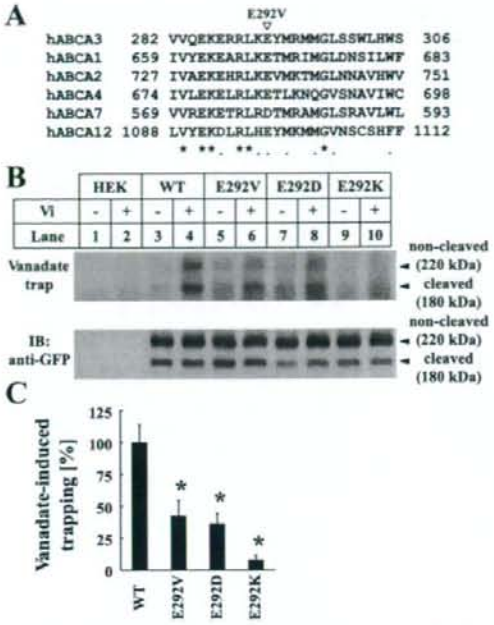

Fig. 4. Alignment of amino acid sequence of intracellular loop 1 (ICL-1) and vanadate-induced nucleotide trapping in site-directed mutant proteins of Glu292. *A*: alignment of amino acid sequence of ICL-1 of some members of the human ABCA subfamily is shown. The amino acid residues that are conserved in 6 transporters and in 4 or 5 transporters are indicated by asterisks and dots, respectively. *B*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP (*lanes 3* and *4*), E292V (*lanes 5* and *6*), E292D (*lanes 7* and *8*), E292K (*lanes 9* and *10*), or untransfected HEK-293 cells (*lanes 1* and *2*) was incubated with 10 μM 8-azido-[α-^{32}P]ATP in the absence or presence of 0.4 mM Vi and 3 mM $MgCl_2$ for 10 min at 37°C. Protein was photoaffinity labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (*top*) and IB using anti-GFP antibody (*bottom*). *C*: radioactivity of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) was quantified by FLA-5000. Radioactivity in the absence of orthovanadate was subtracted from that in the presence of orthovanadate and is expressed after normalization to the level of ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are means ± SD (n = 3). *P < 0.01 vs. WT.

intermediate during ATP hydrolysis was decreased to 37% of that of wild-type protein, as also was the case in E292V mutant protein (Fig. 4*B*, *lanes 5–8*, and *C*). In E292K mutant protein, it was decreased to 4% of that of wild-type protein (Fig. 4*B*, *lanes 9* and *10*, and *C*). These results indicate that not only a negative charge but also an appropriate side chain length of Glu292 at ICL-1 is important for production of a photoaffinity-labeled intermediate during ATP hydrolysis of ABCA3 protein.

Recently, we identified a novel compound heterozygous mutation (maternal T1114A and paternal W1148X) from a Japanese boy with respiratory distress from age 18 mo (37). The T1114A mutation decreased vanadate-induced nucleotide trapping by ABCA3 protein similarly to the T1114M mutation. Because ABCA1, ABCA2, ABCA4, ABCA7, and ABCA12 have conserved Ser residues rather than a Thr residue in putative TM-8 (Fig. 5*A*), loss of the hydroxyl group-containing amino acid might well act on putative TM-8 to hamper communication between transmembrane domains (TMDs) and NBDs, resulting in loss of ATP hydrolysis activity. To clarify this, we substituted Thr1114 with Ser and examined vanadate-induced nucleotide trapping. In vanadate-induced nucleotide trapping by T1114S mutant protein, production of a photoaffinity-labeled intermediate during ATP hydrolysis was found to be similar to that of wild-type protein, whereas that of T1114M and T1114A mutant protein was 56 and 47% of wild-type protein, respectively (Fig. 5*B*, *lanes 5–10*, and *C*). These results indicate that loss of the hydroxyl group of the 1114th amino acid is responsible for the impaired ATP hydrolysis with production of a photoaffinity-labeled intermediate in both the T1114M and T1114A mutant proteins.

Interaction of E690K mutant ABCA3-GFP protein with nucleotides. To investigate the mechanism of enhanced production of a photoaffinity-labeled intermediate during ATP hydrolysis in E690K mutant protein, we performed the trap-

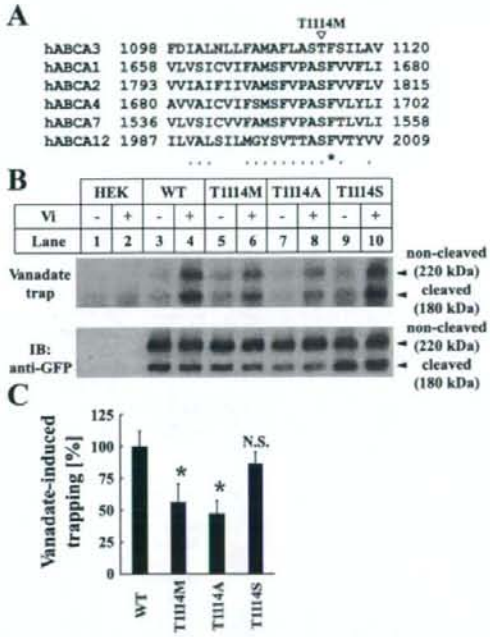

Fig. 5. Alignment of amino acid sequence of the 8th putative transmembrane segment (TM-8) and vanadate-induced nucleotide trapping in site-directed mutant proteins of Thr1114. *A*: alignment of amino acid sequence of putative TM-8 of some members of the human ABCA subfamily is shown. The amino acids residues that are conserved in 6 transporters and in 4 or 5 transporters are indicated by asterisks and dots, respectively. *B*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP (*lanes 3* and *4*), T1114M (*lanes 5* and *6*), T1114A (*lanes 7* and *8*), T1114S (*lanes 9* and *10*), or untransfected HEK-293 cells (*lanes 1* and *2*) was incubated with 10 μM 8-azido-[α-^{32}P]ATP in the absence or presence of 0.4 mM Vi and 3 mM $MgCl_2$ for 10 min at 37°C. Proteins were photoaffinity labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (*top*) and IB using anti-GFP antibody (*bottom*). *C*: radioactivity of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) was quantified by FLA-5000. Radioactivity in the absence of orthovanadate was subtracted from that in the presence of orthovanadate and is expressed after normalization to the level of ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are means ± SD (n = 3). *P < 0.01 vs. WT.

ping experiments using 8-azido-[γ-^{32}P]ATP. In this procedure, a posthydrolyzed trapped nucleotide should not be detected by vanadate-induced trapping because of hydrolytic loss of [γ-^{32}P]PO_4. Indeed, in the presence of orthovanadate, photoaffinity labeling of wild-type and E690K mutant proteins with 8-azido-[γ-^{32}P]ATP was barely detectable (Fig. 6*A*). These data combined with vanadate-induced trapping using 8-azido-[α-^{32}P]ATP indicate that E690K mutant protein hydrolyzes 8-azido-[α-^{32}P]ATP and releases γ-phosphate and that the nucleotide trapped by E690K mutant protein is mostly in the ADP form.

In E690K mutant protein, ATP binding determined by photoaffinity labeling with 8-azido-[γ-^{32}P]ATP was dramatically increased compared with that of wild-type protein (see Fig. 3, *C* and *D*). To determine whether the E690K mutation alters ADP binding, we performed labeling experiments using 8-azido-[α-^{32}P]ADP at 0°C. In this condition, photoaffinity labeling of E690K mutant protein was comparable to that of wild-type ABCA3-GFP protein (Fig. 6, *B* and *C*), suggesting that γ-phosphate of 8-azido-[γ-^{32}P]ATP contributes to enhanced photoaffinity labeling in E690K mutant protein.

Since some mutations in the Glu residues following the Walker B motif have been reported to interfere with the ATP-hydrolysis cycle including the ADP release step (5, 24, 25, 33), we examined release of trapped nucleotides from wild-type and E690K mutant ABCA3-GFP proteins. A 20,000-*g* membrane fraction was first incubated for 10 min at 37°C with 8-azido-[α-^{32}P]ATP in the presence of orthovanadate, and unbound nucleotides were removed by washing. The membrane fraction was reincubated at 37°C for 0–15 min in buffer containing $MgCl_2$ before cross-linking. In wild-type ABCA3-GFP protein, trapped nucleotides were time-dependently reduced by reincubation, and photoaffinity labeling at 5 min was 32% of that at 0 min (Fig. 6, *D* and *E*). In contrast, in E690K mutant protein, trapped nucleotides were more slowly released than in wild-type protein, and photoaffinity labeling at 5 min was 73% of that at 0 min, suggesting that E690K mutant protein forms a more stable inhibitory intermediate after hydrolysis of 8-azido-[α-^{32}P]ATP than wild-type protein. Thus these data further suggest abnormal interaction with nucleotides in E690K mutant protein.

Mutational analysis of Glu690 adjacent to walker B motif in NBD-1. To further clarify the enhanced production of a photoaffinity-labeled intermediate during ATP hydrolysis in E690K mutant, we performed mutational analyses of the Glu690 residue adjacent to the Walker B motif in NBD-1. Because Glu and Lys are negatively and positively charged amino acids, respectively, alteration of charge at the 690th amino acid residue could well be responsible for the abnormal interaction with nucleotides in the E690K mutant. Accordingly, Glu690 was substituted with Asp and Arg, which are negatively and positively charged, respectively. Substitution with Asp and Arg caused a dramatic

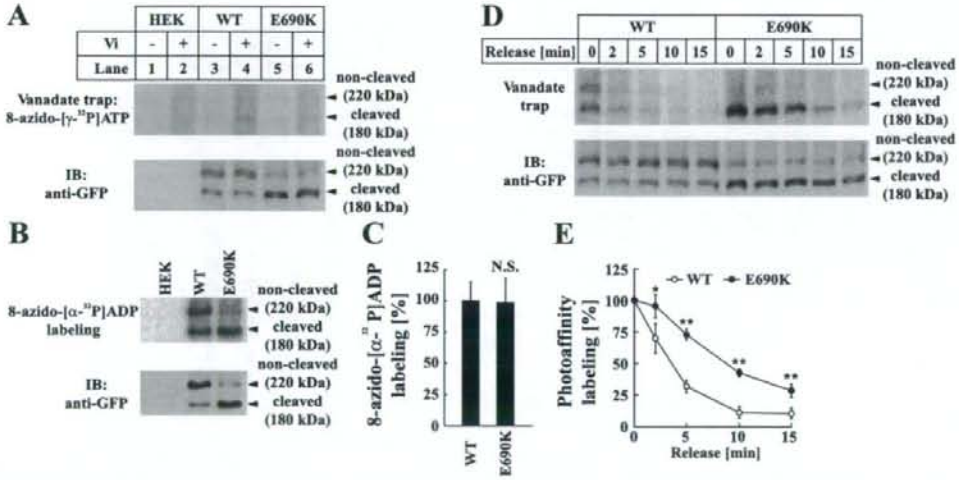

Fig. 6. Interaction of E690K mutant ABCA3-GFP protein with nucleotides. *A*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP (*lanes 3* and *4*) E690K (*lanes 5* and *6*), or untransfected HEK-293 cells (*lanes 1* and *2*) was incubated with 10 μM 8-azido-[γ-^{32}P] ATP in the absence or presence of 0.4 mM Vi and 3 mM $MgCl_2$ for 10 min at 37°C. Protein was photoaffinity labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (*top*) and IB using anti-GFP antibody (*bottom*). *B*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP, E690K, or untransfected HEK-293 cells was incubated with 40 μM 8-azido-[α-^{32}P]ADP and 3 mM $MgCl_2$ for 10 min at 0°C. Protein was photoaffinity labeled with UV irradiation, immunoprecipitated with anti-human ABCA3 antibody, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane. Membrane was analyzed by FLA-5000 (*top*) and IB using anti-GFP antibody (*bottom*). *C*: radioactivity of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) was quantified by FLA-5000 and is expressed after normalization to the level of ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are means ± SD (n = 3). *D*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP or E690K was first incubated for 10 min at 37°C with 10 μM 8-azido-[α-^{32}P]ATP in the presence of orthovanadate, and unbound nucleotides were removed by washing. A membrane fraction was reincubated at 37°C for 0–15 min in a buffer containing $MgCl_2$, before cross-linking. After removal of released nucleotides and cross-linking, protein was electrophoresed on SDS-PAGE and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (*top*) and IB using anti-GFP antibody (*bottom*). *E*: radioactivity of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) was quantified by FLA-5000 and is expressed as a percentage of the 0-min value after normalization to the level of ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are means ± SD (n = 4). *$P < 0.05$; **$P < 0.01$ vs. WT.

decrease in ATP hydrolysis with production of a photoaffinity-labeled intermediate to 11 and 12% of that of wild-type protein, respectively (Fig. 7, *A* and *B*). These results show that both negative charge and side chain length of Glu690 are important for ATP hydrolysis of wild-type ABCA3 protein and that not only positive charge but also side chain length of Lys690 contribute to enhanced production of a photoaffinity-labeled intermediate during ATP hydrolysis in the E690K mutant protein.

DISCUSSION

In the present study, to clarify the phenotypic heterogeneity of lung disease associated with *ABCA3* mutation, we characterized the ABCA3 mutant proteins identified in pILD patients, a more mild disease than fatal surfactant deficiency. Although E292V, E690K, and T1114M mutant proteins were found to traffic to intracellular vesicles, the lipid transport function of E292V mutant protein was partially impaired, and those of E690K and T1114M mutant protein were severely impaired, accompanied by an aberrant catalytic cycle. We recently found that fatal surfactant deficiency due to *ABCA3* mutation comprises defects of abnormal intracellular localization (type I) and normal intracellular localization with decreased ATP hydrolysis of ABCA3 protein (type II) (17). Accordingly, E292V, E690K, and T1114M are type II mutations.

Patients with fatal surfactant deficiency carrying a type I homozygous *ABCA3* mutation (W1142X/W1142X, L101P/L101P, or L1553P/L1553P) or a type I/type II compound heterozygous mutation (L982P/G1221S or Ins1518/L1580P) die within the neonatal period (Table 1) (27). On the other hand, patients carrying a type II/type II *ABCA3* mutation (E292V/T1114M or E292V/E690K) exhibit pILD (4), suggesting that the type II/type II *ABCA3* mutation produces a milder phenotype. Although an exception has been identified in a Japanese patient with a type I/type II *ABCA3* mutation (W1148X/T1114A) (37), the moderately preserved lipid transport function of the E292V mutant protein may underlie the generally milder phenotype of pILD patients. Further analysis using transgenic mice is required to understand the impacts of these mutations on surfactant metabolism in vivo.

Table 1. *Genotype-phenotype correlation for ABCA3 mutation*

ABCA3 Mutation		Age of Symptoms	Phenotype	Ref.
W1142X	*W1142X*	Neonate	FSD	27
L101P	*L101P*	Neonate	FSD	27
L1553P	*L1553P*	Neonate	FSD	27
Ins1518	L1580P	Neonate	FSD	27
L982P	G1221S	Neonate	FSD	27
E292V	T1114M	Neonate	pILD	4
E292V	E690K	5 or 7 yr	pILD	4
W1148X	T1114A	12 mo	pILD	37

Type I and type II ATP binding cassette A3 (*ABCA3*) mutations are shown in italics and roman, respectively. FSD, fatal surfactant deficiency; Ins1518, Ins1518fs/ter1519; pILD, pediatric interstitial lung disease.

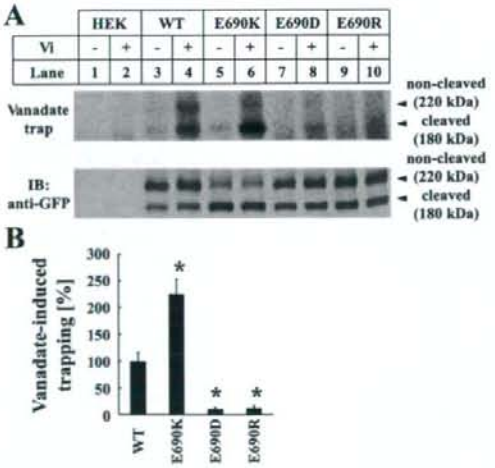

Fig. 7. Vanadate-induced nucleotide trapping in site-directed mutant proteins of Glu690. *A*: 20,000-*g* membrane fraction prepared from HEK-293 cells stably expressing the WT ABCA3-GFP (*lanes 3* and *4*), E690K (*lanes 5* and *6*), E690D (*lanes 7* and *8*), E690R (*lanes 9* and *10*), or untransfected HEK-293 cells (*lanes 1* and *2*) was incubated with 10 μM 8-azido-[α-^{32}P]ATP in the absence or presence of 0.4 mM Vi and 3 mM $MgCl_2$ for 10 min at 37°C. Protein was photoaffinity labeled with UV irradiation after removal of unbound ATP, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane. Membrane was analyzed by autoradiography (*top*) and IB using anti-GFP antibody (*bottom*). *B*: radioactivity of photoaffinity-labeled protein (total 220-kDa noncleaved form plus 180-kDa cleaved form) was quantified by FLA-5000. Radioactivity in the absence of orthovanadate was subtracted from that in the presence of orthovanadate and is expressed after normalization to the level of ABCA3-GFP protein (total 220-kDa noncleaved form plus 180-kDa cleaved form). Data are means ± SD (n = 3). *P < 0.01 vs. WT.

The E292V mutant protein exhibits moderately preserved lipid transport function and vanadate-induced nucleotide trapping, and mutational analysis of Glu292 in ICL-1 indicates the significance of both negative charge and side chain length of Glu292 for ATP hydrolysis with production of a photoaffinity-labeled intermediate in ABCA3 protein. In addition, the recently solved crystal structure of the bacterial ABC transporter Sav1866 suggests that ICLs interact with NBDs and TMDs to transmit conformational changes generated by ATP binding and hydrolysis from NBDs to TMDs (9). Thus the E292V mutation might impede the transmission of conformational changes, resulting in moderate impairment of lipid transport in ABCA3 protein.

The T1114M mutant protein exhibits impaired lipid transport function accompanied by moderately preserved vanadate-induced nucleotide trapping. In addition, mutational analysis of Thr1114 in putative TM-8 shows the importance of the hydroxyl group of the 1114th amino acid residue for ATP hydrolysis with production of a photoaffinity-labeled intermediate in ABCA3 protein. In membrane proteins, the hydroxyl group of Ser and that of Thr are known to form a hydrogen bond with backbone nitrogen to contribute to tight helix packing (10), suggesting that loss of the hydroxyl group from the 1114th amino acid in putative TM-8 might well hamper conformational change of TMDs during ATP-hydrolysis that results in impaired lipid transport of the ABCA3 protein.

The glutamate residue following the Walker B motif in ABC transporters has been suggested to be a catalytic carboxylate that facilitates nucleophilic attack on ATP via a water molecule (13, 23). However, it has been reported that some mutations in the Glu residues interfere with ADP release (5, 24, 25, 33) and tight dimerization of NBDs (29, 30, 31). In E690K mutant

ABCA3 protein, lipid transport function is severely impaired, accompanied by abnormal interaction with nucleotides: enhanced vanadate-induced nucleotide trapping at 37°C, enhanced photoaffinity labeling with 8-azido-[γ-^{32}P]ATP at 0°C, and delayed release of 8-azido-[α-^{32}P]ADP after vanadate-induced nucleotide trapping. Furthermore, mutational analysis of Glu690 indicates that side chain length of Lys690 contributes to enhanced production of a photoaffinity-labeled intermediate during ATP hydrolysis in the E690K mutant protein. To clarify the origin of the abnormal interaction with nucleotides in E690K mutant protein, we modeled the structure of NBD-1 of ABCA3 based on the crystal structure of *E. coli* MalK using SWISS-MODEL (Supplemental Fig. 2). In the wild-type ABCA3 model, the distance from side chain oxygen of Glu690 to γ-phosphate oxygen of ATP is ~7.1 Å, similar to the distance from side chain oxygen of Glu159 in MalK. On the other hand, in the model of E690K mutant, the distance from side chain nitrogen of Lys690 to γ-phosphate oxygen of ATP is ~3.6 Å. One possible interpretation of these biochemical results and modeling is that ionic interaction of Lys690 and γ-phosphate of ATP in the E690K mutant protein may tighten the binding of ATP in NBD-1, resulting in delayed ADP release after ATP hydrolysis, probably in NBD-2. Another possible interpretation is that the interaction of Lys690 with γ-phosphate of ATP may alter the directionality of the adenine moiety of ATP, increasing the efficiency of photoaffinity labeling with 8-azido-ATP. Analysis using purified ABCA3 protein would further clarify the aberrant catalytic cycle and impaired lipid transport in E690K mutant protein.

The cleaved form of ABCA3 protein is predominantly expressed in native lung tissue (11, 18, 36) and shows increased vanadate-induced trapping compared with the noncleaved form of the protein in HEK-293 cells. Furthermore, cleavage of ABCA3 protein at ECD1 is suggested to be important for fully active transport function (unpublished observation). Since the cleaved form was rarely detected in ABCA3 mutant protein that remained localized to endoplasmic reticulum (17), the cleavage of ABCA3 protein may occur in Golgi or post-Golgi compartments. Interestingly, in E690K mutant protein, the amount of 180-kDa cleaved form was increased compared with that of wild-type protein. The increased level of 180-kDa cleaved form E690K mutant protein might be due to the abnormal nucleotide-bound conformation of E690K mutant protein being preferentially cleaved by enzymes within intracellular vesicles, compared with that in wild-type protein. Further studies to determine cleavage sites and the enzymes involved in the cleavage of ABCA3 protein are needed.

In summary, the moderately preserved lipid transport function of E292V mutant protein may be responsible for the milder phenotype in pILD caused by *ABCA3* mutation compared with that in fatal surfactant deficiency. The molecular mechanisms of the impaired lipid transport function of ABCA3 could be key to developing treatment strategies to restore ABCA3 function in patients with *ABCA3* mutation.

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Factors responsible for elevation of 1-h postchallenge plasma glucose levels in Japanese men

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ABSTRACT

The 1-h postchallenge plasma glucose (1-h PG) level is considered to be a good index of the development of glucose intolerance and type 2 diabetes as well as of diabetic complications. In some cases, in Japanese, 1-h PG is elevated despite normal fasting glucose during oral glucose tolerance test (OGTT), but the factors responsible remain unclear. In the present study, subjects with normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG), and isolated impaired glucose tolerance (IGT) were divided into subgroups at 1-h PG of 10.0 mM, and the four indices of insulin secretion and insulin sensitivity were compared. In all three categories, the insulinogenic index in subjects with elevated 1-h PG was remarkably lower than in those without elevated 1-h PG. In addition, the insulinogenic index was the strongest factor in elevated 1-h PG according to the multiple regression analysis. Interestingly, one third of the NGT subjects enrolled in this study had elevated 1-h PG. These subjects showed significantly elevated area under the curve of glucose (G-AUC) compared to NGT subjects without 1-h PG elevation. Thus, elevated 1-h PG in Japanese subjects indicates mildly impaired glucose tolerance due to decreased early-phase insulin secretion.

1. Introduction

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity [1–3]. Some patients with glucose intolerance leading to type 2 diabetes show elevated postchallenge plasma glucose without elevated fasting glucose during oral glucose tolerance test (OGTT) [4–6]. Although elevated 1-h postchallenge plasma glucose involves a different regulatory mechanism than 2-h postchallenge plasma glucose (2-h PG), 1-h postchallenge plasma glucose (1-h PG) is also as reliable an index of glucose tolerance as 2-h PG generally [7,8]. However, the relevance of 1-h PG and 2-h PG for diabetes screening is controversial [9,10]. It has been reported that subjects with 1-h PG higher than 10.0 mmol/l show higher risk of developing diabetes than subjects with lower 1-h PG [11]. In addition, 1-h PG higher than 11.2 mmol/l was found to be an independent risk factor for mortality in cardiovascular disease [12–14]. It was recommended in a

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number of studies that subjects having normal fasting plasma glucose at OGTT together with high 1-h PG are followed as carefully as IGT subjects in cases of higher frequency of elevated HbA1c, hypertension, family history of diabetes, or peripheral vascular involvement [15]. In addition, 1-h PG is used in diagnosis of gestational diabetes mellitus (GDM) and risk of macrosomia and other perinatal complications [16,17].

In the present study, the insulin secretion and insulin sensitivity indices of Japanese subjects undergoing OGTT in three WHO categories, normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG) and isolated impaired glucose tolerance (IGT), subdivided at 1-h PG of 10.0 mmol/l were evaluated and compared.

2. Subjects and methods

2.1. Subjects

We recruited subjects undergoing OGTT because of positive urine glucose test, >5.0% HbA1c level, >5.6 mmol/l fasting plasma glucose level, and family history of diabetes at initial examination for medical check-up at Kyoto University Hospital, Ikeda Hospital, Kansai Electric Power Hospital, Kansai Health Management Center, and Kyoto Preventive Medical Center from 1993 to 2005. Subjects in the three categories of glucose tolerance, NGT ($n = 179$: fasting plasma glucose (FPG) level < 6.1 mmol/l and 2-h PG level < 7.8 mmol/l), isolated IFG ($n = 44$: FPG level of 6.1–7.0 mmol/l and 2-h PG < 7.8 mmol/l), and isolated IGT ($n = 103$: FPG level < 6.1 mmol/l and 2-h PG level of 7.8–11.1 mmol/l) according to the diagnostic criteria of World Health Organization in 1998 [18] were enrolled in the study. All subjects were men with no signs of hypertension, hepatic or renal dysfunction, endocrine or malignant disease, engaging in heavy exercise, history of gastrectomy, or history of medication known to affect glucose metabolism. The study was designed in compliance with the ethics regulations of the Helsinki Declaration. After the subjects fasted overnight for 10–16 h, standard OGTT with 75 g glucose was administered according to the National Diabetes Data Group recommendations [16].

The three WHO categories of glucose tolerance were divided into subgroups at 1-h PG of 10.0 mmol/l in this study: NGT with higher 1-h plasma glucose (NGT-HG: NGT criteria and 1-h PG ≥10.0 mmol/l), NGT with lower 1-h plasma glucose (NGT-LG: NGT criteria and 1-h PG <10.0 mmol/l), isolated IFG with higher 1-h plasma glucose (IFG-HG: IFG criteria and 1-h PG ≥10.0 mmol/l), isolated IFG with lower 1-h plasma glucose (IFG-LG: IFG criteria and 1-h PG <10.0 mmol/l), isolated IGT with higher 1-h plasma glucose (IGT-HG: IGT criteria and 1-h PG ≥10.0 mmol/l), and isolated IGT with lower 1-h plasma glucose (IGT-LG: IGT criteria and 1-h PG <10.0 mmol/l).

2.2. Laboratory examination

Blood samples were collected at 0, 30, 60, and 120 min after OGTT, and plasma glucose and serum insulin levels were measured for all subjects. Plasma glucose and serum insulin levels at 90 min were measured for 75 NGT subjects. Blood samples for measurements of HbA1c, total cholesterol, HDL cholesterol, and triglycerides were drawn after an overnight fast.

The plasma glucose level was measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured by two-site radioimmunoassay (Insulin Riabead II, Dainabot, Tokyo, Japan) as reported previously [19]. Serum total cholesterol and triglycerides levels were measured as reported previously [20].

2.3. Measurement

Basal insulin secretion and sensitivity were evaluated by HOMA β-cell and HOMA-IR [21,22], respectively. Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index [23] and ISI composite [24,25]. The calculations were as follows:

$$\text{HOMA } \beta\text{-cell} = \frac{20 \times \text{fasting serum insulin level (FI) (mU/l)}}{\text{fasting plasma glucose level (FPG) (mmol/l)} - 3.5}$$

$$\text{HOMA-IR} = \frac{\text{FI (mU/l)} \times \text{FPG (mmol/l)}}{22.5}$$

$$\text{Insulinogenic index} = \frac{\text{30-min insulin} - \text{FI (pmol/l)}}{\text{30-min plasma glucose} - \text{FPG (mmol/l)}}$$

$$\text{ISI composite} = \frac{10{,}000}{[\text{FPG (mg/dl)}\ \text{FI (mU/ml)} \times \text{mean OGTT glucose (mg/dl)} \times \text{mean OGTT serum insulin (mU/ml)}]^{0.5}}$$

2.4. Statistical analysis

All analyses were performed using STATVIEW 5 system (Stat View, Berkeley, CA). Differences between two groups were assessed by unpaired *t*-test in terms of age, BMI, plasma glucose level, serum insulin level, HbA1c, triglyceride, total cholesterol, insulinogenic index, ISI composite, HOMA-IR, and HOMA β-cell. We used simple regression analysis and multiple regression analysis for comparison of the relationship between 1-h PG and the indices of insulin secretion and sensitivity. Probability (p) values less than 0.05 were considered statistically significant. Data are presented as mean ± S.E.

3. Results

Table 1 shows the clinical and metabolic characteristics of the six subgroups. NGT-HG had higher average age, BMI, FPG, 2-h PG and HbA1c than NGT-LG. IFG-HG had higher BMI than IFG-LG. IGT-HG had higher BMI, FPG, 2-h PG, 1-h insulin and HbA1c than IGT-LG. There were no significant differences in insulin (fasting and 2-h), triglycerides, total cholesterol and HDL-cholesterol levels between the two subgroups of NGT, isolated IFG, and isolated IGT.

The insulin secretion indices of insulinogenic index and HOMA β-cell indices in the three WHO categories are shown in

Table 1 – Clinical characteristics of the subjects in six subgroups of three WHO categories at 10.0 mmol/l

	NGT		Isolated IFG		Isolated IGT	
	NGT-LG	NGT-HG	IFG-LG	IFG-HG	IGT-LG	IGT-HG
Total N	179		44		103	
N	121	58	14	30	32	71
Age (years)	47.2 ± 1.1	53.1 ± 1.3**	54.4 ± 2.1	51.5 ± 1.9	52.0 ± 1.6	52.2 ± 1.1
BMI (kg/m2)	23.1 ± 1	24.2 ± 0.4*	22.6 ± 0.8	24.2 ± 0.6*	22.8 ± 0.5	24.0 ± 0.3*
FPG (mmol/l)	5.2 ± 0.0	5.6 ± 0.0***	6.3 ± 0.1	6.3 ± 0.0	5.2 ± 0.1	5.6 ± 0.0***
1-h PG(mmol/l)	7.7 ± 0.1	11.5 ± 0.2***	8.2 ± 0.4	12.5 ± 0.4***	7.9 ± 0.3	12.2 ± 0.2***
2-h PG(mmol/l)	5.7 ± 0.1	6.2 ± 0.1*	6.0 ± 0.4	6.5 ± 0.1	8.6 ± 0.1	9.2 ± 0.1**
Fasting insulin(pmol/l)	31 ± 1	30 ± 2	26 ± 2	33 ± 3	34 ± 4	34 ± 2
1-h insulin(pmol/l)	250 ± 18	287 ± 23	287 ± 68	238 ± 29	146 ± 16	221 ± 18*
2-h insulin(pmol/l)	191 ± 16	196 ± 16	133 ± 17	192 ± 27	211 ± 22	254 ± 20
HbA1c(%)	5.1 ± 0.1	5.4 ± 0.1***	5.6 ± 0.1	5.6 ± 0.1	5.2 ± 0.1	5.6 ± 0.1*
Triglycerides(mmol/l)	1.28 ± 0.08	1.47 ± 0.16	1.14 ± 0.17	1.31 ± 0.15	1.74 ± 0.29	1.85 ± 0.25
Total cholesterol(mmol/l)	5.33 ± 0.1	5.31 ± 0.11	5.01 ± 0.3	5.41 ± 0.14	5.3 ± 0.16	5.41 ± 0.1
HDL-cholesterol(mmol/l)	1.45 ± 0.05	1.47 ± 0.07	1.46 ± 0.11	1.51 ± 0.11	1.37 ± 0.10	1.39 ± 0.06

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LG. Data are mean ± S.E.

Fig. 1A and B. The insulinogenic index in the HG groups was remarkably lower than in the LG groups. The insulinogenic index values were 25.6 ± 0.3 vs. 75.9 ± 1.6 (NGT-HG vs. NGT-LG: $p < 0.01$), 23.1 ± 0.5 vs. 67.0 ± 3.5 (IFG-HG vs. IFG-LG: $p < 0.05$) and 22.6 ± 0.3 vs. 56.4 ± 1.9 (IGT-HG vs. IGT-LG: $p < 0.01$). The HOMA-β cell index of the HG group was significantly lower than that of the LG group in NGT and isolated IGT. There was no difference between IFG-HG and IFG-LG in HOMA β-cell index. We also estimated the insulin sensitivity indices using ISI composite and HOMA-IR in the three categories. The ISI composite index represents insulin sensitivity during OGTT, while HOMA-IR represents insulin resistance at fasting state. The ISI composite and the HOMA-IR values were similar in the HG group and the LG group in all three WHO categories. ISI composite values were 7.5 ± 0.6 vs. 9.2 ± 0.4 (NGT-HG vs. NGT-LG: n.s.), 6.9 ± 0.6 vs. 8.0 ± 0.8 (IFG-HG vs. IFG-LG: n.s.) and 7.0 ± 0.4 vs. 7.8 ± 0.7 (IGT-HG vs. IGT-LG: n.s.). HOMA-IR values were 1.2 ± 0.1 vs. 1.2 ± 0.1 (NGT-HG vs. NGT-LG: n.s.), 1.4 ± 0.1 vs. 1.2 ± 0.1 (IFG-HG vs. IFG-LG: n.s.) and 1.4 ± 0.1 vs. 1.4 ± 0.1 (IGT-HG vs. IGT-LG: n.s.).

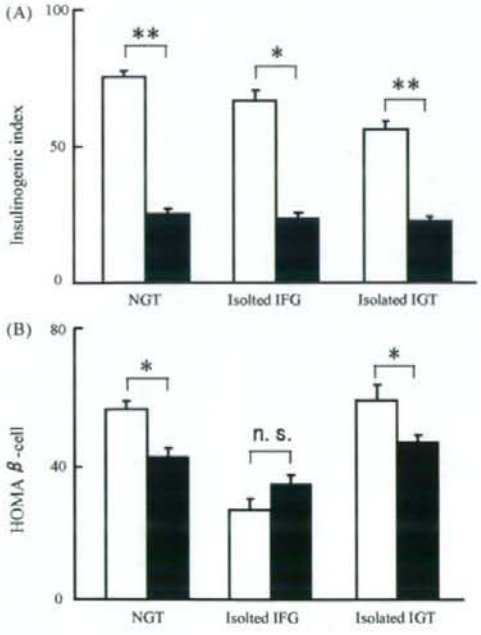

Fig. 1 – Indices of insulin secretion in six subgroups of three WHO categories: (A) insulinogenic index, (B) HOMA β-cell; light bars indicate subjects without elevated 1-h PG, dark bars indicate subjects with elevated 1-h PG. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LG, data are mean ± S.E.

In addition, we then analyzed the relationship between 1-h PG and the indices of insulin secretion and insulin sensitivity. Scattered plots of simple regression analysis between 1-hPG and the four indices are shown in Fig. 2. 1-h PG had a significant correlation with the insulinogenic index, HOMA β-cell, and ISI composite. Multiple regression analysis revealed that HOMA β-cell, ISI composite, and insulinogenic index were the independent factors in the variation of 30.0% in 1-h PG ($p < 0.001$). The correlation coefficients of these indices with 1-h PG in simple regression analysis, and the β values and p-values of multiple regression analysis are shown in Table 2. The insulinogenic index was the strongest factor to explain the 1-h PG levels.

4. Discussion

In the present study, we found that elevated 1-h PG is strongly associated with decreased insulinogenic index, indicating reduced capacity of early-phase insulin secretion [24,25]. The insulinogenic index in NGT-HG became remarkably lower than in NGT-LG at about 20, declining to the absolute levels of IFG-HG and IGT-HG. Multiple regression analysis showed that the insulinogenic index was the strongest factor among the four indices in elevated 1-h PG. These results indicate that decreased insulinogenic index is the major factor in elevated 1-h PG during oral glucose tolerance test. Since NGT-HG showed significantly higher area under the curve of glucose

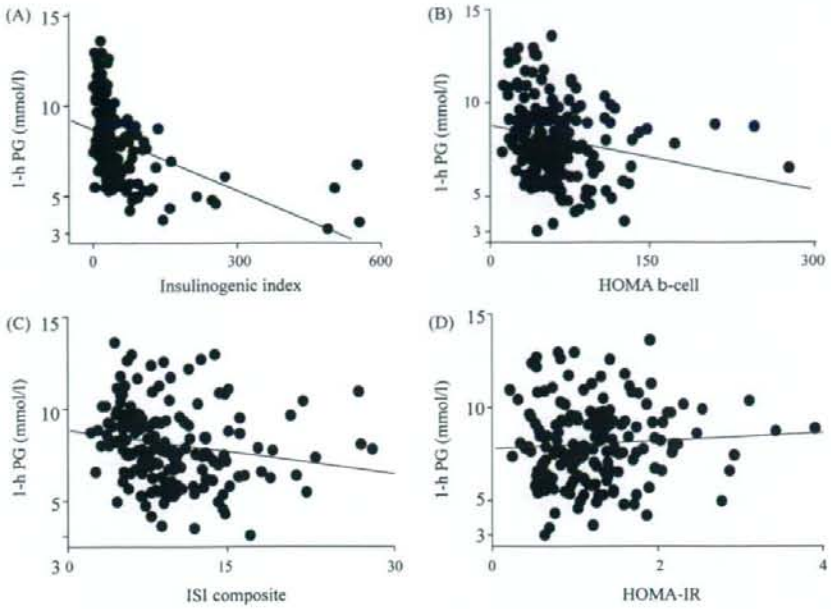

Fig. 2 – Relationship between 1-h PG and indices of insulin secretion and insulin sensitivity; (A) insulinogenic index, (B) HOMA β-cell, (C) ISI composite, (D) HOMA-IR; in insulin secretion, insulinogenic index and HOMA β-cell had significant relationships with 1-h PG ($r = -0.46$, $p < 0.001$, and $r = -0.2$, $p < 0.01$, respectively) In insulin sensitivity, there was a only mild significant relationship between 1-h PG and ISI composite ($r = -0.18$, $p < 0.05$). HOMA-IR had no significant relationship with 1-h PG ($r = 0.06$).

(G-AUC) compared to NGT-LG (19930 ± 256 vs. 15131 ± 181: $p < 0.05$), mildly impaired glucose tolerance due to reduced early-phase insulin secretion may already be present in NGT-HG. In addition, NGT-HG showed normal 2-h PG despite the elevated 1-h PG in the present study. Regarding the serum insulin level during OGTT, the 90 min insulin level in NGT-HG was significantly higher than in NGT-LG subjects (56.4 ± 7.3 vs. 40.3 ± 4.1: $p < 0.05$) in the cases we could analyze. Since late-phase insulin secretion in NGT-HG was sufficient to normalize 2-h PG, the regulatory mechanisms of elevated 1-h PG and 2-h PG are at least partly distinct.

HOMA β-cell measures insulin secretion capacity in the fasting state. HOMA β-cell values in NGT-HG and IGT-HG were significantly lower than those in NGT-LG and IGT-LG, but were similar to those in IFG-LG and IFG-HG. The values of HOMA β-cell is influenced with fasting PG per se. Isolated IFG subjects whose fasting PG levels are higher than those of NGT and isolated IGT had already low HOMA β-cell. It may explain for no significant difference between IFG-HG and IFG-LG in HOMA β-cell. Further studies are necessary to elucidate the other factors to influence HOMA β-cell in isolated IFG subjects.

Table 2 – Relationship of indices of insulin secretion and insulin sensitivity with 1-h PG

	Correlation coefficients	Standardized β	p-Value
Insulinogenic index	−0.47	0.42	<0.001
HOMA β-cell	−0.2	−0.31	<0.05
ISI composite	−0.18	−0.23	<0.05
HOMA-IR	0.06	0.13	n.s.

ISI composite and HOMA-IR are used to estimate insulin sensitivity [23]. We found both indices in LG and HG subjects to be similar in all three WHO categories. However, the insulin sensitivity of these subjects was higher than in Mexican Americans and Caucasians, as previously reported [26–28]. Since Japanese diabetes subjects are less obese than Caucasians, and insulin secretion rather than insulin sensitivity is the more important factor in the progression from NGT to diabetes in Japanese, it is likely that elevated 1-h PG in these subjects is mainly due to decreased early-phase insulin secretion rather than to impaired insulin sensitivity [19,29–31].

The ratio of NGT-HG subjects to total NGT subjects was 33% (58/121), while it was 69% (14/30) and 68% (32/71) for isolated IFG and isolated IGT subjects, respectively. The fact that the ratios increased similarly and progressively from NGT to isolated IFG and isolated IGT also suggests the use of 1-h PG as a marker to detect early stages of impaired glucose tolerance.

In conclusion, we have elucidated that impaired early-phase insulin secretion is strongly associated with an elevated 1-h PG level in Japanese subjects, suggesting that elevated 1-h PG may be a convenient marker to screen for decreased early-phase insulin secretion in early stage glucose intolerance.

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Conflict of interest

There are no conflict of interest.

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Glycemic instability in type 1 diabetic patients: Possible role of ketosis or ketoacidosis at onset of diabetes

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ABSTRACT

Aims: In type 1 diabetic patients, some have glycemic instability while others glycemic stability. We have developed criteria for evaluating glycemic instability and investigated the factors responsible.

Methods: Glycemic instability in 52 type 1 diabetic patients was assessed by the mean amplitude of glycemic excursions (MAGE) and M-value, and clinical characteristics of good, fair and poor control groups were compared.

Results: The median MAGE and M-value was 6.6 mmol/L and 18.7, respectively. Then MAGE ≥6.6 mmol/L and M-value ≥18.7 was defined as poor control. In the 32 patients without detectable C-peptide levels, 18 patients (56%) showed poor control. The frequency of ketosis or ketoacidosis at onset of diabetes was dramatically higher in the poor control group not only in the patients as a whole but also in those without detectable C-peptide levels.

Conclusions: A decreased level of C-peptide is a significant factor in glycemic instability. However, some patients have glycemic stability though β-cell function is completely depleted. The presence of ketosis or ketoacidosis at onset of diabetes may be a factor in later glycemic instability, suggesting the importance of examining patients in detail at onset of diabetes for careful follow-up to prevent progression of acute and chronic complications of diabetes.

1. Introduction

Type 1 diabetes mellitus is characterized by various forms of β-cell destruction, exhibiting various modes of onset [1]: acute-onset ('classical'), slow-onset [2] and fulminant [3]. While the incidence rate and mode of onset may differ among various populations, being 20-fold greater in Finland than in Japan, for example [4], β-cell destruction usually results in their complete loss and insulin-dependent diabetes mellitus (insulin-dependency) [5]. In patients with insulin-dependency, not only does the HbA_{1c} level increase, which represents the mean blood glucose level [6], but, as we previously reported [7], the blood glucose level remains unstable despite all efforts to optimize the use of exogenous insulin. Patients with glycemic

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instability are at increased risk of chronic macroangiopathic complications [8,9] as well as acute complications such as recurrent ketoacidosis [10], life-threatening hypoglycemia unawareness, and even "dead-in-bed syndrome" [11].

To quantify glycemic instability, the mean amplitude of glycemic excursions (MAGE) [12] and M-value [13] are commonly used [14,15]. However, glycemic instability has not been assessed by these indexes in a large population recently though the development of various new drugs has considerably facilitated glycemic control, and there are no established criteria for glycemic instability suitable for clinical use.

The blood glucose levels of some type 1 diabetic patients are as stable as those of type 2 diabetic patients [7]. To examine the distribution of glycemic instability and clarify the factors responsible for glycemic instability is of clinical importance for careful follow-up to prevent onset and progression of both acute and chronic secondary complications of diabetes.

In the present study, we assessed glycemic instability just before discharge after hospital treatment of at least a week period to exclude the influence of poor adherence to diet and exercise therapy, inappropriate use of exogenous insulin, and stressful circumstances of home or work place. We then compared the clinical characteristics and laboratory data of the patients with poor control and those with good or fair control to clarify the factors responsible.

2. Subjects and methods

2.1. Subjects

We examined all of the type 1 and type 2 diabetic patients admitted to Kyoto University Hospital from January, 2003 to July, 2007, except those meeting the exclusion criteria. A total of 52 type 1 diabetic patients (27 women and 25 men) (median age, 49 years; range, 16–80: median duration of diabetes, 6 years; range, 0–37) and 160 type 2 diabetic patients (64 women and 96 men) (median age, 65 years; range, 16–83: median duration of diabetes, 11 years; range, 0–53) were involved. American Diabetes Association Criteria was used as the criteria of type 1 diabetes [5]. Patients with renal insufficiency (Cre $\geq$ 1.5 mg/dl) were excluded, as were those with liver failure, acute illness such as infection, psychological comorbidities such as eating disorders, depression, needle phobia, those taking steroid medication, or inadequately monitoring blood glucose, and those on short-term admission (<7 days). No patients have learning disorders and apparent manipulative behaviour.

2.2. Assessment of glycemic instability

Seven capillary glucose measurements (before meals, 120 min after meals, and at bedtime) for two successive days just before discharge were analyzed to calculate the mean amplitude of glycemic excursions (MAGE) [12] and M-value, the indexes of glycemic instability. For calculating M-value, the modification [16] of the method of Schlichtkrull et al. [13,17] is commonly used: the average of M_{BS}^{BS} values; $M_{BS}^{BS} = |10 \times \log \frac{BS}{120}|^3$, which is the logarithmic transformation of the deviation of glycemia from the arbitrarily selected standard (120 mg/dl). Recently, 90 mg/dl is often used as the selected standard [18], and we used 100 mg/dl to give greater emphasis to hypoglycemia. Thus, in the present study, M-value is defined as the mean of M_{BS}^{BS} of each successive day: $M_{BS}^{BS} = |10 \times \log \frac{BS}{100}|^3$. The MAGE and M-value of the type 1 diabetic patients suggested a criterion for glycemic instability: MAGE $\geq$ the median and M-value $\geq$ the median representing poor control, MAGE < the first quartile and M-value < the first quartile indicating good control, and fair control comprising the middle ranges.

2.3. Factors responsible for glycemic instability

To clarify the factors responsible for glycemic instability, we compared the clinical characteristics and laboratory data of the good or fair groups and the poor control group of the 51 type 1 diabetic patients receiving intensive insulin therapy based on the guidelines by Japan Diabetes Society [19]. Ketosis was established by ketonuria, elevated serum ketones, or both. Serum and urinary C-peptide levels were measured using a commercially available EIA kit (ST AIA-PACK C-peptide, TOSOH corporation, Tokyo, Japan) with a detection limit of 0.1 ng/ml (intra-assay coefficient of variation [CV] 1.3–2.2%, interassay CV 1.7–2.0%) for serum C-peptide and 0.1 ng/dl (intra-assay CV 3.1–3.8%, inter-assay CV 1.9–2.1%) for urinary C-peptide. This kit has good reproductivity even when the level of C-peptide is lower than 1.0 ng/ml [20]. Diabetic sensorimotor distal symmetric polyneuropathy was assessed by pinprick, vibration perception threshold, and ankle reflexes. Autonomic function was evaluated by the coefficient of variation of the R-R interval (CVR-R) during deep breathing monitored on an electrocardiogram, and values below the reference values of healthy subjects [21] were counted abnormal. The presence of diabetic neuropathy was established by at least one abnormal result in the tests described above.

2.4. Statistical analysis

Qualitative variables were compared using Fisher's exact test. The Mann–Whitney U test was used to compare quantitative variables, which were expressed as medians and ranges. Two-sided P values <0.05 were considered statistically significant. Data were analyzed using StatView 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. Criteria for glycemic instability

The median MAGE of type 1 diabetic patients was 6.6 mmol/L, significantly higher than that of type 2 diabetic patients ($P < 0.001$) (Fig. 1A). The first quartile MAGE of type 1 diabetic patients was 4.3 mmol/L and the third quartile was 7.5 mmol/L. The median M-value of type 1 diabetic patients was 18.7, significantly higher than that of type 2 diabetic patients ($P < 0.05$) (Fig. 1B). The first quartile M-value of type 1 diabetic patients was 9.2 and the third quartile was 30.2. MAGE was

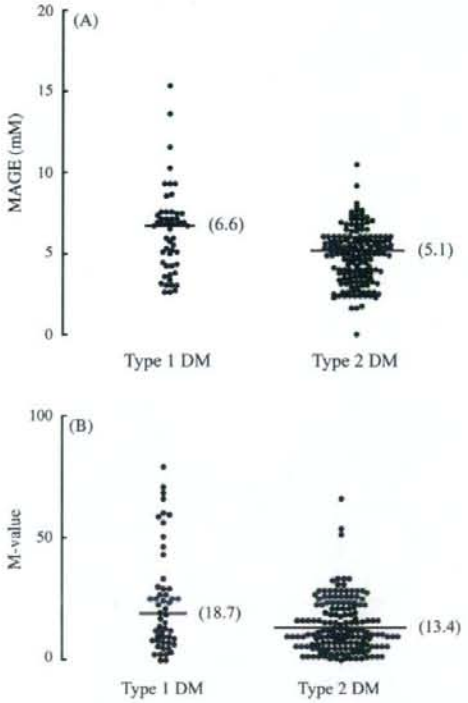

Fig. 1 – (A) MAGE of 52 type 1 diabetic patients and 160 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians. (B) M-value of 52 type 1 diabetic patients and 160 type 2 diabetic patients at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians.

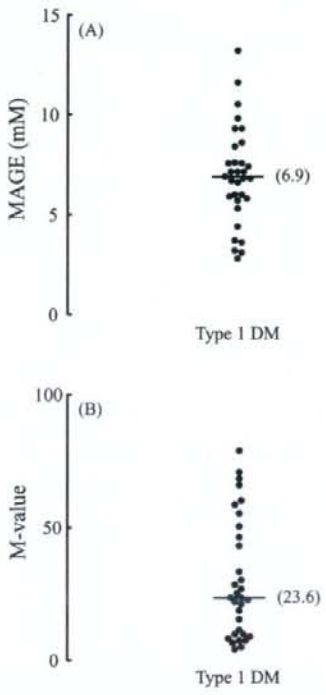

Fig. 2 – (A) MAGE of 32 type 1 diabetic patients without detectable C-peptide levels at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians. (B) M-value of 32 type 1 diabetic patients without detectable C-peptide levels at discharge who were admitted to Kyoto University Hospital. Horizontal lines represent medians.

significantly correlated with M-value ($r = 0.71$, $P < 0.0001$). Then MAGE≥6.6 mmol/L and M-value ≥18.7 was defined as poor control, MAGE <4.3 mmol/L and M-value <9.2 good control, and the middle ranges fair control. By these criteria, 57.7% of the type 1 diabetic patients exhibited good or fair control (good: 15.4%, fair: 42.3%, respectively), while 90.0% of the type 2 diabetic patients exhibited good or fair control (good: 20.6%, fair: 69.4%, respectively). Thus, type 1 diabetic patients exhibited significantly greater glycemic instability than type 2 diabetic patients ($P < 0.001$).

3.2. *Clinical characteristics and laboratory data in 51 type 1 diabetic patients*

Clinical characteristics and laboratory data of the good or fair groups vs. the poor control group in 51 type 1 diabetic patients are shown in Table 1. There were no significant differences between the good or fair groups and the poor control group regarding age, sex, BMI, duration of diabetes, IA-2 antibodies, insulin antibodies, HbA_{1c} level, the state of diabetic complications and thyroid function at present admission, age at onset, and GAD antibodies at onset. Serum C-peptide at prestimulation, post glucagon stimulation and urinary C-peptide at present admission were significantly lower in the poor control group than in the good or fair groups ($P < 0.05$). The frequency of ketosis or ketoacidosis at onset of diabetes was significantly higher in the poor control group compared to the good or fair groups ($P < 0.01$; risk ratio [RR] 3.5 [95% CI 1.4–9.0]). In other words, in those with ketosis or ketoacidosis at onset of diabetes, glycemic instability was markedly higher than in those without ketosis or ketoacidosis at onset of diabetes (14 of 23 vs. 4 of 23; $P < 0.01$; [RR] 3.5 [95% CI 1.4–9.0]). There were no significant differences regarding the frequency of ketosis or ketoacidosis at onset of diabetes between those with detectable C-peptide levels and those without (11 of 18 vs. 12 of 28; $P = 0.37$). The frequency of positive GAD antibodies (≥1.5 U/ml) at present admission was significantly lower in the poor control group than in the good or fair groups ($P < 0.05$; [RR] 2.3 [95% CI 1.1–4.8]).

3.3. *Glycemic instability in type 1 diabetic patients without detectable C-peptide levels*

Thirty-two type 1 diabetic patients without detectable serum C-peptide levels at post glucagon stimulation and without detectable urinary C-peptide levels in 24-h urine collections

Table 1 – Clinical characteristics of type 1 diabetic patients

Glycemic instability	Good or fair control	Poor control	P-value or RR (95% CI) good or fair vs. poor
	MAGE <6.6 or M-value <18.7	MAGE ≥6.6 and M-value ≥18.7	
Clinical characteristics at present admission			
Age (years)	49 (16–80)	43 (18–79)	0.76
Sex (female/male)	16/13	11/11	0.78
BMI (kg/m^2)	21.0 (17.8–26.9)	20.8 (16.1–25.3)	0.95
Duration of diabetes (years)	6 (0–37)	7.5 (0–31)	0.95
GAD antibodies (<1.5/≥1.5 U/ml)	11/17	16/6	0.024
			2.3 (1.1–4.8)
IA-2 antibodies (<0.4/≥0.4 U/ml)	7/3	6/2	0.99
Insulin antibodies (%)	10.2 (3.7–77.6)	9.8 (6.6–89.6)	0.67
HbA_{1c} (%)	8.1 (5.6–16.3)	8.3 (5.0–11.6)	0.73
Serum C-peptide at prestimulation (ng/ml)	0 (0–0.90)	0 (0–0.43)	0.023
Serum C-peptide at post glucagon stimulation (ng/ml)	0.15 (0–2.0)	0 (0–0.92)	0.010
Serum ΔC-peptide (ng/ml)	0.05 (0–1.63)	0 (0–0.49)	0.010
Urinary C-peptide (μg/day)	0 (0–57.7)	0 (0–21.0)	0.019
Diabetic retinopathy (NDR/NPDR/PDR)	22/3/4	14/8/0	0.78
Diabetic nephropathy (normoalbuminuria/ microalbuminuria/proteinuria)	25/3/1	17/5/0	0.59
Diabetic neuropathy (negative/positive)	14/15	8/14	0.27
CVR-R (%) (normal/abnormal)	11/10	8/6	0.99
Ankle brachial pressure index (ABI)	1.10 (0.88–1.24)	1.11 (0.72–1.23)	0.86
Carotid intima-media thickness (mm)	0.9 (0.6–1.5)	0.8 (0.7–1.6)	0.69
TSH (μU/ml)	1.80 (0.59–8.02)	1.31 (0–54.8)	0.62
Free T_4 (ng/dl)	1.22 (0.89–1.90)	1.14 (0.57–2.69)	0.42
Clinical characteristics at onset			
Age at onset (years)	40 (7–78)	34 (10–74)	0.52
Ketosis or ketoacidosis (negative/positive)	19/9	4/14	0.0058
			3.5 (1.4–9.0)
GAD antibodies (<1.5/≥1.5 U/ml)	3/18	6/8	0.11
			2.2 (1.0–4.5)

Data are median (range) or number of patients. Serum C-peptide, urinary C-peptide, GAD antibodies and IA-2 antibodies below detection limits are expressed as 0.

were then selected. Of these patients, 18 patients (56%) showed poor control, and the other 14 patients (44%) showed good or fair control (MAGE <6.6 mmol/L in 12 patients, M-value <18.7 in 12 patients) (Fig. 2A and B). In contrast, of the 19 patients with detectable C-peptide levels, 15 (79%) showed good or fair control, and the other 4 (21%) showed poor control.

The good or fair and poor control groups in type 1 diabetic patients without detectable C-peptide levels were then compared. The frequency of ketosis or ketoacidosis at onset was dramatically higher in the poor control group than in the good or fair groups (11 [73%] of 15 vs. 1 [8%] of 13; $P < 0.001$; [RR] 3.7 [95% CI 1.5–8.7]). In other words, of the 13 patients without detectable C-peptide levels but with glycemic stability, 12 patients did not have ketosis or ketoacidosis at onset. The frequency of positive GAD antibodies at present admission was significantly lower in the poor control group than in the good or fair groups (3 [17%] of 18 vs. 8 [62%] of 13; $P < 0.05$; [RR] 2.8 [95% CI 1.0–7.5]).

3.4. *Glycemic instability in acute-onset type 1 diabetic patients*

In the 51 type 1 diabetic patients, 38 patients had acute-onset ('classical') type 1 diabetes, 6 patients fulminant type 1 diabetes [3], and 2 patients latent autoimmune diabetes of adults [2], and 5 patients unknown. Thirty-eight acute-onset type 1 diabetic patients were then examined to exclude the influence of fulminant type 1 diabetes, because the presence of ketosis or ketoacidosis at onset of diabetes, which has been found to be involved in glycemic instability in the present study, is a characteristic of fulminant type 1 diabetes [3], and glycemic instability in fulminant type 1 diabetes was markedly greater than in acute-onset type 1 diabetes (good: 0 of 6, fair: 1 of 6, poor: 5 of 6 vs. good: 6 of 38, fair: 18 of 38, poor: 14 of 38, $P < 0.05$). In acute-onset type 1 diabetic patients, the frequency of ketosis or ketoacidosis at onset was significantly higher in the poor control group than in the good or fair groups (8 [73%] of 11 vs. 7 [29%] of 24; $P < 0.05$; [RR] 3.6 [95% CI 1.1–11.2]).

4. Discussion

It has been reported that factors that influence glycemic instability are deficiency of endogenous insulin secretion [22,23], abnormal response of insulin-counteracting hormones [22,24,25], poor compliance with diet and exercise therapy, stressful life circumstances [26], and inappropriate use of exogenous insulin [27,28].

In the present study, we assessed glycemic instability by MAGE and M-value in 52 type 1 and 160 type 2 diabetic

patients, and proposed criteria for glycemic instability suitable for clinical use. MAGE and M-value of most of the type 2 diabetic patients showed good or fair glycemic stability.

Analysis of the 51 type 1 diabetic patients showed that serum C-peptide at post glucagon stimulation and urinary C-peptide at present admission were significantly lower in the poor control group than in the good or fair groups. In addition, of the 32 patients without detectable C-peptide levels, 18 (56%) showed poor control. These results indicate that decreased endogenous insulin secretion is a significant factor in glycemic instability. However, of the 32 patients without detectable C-peptide levels, 14 (44%) exhibited good or fair control. In addition, of these patients, MAGE of 6 (19%) and the M-value of 10 (31%) were lower than the median values of the type 2 diabetic patients. These results demonstrate that some patients with type 1 diabetic patients have glycemic stability though β-cell function is completely depleted.

The present study suggests that the presence of ketosis or ketoacidosis at onset of diabetes is a factor in later glycemic instability. This is not simply due to the including of fulminant type 1 diabetic patients, because analysis of acute-onset type 1 diabetic patients also showed that the frequency of ketosis or ketoacidosis at onset was significantly higher in the poor control group than in the good or fair groups. Moreover, analysis of the type 1 diabetic patients without detectable C-peptide levels also showed that the frequency of ketosis or ketoacidosis at onset was dramatically higher in the poor control group than in the good or fair groups. The same tendency of the higher frequency of ketosis or ketoacidosis at onset in the poor control group was shown in the patients with detectable C-peptide levels. These results suggest that the presence of ketosis or ketoacidosis at onset is a factor in later glycemic instability, and other factors than C-peptide also exist as the underlying mechanism besides decreased levels of C-peptide.

Then, what is the mechanism whereby ketosis or ketoacidosis at onset of diabetes leads to later glycemic instability? One possibility is decreased β-cell functional reserve as mentioned above. Other factors than C-peptide underlying the mechanism may be the increased levels of counter-regulatory hormones inducing insulin resistance, including glucagon, epinephrine, cortisol and growth hormone [29,30]. Glucagon levels are markedly elevated in certain patients with ketoacidosis including those having preserved endogenous insulin secretion [30]. In addition, not only insulin-dependent diabetic patients but also noninsulin-dependent diabetic patients with hyperglycemia display paradoxical hyperglucagonemia, which contributes to postprandial hyperglycemia and glycemic instability [24,31].

In addition, there have been no studies of type 1 diabetic patients to clarify the association of GAD antibodies with glycemic instability. The present study indicates that negative GAD antibodies at onset may contribute to later glycemic instability. This may be because there is an inverse relation between the level of GAD antibodies and β-cell-destructive T-cell responses [32] or partly because patients with fulminant type 1 diabetes were included.

The present study has limitations in that this is a cross-sectional study and we have little C-peptide data at onset of diabetes. However, the frequency of ketosis or ketoacidosis at onset was clearly higher in the poor control group than in the good or fair groups not only in the patients as a whole but also in those without detectable C-peptide levels at present admission.

In summary, we confirmed that a decreased level of C-peptide is a significant factor in glycemic instability. However, the present study showed that some patients with type 1 diabetic patients have glycemic stability though β-cell function is completely depleted. The present study suggested that ketosis or ketoacidosis at onset of diabetes is a factor in later glycemic instability. In addition, GAD antibodies at onset may also influence later glycemic instability. These results suggest the importance of examining patients in detail at the onset of diabetes. Patients with glycemic instability are at increased risk of both acute and chronic complications of diabetes, so patients exhibiting ketosis or ketoacidosis at onset of type 1 diabetes should be carefully monitored.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ARTICLE

Src activation generates reactive oxygen species and impairs metabolism–secretion coupling in diabetic Goto–Kakizaki and ouabain-treated rat pancreatic islets

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Abstract
Aims/hypothesis Na^+/K^+-ATPase inhibition by ouabain suppresses ATP production by generating reactive oxygen species (ROS) and impairs glucose-induced insulin secretion from pancreatic islets. To clarify the signal-transducing function of Na^+/K^+-ATPase in decreasing ATP production by the generation of ROS in pancreatic islets, the involvement of Src was examined. In addition, the significance of Src activation in diabetic islets was examined.
Methods Isolated islets from Wistar rats and diabetic Goto–Kakizaki (GK) rats (a model for diabetes) were used. ROS was measured by 5-(and 6)-chloromethyl-2′,7′-dichlorofluorescein fluorescence using dispersed islet cells. After lysates were immunoprecipitated by anti-Src antibody, immunoblotting was performed.
Results Ouabain caused a rapid Tyr^{418} phosphorylation, indicating activation of Src in the presence of high glucose. The specific Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) restored the ouabain-induced decrease in ATP content and the increase in ROS production. Both PP2 and ROS scavenger restored the impaired insulin release and impaired ATP elevation in GK islets, but had no such effect in control islets. PP2 reduced the high glucose-induced increase in ROS generation in GK islet cells but had no effect on that in control islet cells. Moreover, ouabain had no effect on ATP content and ROS production in the presence of high glucose in GK islets.
Conclusions/interpretation These results indicate that Src plays a role in the signal-transducing function of Na^+/K^+-ATPase, in which ROS generation decreases ATP production in control islets. Moreover, ROS generated by Src activation plays an important role in impaired glucose-induced insulin secretion in GK islets, in which Src is endogenously activated independently of ouabain.

Keywords ATP · GK rat · Na^+/K^+-ATPase · Pancreatic islet · ROS · Src

Abbreviations

$\Delta\Psi_m$	change in mitochondrial membrane potential
CM-DCF	5-(and 6)-chloromethyl-2′,7′-dichlorofluorescein
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GK	Goto–Kakizaki
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide
KRBB	Krebs Ringer bicarbonate buffer
ROS	reactive oxygen species
PP2	4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine

Introduction

In pancreatic beta cells, intracellular glucose metabolism regulates exocytosis of insulin granules according to metabolism–secretion coupling, in which glucose-induced

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mitochondrial ATP production plays an essential role [1]. Since depletion of mitochondrial DNA abolishes the glucose-induced ATP elevation, mitochondria clearly are a major source of ATP production in pancreatic beta cells [2, 3]. Glucose-induced insulin secretion from beta cells is often impaired by exposure to high concentrations of fuels including glucose, NEFAs and ketone bodies, and by administration of diabetogenic pharmacological agents, all of which involve impaired glucose-induced ATP elevation in beta cells [4–11]. Thus, reduced mitochondrial ATP production plays an important role in impaired glucose-induced insulin secretion.

Among the various agents that impair metabolism–secretion coupling in beta cells, the effects of reactive oxygen species (ROS) on glucose-induced insulin secretion have been extensively examined. Exposure to exogenous hydrogen peroxide (H_2O_2), the most abundant ROS, reduces glucose-induced insulin secretion by impairing mitochondrial metabolism in beta cells. Transient exposure to H_2O_2 suppresses the hyperpolarisation of mitochondrial membrane potential [12], the increment in insulin secretion, and the increase in ATP content induced by glucose in pancreatic beta cells [12, 13].

However, little is known of the role of endogenous ROS in impaired glucose-induced insulin secretion. Recent studies have shown that mitochondria produce endogenous ROS in beta cells under physiological and pathophysiological conditions. Exposure to high glucose increases mitochondrial ROS production [14, 15], and the superoxide content of islets from Zucker diabetic fatty rats is higher than that from Zucker lean control islets under a basal level of glucose but are relatively insensitive to high glucose [14].

Ouabain, a well-known specific inhibitor of Na^+/K^+-ATPase, decreases glucose-induced insulin release in the second phase [16]. We have found that ouabain decreases glucose-induced insulin release by reducing ATP content [17]. In addition, high glucose-induced hyperpolarisation of mitochondrial membrane potential was inhibited by ouabain. Furthermore, ouabain induced mitochondrial ROS production that was blocked by myxothiazol, an inhibitor of site III of the mitochondrial respiratory chain. Interestingly, these phenomena also occurred in Ca^{2+}- or Na^+-depleted conditions. An antioxidant, α-tocopherol, blocked the ouabain-induced ROS increase as well as the suppressive effect of ouabain on ATP production and insulin release. However, ouabain did not directly affect ATP production from the mitochondrial fraction. These results suggest that ouabain suppresses mitochondrial ATP production by generating mitochondrial ROS via signal transduction, independently of the intracellular cationic alternation, and has a suppressive effect on insulin secretion.

However, the details of Na^+/K^+-ATPase-mediated signal transduction in suppressing ATP production by the generation of mitochondrial ROS in pancreatic islets remain unknown. The binding of ouabain to Na^+/K^+-ATPase has been shown to activate Src, a non-receptor protein-tyrosine kinase, subsequently enhancing mitochondrial ROS production in cardiac myocytes [18–20]. In the present study, we investigated the involvement of Src in the signal-transducing function of Na^+/K^+-ATPase that reduces ATP production by generating mitochondrial ROS in pancreatic islets. In addition, the role of Src activation in impaired glucose-induced insulin secretion from diabetic islets was examined.

Methods

Animals Male Wistar and Goto–Kakizaki (GK) rats were obtained from Shimizu (Kyoto, Japan). The animals were fed standard laboratory chow ad libitum and allowed free access to water in an air-conditioned room with a 12 h light:12 h darkness cycle until used in the experiments. All experiments were carried out with rats aged 8–12 weeks. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University.

Islet isolation and culture Islets of Langerhans were isolated from Wistar and GK rats by collagenase digestion as described previously [21]. Isolated islets were cultured for 12 h in RPMI 1640 medium containing 10% (vol./vol.) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 5.5 mmol/l glucose, at 37°C in humidified air containing 5% CO_2.

Solutions The medium used for islet isolation and preincubation of intact islets was Krebs Ringer bicarbonate buffer containing (in mmol/l) 129.4 NaCl, 3.7 KCl, 2.7 $CaCl_2$, 1.3 KH_2PO_4, 1.3 $MgSO_4$, 24.8 $NaHCO_3$ (equilibrated with 5% CO_2–95% O_2, pH 7.4), and 0.2% (vol./vol.) BSA, hereafter referred to as KRBB. Ca^{2+}-free media were prepared with Ca^{2+}-free KRBB plus 1 mmol/l EGTA and 10 mmol/l HEPES (Ca^{2+}-free KRBB).

Measurement of ATP content After groups of ten islets were preincubated in KRBB with 2.8 mmol/l glucose for 30 min, they were batch-incubated for the indicated times in 0.5 ml Ca^{2+}-free KRBB with 2.8 or 16.7 mmol/l glucose with or without test materials. 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3, 4-d]pyrimidine (PP2) and α-tocopherol plus ascorbate were also included during preincubation. After immediate addition of $HClO_4$, sonication in ice-cold water for 3 min, and centrifugation, part of the supernatant fraction was mixed with HEPES and Na_2CO_3 and the ATP

content in islets was determined by luminometry as previously described [22].

Fluorescence measurement of ROS production and change in mitochondrial membrane potential ROS production and change in mitochondrial membrane potential ($\Delta\Psi_m$) in dispersed islet cells under Ca^{2+}-free conditions were measured by 5-(and 6)-chloromethyl-2′,7′-dichlorofluorescein (CM-DCF) fluorescence and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) fluorescence, respectively, as previously reported [17]. Fluorescence was corrected by subtracting parallel blanks in which islet cells were not loaded with probes, and is presented as a ratio with respect to the value at time zero.

Measurement of phosphorylation of Src Activation of Src in islets was determined by Western blotting after immunoprecipitation. After preincubation in KRBB containing 2.8 mmol/l glucose, islets were exposed to ouabain in Ca^{2+}-free KRBB or KRBB with 16.7 mmol/l glucose for the indicated times. After washing with ice-cold PBS, the islets were solubilised in ice-cold lysis buffer containing 10 mmol/l Tris–HCl (pH 7.2), 100 mmol/l NaCl, 1 mmol/l EDTA, 5 mmol/l sodium pyrophosphate, 0.5% sodium deoxycholate, 1% Nonidet P-40, protease inhibitor cocktail tablet (Roche, Penzberg, Germany) and phosphatase inhibitor cocktail set II (Calbiochem, Darmstadt, Germany) and sonicated. Cell lysates were centrifuged (560,000×*g* for 10 min at 4°C) to obtain crude cell extracts. Protein content of the supernatant was measured and adjusted by the Bradford method. The supernatant was mixed with 4 μg monoclonal anti-Src antibody (mouse monoclonal IgG_1, clone GD11; Upstate, Lake Placid, NY, USA) and 30 μl of washed Protein-G agarose beads, and gently rotated for 4 h at 4°C. After washing three times with ice-cold lysis buffer, immunoprecipitates were dissolved in 30 μl SDS-PAGE sample buffer (50 mmol/l Tris–HCl [pH 6.8], 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 1% bromophenol blue) and boiled for 5 min at 95°C. The samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). After blocking with PBS containing 0.1% Tween 20 and 5% BSA (blocking buffer) overnight at 4°C, blotted membranes were incubated overnight with rabbit polyclonal anti-Src antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C in blocking buffer, and subsequently with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Tokyo, Japan) for 1 h prior to detection using ECL Plus (Amersham Biosciences). In the same membrane, the process was repeated for the following primary phosphospecific antibodies: rabbit polyclonal antibody to Src phosphorylated at Tyr^{418} (pY^{418}Src) or rabbit polyclonal antibody to Src phosphorylated at Tyr^{529} (pY^{529}Src; Biosource, Camarillo, CA, USA) and mouse polyclonal anti-phosphotyrosine antibody (pY; clone 4G10; Upstate). Anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) was used to detect the mouse primary antibody.

Measurement of glucose oxidation Glucose oxidation was measured as previously described [8]. Cultured islets were preincubated in KRBB with 2.8 mmol/l glucose in the presence or absence of Src inhibitor and antioxidants at 37° C for 30 min. Twenty-five islets in a small tube were incubated at 37°C for 90 min in 150 μl Ca^{2+}-free KRBB containing 2.8 or 16.7 mmol/l glucose, test materials, and [U-^{14}C]glucose (1.85×10^4 Bq per tube) (Amersham, Buckinghamshire, UK). After 90 min incubation, the reaction was stopped, and the dpm of trapped $^{14}CO_2$ in the hydroxide of hyamine 10-X (Packard, Meriden, CT, USA) was counted.

Measurement of insulin release, insulin content and DNA content Insulin release from cultured islets was monitored using static incubation as described previously [17]. After an aliquot of incubation medium for insulin assay was taken, the islets remaining were lysed to determine insulin and DNA contents as described previously [22].

Materials RPMI 1640 medium, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), α-tocopherol and L-ascorbic acid were purchased from Sigma (St Louis, MO, USA). Luciferin-luciferase was obtained from Turner Designs (Sunnyvale, CA, USA). CM-DCFH diacetate and JC-1 were purchased from Invitrogen (Eugene, OR, USA). PP2, herbimycin A and SU6656 were purchased from Calbiochem (La Jolla, CA, USA). All other agents including ouabain were obtained from Nacalai Tesque (Kyoto, Japan).

Statistical analysis Results are expressed as means±SE. Statistical significance was evaluated by an unpaired Student's t test. $p<0.05$ was considered significant.

Results

Effect of ouabain on ATP content Exposure to 16.7 mmol/l glucose for 15 min increased ATP content compared with that in the presence of 2.8 mmol/l glucose (at 15 min, 16.7 mmol/l glucose: 17.1±0.9 vs 2.8 mmol/l glucose: 8.5±0.2 pmol/islet; $p<0.01$; Fig. 1a). For the 60 min incubation, ATP content remained high in the presence of 16.7 mmo/l glucose compared with that in the presence of 2.8 mmol/l glucose. Exposure to 1 mmol/l ouabain for