed and approved the final version of the manuscript.

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Clinical Study

Fat Restriction Is Associated with Impaired Quality of Life in Patients with Ulcerative Colitis and Crohn's Disease

A. Kuwabara,[1,2] H. Nakase,[3] H. Tsuji,[4] K. Shide,[4] T. Chiba,[3] N. Inagaki,[4] and K. Tanaka[2]

[1] *Department of Health and Nutrition, Osaka Shoin Women's University, 4-2-26 Hishiyanishi, Higashiosaka-shi, Osaka 577-8550, Japan*
[2] *Department of Food and Nutrition, Kyoto Women's University, 35 Imakumano-kitahiyoshi-cho, Higashiyama-ku, Kyoto 605-8501, Japan*
[3] *Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin kawahara-cho, Sakyo-ku Kyoto, 606-8507, Japan*
[4] *Department of Diabetes and Clinical Nutrition, Kyoto University Graduate School of Medicine, 54 Shogoin kawahara-cho, Sakyo-ku Kyoto, 606-8507, Japan*

Correspondence should be addressed to K. Tanaka, ktanaka@zeus.eonet.ne.jp

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Inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease, is reported to be associated with impaired health-related quality of life (QOL). Although decreased QOL in these subjects has been reported to be associated with various factors, the effect of nutritional therapy, especially nutrients intake on QOL has received less attention. In this study, we evaluated the various factors including nutrients intake on QOL using SF-8 in 64 patients with IBD. Patients with IBD seem to have decreased QOL especially in the mental aspects. The percentage energy intake from fat of total energy fat intake (% energy) of the whole subjects, was lower than those of the annual National Nutrition Survey in Japan. Multiple regression analyses revealed that fat intake (% energy) was a significant predictor for mental component summary. In conclusion, fat restriction contributes to impaired QOL especially in the mental aspects in IBD patients.

1. Introduction

Inflammatory bowel disease (IBD); ulcerative colitis (UC) and Crohn's disease, is reported to be associated with impaired health-related quality of life (HR-QOL). In this paper, HR-QOL will be simply designated as QOL. Decreased QOL in these subjects has been reported to be related to various factors such as age, gender [1, 2], treatment effects [3], disease activity, and social environment [4]. However, the effect of nutritional therapy on the QOL of IBD patients has received less attention, most of which is devoted to the parenteral nutrition therapy, not the nutritional therapy in general [5, 6].

Since excessive fat intake is considered to worsen the inflammation in the intestine, its restriction has traditionally been employed in Japan as the oral nutritional therapy for IBD patients, especially for those with CD, which, however, has its own pros and cons.

Recently, we have studied the possible involvement of hypovitaminosis D and K in the development of osteoporosis in IBD patients [7]. In face of apparently sufficient intake of these vitamins, their plasma levels were quite low in these patients. Paradoxically, plasma concentrations of vitamin D and K were correlated with the fat intake but not with their intake of these vitamins. These results were more prominent in patients with CD than those with UC. Then it was concluded that fat-soluble substances such as vitamin D and K were not effectively absorbed from the intestine without concomitant intake of enough fat.

Through this paper, we were interested in what fat restriction means from the patients' perspectives and studied

the effect of fat restriction on the QOL of IBD subjects in this paper.

2. Subjects and Methods

2.1. Subjects. Study subjects were 64 patients with IBD attending the gastroenterology clinic at the Kyoto University Hospital; 33 with CD (19 men/14 women) and 31 with UC (20 men/11 women). Detailed information was given and written consent was obtained. The study protocol was approved by the ethical committee of the Kyoto Women's University. Almost all patients (27/33 in CD and 28/33 in UC) were receiving 5-aminosalicylic acid. Glucocorticoid therapy was given to four and two patients with CD and UC, respectively. Immunosuppressive drug therapy was performed in 25 and 4 patients with CD and UC, respectively. Eight patients with CD, but none with UC, were on combined therapy of infliximab, synthetic glucocorticoid, and immunosuppressive drug. Fifteen patients with CD and one with UC were on enteral or total parenteral nutrition therapy, respectively.

2.2. Methods

2.2.1. Dietary Information. Dietary information was obtained from food intake records in 2 weekdays by the patients. By calculating these records, their energy and nutrients intakes were obtained by computer software program (Healthy Maker Pro 501, Mushroom soft Corp.).

2.2.2. QOL Measurement. QOL was assessed using the Japanese Short Form Health Survey (SF-8), a widely used generic questionnaire [8]. Eight subscales are obtained; physical function (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social function (SF), role emotional (RE), and mental health (MH). RP and RE refer to the limitations due to physical or emotional reasons, respectively. They are also summarized into two summary scores: physical component summary (PCS) and mental component summary (MCS). Data are transformed to deviation scores based on Japanese norms [8]. Higher scores indicate better QOL, with 50 corresponding to the national norms.

2.2.3. Statistical Analyses. Statistical analyses were performed using SPSS 17.0J for Windows (SPSS. Japan Inc., Tokyo, Japan). Comparison of data from IBD patients with Japanese norms was done by one-sample t test. The difference between two independent groups was analyzed by unpaired t test or Mann-Whitney test depending on normality. Correlations between two independent variables were analyzed by Pearson's or Spearman's correlations. Multiple regression analysis was performed to determine independent factors for QOL scores in IBD patients.

3. Result

3.1. Background Profiles and Biochemical Indices. The baseline characteristics of the patients are shown in Table 1.

Table 1: Background profiles and results from blood tests in patients with CD and UC.

	CD	UC	P value
Age (y)	35.6 ± 7.3	41.7 ± 17.3	.343[a]
Sex (F/M)	19/14	20/11	—
Disease duration (y)	13.7 ± 7.4	6.8 ± 4.8	<.001[b]
Body mass index (kg/m^2)	19.5 ± 2.3	21.1 ± 3.3	.025[b]
Disease location (involving small bowel/not involving small bowel)	30/2	0/31	—
Glucocorticoid therpy	4	2	—
Immunosuppressive therapy	25	4	—
Immunopotentiating therapy (TNF-α)	8	0	—
Enteral or total parenteral nutrition therapy	15	1	—
C-reactive protein (g/dl)	0.6 ± 1.0	0.3 ± 0.6	.135[b]
Albumin (g/dl)	3.9 ± 0.4	4.3 ± 0.3	<.001[b]
Total cholesterol (mg/dl)	126.9 ± 25.0	177.1 ± 40.3	<.001[b]

Values represent mean ± SD. Comparison of indices between patients with CD and those with UC was done by unpaired t test[a] or Mann-Whitney test[b] depending on normality.

CD patients had significantly longer disease duration and lower BMI than UC patients. While nutritional indices such as serum albumin and total cholesterol were lower in CD subjects, there was no significant difference in C-reactive protein which is an inflammatory parameter between these groups. Most of patients were in remission.

3.2. Energy and Nutrients Intake in CD and UC Patients. Food intake could be evaluated in 62 patients (31 with CD and 31 with UC). Energy and nutrients intake in these patients is shown in Table 2. Fourteen patients with CD were on enteral nutrition, and each one of subjects with CD and UC was on total parental nutrition. Although the energy intake was not significantly different between the two groups, fat intake was significantly lower in CD patients than UC subjects. The annual National Nutrition Survey in Japan (NNS-J) in 2008 showed that in subjects of 30–39 or 40–49, years of age including both genders [9], the daily fat intake (% energy) was 26.5% or 25.6%, respectively. These were significantly higher than those of IBD subjects in this study (P = .001; data not shown). Subjects with enteral or parental nutrition had fat intake only approximately half of that in subjects with oral intake (data not shown). The percentage energy intake from protein, fat, and carbohydrates was significantly different between CD and UC subjects.

Table 2: Comparison of nutrient intakes in CD and UC patients.

		IBD (n = 62)	CD (n = 31)	UC (n = 31)	P value
Energy	Intake (kcal)	1816 ± 465 (1804)	1847 ± 392 (1842)	1785 ± 533 (1764)	NS
Protein	Intake (g)	66.0 ± 21.8 (63.5)	71.0 ± 20.6 (67.2)	60.9 ± 22.0 (61.6)	NS
Fat	Intake (g)	44.7 ± 21.6 (43.0)	38.7 ± 17.6 (37.4)	50.6 ± 23.6 (48.1)	$P < .05$
Carbohydrates	Intake (g)	275.4 ± 91.6 (268.6)	298.3 ± 93.1 (275.7)	252.4 ± 85.4 (254.9)	$P < .05$
Protein (% energy)		14.4 ± 2.7 (14.2)	15.0 ± 2.2 (15.6)	13.5 ± 2.9 (13.6)	$P < .001$
Fat (% energy)		22.4 ± 9.6 (24.6)	19.5 ± 8.9 (18.9)	25.2 ± 9.5 (26.8)	$P < .001$
Carbohydrates (% energy)		63.2 ± 9.6 (62.4)	65.2 ± 8.6 (64.0)	56.5±9.5 (60.5)	$P < .001$

Data are expressed as mean ± SD with the values in parentheses showing the median. Comparison of indices between patients with CD and those with UC was done by unpaired t test

Table 3: Dimensional SF-8 scores in patients with CD and UC.

	IBD (n = 64)	CD (n = 33)	UC (n = 31)
PF	50.1 ± 4.7 (53.6)	50.1 ± 4.5 (53.6)	50.0 ± 5.0 (53.6)
RP	*48.2 ± 6.8 (48.5)	48.7 ± 5.3 (48.5)	47.7 ± 8.1 (48.5)
BP	50.8 ± 7.6 (51.8)	50.5 ± 6.8 (51.8)	51.2 ± 8.5 (51.8)
GH	*47.8 ± 7.5 (50.7)	*47.7 ± 6.5 (50.7)	47.8 ± 8.5 (50.7)
VT	49.6 ± 6.5 (54.5)	48.4 ± 5.7 (45.3)	51.0 ± 7.1 (54.5)
SF	**46.2 ± 8.3 (45.2)	*46.9 ± 7.2 (45.2)	*45.5 ± 9.4 (45.2)
RE	*48.3 ± 6.4 (49.1)	48.0 ± 6.5 (49.1)	48.6 ± 6.5 (49.1)
MH	**47.3 ± 6.5 (45.0)	*46.8 ± 7.5 (45.0)	*47.8 ± 5.4 (50.3)
PCS	49.0 ± 6.7 (49.1)	49.2 ± 5.4 (49.0)	48.9 ± 7.9 (50.0)
MCS	***46.1 ± 6.6 (46.5)	**45.7 ± 7.1 (46.6)	**46.6 ± 6.0 (46.5)

Data are expressed as mean ±SD with median in the parentheses. One-sample t test was used for comparison between Japanese norms and scores of CD or UC patients. The asterisk denotes the significant difference (*$P >$ .05; **$P >$ .01; ***$P >$ .001).

3.3. QOL Assessment. In Table 3 is shown the eight subscales and two summary scores of SF-8 in subjects with IBD patients. Since data are expressed as the deviation values normalized by the Japanese normative values, the value "50" correponds to Japanese norm. Subscales such as RP, GH, SF, MH, and MCS were significantly lower than the Japanese norms.

Table 3 shows the comparison between CD and UC subjects. There were no significant differences in the eight subscales and two summary scores except for lower VT in CD patients than in those with UC.

3.4. Correlations between PCS/MCS Scores and Clinical Characteristics, Biochemical Markers, and Nutritients Intakes. We analyzed the correlation between these summary scores and biochemical indices, fat intake expressed as the percentage energy intake from fat of total energy, fat intake (% energy) (Table 4). Fat intake (% energy) was significantly correlated with MCS in CD patients. There was significant but weak, correlation between PCS and serum albumin and MCS and BMI in UC patients. In the whole subjects, BMI was significantly correlated with PCS, and fat intake (% energy) was associated with MCS.

3.5. Multiple Regression Analysis for Variable Associated with PCS/MCS Scores. Then multiple regression analyses were done to study the determinant(s) of the subjects' PCS and MCS (Table 5). Variables included in the analysis were types of disease (CD/UC), BMI, serum concentrations of Alb, and fat intake (% energy). BMI was the significant predictor of PCS score (βcoefficient 0.29, P = .023) whereas fat intake was the only significant determinant of MCS score (βcoefficient 0.29, P = .027).

4. Discussion

Recently, various questionnaires have been developed for QOL evaluation, both generic and disease targeted [10]. Generic ones, by their definition, only consist of questions related to the subjects' general status and do not include the questions related to the features which are specific to a certain disease. Therefore, they are applicable to such studies as comparing the impact on QOL by various diseases or even to the evaluation of healthy subjects. In contrast, disease-targeted ones include items specific to a certain disease. They can be more sensitive than the generic ones in detecting the QOL impairment closely related to a certain disease state but are not applicable to the evaluation of patients with other diseases. Various disease-targeted questionnaires have been developed for IBD subjects; the most well known of which would be IBDQ (inflammatory bowel disease questionnaire) including many items related to the patients' gastroenterological problems [11]. Since the purpose of our current work was to study the effects of nutritional therapy on the patients' QOL, we considered it more appropriate to evaluate the patients' QOL using the generic questionnaire.

SF-36 is one of the most commonly used generic questionnaires, and SF-8, used in this study, is the shortened one. Eight subscales, two summary scores are obtained, and expressed as the deviation values, which are normalized by the nations' normative value. Many previous papers on the QOL of IBD patients using SF-36 seem to have handled the data improperly [2, 4]. For example, Bernklev and Andersson expressed their data as the 0–100 scale scores [2, 4], which

Table 4: Correlations between PCS/MCS scale scores and clinical characteristics, biochemical markers, and fat intake as proportion of total energy intake.

		IBD ($n = 64$)		CD ($n = 33$)		UC ($n = 31$)	
		PCS	MCS	PCS	MCS	PCS	MCS
Diseaae duration (y)	r	0.012	−0.175	0.070	−0.221	−0.085	−0.066
Body mass index (kg/m^2)	r	0.261*	0.088	0.144	−0.075	0.248	0.415*
C-reactive protein (g/dl)	r	−0.083	0.075	−0.058	0.196	−0.116	−0.045
Albumin (g/dl)	r	0.235	0.082	0.092	0.064	0.424*	0.059
Total cholesterol (mg/dl)	r	0.033	0.196	−0.132	0.169	0.174	0.249
Fat intake(% energy)	r	0.175	0.287*	0.146	0.458***	0.238	0.109

The asterisk denotes the value is significant correlation (*$P < .05$, **$P < .01$, ***$P < .001$) by Pearson's correlation or Spearman's correlation.

Table 5: Multiple regression analyses for the predictor(s) of PCS and MCS scores in IBD patients.

	PCS score		MCS score	
	$r^2 = 0.086$	$P = .023$	$r^2 = 0.081$	$P = .027$
	β	P	β	P
CD/UC (1;CD, 2;UC)	−0.141	.283	−0.059	.657
BMI	0.293	.023	0.069	.594
Alb	0.141	.309	0.024	.855
Fat intake (% total energy)	0.121	.347	0.285	.027

Abbreviations are as follow: β for β coefficient and P for P value. Determinants of independent predictors for PCS/MCS scores were analyzed by multivariate analysis with stepwise method. Variables included were CD/UC, BMI, serum albumin concentration, and fat intake (% total energy)

can be misleading [12]. In the present paper, data were analyzed according to the authorized instruction.

In this study, subscales such as RP, GH, SF, RE, MH, and MCS were significantly lower than the Japanese norms. Decreased RP in face of normal PF is conceivable considering that the patients do not have severe physical impairment but have some limitation in their daily activities by reasons such as the bowel habit problem. Impaired SF would be also conceivable from the similar viewpoint. As a whole, patients with IBD seem to have decreased QOL especially in the mental aspects.

Then, we have analyzed variables associated with PCS and MCS. There were substantial differences in the objective clinical features of patients with CD and UC. For example, CD patients had longer disease duration and lower nutritional status than those of UC subjects. Nevertheless, there were no significant differences in 7 out of 8 dimensions between the two conditions. Namely, QOL which represents the patients' subjective evaluation of their health states seems to be impaired in both CD and UC patients.

Then, we have studied the determinants for PCS and MCS. PCS score was correlated with indices representing their nutritional status such as BMI ($r = 0.261$, $P < .05$) and albumin with marginal significance ($r = 0.235$, $P = .066$). In contrast, none of these factors were significantly correlated with MCS. Thus, it was considered unlikely that disease activities or other clinical features alone could account for the impaired mental aspects of QOL in these subjects. The association of QOL with mental aspects of the subjects has been previously reported. Boye et al. reported that neuroticism was a significant predictor for mental and vitality subscales of SF-36 in IBD patients using multiple regression analyses controlled for gender, age, and clinical disease activity [13]. Martin also reported that QOL was not closely correlated with the clinical features in CD patients [14]. These results, together with our current findings, suggest that mental aspects can more strongly affect QOL than clinical ones in IBD patients.

Theoretically, it is well known that the QOL scores in subjects with disabilities are higher than those anticipated from their objective physical impairment (disability paradox) [15]. This phenomenon is because subjects with long-term disabilities change their internal standard and make the adaptation to their actual status (response shift) [16].

Next, we have made a hypothesis that nutrients intake such as fat restriction may contribute to the impairment of mental aspects of QOL in these subjects. Although CD patients had lower fat intake than UC subjects, fat intake (% energy) of the whole subjects was significantly lower than those of the NNS-J.

Then, we have analyzed the association between these summary scores and their fat intake (% energy). Fat intake (% energy) was significantly associated with MCS, but not with PCS in patients with IBD. When CD and UC patients were separately analyzed, the correlation coefficients was almost the same, but not statistically significant anymore, probably due to the smaller number of study subjects. We then have performed the multivariate analysis. Of the various factors included for analysis types of disease (CD/UC), BMI, serum albumin, fat intake (% energy), BMI, and fat intake (% energy) were the only significant determinants of PCS and MCS, respectively. Since many IBD patients are young, they are quite likely to favor foods rich in fat. Nevertheless, fat

restriction is the common practice in the nutritional therapy for IBD. It is quite conceivable that fat restriction impairs the mental and social aspects of QOL, and enteral nutrition will make the matter even worse. Of interest, but not apparently compatible with our findings, is the report by Kuriyama et al. They reported that enteral nutrition improved the health-related quality of life of Crohn's disease patients with long-term disease duration, and enteral nutrition was an independent factor for bowel symptoms and systemic symptoms [17]. In their study, IBDQ was employed for the assessment of QOL, which is an IBD-targeted questionnaire with many items related to the patients' gastroenterological problems. Thus it is likely that only the physical aspects of QOL were detected, and mental aspects were overlooked in their study.

Two additional considerations might be added to the current finding: decreased QOL in IBD patients and its association with fat restriction. First, considering the response shift, actual detrimental effect of fat restriction on the mental aspects of QOL might be even greater. Second, the adaptation process seems to be only partial. Chronic pain is known to be associated with response shift [18]. However, the association of fat restriction with impaired mental aspects of QOL was obvious in the current study. Since food intake is one of the most fundamental requirements, it is likely that subjects with fat restriction cannot easily adapt to a situation with long-term fat-restricted diet.

In conclusion, fat restriction exerts undesirable effects on IBD patients in two different ways: decreased intestinal absorption of fat-soluble substances such as vitamin D and K and impaired QOL especially in the mental aspects.

Conflict of interests

None of the authors have any conflict of interests.

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ORIGINAL ARTICLE

Exendin-4 Suppresses Src Activation and Reactive Oxygen Species Production in Diabetic Goto-Kakizaki Rat Islets in an Epac-Dependent Manner

Eri Mukai,[1,2] Shimpei Fujimoto,[1] Hiroki Sato,[1] Chitose Oneyama,[3] Rieko Kominato,[1] Yuichi Sato,[1] Mayumi Sasaki,[1] Yuichi Nishi,[1] Masato Okada,[3] and Nobuya Inagaki[1,4]

OBJECTIVE—Reactive oxygen species (ROS) is one of most important factors in impaired metabolism secretion coupling in pancreatic β-cells. We recently reported that elevated ROS production and impaired ATP production at high glucose in diabetic Goto-Kakizaki (GK) rat islets are effectively ameliorated by Src inhibition, suggesting that Src activity is upregulated. In the present study, we investigated whether the glucagon-like peptide-1 signal regulates Src activity and ameliorates endogenous ROS production and ATP production in GK islets using exendin-4.

RESEARCH DESIGN AND METHODS—Isolated islets from GK and control Wistar rats were used for immunoblotting analyses and measurements of ROS production and ATP content. Src activity was examined by immunoprecipitation of islet lysates followed by immunoblotting. ROS production was measured with a fluorescent probe using dispersed islet cells.

RESULTS—Exendin-4 significantly decreased phosphorylation of Src Tyr416, which indicates Src activation, in GK islets under 16.7 mmol/l glucose exposure. Glucose-induced ROS production (16.7 mmol/l) in GK islet cells was significantly decreased by coexposure of exendin-4 as well as PP2, a Src inhibitor. The Src kinase–negative mutant expression in GK islets significantly decreased ROS production induced by high glucose. Exendin-4, as well as PP2, significantly increased impaired ATP elevation by high glucose in GK islets. The decrease in ROS production by exendin-4 was not affected by H-89, a PKA inhibitor, and an Epac-specific cAMP analog (8CPT-2Me-cAMP) significantly decreased Src Tyr416 phosphorylation and ROS production.

CONCLUSIONS—Exendin-4 decreases endogenous ROS production and increases ATP production in diabetic GK rat islets through suppression of Src activation, dependently on Epac. ***Diabetes*** **60:218–226, 2011**

From the [1]Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto, University, Kyoto, Japan; the [2]Japan Association for the Advancement of Medical Equipment, Tokyo, Japan, the [3]Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; and the [4]Core Research for Evolutional Science and Technology of Japan Science and Technology Cooperation, Kyoto, Japan.

Corresponding author: Shimpei Fujimoto, fujimoto@metab.kuhp.kyoto-u.ac.jp.

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In pancreatic β-cells, glucose metabolism regulates exocytosis of insulin granules through metabolism secretion coupling, in which glucose-induced ATP production in mitochondria plays an essential role (1). Impairment of mitochondrial ATP production causes reduced glucose-induced insulin secretion.

Reactive oxygen species (ROS) is one of the most important factors that impair metabolism secretion coupling in β-cells. Exposure to exogenous hydrogen peroxide (H_2O_2), the most abundant ROS, reduces glucose-induced insulin secretion by impairing mitochondrial metabolism in β-cells (2,3). However, little is known of the role of endogenous ROS in impaired glucose-induced insulin secretion from β-cells. Some studies (4,5) have shown that endogenous ROS is produced in mitochondria by exposure to high glucose. In Zucker diabetic fatty rats, the superoxide content of islets at basal glucose levels is higher than that in Zucker lean control rats (4). Furthermore, we recently reported that high glucose–induced ROS production in islet cells is elevated in diabetic Goto-Kakizaki (GK) rats compared with control Wistar rats (6). Thus, endogenous ROS production is elevated in β-cells under diabetic pathophysiological conditions.

Although the mechanism of endogenous ROS production in β-cells in the diabetic state remains largely unknown, we have reported that Src (c-Src) plays an important role in the signal transduction that produces ROS (6). Src is a nonreceptor tyrosine kinase that is associated with the cell membrane and plays important roles in various signal transductions, and its activity is regulated by intramolecular interactions that depend on tyrosine phosphorylation (7,8). Phosphorylation of Tyr527 (Tyr529 in humans), which is located near the C terminus of Src, is brought about by COOH terminal Src kinase (Csk), a negative regulator of Src (9), and holds the kinase in the inactive form. Dephosphorylation of Tyr527 followed by disruption of the intramolecular interaction allows phosphorylation of Tyr416 (Tyr418 in humans) at the kinase domain, resulting in Src activation. In our previous report (6), PP2, a selective Src inhibitor, decreased high-glucose–induced ROS production in GK islet cells, in contrast to the lack of any effect of the agent in Wistar islet cells, suggesting that Src may be activated in the diabetic condition and cause elevation of ROS production in the presence of high glucose.

Glucagon-like peptide (GLP)-1 is one of the incretin peptides released from the intestine in response to nutrient ingestion that augments glucose-induced insulin secretion from β-cells (10,11). GLP-1 binding to the GLP-1 receptor, a member of the G protein–coupled receptor

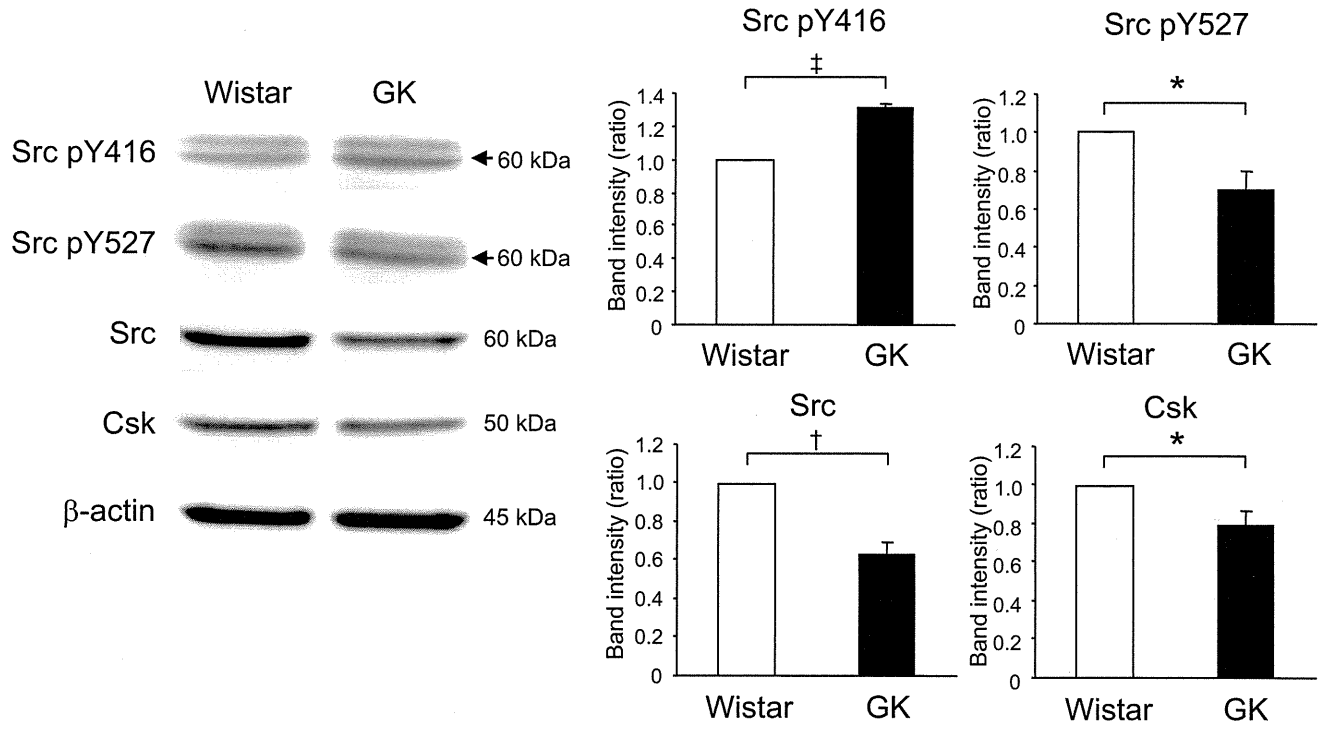

FIG. 1. Comparison of expression of Src between fresh Wistar and GK islets. Fresh islets were lysated and subjected to immunoblot analyses. Blots (50 μg of protein) were probed with anti–phospho-Src (Tyr^{416}), anti–phospho-Src (Tyr^{527}), anti-Src, or anti-Csk. The same blots were stripped and reprobed with anti–β-actin, respectively. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to Wistar islet value corrected by β-actin level (means ± SE). *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. Representative blot panels of three to five independent experiments are shown.

(GPCR) superfamily, induces activation of adenylyl cyclase and elevation of intracellular cAMP levels, which elicits protein kinase A (PKA)-dependent signal transduction. Recently, Epac (also known as cAMP-GEF [guanine nucleotide exchange factor]) has been shown to be a novel cAMP sensor in the PKA-independent pathway (12,13). In β-cells, one member of the Epac family, Epac2, has an important role in insulin secretion, especially in regulation of exocytosis of insulin granules (14,15). Previous studies have shown that GLP-1 also has beneficial long-term effects on diabetic β-cells, including induction of β-cell proliferation (16,17), enhanced resistance to apoptosis (17,18), and amelioration of endoplasmic reticulum stress (19). Furthermore, increased ROS in diabetic *db/db* mouse islets is decreased by treatment with an inhibitor of dipeptidyl peptidase IV that delays the degradation of GLP-1 (20).

In the present study, we investigated whether the GLP-1 signal directly ameliorates endogenous ROS production in diabetic GK islets using exendin-4, a GLP-1 receptor agonist. In particular, we focused on clarifying regulation of Src activity by GLP-1 signaling. We describe here both a novel effect and a mechanism of GLP-1 signaling that acutely decreases ROS production by high glucose through suppression of Src activation PKA independently and Epac dependently.

RESEARCH DESIGN AND METHODS

Male Wistar and GK rats were obtained from Shimizu (Kyoto, Japan). All experiments were carried out with rats that were aged ~7–8 weeks. Nonfasting blood glucose levels were ~160–240 mg/dl in the GK rats and ~70–120 mg/dl in the Wistar rats used in the experiments. The animals were maintained and used in accordance with the guidelines of the animal care committee of Kyoto University.

Islet preparation. Pancreatic islets were isolated from Wistar and GK rats by the collagenase digestion technique (6). Isolated islets were washed with Krebs Ringer bicarbonate buffer (KRBB) (in mmol/l: 129.4 NaCl, 5.2 KCl, 2.7 $CaCl_2$, 1.3 KH_2PO_4, 1.3 $MgSO_4$, and 24.8 $NaHCO_3$ [equilibrated with 5% CO_2/95% O_2, pH 7.4]) containing 2.8 mmol/l glucose and cultured for ~20 h in RPMI-1640 medium containing 5.5 mmol/l glucose and 10% FCS. Cultured islets were preincubated for 30 min at 37°C in KRBB supplemented with 0.2% BSA and 10 mmol/l HEPES (KRBB medium) containing 2.8 mmol/l glucose and incubated for the indicated times at 37°C in KRBB medium containing 16.7 mmol/l glucose with or without test materials.

Retroviral-mediated gene transfer. Production of retroviral vectors with pCX4 was performed as previously described (21). Src kinase–negative mutant (K295M) was subcloned into pCX4pur (22). Gene transfer experiments of islets were carried out by an in vivo gene transduction method (23). Briefly, after rats were anesthetized and subjected to laparotomy, the hepatic artery with the portal vein and the splenic artery were ligated. The upper side of the celiac artery that branches from the abdominal aorta was clamped, and 100 μl of retroviral vector suspension was injected into the lower side of the clamped point of the artery. The pancreatic islets were then isolated and cultured for 48 h before the experiment. Gene expression using green fluorescent protein–expressing vector was effective in the inside of the islets, as previously reported (23).

Immunoprecipitation and immunoblotting. Fresh or incubated islets were lysed in ice-cold lysis buffer (10 mmol/l Tris [pH 7.2], 100 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Complete; Roche, Mannheim, Germany), phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany), and 5 mmol/l sodium pyrophosphate. For determination of Src activation, lysates were centrifuged at 560,000*g* for 10 min at 4°C, and the supernatant (~2 mg of protein content/2,500 islets) was mixed with 4 μg mouse monoclonal anti-Src antibody (clone GD11; Upstate, Billerica, MA) and 30 μl washed protein G Sepharose (GE Healthcare, Uppsala, Sweden) followed by gentle rotation for 4 h at 4°C. Immunoprecipitates or islet lysates (50 μg) were subjected to immunoblotting as previously described (23). Primary antibodies used were rabbit anti–phospho-Src (Tyr416) and anti–phospho-Src (Tyr527) from Biosource (Camarillo, CA); rabbit anti-Src, anti-Csk, anti-Epac2, extracellular signal–regulated kinase (ERK) 1/2, and mouse anti–phospho-ERK1/2 (Thr202/Tyr204) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Rap1 from Upstate; rabbit anti–phospho-Akt (Ser473) and anti-Akt from Cell

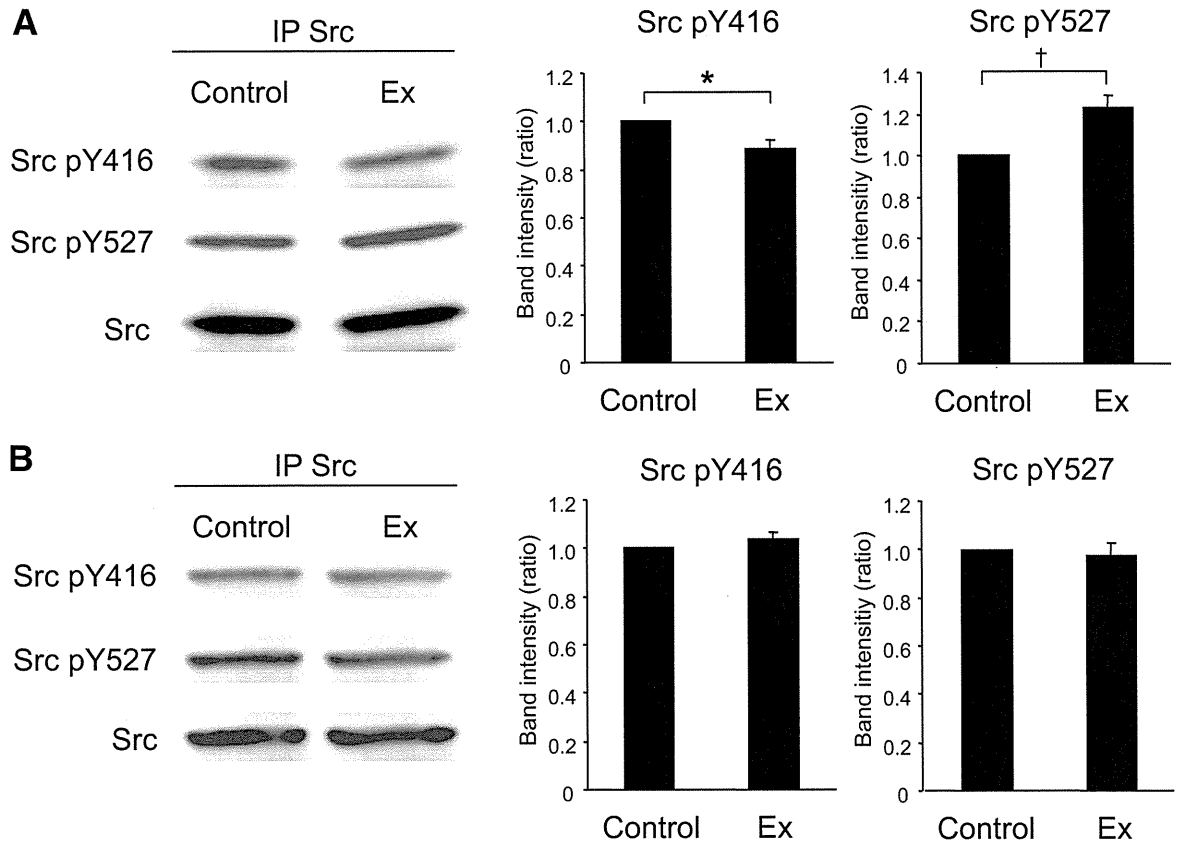

FIG. 2. Exendin-4 suppresses Src activity at high glucose in GK islets. Effects of exendin-4 on Src activity at high glucose in GK (*A*) and Wistar (*B*) islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 16.7 mmol/l glucose with or without 100 nmol/l exendin-4 for 10 min. Islet lysates (~2 mg of protein) were immunoprecipitated with anti-Src antibody and subjected to immunoblot analyses. Blots were probed with anti–phospho-Src (Tyr^{416}), anti–phospho-Src (Tyr^{527}), or anti-Src by stripping and reprobing of the same blots. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to control value corrected by Src level (means ± SE). *$P < 0.05$; †$P < 0.01$. Representative blot panels of four (*A*) or three (*B*) independent experiments are shown.

Signaling (Danvers, MA); and mouse anti–β-actin from Sigma (St. Louis, MO). Secondary antibodies used were horseradish peroxidase–conjugated anti-rabbit and mouse antibody (GE Healthcare). Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).

Measurement of ROS production. ROS production in islet cells was measured by 2′,7′-dichlorofluorescein fluorescence (6). Briefly, cultured islets were dispersed using 0.05% trypsin/0.53 mmol/l EDTA (Invitrogen, Carlsbad, CA) and PBS. Dispersed islet cells were preincubated in KRBB medium containing 2.8 mmol/l glucose and 10 μmol/l 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen) for 20 min at 37°C. After a 60-min incubation in 400 μl KRBB medium containing 16.7 mmol/l glucose with or without test materials, fluorescence was measured using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan), with excitation wavelength at 505 nm and emission wavelength at 540 nm. Fluorescence was corrected by subtracting parallel blanks and represented by fold increases of the value at time zero.

Measurement of ATP content. ATP content in islets was determined by luminometry as previously described (6). Briefly, after preincubation, groups of 10 islets were batch incubated for 30 min in KRBB medium containing 2.8 or 16.7 mmol/l glucose with or without test materials. Incubation was stopped immediately by addition of $HClO_4$ and sonication in ice-cold water for 10 min. They were then centrifuged, and a fraction of the supernatant was mixed with HEPES and Na_2CO_3. The ATP content in the supernatant of islet lysates was measured using ENLITEN luciferase/luciferin reagent (Promega, Madison, WI) with a luminometer (GloMax 20/20n; Promega).

Materials. Exendin-4 and forskolin were purchased from Sigma. PP2 was purchased from Tocris (Ellisville, MO). PP3, H-89, myristoylated PKA inhibitor amide14–22 (PKI), LY294002, wortmannin, PD98059, and AG1478 were purchased from Calbiochem. Dibutyryl cAMP was purchased from Daiichisankyo (Tokyo, Japan). 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP (8CPT-2Me-cAMP) was purchased from Biolog Life Science (Bremen, Germany).

Statistical analysis. Data are expressed as means ± SE. Statistical significance of difference was evaluated by the unpaired Student *t* test. $P < 0.05$ was considered significant.

RESULTS

Comparison of expression of Src between Wistar and GK islets. To examine whether the expression levels of Src in GK islets differ from those in Wistar islets, immunoblotting using fresh islets was performed. As shown in Fig. 1*A*, the level of Src pY416, which indicates activation of Src, in GK islets was significantly higher than that in Wistar islets. The levels of Src pY527, total Src, and Csk in GK islets were significantly lower than those in Wistar islets. The levels of other Src family kinases (SFKs) were similar in Wistar and GK islets, whereas the expression of Fgr was very low and that of Fyn was undetectable (supplementary Fig. 1 in the online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0021/DC1). Results of immunoblotting using islets cultured for 20 h in the presence of 5.5 mmol/l glucose (supplementary Fig. 2) were similar to those shown in Fig. 1*A*.

Exendin-4 suppresses Src activity in GK islets. To investigate whether exendin-4 regulates Src activity, phosphorylation of Src was examined by immunoprecipitation and immunoblotting. As shown in Fig. 2*A*, Src pY416 was

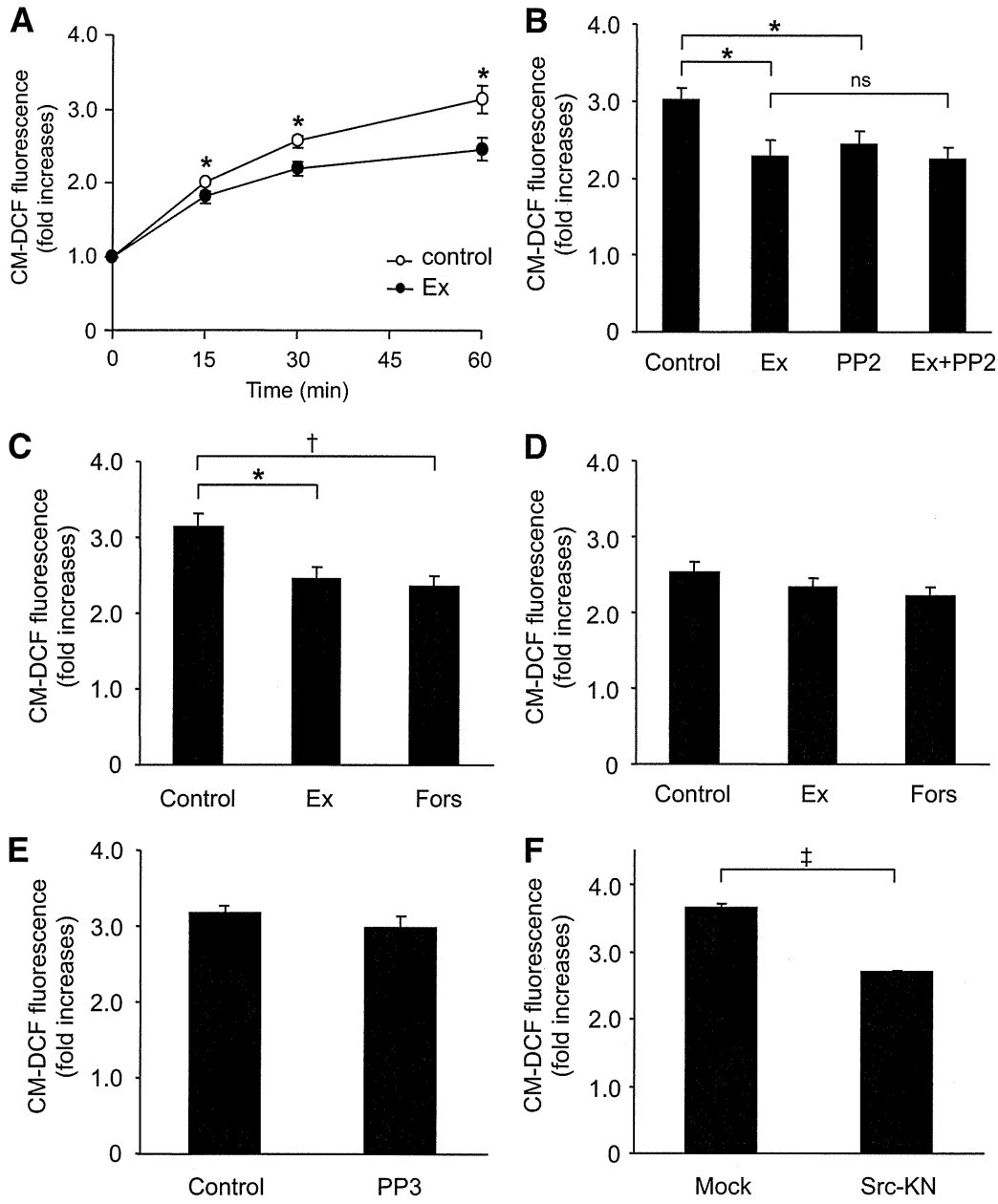

FIG. 3. Exendin-4 decreases ROS production at high glucose in GK islet cells. *A*: Time course of high-glucose–induced ROS production with or without 100 nmol/l exendin-4 in GK islet cells. After preincubation in the presence of 2.8 mmol/l glucose and 10 μmol/l CM-H_2DCFDA for 20 min, dispersed islet cells were incubated in the presence of 16.7 mmol/l glucose with (●) or without (○) 100 nmol/l exedin-4 for 60 min. Fluorescence is represented as fold increases against the value at time zero. Data are expressed as means ± SE (n = 5–7). *P < 0.05 vs. control. *B*: Effects of exendin-4 and PP2 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 4–6). *P < 0.05. *C*: Effects of exendin-4 and forskolin on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 5–6). *P < 0.05; †P < 0.01. *D*: Effects of exendin-4 and forskolin on high-glucose–induced ROS production at 60 min in Wistar islet cells. Data are expressed as means ± SE (n = 3–4). *E*: Effects of PP3 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 3). *F*: Effect of Src-KN on high-glucose–induced ROS production at 60 min in GK islet cells. Retroviral (empty vector and Src-KN vector)-mediated gene transfer to islets was carried out by in vivo gene transduction method, as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE (n = 3). ‡P < 0.001.

significantly decreased by 100 nmol/l exendin-4 in the presence of 16.7 mmol/l glucose in GK islets. Exendin-4 also significantly increased Src pY527 in GK islets in the same condition. On the other hand, exendin-4 did not affect Src pY416 or pY527 at high glucose in Wistar islets (Fig. 2*B*). Both Src pY416 and pY527 were not altered by change in glucose concentration in GK or Wistar islets (supplementary Fig. 3).

Exendin-4 decreases ROS production in GK islet cells. We then investigated whether exendin-4 ameliorates endogenous ROS production at high glucose in GK islet cells. A total of 16.7 mmol/l glucose exposure induced ROS production in GK islet cells (Fig. 3*A*). Coexposure of exendin-4 significantly decreased ROS production in the presence of 16.7 mmol/l glucose at 15, 30, and 60 min. A total of 10 μmol/l PP2, a Src inhibitor, significantly de-

creased high-glucose–induced ROS production (Fig. 3*B*), but PP3, the inactive PP2 analog, did not affect it (Fig. 3*E*). Exendin-4 did not further decrease ROS production in the presence of PP2 (Fig. 3*B*), suggesting that the effect of exendin-4 is via the Src signal. The decrease in high-glucose–induced ROS production also was observed in the presence of 10 μmol/l forskolin, an adenylyl cyclase activator (Fig. 3*C*). High-glucose–induced ROS production in Wistar islet cells was lower than that in GK islet cells and was not changed by addition of exendin-4 or forskolin (Fig. 3*D*). To confirm that Src is actually involved in ROS production, we measured ROS production in GK islets expressing a kinase-negative form of Src (Src-KN) by retroviral vector. ROS production in Src-KN–expressing islets was significantly lower than that in control (Fig. 3*F*), demonstrating that Src regulates ROS production in GK islets.

Exendin-4 increases ATP content in GK islets. In Wistar islets, 16.7 mmol/l glucose-exposure significantly increased ATP content compared with that in the presence of 2.8 mmol/l glucose, as shown in Fig. 4*B*. Exendin-4, PP2, or exendin-4 plus PP2 did not affect the ATP content in the presence of 16.7 mmol/l glucose in Wistar islets. The ATP content in GK islets exposed to 16.7 mmol/l glucose was not increased compared with that in the presence of 2.8 mmol/l glucose (Fig. 4*A*). Exendin-4 as well as PP2 significantly increased the ATP content in the presence of 16.7 mmol/l glucose. Further increase of ATP content by combined exendin-4 and PP2 was not observed.

The effects of exendin-4 are dependent on Epac. We then investigated whether the decrease in ROS production by exendin-4 is dependent on PKA. As shown in Fig. 5*A*, decreased ROS production by exendin-4 or forskolin was not affected by 10 μmol/l H-89 or PKI, a PKA inhibitor, indicating that the effect is PKA independent. Not only dibutyryl cAMP, a general cAMP analog, but also 8CPT-2Me-cAMP, an Epac-specific cAMP analog, decreased ROS production (Fig. 5*C*). Epac possesses guanine nucleotide exchange factor activity toward Rap1, a member of the Ras superfamily of small GTPases. Epac2 and Rap1 proteins were expressed similarly in both Wistar and GK islets (Fig. 5*B*). To determine involvement of Epac in Src activation, Src phosphorylation was examined. Src pY416 was significantly decreased by 8CPT-2Me-cAMP (Fig. 5*D*).

A downstream pathway of Src is PI3K/Akt signaling. Src signalings toward downstream proteins are complex, but one of the typical pathways is phosphatidylinositol 3 kinase (PI3K)/Akt signaling (8). We therefore examined the involvement of PI3K/Akt signaling on ROS production. A total of 50 μmol/l LY294002 and 0.5 μmol/l wortmannin, both of which are PI3K inhibitors, significantly decreased ROS production in GK islets (Fig. 6*A*). Exendin-4 and PP2 both significantly decreased phosphorylation of Akt in GK islets (Fig. 6*B*) but not in Wistar islets (Fig. 6*C*). Considering these findings together, PI3K/Akt signaling that produces ROS is located downstream of Src activation. We also examined the involvement of mitogen-activated protein kinase signaling, another downstream pathway of Src. A total of 50 μmol/l PD98059, a MAPK-ERK kinase inhibitor, did not affect ROS production in GK islets (Fig. 6*D*), and neither exendin-4 nor PP2 affected phosphorylation of ERK (Fig. 6*E*). Several GPCR agonists have been shown to induce transactivation of epidermal growth factor receptor (EGFR) (24,25) by a mechanism involving Src (25–27) and frequently subsequent PI3K/Akt signaling (25,28). Therefore, involvement of EGFR transactivation on regulation of ROS production was examined. A total of 0.5 μmol/l AG1478, an EGFR kinase inhibitor, significantly decreased ROS production (Fig. 6*F*).

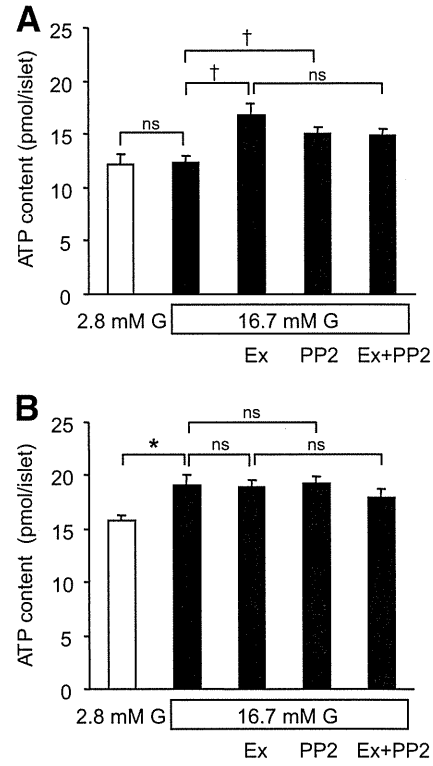

FIG. 4. Exendin-4 increases ATP content at high glucose in GK islets. Effects of exendin-4 and PP2 on ATP content in the presence of high glucose for 30 min in GK (*A*) and Wistar (*B*) islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 2.8 or 16.7 mmol/l glucose with or without 100 nmol/l exendin-4, 10 μmol/l PP2, or both for 30 min. Data are expressed as means ± SE (n = 7–8). *P < 0.05; †P < 0.01.

DISCUSSION

We previously reported that endogenous ROS production by high glucose in diabetic GK islets is elevated compared with that in control Wistar islets and is effectively ameliorated by Src inhibition, suggesting that Src may be activated in GK islets (6). In the present study, we first investigated whether Src activity is altered in GK islets. Immunoblotting analysis revealed that the level of Src pY416, which indicates the level of Src activation, is higher in GK islets than that in Wistar islets, despite lower levels of total Src, Src pY527, and Csk. The lower level of total Src seems to be a consequence of Src activation. Targeted degradation of active forms of Src is brought about by ubiquitination (29). The protooncogene c-Cbl, recently found to be an E3 ubiquitin ligase, mediates ubiquitination of activated Src (30). These reports suggest that increased degradation of activated Src may result in a lower level of total Src in GK islets. In addition, a lower level of Csk might cause a lower activity of the kinase in GK islets. However, Src activity is not directly regulated through phosphorylation of Tyr527 by Csk (8), and a subtle decrease in Csk activity is not believed to contribute to regulation of Src activity because of the excess amount of expression of Csk. This is supported by the findings that heterozygous disruption of ubiquitously expressed Csk