Gotoh N, Nakanishi H, Hayashi H, Yamada R, Otani A, Tsujikawa A, Yamashiro K, Tamura H, Saito M, Saito K, Iida T, Matsuda F, Yoshimura N.	*ARMS2 (LOC387715)* variants in Japanese patients with exudative age-related macular degenerationand polypoidal choroidal vasculopathy.	***Am. J. Ophthalmol.***	147(6)	1037-1041	2009
Tocharoentanaphol C, Promso S, Zelenika D, Lowhnoo T, Tongsima S, Sura T, Chantratita W, Matsuda F, Mooney S, Sakuntabhai A.	Evaluation of resequencing on number of tag SNPs of 13 atherosclerosis-related genes in Thai population.	***J. Hum. Genet.***	53(1)	74-86	2008
Hung RJ, McKay JD, Gaborieau V, Boffetta P, Hashibe M, Zaridze D, Mukeria A, Szeszenia-Dabrowska N, Lissowska J, Rudnai P, Fabianova E, Mates D, Bencko V, Foretova L, Janout V, Chen C, Goodman G, Field JK, Liloglou T, Xinarianos G, Cassidy A, McLaughlin J, Liu G, Narod S, Krokan HE, Skorpen F, Elvestad MB, Hveem K, Vatten L, Linseisen J, Clavel-Chapelon F, Vineism P, Bueno-de-Mesquita HB, Lund E, Martinez C, Bingham S, Rasmuson T, Hainaut P, Riboli E, Ahrens W, Benhamou S, Lagiou P, Trichopoulos D, Holcátová I, Merletti F, Kjaerheim K, Agudo A, Macfarlane G, Talamini R,	A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25.	***Nature***	452(7187)	633-637	2008

Simonato L, Lowry R, Conway DI, Znaor A, Healy C, Zelenika D, Boland A, Delepine M, Foglio M, Lechner D, Matsuda F, Blanche H, Gut I, Heath S, Lathrop M, Brennan P.					
Gotoh N, Yamada R, Matsuda F, Yoshimura N, Iida, T.	Manganase superoxide dismutase gene (*SOD2*) polymorphism and exudative age-related macular degeneration in the Japanese population.	***Am. J. Ophthalmol.***	146(1)	146-147	2008
Trégouet DA, Groop PH, McGinn S, Forsblom C, Hadjadj S, Marre M, Parving HH, Tarnow L, Telgmann R, Godefroy T, Nicaud V, Rousseau R, Parkkonen M, Hoverfält A, Gut I, Heath S, Matsuda F, Cox R, Kazeem G, Farrall M, Gauguier D, Brand-Herrmann	G/T substitution in intron-1 of UNC13B gene is associated with increased risk of nephropathy in patients with type 1 diabetes.	***Diabetes***	57(10)	2843-2850	2008

SM, Cambien F, Lathrop M, Vionnet N. For the EURAGEDIC Consortium.					
The SEARCH Collaborative Group, Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F, Gut I, Lathrop M, Collins R.	SLCO1B1 variants and statin-induced myopathy--a genomewide study.	*N. Engl. J. Med.*	359(8)	789-799	2008
Yamano E, Isowa T, Nakano Y, Matsuda F, Hashimoto-Tamaoki T, Ohira H, Kosugi S.	Association study between reward dependence temperament and a polymorphism in the phenylethanolamine N-methyltransferase gene in a Japanese female population.	*Compr. Psychiatry.*	49(5)	503-507	2008
Gotoh N, Yamada R, Nakanishi H, Saito M, Iida T, Matsuda F, Yoshimura N.	Correlation between CFH Y402H and HTRA1 rs11200638 genotype to typical exudative age-related macular degeneration and polypoidal choroidal vasculopathy phenotype in the Japanese population.	*Clin. Experiment. Ophthalmol.*	36(5)	437-442	2008

McKay JD, Hung RJ, Gaborieau V, Boffetta P, Chabrier A, Byrnes G, Zaridze D, Mukeria A, Szeszenia-Dabrowska N, Lissowska J, Rudnai P, Fabianov E, Mates D, Bencko V, Foretova L, Janout V, McLaughlin J, Shepherd F, Montpetit A, Narod S, Krokan HE, Skorpen F, Elvestad MB, Vatten L, Njølstad I, Axelsson T, Chen C, Goodman G, Barnett M, Loomis MM, Lubiński J, Matyjasik J, Lener M, Oszutowska D, Field J, Liloglou T, Xinarianos G, Cassidy A; EPIC Study, Vineis P, Clavel-Chapelon F, Palli D, Tumino R, Krogh V, Panico S, González CA, Ramón Quirós J, Martínez C, Navarro C, Ardanaz E, Larrañaga N, Kham KT, Key T, Bueno-de-Mesquita HB, Peeters PH, Trichopoulou A, Linseisen J, Boeing H, Hallmans G, Overvad K, Tjønneland A, Kumle M, Riboli E, Zelenika D, Boland A, Delepine M, Foglio M, Lechner D, Matsuda F, Blanche H, Gut I, Heath S, Lathrop M, Brennan P.	Lung cancer susceptibility locus at 5p15.33.	*Nat. Genet.*	40(12)	1404-1406	2008

Taniguchi T, Kobayashi H, Nishikawa K, Iida E, Michigami Y, Morimoto E, Yamashita R, Miyagi K, Okamoto M.	Diffusion-weighted magnetic resonance imaging in autoimmune pancreatitis.	*Jpn. J. Radiol.*	27(3)	138-142	2009
Taniguchi T, Hamasaki A, Okamoto M.	Subclinical hypercortisolism in hospitalized patients with type 2 diabetes mellitus.	*Endocr. J.*	55(2)	429-432	2008
Koshiyama H, Taniguchi A, Tanaka K, Kagimoto S, Nakamura Y, Iwakura A, Kuroe A, Ohya M, Yamamoto T, Fujimoto S, Hamamoto Y, Honjo S, Ikeda H, Nabe K, Tsuda K, Inagaki N, Seino Y, Fujioka Y, Hirata K, Kume N.	Effect of pitavastatin on lipid profile and high-sensitivy CRP in Japanese subjects with hypercholesterolemia: Kansai Investigation of Statin for Hyperlipidemic Intervention in Metabolism and Endocrinology (KISHIMEN) Investigators.	*J. Atheroscler Thromb.*	15(6)	345-350	2008
Matsunaga T, Gu N, Yamazaki H, Adachi T, Yasuda K, Moritani T, Tsuda K, Nishiyama T, Nonaka M.	Association of estrogen receptor-alpha gene polymorphisms with cardiac autonomic nervous activity in healthy young Japanese males.	*Clin. Chim. Acta.*	411(7-8)	505-509	2010
Matsunaga T, Gu N, Yamazaki H, Tsuda M, Adachi T, Yasuda K, Moritani T, Tsuda K, Nonaka M, Nishiyama T.	Association of UCP2 and UCP3 polymorphisms with heart rate variability in Japanese men.	*J. Hypertens.*	27(2)	305-313	2009
Gu N, Tsuda M, Matsunaga T, Adachi T, Yasuda K, Ishihara A, Tsuda K.	Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells.	*Clin. Exp. Pharmacol. Physiol.*	35(12)	1433-1439	2008

BJ www.biochemj.org

Biochem. J. (2011) 435, 421–430 (Printed in Great Britain) doi:10.1042/BJ20101708

Role of mitochondrial phosphate carrier in metabolism–secretion coupling in rat insulinoma cell line INS-1

Yuichi NISHI, Shimpei FUJIMOTO[1], Mayumi SASAKI, Eri MUKAI, Hiroki SATO, Yuichi SATO, Yumiko TAHARA, Yasuhiko NAKAMURA and Nobuya INAGAKI

Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606–8507, Japan

In pancreatic β-cells, glucose-induced mitochondrial ATP production plays an important role in insulin secretion. The mitochondrial phosphate carrier PiC is a member of the SLC25 (solute carrier family 25) family and transports P_i from the cytosol into the mitochondrial matrix. Since intramitochondrial P_i is an essential substrate for mitochondrial ATP production by complex V (ATP synthase) and affects the activity of the respiratory chain, P_i transport via PiC may be a rate-limiting step for ATP production. We evaluated the role of PiC in metabolism–secretion coupling in pancreatic β-cells using INS-1 cells manipulated to reduce PiC expression by siRNA (small interfering RNA). Consequent reduction of the PiC protein level decreased glucose (10 mM)-stimulated insulin secretion, the ATP:ADP ratio in the presence of 10 mM glucose and elevation of intracellular calcium concentration in response to 10 mM glucose without affecting the mitochondrial membrane potential ($\Delta\psi_m$) in INS-1 cells. In experiments using the mitochondrial fraction of INS-1 cells in the presence of 1 mM succinate, PiC down-regulation decreased ATP production at various P_i concentrations ranging from 0.001 to 10 mM, but did not affect $\Delta\psi_m$ at 3 mM P_i. In conclusion, the P_i supply to mitochondria via PiC plays a critical role in ATP production and metabolism–secretion coupling in INS-1 cells.

Key words: inorganic phosphate (P_i), insulin secretion, mitochondria, mitochondrial phosphate carrier (PiC), small interfering RNA (siRNA), solute carrier family 25 (SLC25).

INTRODUCTION

Glucose stimulates insulin secretion by both triggering and amplifying signals in pancreatic β-cells [1]. The triggering pathway includes entry of glucose into β-cells, acceleration of glycolysis in the cytosol and mitochondrial metabolism of products derived from glycolysis, increase in ATP content and ATP/ADP ratio, closure of ATP-sensitive K^+ channels (K_{ATP} channels), membrane depolarization, opening of VDCCs (voltage-dependent Ca^{2+} channels), increase in Ca^{2+} influx through VDCCs, rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and exocytosis of insulin granules. Glucose also exerts its effects by increasing Ca^{2+} efficacy in stimulation–secretion coupling via an amplifying pathway, owing at least in part to the direct effect of increased ATP derived from glucose metabolism on exocytosis. Since depletion of mitochondrial DNA abolishes the glucose-induced ATP elevation, mitochondria are clearly a major source of ATP production in pancreatic β-cells [2,3]. Collectively, in pancreatic β-cells, intracellular glucose metabolism regulates exocytosis of insulin granules according to metabolism–secretion coupling in which glucose-induced mitochondrial ATP production plays an important role.

Almost all of the mitochondrial carrier proteins are embedded in the inner membranes of mitochondria, where they transport solutes across the membrane. They belong to the SLC25 (solute carrier family 25) group of proteins [4]. Several members of the SLC25 group have been reported to play roles in GSIS (glucose-stimulated insulin secretion) in pancreatic β-cells. Overexpression or silencing of AGC1 (aspartate/glutamate carrier 1; SLC25A12 or Aralar1) has been reported to increase or reduce GSIS in INS-1E cells respectively [5,6]. Overexpression of UCP2 (uncoupling protein 2; SLC25A8) by adenovirus vector is known to inhibit GSIS from rat islets [7], whereas GSIS from islets of UCP2-deficient mice is enhanced compared with that from control islets [8]. In addition, down-regulation of OGC (2-oxoglutarate carrier; SLC25A11), CIC (citrate/isocitrate carrier; SLC25A1) and GC1 (glutamate carrier 1; SLC25A22) by siRNA (small interfering RNA) suppress GSIS [9–11].

The mitochondrial phosphate carrier PiC (SLC25A3) is a member of the SLC25 family and transports P_i from the cytosol into the mitochondrial matrix. The *PiC* gene has 9 exons; the 3rd and the 4th exons are called exon 3A and exon 3B respectively. These two exons are alternatively spliced and two isoforms of PiC, PiC-A and PiC-B, are generated [12]. They differ considerably in their kinetic parameters as previously shown in a study using a reconstitution system [13]. The K_m of PiC-A for P_i on the external membrane surface is 3-fold that of PiC-B (PiC-A: ~2.2 mM; PiC-B: ~0.78 mM). The K_m on the internal surface is much higher (PiC-A: ~9.7 mM; PiC-B: ~6.3 mM) than K_m on the external membrane surface. The maximum transport rate of PiC-A is approximately a third that of PiC-B. These isoforms also differ in their tissue distribution. PiC-A is expressed in skeletal muscle and cardiac muscle, whereas PiC-B is expressed ubiquitously [13,14]. A case study of patients with PiC-A deficiency who suffered from lactic acidosis, heart failure and muscle weakness and died within the first year of life, demonstrates the critical significance of this carrier [15].

Since intramitochondrial P_i is an essential substrate for mitochondrial ATP production by complex V (ATP synthase) and affects activity of the respiratory chain [16], the supply of P_i from

Abbreviations used: AAC, ATP/ADP carrier; DAPP, diadenosine pentaphosphate; DIC, dicarboxylate carrier; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs-Ringer bicarbonate Hepes buffer; RT, reverse transcription; siRNA, small interfering RNA; SLC25, solute carrier family 25; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

[1] To whom correspondence should be addressed (email fujimoto@metab.kuhp.kyoto-u.ac.jp).

cytosol to mitochondrial matrix via PiC may be a rate-limiting step for ATP production. However, precise detection of PiC and its significance in metabolism–secretion coupling in pancreatic β-cells has not been reported previously. In the present study, the role of PiC in metabolism–secretion coupling in pancreatic β-cells is evaluated using INS-1 cells manipulated to reduce PiC expression.

EXPERIMENTAL

Materials

ATP, ADP, poly-L-ornithine, DAPP (diadenosine pentaphosphate), Safranin O, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), ATP sulfurylase and Na_2MoO_4 were purchased from Sigma. Hepes, KCl, EGTA, sodium pyruvate, $MgSO_4$, NaH_2PO_4, $CaCl_2$, glucose, NaCl, $NaHCO_3$, $HClO_4$, Na_2CO_3, pyruvate kinase, BSA, KOH, potassium gluconate and KH_2PO_4 were purchased from Nacalai. 2-mercaptoethanol, penicillin, streptomycin and mouse monoclonal antibodies to the subunits of the mitochondrial respiratory chain complexes were purchased from Invitrogen. Luciferin-luciferase was purchased from Promega.

Cell culture

INS-1 (rat insulinoma) cells were cultured in RPMI 1640 medium containing 11.1 mM glucose (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere (5 % CO_2 and 95 % air). COS-7 (African green monkey kidney) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere (5 % CO_2 and 95 % air).

siRNA transfection

Stealth™ siRNAs were synthesized by Invitrogen. The sequences of siRNAs specific for both rat PiC-A and PiC-B were: 5′-AAAUAUGCCCUUGUACUUCUGAGGG-3′ and 5′-CCCUCAGAAGUACAAGGGCAUAUUU-3′ designated as PiC siRNA1 and 5′-GAACACCUAUCUGUGGCGUACAUCA-3′ and 5′-UGAUGUACGCCACAGAUAGGUGUUC-3′ designated as PiC siRNA2. The sequences of control siRNAs were: 5′-ACCAACAACAGUUUGGGAAUAGGGA-3′ and 5′-UCCCUAUUCCCAAACUGUUGUUGGU-3′. Cultured INS-1 cells were trypsinized, suspended with RPMI 1640 medium without antibiotics, mixed with Opti-MEM (Invitrogen) containing siRNA and Lipofectamine™ 2000 (Invitrogen), plated on dishes or wells and then incubated at 37 °C in a CO_2 incubator. The final amounts of INS-1 cells, RPMI 1640, Opti-MEM, siRNA and Lipofectamine™ 2000 were 1×10^6 cells/ml, 75 % (v/v), 25 % (v/v), 80 nM and 0.3 % respectively. Medium was replaced with RPMI 1640 3–4 h after transfection. All experiments using siRNA-transfected INS-1 cells were performed 48 h after transfection unless otherwise noted.

Isolation of total RNA and quantitative RT (reverse transcription)–PCR

Total RNA was isolated from cardiac muscle, brain, skeletal muscle, kidney, liver and lung of Wistar rats using TRIzol® (Invitrogen) and from islets of Wistar rats and INS-1 cells using RNeasy mini kit (Qiagen). Animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University. Islets were isolated by collagenase digestion [17]. cDNA was prepared by reverse transcriptase (Superscript II; Invitrogen) with an oligo(dT) primer. The rat sequences of forward and reverse primers to detect PiC-A, PiC-B, total PiC (PiC-A plus PiC-B) and β-actin (as an inner control) are shown in Table 1. AmpliTaq Gold (Applied Biosystems) was used as a DNA polymerase for RT–PCR. SYBR Green PCR Master Mix (Applied Biosystems) was prepared for the quantitative RT–PCR run. The thermal cycling conditions were denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 30s and 60 °C for 30s.

Table 1 Primer sequences used in RT–PCR and quantitative RT–PCR

Name	Forward	Reverse
PiC-A	5′-AGCTGGTGCACGATGTGTCG-3′	5′-TTCCTCCGAGTCCACAGAGG-3′
PiC-B	5′-AGCTGGTGCACGATGTGTCG-3′	5′-CCACCAAAGCCACACAGTGC-3′
Total PiC (PiC-A+PiC-B)	5′-AGAGCAGCTGGTTGTGACAT-3′	5′-ACACCTCTAAAGCCAAGCCT-3′
β-actin	5′-CAATGAGCGGTTCCGATGCC-3′	5′-AATGCCTGGGTACATGGTGG-3′

Plasmid construction and transfection

The cDNA fragment of rat PiC-B was obtained from rat islets by RT–PCR and cloned into the pHMCA5 vector. pHMCA5-PiC-B was transfected into COS-7 and INS-1 cells using FuGENE™ 6 transfection reagent (Roche) and Lipofectamine™ 2000 respectively.

Immunoblot analysis

Rabbit antibody against the rat PiC peptide PPEM-PESLKKKLGLTE corresponding to C-terminal residues was originally raised. For immunoblotting, cells were washed with PBS containing protease inhibitor (Complete; Roche), suspended in 1 ml of PBS containing protease inhibitor and homogenized. Protein (50 μg per sample) was separated on a 15 % polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with TBS (Tris-buffered saline; 10 mM Tris/HCl and 100 mM NaCl, pH 7.5) containing 0.1 % Tween 20 and 5 % skimmed milk (blocking buffer) at room temperature (25 °C) for 2 h, blotted membranes were incubated overnight at 4 °C with anti-PiC antibody at 1:500 dilution, anti-DIC (dicarboxylate carrier) antibody (Novus Biologicals) at 1:100 dilution, mouse monoclonal anti-complex I (39 kDa subunit) antibody, anti-complex III (core II) antibody, anti-complex IV (subunit I) antibody or anti-complex V (subunit α) of mitochondrial respiratory chain antibody at 1:1000 dilution in blocking buffer, and subsequently with anti-rabbit (for PiC and DIC) or anti-mouse (for respiratory chain proteins) IgG horseradish peroxidase-conjugated secondary antibody (GE Healthcare) diluted 1:5000 at room temperature for 2 h prior to detection using ECL (GE Healthcare). In the same membrane, the process was repeated for β-actin at 1:1000 dilution of the antibody. Band intensities were quantified with Multi Gauge software (Fujifilm).

Insulin secretion

For insulin secretion assays, INS-1 cells cultured on 24-well plates coated with 0.001 % poly-L-ornithine were washed with KRBH (Krebs-Ringer bicarbonate Hepes buffer) composed of

140 mM NaCl, 3.6 mM KCl, 0.5 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 0.1 % BSA and 10 mM Hepes (pH 7.4) with 2 mM glucose, preincubated at 37 °C for 30 min in KRBH with 2 mM glucose, and then incubated at 37 °C for 30 min in KRBH with 2 mM glucose, 10 mM glucose or 2 mM glucose plus 30 mM KCl. Insulin concentrations were determined by RIA using rat insulin as a standard as previously described [17].

Adenine nucleotides

ATP and ADP contents were determined as previously described [18,19] with some modifications. Briefly, INS-1 cells were cultured, washed and preincubated as described above and incubated with KRBH with 2 mM glucose, 10 mM glucose or 2 mM glucose plus 30 mM KCl at 37 °C for 30 min. Incubation was stopped by the addition of $HClO_4$. The contents of wells were sonicated [three pulses of 3 s duration using a Handy Sonic UR-20P instrument (TOMY SEIKO) on ice] and transferred into glass tubes. The tubes were then centrifuged, and a fraction of the supernatant was neutralized with Hepes and Na_2CO_3. The ATP concentration was measured by luciferin-luciferase assay. After ATP in the neutralized extract was irreversibly converted to AMP with ATP sulfurylase in the presence of Na_2MoO_4, ADP in the reactant was converted to ATP with pyruvate kinase and was determined by luciferin-luciferase assay as the difference between the measurements with and without pyruvate kinase.

Intracellular calcium concentration ($[Ca^{2+}]_i$) and mitochondrial membrane potential ($\Delta\psi_m$) in living cells

INS-1 cells were seeded on to glass coverslips coated with 0.001 % poly-L-ornithine and cultured 48 h before measurements were made. For measurements of $[Ca^{2+}]_i$, cultured INS-1 cells were loaded with 5 μM Fura-PE3/AM (Calbiochem) at 37 °C for 90 min, placed in a heat-controlled chamber on the stage of an inverted microscope kept at 36 ± 1 °C, superfused with KRBH containing 2 mM glucose, and subsequently exposed to the buffer containing 10 mM glucose or 30 mM KCl. The cells were excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by CCD camera (Micro Max 5 MHz System, Roper Industries, Trenton, NJ). The images were analysed with the Meta Fluor image analyzing system (Universal Imaging). The 340 nm (F340) and 380 nm (F380) fluorescence signals were detected every 15 s, and ratios (F340/F380) were calculated. For $\Delta\psi_m$ measurements, the same protocol as above was used except that cultured cells were loaded with 10 μg/ml rhodamine 123 (Invitrogen) at 37 °C for 30 min and fluorescence excited at 490 nm and emitted at 530 nm every 20 s was monitored.

ATP production and $\Delta\psi_m$ in mitochondrial fraction

Measurement of ATP production from the mitochondrial fraction was performed as previously described [18] with minor modifications. Firstly, INS-1 cells were homogenized in solution A consisting of 50 mM Hepes, 100 mM KCl, 1.8 mM ATP, 1 mM EGTA, 2 mM $MgCl_2$ and 0.5 mg/ml BSA (electrophoretically homogeneous) with the pH adjusted to 7.00 at 37 °C with KOH. After precipitation of cell debris and nuclei by 800 *g* centrifugation for 3 min, the supernatant was centrifuged more rapidly (10000 *g* for 3 min) to obtain a pellet containing the mitochondrial fraction. The precipitation, diluted by 200 μl of solution A, was centrifuged again and rinsed three times in solution B, consisting of 20 mM Hepes, 1 mM EGTA, 12 mM NaCl, 0.3 mM $MgCl_2$, 130 mM potassium gluconate and 0.5 mg/ml BSA (electrophoretically homogeneous) with the pH adjusted to 7.10 with KOH. The mitochondrial fraction in 500 μl of solution B was kept on ice until use. To measure ATP production by oxidative phosphorylation, the reaction was started by adding mitochondrial suspension to prewarmed solution B (37 °C) containing mitochondrial substrates with or without respiratory chain inhibitors, 50 μM ADP, 1 μM DAPP and various levels of P_i. DAPP, a specific inhibitor of adenylate kinase, was used to measure ATP production by oxidative phosphorylation exclusively. After the reaction was stopped, the ATP concentration in the solutions was measured by adding luciferin-luciferase solution with a bioluminometer. ATP production was corrected by mitochondrial protein content. Measurement of $\Delta\psi_m$ was performed as previously described [20] with some modifications. Fluorescence was successively monitored using a spectrofluorophotometer (RF 5000; Shimadzu) with an excitation wavelength of 495 nm and emission at 586 nm, and with stirring solution B supplemented with 3 mM KH_2PO_4, 50 μM ADP and 2.5 μM Safranin O applied in a glass cuvette at 37 °C. Mitochondria, succinate and FCCP were added to the solution in this order and final concentrations were 50 μg/ml, 1 mM and 200 nM respectively.

Statistical analysis

The data are expressed as means ± S.E.M. Statistical significance was calculated by unpaired Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Expression of PiC mRNA in pancreatic β-cells

Tissue distribution of PiC was evaluated by RT–PCR (Figure 1A). PiC-B was expressed ubiquitously whereas PiC-A was expressed clearly in cardiac muscle and skeletal muscle as previously reported [13,14] and obscurely in rat islets and INS-1 cells. These results indicate that PiC-B was dominantly expressed in pancreatic β-cells.

Evaluation of anti-PiC antibody

The cell lysates of COS-7 cells transfected with pHMCA5-PiC or pHMCA5-null, INS-1 cells transfected with pHMCA5-null, intact INS-1 cells and rat islets were electrophoresed and immunoblotted using the anti-PiC antibody. As shown in Figure 1(B), the band at ~30 kDa, which was not detected in COS-7 cells transfected with pHMCA5-null, was detected in COS-7 cells transfected with pHMCA5-PiC, INS-1 cells transfected with pHMCA5-null, intact INS-1 cells and rat islets. This observation is consistent with a previous report that rat PiC was detected at ~30 kDa by an antibody originally raised using the C-terminal amino acids as the antigen peptide [21].

Silencing effects of PiC siRNAs on INS-1 cells

Quantitative RT–PCR assays using primers for total PiC (PiC-A plus PiC-B, Table 1) and immunoblotting using anti-PiC antibody revealed ~70 % reduction of PiC mRNA expression and ~40 % reduction of the protein expression in INS-1 cells 48 h after both PiC siRNA1 and 2 transfection respectively (Figures 1C and 1D). Time-dependent reduction of PiC protein expression (~25 %, ~40 % and ~50 % reduction at 24 h, 48 h and 72 h after siRNA1 and 2 transfection) implies long half-life of PiC, which causes low efficacy of suppression (Figure 1D). Transfection of control siRNA did not affect the expression of PiC in INS-1 cells at both mRNA and protein levels. Protein expressions of DIC,

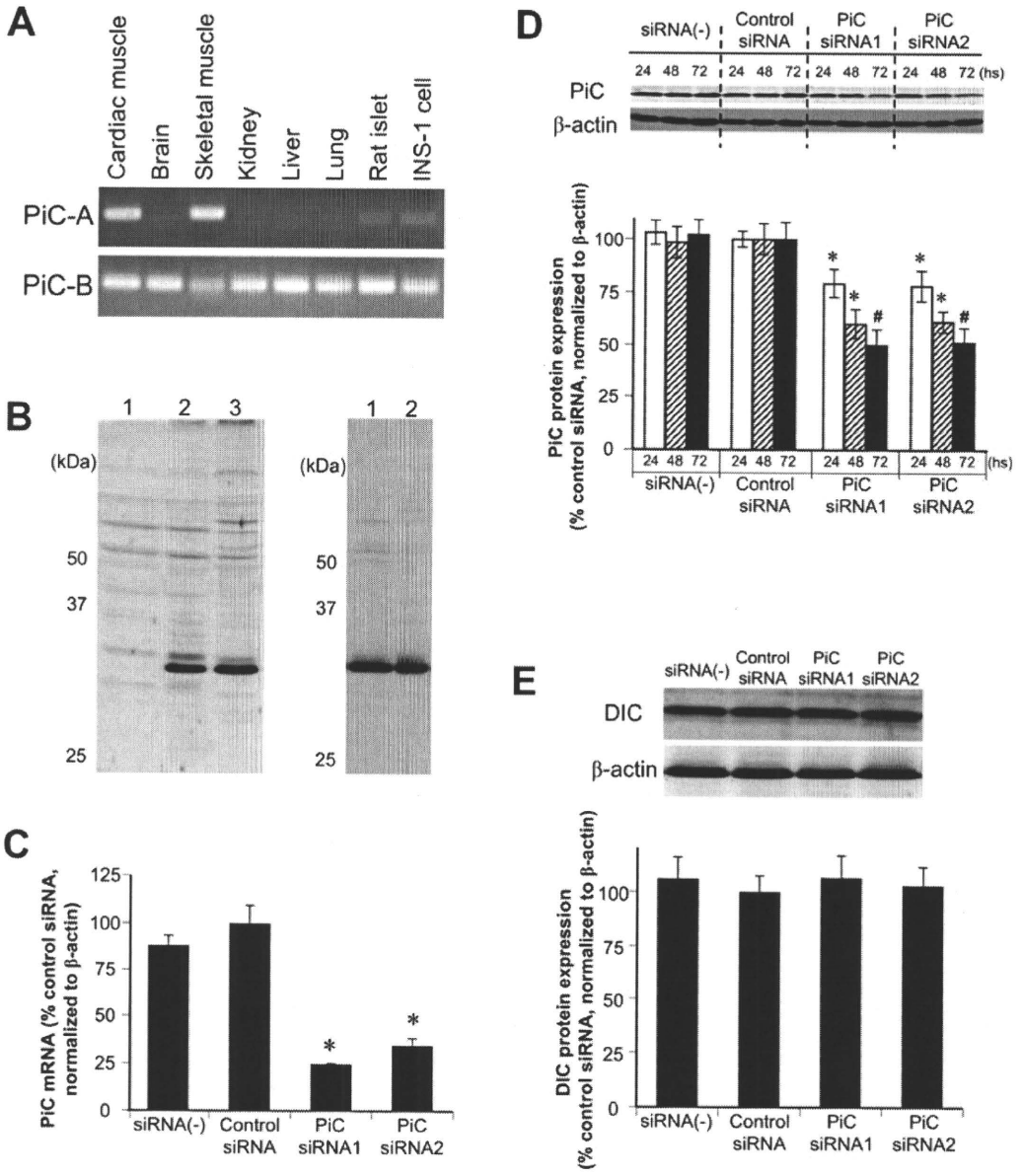

Figure 1 Detection of rat PiC and silencing effects of PiC siRNAs on INS-1 cells

(**A**) RT–PCR detection of PiC mRNA expression in various rat tissues and INS-1 cells. PiC mRNA expressions in cardiac muscle, brain, skeletal muscle, kidney, liver, lung and islets of Wistar rat and INS-1 cells were evaluated with RT–PCR using primers of specific sequences for PiC-A and PiC-B. Product sizes are 141 bp for PiC-A and 136 bp for PiC-B. (**B**) Evaluation of anti-PiC antibody by immunoblot analysis. Left panel: whole cell lysates from COS-7 cells transfected with pHMCA5-null (lane 1), COS-7 cells transfected with pHMCA5-PiC (lane 2) and INS-1 cells transfected with pHMCA5-null (lane 3) were electrophoresed and immunoblotted with anti-PiC antibody. Right panel: whole cell lysates from INS-1 cells (lane 1) and rat islets (lane 2) were electrophoresed and immunoblotted with anti-PiC antibody. Molecular mass in kDa is given on the left-hand side of each panel. (**C**) Effects of transfection of PiC siRNAs on the expression of PiC mRNA was evaluated with quantitative RT–PCR using a pair of primers recognizing both PiC-A and PiC-B (total PiC). Data were normalized using β-actin mRNA. $n = 3$ in each group. $^*P < 0.01$ compared with control siRNA. (**D**) Immunoblot analysis of PiC expression revealed that PiC siRNAs reduced PiC expression in INS-1 cells. Time (h) after siRNA transfection is indicated. Data were normalized by the expression of β-actin. $n = 4$ in each bar. $^*P < 0.05$ and $\#P < 0.01$ compared with control siRNA. (**E**) Effects of PiC silencing on expression of DIC. Whole INS-1 cell lysate was electrophoresed and immunoblotted using antibodies against DIC. Quantification data were obtained from four independent experiments and normalized with β-actin levels.

another P_i carrier, were not affected by siRNA1 and 2 transfection (Figure 1E).

Effects of PiC down-regulation on glucose- and depolarization-stimulated insulin secretion

Down-regulation of PiC decreased GSIS (10 mM glucose) in INS-1 cells, as shown in Figure 2. A reduction in GSIS of 61 % by PiC siRNA1 and 47 % by PiC siRNA2 was observed. K^+ (30 mM)-stimulated insulin secretion was also reduced: the reduction was 27 % by PiC siRNA1 and 23 % by PiC siRNA2, which were milder than those of GSIS (Figure 2). Insulin secretion in the basal glucose state (2 mM) was not affected by PiC siRNA1, but was slightly increased by PiC siRNA2. Transfection of control siRNA did not affect GSIS in INS-1 cells.

Effects of PiC down-regulation on adenine nucleotides

Down-regulation of PiC increased ADP and decreased the ATP:ADP ratio, whereas it did not significantly affect ATP in

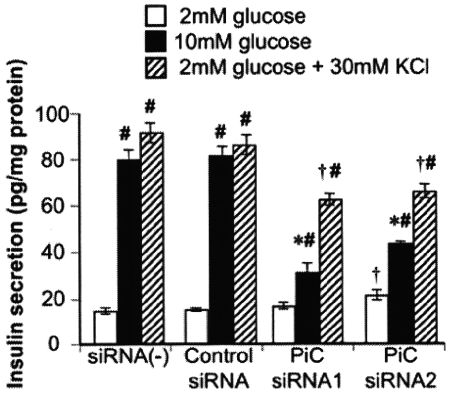

Figure 2 Effects of PiC down-regulation on glucose- or KCl-stimulated insulin secretion

INS-1 cells were incubated for 30 min with 2 mM glucose, 10 mM glucose or 2 mM glucose and 30 mM K^+, and insulin secretion was measured. Data were obtained from six independent experiments normalized by protein concentration. Error bars are means ± S.E.M. *$P < 0.01$ and †$P < 0.05$ compared with control siRNA at the corresponding condition. #$P < 0.01$ compared with corresponding 2 mM glucose.

the presence of 10 mM glucose in INS-1 cells (Table 2A). ATP, ADP and the ATP:ADP ratio at 2 mM glucose was not altered by silencing PiC (Tables 2A and 2B). Depolarization evoked by 30 mM K^+ in the presence of 2 mM glucose decreased the ATP:ADP ratio in both control and PiC down-regulated cells, whereas suppression of the ATP:ADP ratio was lower in PiC down-regulated cells compared with control cells (Table 2B).

Effects of PiC down-regulation on $[Ca^{2+}]_i$ and $\Delta\psi_m$ in living cells

Fluorescence signals of Fura-PE3 revealed that elevation of $[Ca^{2+}]_i$ in response to a stimulating level of 10 mM glucose was decreased and delayed by PiC down-regulation compared with that in control (Figure 3A). Average values calculated using the data from Figure 3(A) also indicate that PiC siRNA reduced the mean $[Ca^{2+}]_i$ at 10 mM glucose (PiC siRNA1, 0.864 ± 0.004 compared with control siRNA, 0.896 ± 0.003; $P < 0.01$) whereas there was no significant change at basal (2 mM) glucose (PiC siRNA1, 0.846 ± 0.004; control siRNA, 0.857 ± 0.003), as shown in Figure 3(B). Elevation of $[Ca^{2+}]_i$ in response to 30 mM K^+ was slightly decreased by PiC siRNA1 (average value of Fura-PE3 fluorescence ratio was 0.968 ± 0.005, compared with a control siRNA ratio of 0.991 ± 0.005, $P < 0.01$) without affecting basal value (control siRNA, 0.857 ± 0.006; siRNA1, 0.854 ± 0.004) as shown in Figures 3(C) and 3(D). Fluorescence measurement using rhodamine 123 demonstrated that the mitochondrial membrane in INS-1 cells was hyperpolarized by raising glucose from 2 to 10 mM and prominently depolarized by FCCP, and that PiC down-regulation did not affect glucose-induced hyperpolarization and total depolarization after FCCP exposure of $\Delta\psi_m$ throughout the measurement (Figure 3E).

Effects of PiC down-regulation on ATP production and $\Delta\psi_m$ in mitochondrial fraction

ATP production by mitochondria from INS-1 cells transfected with control or PiC siRNAs in the presence of 1 mM succinate and various concentrations of P_i ($[P_i]$) is shown in Figure 4(A). PiC down-regulation decreased mitochondrial ATP production by 50–60 % at $[P_i]$ ranging from 0.001 to 10 mM. ATP production in all groups reached maximum rates above ~3 mM of $[P_i]$, which indicates that the PiC amount regulates the maximal rate of mitochondrial ATP production. On the other hand, K_m values of $[P_i]$ for ATP production were similar (~0.05 mM). Mitochondrial ATP production in the presence of various mitochondrial substrates and inhibitors of the respiratory chain is shown in Table 3. ATP production in the presence of succinate was completely inhibited by antimycin A, a complex III inhibitor, in both control and PiC down-regulated INS-1 cells. PiC siRNAs decreased ATP production in the presence of pyruvate and malate by 42–58 %, succinate plus rotenone by 46–62 % and TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) plus ascorbate by 61–62 %, showing that ATP production by electrons rendered at complex I, complex II and complex IV is suppressed to a similar degree. In spite of significant down-regulation of ATP production, PiC down-regulation did not affect $\Delta\psi_m$ of isolated mitochondria measured with Safranin O in the presence of succinate (Figure 4B).

Effects of PiC down-regulation on expression of mitochondrial respiratory chain proteins

Immunoblotting using lysates of whole INS-1 cells revealed that transfection of PiC siRNAs did not change the expression of complex I, III, IV or V of mitochondrial respiratory chain proteins (Figure 5).

DISCUSSION

In the present study, the mitochondrial phosphate carrier (PiC) was revealed to play an important role in metabolism–secretion coupling of pancreatic β-cells by using INS-1 cells and PiC siRNA. PiC down-regulation brings about reduction in mitochondrial ATP production by mitochondrial fuels, resulting in reduced glucose-induced $[Ca^{2+}]_i$ elevation and impaired GSIS.

In pancreatic β-cells, ATP increase is slight and ADP decrease is prominent via an increase in glucose levels beyond the triggering level of insulin secretion. In addition, the ATP/ADP ratio is well-correlated with GSIS rather than the absolute value of ATP [22,23]. PiC down-regulation decreased the ATP/ADP ratio in the presence of high glucose, which causes insufficient closure of K_{ATP} channels, a decrease in $[Ca^{2+}]_i$ elevation by glucose (Figures 3A and 3B), and suppression of GSIS (Figure 2).

Insulin secretion at 10 mM glucose was similar to that at 30 mM K^+ and 2 mM glucose in the control samples. In contrast, in PiC down-regulated INS-1 cells, GSIS is lower than depolarization-induced insulin secretion, which suggests specific effects of PiC on metabolism–secretion coupling (Figure 2). However, ~25 % suppression of depolarization-induced insulin secretion, which is modest compared with GSIS, was observed in PiC down-regulated INS-1 cells. Measurements revealed that $[Ca^{2+}]_i$ in the presence of 2 mM glucose and 30 mM K^+ was reduced by PiC down-regulation (Figures 3C and 3D), which plays a role in reduced depolarization-induced insulin secretion by PiC down-regulation. Depolarization reduced the ATP/ADP ratio in the presence of a basal level of glucose in control samples, which accords with a previous study where an increase in $[Ca^{2+}]_i$ causes a larger consumption than production of ATP [24] (Table 2B). The ATP/ADP ratio was also reduced by depolarization at 2 mM glucose in PiC down-regulated INS-1 cells, although the suppression was lower than that in control samples, which may reflect a smaller elevation of $[Ca^{2+}]_i$ than in the control. In addition, in contrast with a significant suppression of the ATP/ADP ratio at high glucose concentrations by PiC down-regulation, in the presence of a basal

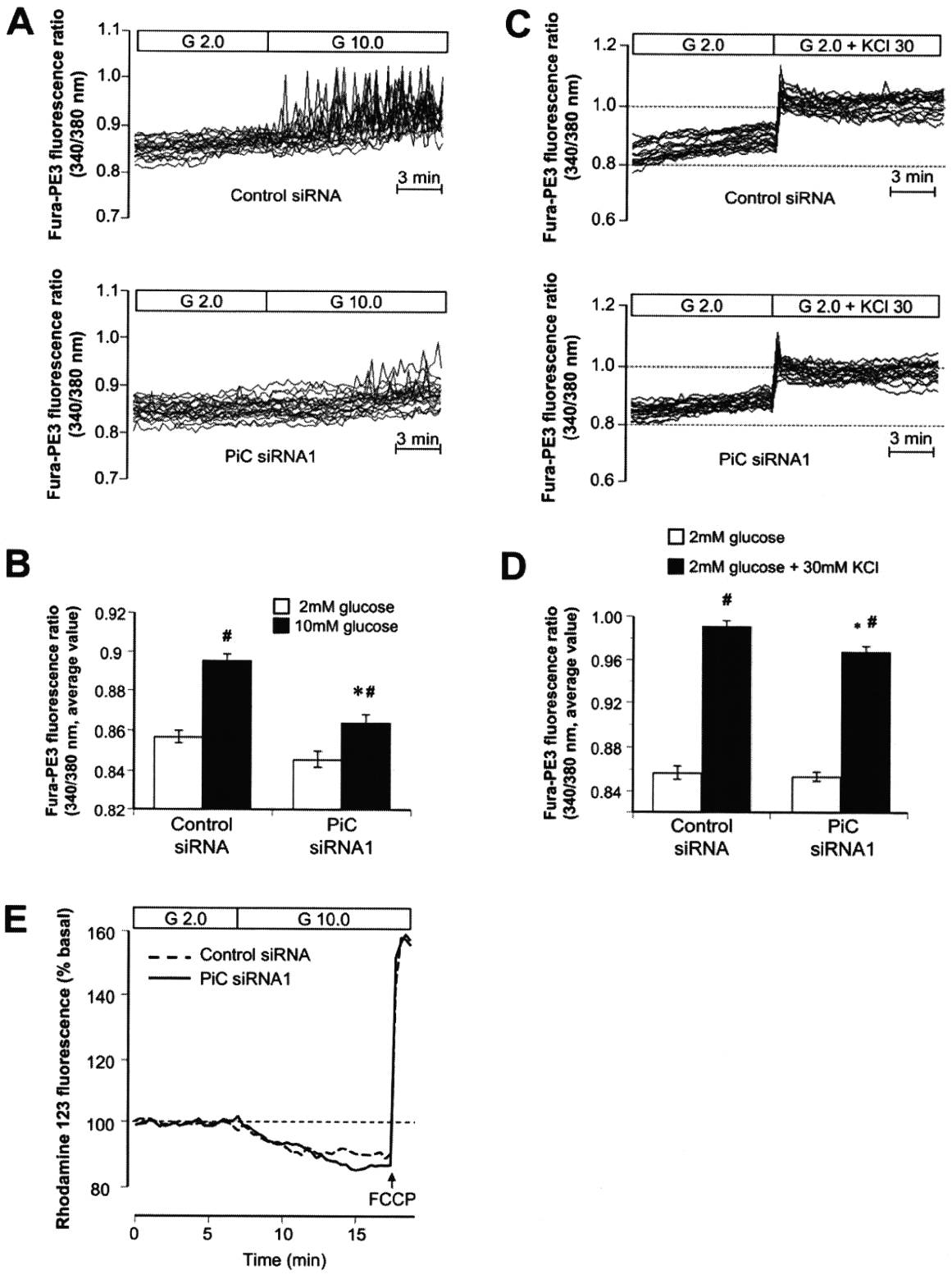

Figure 3 Effects of PiC down-regulation on $[Ca^{2+}]_i$ and $\Delta\psi_m$ in living cells

(**A**) $[Ca^{2+}]_i$ elevation and oscillation induced by raising glucose (G) from 2 to 10 mM were monitored in INS-1 cells transfected with PiC siRNA. Traces of Fura-PE3 fluorescence ratio (340/380 nm) were obtained from 20 cells of each group. (**B**) Average values calculated from the data from (**A**). $^*P < 0.01$ compared with control siRNA at 10 mM glucose. $\#P < 0.01$ compared with corresponding 2 mM glucose. (**C**) $[Ca^{2+}]_i$ elevation induced by 30 mM KCl was monitored in INS-1 cells transfected with PiC siRNA. Traces of Fura-PE3 fluorescence ratio (340/380 nm) were obtained from 20 cells of each group. (**D**) Average values calculated from the data from (**C**). $^*P < 0.05$ compared with control siRNA at 30 mM KCl. $\#P < 0.01$ compared with corresponding 2 mM glucose. (**E**) $\Delta\psi_m$ monitored by rhodamine 123 fluorescence in INS-1 cells. Data were corrected with the average values of fluorescence under basal glucose (2 mM) conditions. $n = 10$. Error bars are means±S.E.M.

Table 2 Effects of PiC down-regulation on adenine nucleotides

(A)

	Control siRNA		PiC siRNA1		PiC siRNA2	
Glucose (mM)	2	10	2	10	2	10
ATP (nmol/mg protein)	65.2 ± 3.8	77.4 ± 3.8*	64.4 ± 1.0	71.8 ± 3.3*	66.2 ± 1.7	80.0 ± 5.5*
ADP (nmol/mg protein)	10.2 ± 0.6	3.9 ± 0.4†	10.6 ± 0.6	6.4 ± 0.9*‡	9.7 ± 0.6	6.1 ± 1.2*‡
ATP/ADP	6.5 ± 0.6	20.5 ± 2.0†	6.1 ± 0.4	12.0 ± 1.7*‡	6.9 ± 0.5	13.9 ± 1.4*‡

(B)

	Control siRNA			PiC siRNA1	
Glucose (mM)	2	2	2	2	2
K^+ (mM)	3.6	30	3.6	3.6	30
Antimycin A (μM)	0	0	1	0	0
ATP (nmol/mg protein)	65.5 ± 3.4	48.6 ± 1.9*	2.5 ± 0.1†	64.4 ± 4.7	61.2 ± 1.8‡
ADP (nmol/mg protein)	10.1 ± 0.1	11.2 ± 0.2*	10.5 ± 0.1*	9.9 ± 0.2	10.5 ± 0.3*‡
ATP/ADP	6.5 ± 0.3	4.4 ± 0.2†	0.2 ± 0.0†	6.5 ± 0.5	5.8 ± 0.2*§

*$P < 0.05$ and †$P < 0.01$ compared with basal condition (2 mM glucose). ‡$P < 0.05$ and §$P < 0.01$ compared with control siRNA. Data were obtained from four independent experiments.

Table 3 Silencing effects of PiC siRNAs on ATP production from mitochondrial fraction of INS-1 cells

	Mitochondrial ATP production (μmol/10 min per mg of protein)		
Experimental conditions	Control siRNA	PiC siRNA1	PiC siRNA2
1 mM succinate	1.28 ± 0.02	0.61 ± 0.02*	0.46 ± 0.01*
1 mM succinate + 1 μM rotenone	1.03 ± 0.09	0.56 ± 0.03*	0.39 ± 0.01*
1 mM succinate + 1 μM antimycin A	0.03 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
1 mM pyruvate + 1 mM malate	0.41 ± 0.03	0.24 ± 0.01*	0.17 ± 0.00*
0.5 mM TMPD + 2 mM ascorbate	3.43 ± 0.09	1.33 ± 0.03*	1.27 ± 0.01*

*$P < 0.01$ compared with control siRNA. Data were obtained from three independent experiments.

level of glucose, PiC down-regulation did not affect the ATP/ADP ratio in INS-1 cells. An incomplete compensatory effect derived from PiC down-regulation, which is valid in a basal supply of substrate to mitochondria but deteriorates in an accelerated supply at high glucose, might save ATP consumption and maintain the basal ratio of ATP/ADP.

PiC, which is required for mitochondrial ATP production, has two isoforms. PiC-A is expressed in skeletal and cardiac muscle whereas PiC-B is expressed ubiquitously. AAC (ATP/ADP carrier), which is also required for mitochondrial ATP production, has isoforms including AAC1 (SLC25A4), AAC2 (SLC25A5) and AAC3 (SLC25A6). Interestingly, these isoforms, except AAC2, expression of which is absent or scarce in most tissues, distribute similarly to the PiC isoforms: AAC1 is expressed in skeletal and cardiac muscle, and AAC3 is expressed ubiquitously. These distributions imply that ubiquitously-expressed PiC-B and AAC3 may meet stable energy requirement, and PiC-A and AAC1, which are expressed exclusively in muscle, meet higher and prompt energy demands for muscle contraction. In the present study, we demonstrate that PiC-B is the dominant isoform of PiC whereas PiC-A is scarcely expressed in INS-1 cells and rat islets (Figure 1A), which may reflect less prompt energy demand in β-cells compared with that in muscles.

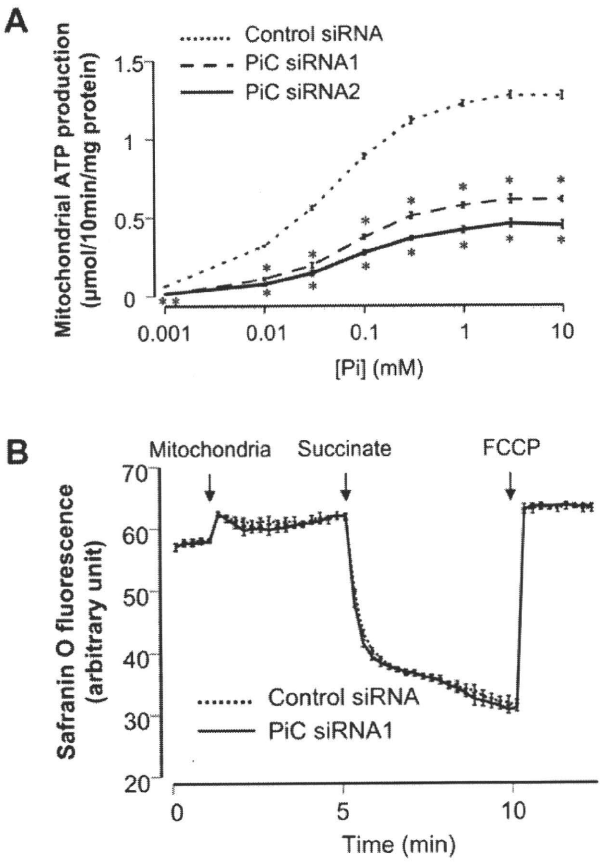

Figure 4 Effects of PiC down-regulation on ATP production and $\Delta\psi_m$ in mitochondrial fraction isolated from INS-1 cells

(**A**) Effects of PiC down-regulation on mitochondrial ATP production at various phosphate concentrations. ATP production was evaluated in mitochondria isolated from INS-1 cells in the presence of 50 μM ADP, 1 μM DAPP and 1 mM succinate with various concentrations of P_i indicated in the Figure. $n = 3$ in each plot. *$P < 0.01$ compared with control siRNA. (**B**) $\Delta\psi_m$ monitored by Safranin O fluorescence. Mitochondria (50 μg/ml), succinate (1 mM) and FCCP (200 nM) were added to the solution containing Safranin O at the points indicated with arrows. $n = 4$ in each group.

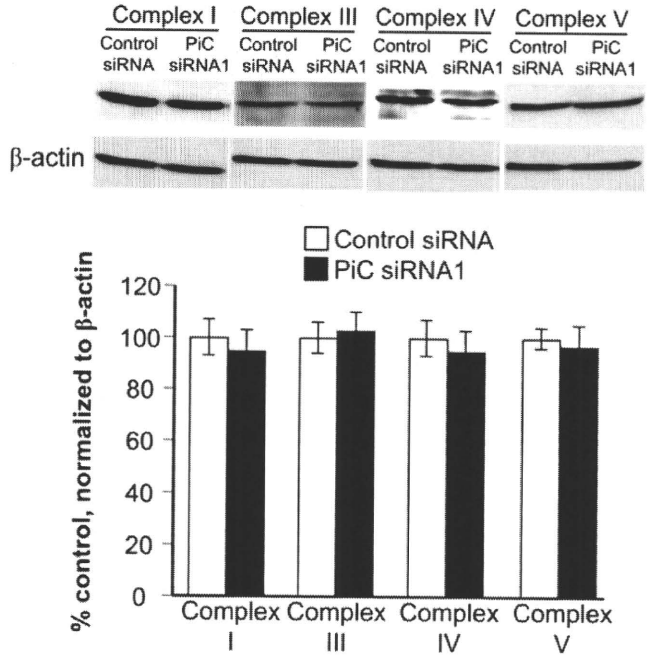

Figure 5 Effects of PiC silencing on expression of mitochondrial respiratory chain proteins

Lysates of whole INS-1 cells were electrophoresed and immunoblotted using antibodies against complex I, III, IV and V. Quantification data were obtained from four independent experiments and normalized with β-actin levels. Error bars are means±S.E.M.

Mitochondrial ATP is produced by complex V (ATP synthase), which is driven by protonmotive force generated by proton extrusion during transport of high-energy electrons in the respiratory chain. In the present study, mitochondrial ATP production in the presence of mitochondrial fuel increased according to the raised extramitochondrial phosphate concentration ($[P_i]_e$), and reached maximum rate above ~3 mM of $[P_i]_e$, which was decreased by 50–60 % without affecting the K_m value of $[P_i]_e$ for ATP production by ~40 % reduction in PiC protein. The physiological intracellular $[P_i]$ in heart determined by methods including ^{31}P NMR is ~1 mM at rest and increases to ~10 mM depending on the metabolic state [25–27]. Levels of P_i in islets are ~20 mmol/kg of dry weight tissue [28], which corresponds to ~10 mM by conversion [29]. Taken together, the rate of mitochondrial ATP production might be barely affected by a physiological change of $[P_i]_e$ but be evidently affected by alteration of the amount of PiC protein. In addition, reduction in ATP production by down-regulation of PiC also suggests that compensatory supply of P_i to mitochondria by other mitochondrial phosphate carriers including DIC (SLC25A10) [30,31] does not occur, which is supported by no apparent effect of PiC down-regulation on DIC expression (Figure 1E). These results accord with the first description that PiC dysfunction impairs the synthesis of ATP [15].

Intramitochondrial P_i is thought to affect oxidative phosphorylation at multiple sites [16]. To find specific defective sites in the respiratory chain in PiC down-regulated INS-1 cells, mitochondrial ATP production was examined in the presence of various substrates and inhibitors. Pyruvate and malate, which are metabolized in mitochondria to generate NADH, render electrons at complex I. In the presence of rotenone, a complex I inhibitor, succinate renders electrons directly to complex II via $FADH_2$. TMPD is an artificial electron donor that can transfer electrons to cytochrome *c*. TMPD reduced by ascorbate renders electrons to cytochrome *c*, which transfers electrons to complex IV. Reduction of ATP production by down-regulation of PiC in the presence of pyruvate plus malate, succinate plus rotenone and TMPD plus ascorbate were all suppressed similarly by 50–60 % (Table 3). These results indicate that reduction in ATP production by down-regulation of PiC may well be derived from a defective site downstream of complex IV and that a defective site upstream of complex IV, if present, does not play a prominent role. Moreover, immunoblotting revealed that expressions of respiratory chain proteins including complex I, III, IV and V were not affected by PiC silencing. Considered together, silencing of PiC seems to suppress mitochondrial ATP production not by affecting mitochondrial biogenesis, but by restricting P_i supply to complex V.

Inhibition of complex V by oligomycin reduces ATP production with hyperpolarization of $\Delta\psi_m$ [32,33], which may be derived from the fact that complex V is a protonophore and its inhibition affects electrogenic H^+ influx to mitochondria specifically and directly affects $\Delta\psi_m$. In contrast, PiC is electroneutral due to symport of H^+ and negatively charged P_i or antiport of OH^- and negatively charged P_i and does not directly affect $\Delta\psi_m$. ATP generation in complex V is driven by protonmotive force (Δp), which has two components: electrical membrane potential ($\Delta\psi_m$) and the difference between the cytosolic and matrix pH (ΔpH) [34,35]. P_i plays regulatory roles in oxidative phosphorylation by affecting Δp. An increase in $[P_i]_e$ reduces ΔpH [34,36–38] due to an increase in co-transport of P_i and protons from cytosol into mitochondrial matrix through PiC. On the other hand, $\Delta\psi_m$ is increased by an increase in $[P_i]_e$ of less than ~2 mM, but reaches a plateau at $[P_i]_e$ above ~2 mM [16,34,38]. Increases in $\Delta\psi_m$ owing to increases in $[P_i]_e$ is not fully elucidated, but some explanations are proposed. An electroneutral influx of protons (H^+) accompanying negatively charged substrates such as P_i does not directly affect $\Delta\psi_m$ but produces a reduction in ΔpH that promotes proton extrusion by the respiratory chain to maintain Δp and eventually increases $\Delta\psi_m$ [35]. Bose et al. [16] provided another explanation: an increase in the influx of P_i activates intramitochondrial NADH production and NADH supply to complex I and also promotes the ability to generate Δp by improving the coupling of electron transport between cytochrome *b* and cytochrome *c*, which eventually increases $\Delta\psi_m$. Interestingly, in the present study, ~40 % reduction in the protein level of PiC did not affect glucose-induced hyperpolarization of the mitochondrial inner membrane in spite of a reduction in ATP production. It is possible that the reduction in P_i influx by down-regulation of PiC in the present study is within a range of P_i influx which does not affect $\Delta\psi_m$ as with higher $[P_i]_e$. In addition, our results were derived from sustained down-regulation of P_i influx to mitochondria, as experiments were performed 48 h after transfection of PiC siRNA in contrast with the acute alteration of P_i influx by manipulation of $[P_i]_e$ in previous studies, which may permit adaptation of $\Delta\psi_m$ to maintain Δp.

It has been generally reported that the contribution of $\Delta\psi_m$ to Δp is 80–85 % [34,35,37–45] or more [16] and that of ΔpH is relatively small, which indicates that the alteration in Δp by down-regulation of PiC in the present study is small considering the non-detectable affect on $\Delta\psi_m$. Therefore the supply of P_i to complex V may well be a critical rate-limiting step for ATP production independent of Δp. The results in the present study demonstrate the critical role of P_i influx to mitochondria in ATP production and metabolism–secretion coupling in pancreatic β-cells.

AUTHOR CONTRIBUTION

Yuichi Nishi researched data, contributed to the discussion, wrote the manuscript and revised/edited the manuscript. Shimpei Fujimoto contributed to the discussion, wrote the manuscript and revised/edited the manuscript. Mayumi Sasaki, Eri Mukai, Hiroki Sato, Yuichi Sato, Yumiko Tahara and Yasuhiko Nakamura researched data. Nobuya Inagaki contributed to the discussion and revised/edited the manuscript.

ACKNOWLEDGEMENTS

We greatly appreciate the gifts of INS-1 cells from Dr Nobuo Sekine (Tokyo Kosei Nenkin Hospital, Tokyo, Japan) and pHMCA5 vector from Dr Hiroyuki Mizuguchi (National Institute of Biomedical Innovation, Ibaraki, Japan). We thank Mr Shinsaku Akagi, Mr Takuro Yamaguchi, Ms Chiyo Kotake and Ms Sara Yasui for technical assistance and Mr Eiji Yoshihara for helpful advice on siRNA transfection.

FUNDING

This study was supported by a Research Grant on Nanotechnical Medicine from the Ministry of Health, Labour, and Welfare of Japan, Scientific Research Grants, a grant from Innovation Cluster Kansai project of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Cooperation.

REFERENCES

1 Maechler, P. and Wollheim, C. B. (2001) Mitochondrial function in normal and diabetic β-cells. Nature **414**, 807–812
2 Kennedy, E. D., Maechler, P. and Wollheim, C. B. (1998) Effects of depletion of mitochondrial DNA in metabolism secretion coupling in INS-1 cells. Diabetes **47**, 374–380
3 Tsuruzoe, K., Araki, E., Furukawa, N., Shirotani, T., Matsumoto, K., Kaneko, K., Motoshima, H., Yoshizato, K., Shirakami, A., Kishikawa, H. et al. (1998) Creation and characterization of a mitochondrial DNA-depleted pancreatic β-cell line: impaired insulin secretion induced by glucose, leucine, and sulfonylureas. Diabetes **47**, 621–631
4 Palmieri, F. (2004) The mitochondrial transporter family (SLC25): physiological and pathological implications. Pflugers Arch. **447**, 689–709
5 Rubi, B., del Arco, A., Bartley, C., Satrustegui, J. and Maechler, P. (2004) The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. J. Biol. Chem. **279**, 55659–55666
6 Casimir, M., Rubi, B., Frigeric, F., Chaffard, G. and Maechler, P. (2009) Silencing of the mitochondrial NADH shuttle component aspartate-glutamate carrier AGC1/Aralar1 in INS-1E cells and rat islets. Biochem. J. **424**, 459–466
7 Chan, C. B., MacDonald, P. E., Saleh, M. C., Johns, D. C., Marban, E. and Wheeler, M. B. (1999) Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. Diabetes **48**, 1482–1486
8 Zhang, C. Y., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y. B. et al. (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes. Cell **105**, 745–755
9 Odegaard, M. L., Joseph, J. W., Jensen, M. V., Lu, D., Ilkayeva, O., Ronnebaum, S. M., Becker, T. C. and Newgard, C. B. (2010) The mitochondrial 2-oxoglutarate carrier is part of a metabolic pathway that mediates glucose- and glutamine-stimulated insulin secretion. J. Biol. Chem. **285**, 16530–16537
10 Joseph, J. W., Jensen, M. V., Ilkayeva, O., Palmieri, F., Alarcon, C., Rhodes, C. J. and Newgard, C. B. (2006) The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion. J. Biol. Chem. **281**, 35624–35632
11 Casimir, M., Lasorsa, F. M., Rubi, B., Caille, D., Palmieri, F., Meda, P. and Maechler, P. (2009) Mitochondrial glutamate carrier GC1 as a newly identified player in the control of glucose-stimulated insulin secretion. J. Biol. Chem. **284**, 25004–25014
12 Dolce, V., Iacobazzi, V., Palmieri, F. and Walker, J. E. (1994) The sequences of human and bovine genes of the phosphate carrier from mitochondria contain evidence of alternatively spliced forms. J. Biol. Chem. **269**, 10451–10460
13 Fiermonte, G., Dolce, V. and Palmieri, F. (1998) Expression in *Escherichia coli*, functional characterization, and tissue distribution of isoforms A and B of the phosphate carrier from bovine mitochondria. J. Biol. Chem. **273**, 22782–22787
14 Dolce, V., Fiermonte, G. and Palmieri, F. (1996) Tissue-specific expression of the two isoforms of the mitochondrial phosphate carrier in bovine tissues. FEBS Lett. **399**, 95–98
15 Mayr, J. A., Merkel, O., Kohlwein, S. D., Gebhardt, B. R., Böhles, H., Fötschl, U., Koch, J., Jaksch, M., Lochmüller, H. and Horváth, R. et al. (2007) Mitochondrial phosphate-carrier deficiency: a novel disorder of oxidative phosphorylation. Am. J. Hum. Genet. **80**, 478–484
16 Bose, S., French, S., Evans, F. J., Joubert, F. and Balaban, R. S. (2003) Metabolic network control of oxidative phosphorylation: multiple roles of inorganic phosphate. J. Biol. Chem. **278**, 39155–39165
17 Fujimoto, S., Ishida, H., Kato, S., Okamoto, Y., Tsuji, K., Mizuno, N., Ueda, S., Mukai, E. and Seino, Y. (1998) The novel insulinotropic mechanism of pimobendan: direct enhancement of the exocytotic process of insulin secretory granules by increased Ca^{2+} sensitivity in β-cells. Endocrinology **139**, 1133–1140
18 Takehiro, M., Fujimoto, S., Shimodahira, M., Shimono, D., Mukai, E., Nabe, K., Radu, R. G., Kominato, R., Aramaki, Y., Seino, Y. and Yamada, Y. (2005) Chronic exposure to β-hydroxybutyrate inhibits glucose-induced insulin release from pancreatic islets by decreasing NADH contents. Am. J. Physiol. **288**, E372–E380
19 Schultz, V., Sussman, I., Bokvist, K. and Tornheim, K. (1993) Bioluminometric assay of ADP and ATP at high ATP/ADP ratios: assay of ADP after enzymatic removal of ATP. Anal. Biochem. **215**, 302–304
20 Votyakova, T. V. and Reynolds, I. J. (2001) $\Delta\psi_m$-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. J. Neurochem. **79**, 266–277
21 Leung, A. W., Varanyuwatana, P. and Halestrap, A. P. (2008) The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. J. Biol. Chem. **283**, 26312–26323
22 Detimary, P., Gilon, P., Nenquin, M. and Henquin, J. C. (1994) Two sites of glucose control of insulin release with distinct dependence on the energy state in pancreatic B-cells. Biochem. J. **297**, 455–461
23 Detimary, P., Van den Berghe, G. and Henquin, J. C. (1996) Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. J. Biol. Chem. **271**, 20559–20565
24 Detimary, P., Gilon, P. and Henquin, J. C. (1998) Interplay between cytoplasmic Ca^{2+} and the ATP/ADP ratio: a feedback control mechanism in mouse pancreatic islets. Biochem. J. **333**, 269–274
25 Katz, L. A., Swain, J. A., Portman, M. A. and Balaban, R. S. (1988) Intracellular pH and inorganic phosphate content of heart *in vivo*: a ^{31}P-NMR study. Am. J. Physiol. **255**, H189–H196
26 Katz, L. A., Swain, J. A., Portman, M. A. and Balaban, R. S. (1989) Relation between phosphate metabolites and oxygen consumption of heart *in vivo*. Am. J. Physiol. **256**, H265–H274
27 Bunger, R., Mallet, R. T. and Hartman, D. A. (1989) Pyruvate-enhanced phosphorylation potential and inotropism in normoxic and postischemic isolated working heart. Near-complete prevention of reperfusion contractile failure. Eur. J. Biochem. **180**, 221–233
28 Ghosh, A., Ronner, P., Cheong, E., Khalid, P. and Matschinsky, F. M. (1991) The role of ATP and free ADP in metabolic coupling during fuel-stimulated insulin release from islet beta-cells in the isolated perfused rat pancreas. J. Biol. Chem. **266**, 22887–22892
29 Erecińska, M., Bryła, J., Michalik, M., Meglasson, M. D. and Nelson, D. (1992) Energy metabolism in islets of Langerhans. Biochim. Biophys. Acta **1101**, 273–295
30 Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M. (1971) Kinetic study of the dicarboxylate carrier in rat liver mitochondria. Eur. J. Biochem. **22**, 66–74
31 Crompton, M., Palmieri, F., Capano, M. and Quagliariello, E. (1974) The transport of sulphate and sulphite in rat liver mitochondria. Biochem. J. **142**, 127–137
32 Brown, G. C., Lakin-Thomas, P. L. and Brand, M. D. (1990) Control of respiration and oxidative phosphorylation in isolated rat liver cells. Eur. J. Biochem. **192**, 355–362
33 Valdez, L. B., Zaobornyj, T. and Boveris, A. (2006) Mitochondrial metabolic states and membrane potential modulate mtNOS activity. Biochim. Biophys. Acta **1757**, 166–172
34 Dzbek, J. and Korzeniewski, B. (2008) Control over the contribution of the mitochondrial membrane potential ($\Delta\psi$) and proton gradient (ΔpH) to the protonmotive force (Δp). *In silico* studies. J. Biol. Chem. **283**, 33232–33239
35 Martin, D. B. (1995) Bioenergetics. In A Practical Approach (Brown, G. C. and Cooper, C. E., eds), pp. 39–62, Oxford University Press, Oxford
36 Oliveira, G. A. and Kowaltowski, A. J. (2004) Phosphate increases mitochondrial reactive oxygen species release. Free Radic. Res. **38**, 1113–1118
37 Kunz, W., Gellerich, F. N., Schild, L. and Schönfeld, P. (1988) Kinetic limitations in the overall reaction of mitochondrial oxidative phosphorylation accounting for flux-dependent changes in the apparent $\Delta G^{ex}{}_P/\Delta\mu H^+$ ratio. FEBS Lett. **233**, 17–21
38 Nicholls, D. G. (1974) The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. Eur. J. Biochem. **50**, 305–315
39 Duszyński, J., Bogucka, K. and Wojtczak, L. (1984) Homeostasis of the protonmotive force in phosphorylating mitochondria. Biochim. Biophys. Acta **767**, 540–547

40 Ouhabi, R., Rigoulet, M., Lavie, J. L. and Guérin, B. (1991) Respiration in non-phosphorylating yeast mitochondria. Roles of non-ohmic proton conductance and intrinsic uncoupling. Biochim. Biophys. Acta **1060**, 293–298
41 Czyż, A., Szewczyk, A., Nałcz, M. J. and Wojtczak, L. (1995) The role of mitochondrial potassium fluxes in controlling the protonmotive force in energized mitochondria. Biochem. Biophys. Res. Commun. **210**, 98–104
42 Rigoulet, M., Fraisse, L., Ouhabi, R., Guerin, B., Fontaine, E. and Leverve, X. (1990) Flux-dependent increase in the stoichiometry of charge translocation by mitochondrial ATPase/ATP synthase induced by almitrine. Biochim. Biophys. Acta **1018**, 91–97
43 Hafner, R. P., Brown, G. C. and Brand, M. D. (1990) Analysis of the control of respiration rate, phosphorylation rate, proton leak rate and protonmotive force in isolated mitochondria using the 'top-down' approach of metabolic control theory. Eur. J. Biochem. **188**, 313–319
44 Lambert, A. J. and Brand, M. D. (2004) Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem. J. **382**, 511–517
45 Nobes, C. D., Brown, G. C., Olive, P. N. and Brand, M. D. (1990) Non-ohmic proton conductance of the mitochondrial inner membrane in hepatocytes. J. Biol. Chem. **265**, 12903–12909

Received 19 October 2010/21 January 2011; accepted 25 January 2011
Published as BJ Immediate Publication 25 January 2011, doi:10.1042/BJ20101708

Utility of indices using C-peptide levels for indication of insulin therapy to achieve good glycemic control in Japanese patients with type 2 diabetes

Shogo Funakoshi[1], Shimpei Fujimoto[1]*, Akihiro Hamasaki[1], Hideya Fujiwara[1], Yoshihito Fujita[1], Kaori Ikeda[1], Shiho Takahara[1], Kazuaki Nagashima[1], Masaya Hosokawa[1], Yutaka Seino[2], Nobuya Inagaki[1]

ABSTRACT

Aims/Introduction: Type 2 diabetes is progressive in that therapy must be altered over time, which is partly as a result of the progressive loss of pancreatic β-cell function. To elucidate the relationship between residual endogenous insulin secretion and the necessity of insulin therapy to achieve good glycemic control, indices using serum C-peptide immunoreactivity (CPR) were analyzed in patients with type 2 diabetes.

Materials and Methods: The data of 201 Japanese patients with type 2 diabetes who achieved the target of glycemic control during admission were analyzed retrospectively. Indices using CPR including fasting CPR (FCPR), CPR 6 min after intravenous injection of glucagon (CPR-6 min), increment of CPR (ΔCPR), secretory unit of islet in transplantation index (SUIT) and C-peptide index (CPI) were compared between the group requiring insulin (insulin group) and the group not requiring insulin (non-insulin group). A receiver–operator characteristic (ROC) curve was made, and optimal cut-off point and likelihood ratio were determined for each index.

Results: All indices of CPR were lower in the insulin group compared with those in the non-insulin group. Likelihood ratios at the optimal point of FCPR, CPR-6 min, ΔCPR, SUIT, and CPI were 2.0, 2.1, 1.6, 2.3 and 2.8, respectively. Optimal cut-off point of CPI was 1.1 ng/mg. Sensitivity and specificity at optimal point of CPI were 61 and 78%, respectively.

Conclusions: The advantage of CPI of the indices of CPR to select insulin therapy to achieve good glycemic control was shown, but limitations of the predictive abilities of the indices using CPR should be taken into account. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00096.x, 2011)

KEY WORDS: C-peptide, Insulin therapy, Glycemic control

INTRODUCTION

Type 2 diabetes is a heterogeneous disease characterized by insulin resistance and defective insulin secretion[1], and is progressive in that therapy must be altered over time. Initially on diagnosis, diet and exercise are generally adequate to achieve good glycemic control; oral hypoglycemic agents (OHA) are required later, when patients cannot achieve control with diet and exercise alone. Daily insulin injection is indicated when patients are unable to achieve control with a combination of oral agents, diet and exercise[2,3]. Insulin therapy is required in these patients not for survival, as is found in type 1 diabetes, but for good glycemic control[4]. This requirement is, at least in part, as a result of the progressive loss of pancreatic β-cell function. The results of the United Kingdom Progressive Diabetes Study (UKPDS) shows that pancreatic β-cell function (%β), assessed by Homeostasis Model Assessment (HOMA) in patients allocated to diet or OHA, decreased approximately 25% in 5 years[5]. A decline in endogenous insulin secretion over more than several decades of diabetes was observed in a cross-sectional study[6].

Determination of fasting serum C-peptide level and stimulated serum C-peptide level by intravenous glucagon is used widely to assess endogenous insulin secretory reserves[7–10]. There are several reports regarding the correlation between levels of residual endogenous insulin secretion and the choice of insulin therapy to achieve glycemic control[11–14]. However, in these studies, because the glycemic goal was not described clearly or was inappropriate, patients with insufficient glycemic control by the selected mode of therapy were sometimes included.

[1]Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, and [2]Kansai Electric Power Hospital, Osaka, Japan
*Corresponding author. Shimpei Fujimoto Tel.: +81-75-751-3560
Fax: +81-75-751-4244 E-mail address: fujimoto@metab.kuhp.kyoto-u.ac.jp
Received 9 September 2010; revised 12 November 2010; accepted 24 November 2010

In the present study, to evaluate the clinical significance of measures of serum C-peptide in achieving good glycemic control, we retrospectively analyzed the use of indices of endogenous insulin secretion in type 2 diabetes patients admitted to our hospital. Using data of patients who achieved the target of glycemic control during the period of admission, the patients were divided into two groups: one that achieved good control without the use of insulin (non-insulin group) and the other that required the use of insulin (insulin group), and the indices using serum C-peptide were compared between them. Optimal values and the utility of indices using serum C-peptide to select insulin therapy to achieve good glycemic control were analyzed.

MATERIALS AND METHODS

Subjects

A total of 746 Japanese patients with type 2 diabetes admitted between 2003 and 2009 to Kyoto University Hospital for poor glycemic control were enrolled in the present study. Type 2 diabetes mellitus was diagnosed based on the criteria of the American Diabetes Association (ADA)[15]. As indicated in Figure S1, 76 patients including those with pancreatic disease and liver disease, those taking diabetogenic medication and pregnant women were excluded. A total of 40 patients with incomplete clinical examinations also were excluded, and 66 patients with serum creatinine ≥1.3 mg/dL were excluded, as serum C-peptide immunoreactivity (CPR) is elevated by decreased renal function[16]. The data of 90 patients taking oral hypoglycemic agents (OHA) plus insulin at discharge were excluded. Good control was defined as mean preprandial capillary plasma glucose level <130 mg/dL, according to the glycemic control recommendation of ADA[17]. The 474 patients were divided into two groups: 201 patients who achieved good glycemic control (achieved group) and 273 patients who did not (non-achieved group). As shown in Figure S2, of the 201 patients in the achieved group, 47, 107, 38 and nine patients were treated with diet alone, OHA, insulin and insulin plus OHA at admission, respectively. At discharge, 24, 95 and 82 patients were treated with diet alone, OHA and insulin, respectively. Patients treated with diet alone and OHA at discharge comprised the non-insulin group; patients treated with insulin at discharge comprised the insulin group. A total of 166 patients of the 474 patients in the achieved or non-achieved group at discharge who could be confirmed within 6 months after discharge to achieve <7.4% in HbA_{1c}, which excludes 'not good' and 'poor' for assessment of glycemic control in the treatment guide for diabetes of the Japan Diabetes Society (JDS guide)[18], were re-analyzed to determine the cut-off point for C-peptide index (CPI) for longer duration of glycemic control. Of the 201 patients in the achieved group at discharge, 85 were excluded as a result of readmission or alteration to the mode of therapy, or were not followed as outpatients due to a change of hospital. Of the remaining 116 patients, 90 showed <7.4% HbA_{1c} within 6 months after discharge. Of the 273 patients in the non-achieved group at discharge, 137 were excluded as a result of readmission or alteration to the mode of therapy, or were not followed as outpatients due to a change of hospital. In the remaining 136 patients, 76 achieved <7.4% HbA_{1c} within 6 months after discharge. In these 166 patients, analysis of optimal values and the utility of CPI during admission was carried out.

Methods

On the first day in hospital, medical history, physical examination and laboratory evaluation including glycosylated hemoglobin were carried out. HbA_{1c} was measured using HPLC (HA-8180; Arcray, Kyoto, Japan). The HbA_{1c} (%) value was estimated as an National Glycohemoglobin Standardization Program equivalent (%) calculated by the formula: HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (National Glycohemoglobin Standardization Program)[19]. β-cell function was evaluated within 1 week after overnight fast by glucagon test measuring CPR before (fasting CPR [FCPR]) and 6 min after i.v. injection of 1 mg glucagon (CPR-6 min)[7], as this test is valid in patients taking insulin therapy. Increment of CPR (ΔCPR) was obtained by subtracting FCPR from CPR-6 min. SUIT index (SUIT) (%) was calculated by the formula: 1500 × FCPR (ng/mL)/(fasting plasma glucose [FPG; mg/dL] − 61.7)[20]. CPI (ng/mg) was calculated by the formula: 100 × FCPR (ng/mL)/FPG (mg/dL). Serum CPR was measured by immunoenzymometric assay (EIA; ST AIA-PACK C-Peptide, Toso corporation, Tokyo, Japan). In patients taking OHA, medication was stopped for the glucagon test, but was maintained until 1 day before to prevent hyperglycemia during the test[6]. Fasting plasma glucose was measured by the glucose oxidase method when the glucagon test was carried out. Patients were treated according to the JDS guide[18]. Treatment policy including diet therapy, exercise therapy, pharmacotherapy and education for each patient was determined by Japanese Board Certified Diabetologists certified by the Japan Diabetes Society. Patients took medical nutritional therapy (25–30 kcal/kg of standard bodyweight/day consisting of 58% carbohydrate, 18% protein and 24% fat energy intake percentages) with counseling by a registered dietitian. Preprandial capillary plasma glucose levels were monitored three t.i.d. during hospitalization. The study protocol was approved by the ethics committee of Kyoto University.

Statistical analysis

Statistical analysis was carried out with the Stat View 5.0 system (SAS institute, Cary, NC, USA). Data are presented as mean ± SE unless otherwise stated. Clinical parameters among the two groups were compared by Mann–Whitney *U*-test. *P*-values <0.01 were considered statistically significant. Histograms and receiver–operator characteristic (ROC) curve were made for FCPR, CPR-6 min, ΔCPR, SUIT and CPI respectively, and sensitivity, specificity, cut-off values, area under the ROC curve (AUC) and the likelihood ratio were calculated.

RESULTS

Clinical profiles of patients with mean preprandial capillary plasma glucose levels at discharge of <130 mg/dL (achieved group) and ≥130 mg/dL (non-achieved group), respectively, are shown in Table 1. Patients of the non-achieved group were older, had lower body mass index at admission, higher mean preprandial capillary plasma glucose level both at admission and at discharge, longer years from diagnosis and lower endogenous insulin secretion indices than those of the achieved group. The clinical stages of diabetic nephropathy and retinopathy were more progressed in the non-achieved group than those in the achieved group. The relationships between indices using serum C-peptide and selected modes of therapy at discharge were analyzed based on the data of the achieved group.

The clinical profiles of patients not requiring insulin for good glycemic control (non-insulin group) and those requiring insulin (insulin group) are shown in Table 2. The patients of the insulin group were older, has lower body mass index, higher HbA_{1c} at admission, higher mean preprandial capillary plasma glucose level at admission, longer years from diagnosis and lower endogenous insulin secretion indices compared with those of the non-insulin group. As shown in Figure S2, the mode of therapy in 41 patients was altered from diet alone or OHA to insulin during admission. The average number of hospital days before altering the therapeutic mode of these patients was 3.1 ± 3.4 (mean ± SD). The reasons for the change to insulin therapy were the necessity of tight glycemic control before operation in five patients, marked hyperglycemia (a fasting plasma glucose level of 250 mg/dL or above, or a causal plasma glucose of 350 mg/dL or above)[21] or both the presence of hyperglycemia and ketosis in 11 patients, and persistent hyperglycemia with OHA in 25 patients. HbA_{1c} at admission of these patients was 10.2 ± 2.2% (mean ± SD). In five patients, the mode of therapy was altered from insulin to OHA. The average number of hospital days before this change was 7.6 ± 4.3 (mean ± SD); the reason was improved glycemic control despite a decrease in the required dosage of insulin. HbA_{1c} at admission of these patients was 10.1 ± 4.4% (mean ± SD). Another patient treated with OHA plus insulin at admission was changed to OHA alone after nine hospital days because of improved glycemic control. Of the 113 patients with therapy of diet alone or OHA both at admission and at discharge, 19 transiently used insulin during the period of admission.

The category of OHA at discharge is shown in Table S1a. In 95 patients treated with OHA, 60 and 29 patients were prescribed sulfonylurea alone or in combination, and biguanide alone or in combination, respectively. In the insulin group, 50 of 86 patients were given premixed insulin b.i.d. at discharge. As shown in Table S1b, the prescribed daily dosages of gliclazide, glimepiride and metformin required were <80, 4 and 750 mg,

Table 1 | Clinical profiles of patients who achieved good glycemic control

	Achieved	Non-achieved	*P*
No. subjects	201	273	
Duration of hospitalization (days)	22.0 ± 0.7	23.6 ± 0.7	0.1115
Age (years)	60.2 ± 0.9	64.5 ± 0.7*	0.0002
Male/female	127/74	159/114	
Systolic blood pressure (mmHg)	124.5 ± 1.0	126.9 ± 1.1	0.1076
Diastolic blood pressure (mmHg)	74.6 ± 0.7	73.6 ± 0.6	0.2653
BMI (kg/m^2)	25.2 ± 0.3	23.8 ± 0.3*	0.0005
HbA_{1c} at admission (%)	9.5 ± 0.1	9.8 ± 0.1	0.0776
PG at admission (mg/dL)	181.1 ± 4.7	209.5 ± 3.9*	<0.0001
PG at discharge (mg/dL)	112.2 ± 0.9	163.2 ± 1.9*	<0.0001
Years from diagnosis	9.1 ± 0.6	13.5 ± 0.6*	<0.0001
FCPR (ng/mL)	1.87 ± 0.06	1.65 ± 0.05*	0.0054
CPR-6 min (ng/mL)	3.99 ± 0.14	3.41 ± 0.10*	0.0006
ΔCPR (ng/mL)	2.12 ± 0.09	1.76 ± 0.07*	0.0011
SUIT (%)	40.6 ± 1.9	32.4 ± 2.0*	0.0043
CPI (ng/mg)	1.34 ± 0.05	1.09 ± 0.04*	<0.0001
Clinical stage of nephropathy (normal/microalbuminuria/macroalbuminuria)	129/56/16 (64/28/8)	133/80/60 (49/29/22)	
Clinical stage of retinopathy (NDR/mild NPDR/moderate NPDR/severe NPDR/PDR)	141/25/26/4/5 (71/12/13/2/2)	112/53/45/22/41 (41/20/16/8/15)	

Data are presented as mean ± SE. *P < 0.01 versus achieved. Achieved group: mean preprandial capillary plasma glucose levels at discharge <130 mg/dL compared with those who did not achieve good glycemic control (non-achieved group ≥130 mg/dL). BMI, body mass index; CPI, C-peptide index; ΔCPR, increment of C-peptide immunoreactivity; CPR-6 min, C-peptide immunoreactivity 6 min after intravenous injection of glucagon; FCPR, fasting C-peptide immunoreactivity; NDR, no diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; PG, mean preprandial capillary plasma glucose level; SUIT, secretory unit of islet in transplantation index. Numbers in parentheses indicate percentages.

Table 2 | Clinical profiles of patients who achieved good glycemic control without requiring the use of insulin and those requiring insulin to achieve good glycemic control

	Non-insulin	Insulin	*P*
No. subjects	119	82	
Male/female	82/37	45/37	
Age (years)	58.4 ± 1.1	62.9 ± 1.3*	0.0099
Systolic blood pressure (mmHg)	124.4 ± 1.4	126.4 ± 1.7	0.3598
Diastolic blood pressure (mmHg)	77.3 ± 1.0	73.3 ± 1.3	0.0135
BMI (kg/m^2)	26.0 ± 0.4	24.0 ± 0.4*	0.0019
HbA_{1c} at admission (%)	9.2 ± 0.2	10.0 ± 0.2*	0.0050
PG at admission (mg/dL)	163.2 ± 5.0	206.9 ± 8.0*	<0.0001
PG at discharge (mg/dL)	110.9 ± 1.2	114.2 ± 1.3	0.0602
Years from diagnosis	7.8 ± 0.6	10.9 ± 1.0*	0.0052
FCPR (ng/mL)	2.06 ± 0.07	1.61 ± 0.09*	0.0001
CPR-6 min (ng/mL)	4.48 ± 0.18	3.29 ± 0.19*	<0.0001
ΔCPR (ng/mL)	2.43 ± 0.12	1.68 ± 0.12*	<0.0001
SUIT (%)	47.2 ± 2.5	31.1 ± 2.7*	<0.0001
CPI (ng/mg)	1.57 ± 0.07	1.06 ± 0.06*	<0.0001

Data are presented as mean ± SE. *P < 0.01 versus non-insulin. Good glycemic control: mean preprandial capillary plasma glucose levels at discharge <130 mg/dL.

BMI, body mass index; CPI, C-peptide index; ΔCPR, increment of C-peptide immunoreactivity; CPR-6 min, C-peptide immunoreactivity 6 min after intravenous injection of glucagon; FCPR, fasting C-peptide immunoreactivity; PG, mean preprandial capillary plasma glucose level; SUIT, secretory unit of islet in transplantation index.

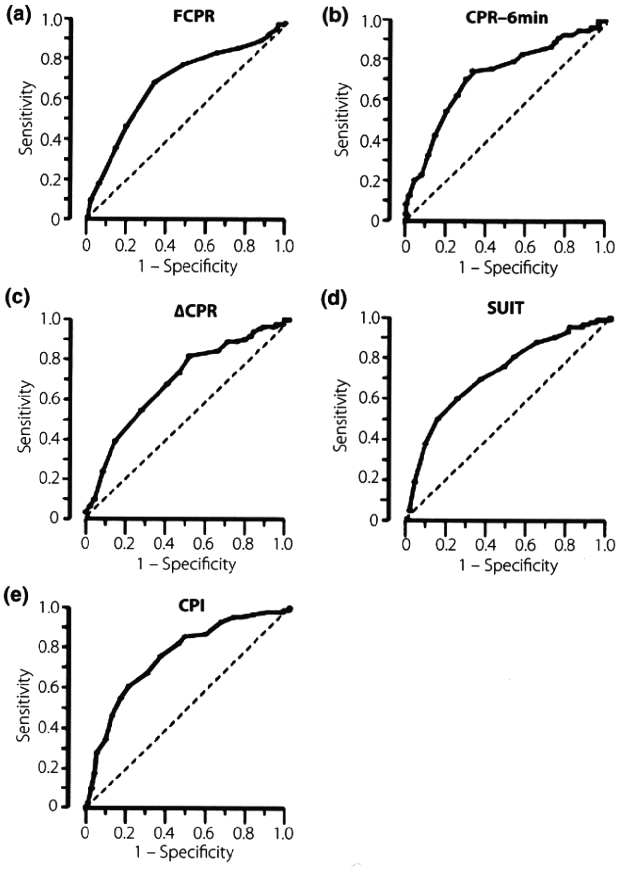

Figure 1 | Receiver–operator characteristic curves of (a) fasting C-peptide immunoreactivity (FCPR), (b) CPR 6 min after intravenous injection of glucagon (CPR-6 min), (c) increment of CPR (ΔCPR), (d) secretory unit of islet in transplantation index (SUIT) and (e) C-peptide index (CPI) of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge.

respectively in almost all (more than 95%) patients. Daily insulin dosage was 22.0 ± 11.1 U (mean ± SD) in the insulin group.

In Figure S3, peak relative frequency of indices using CPR of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge in the insulin group and the non-insulin group, respectively, is shown (FCPR: 1.50–1.75, 2.00–2.25 ng/mL; CPR-6 min: 2.75–3.00, 4.00–4.25 ng/mL; ΔCPR: 1.25–1.50, 1.25–1.50 plus 2.25–2.50 ng/mL; SUIT: 15–20, 25–30 plus 35–40 plus 45–50%; and CPI: 0.8–0.9, 1.5–1.6 ng/mg). According to ROC curves of indices using CPR shown in Figure 1, AUC, cut-off values and values at optimal cut-off points including sensitivity, specificity and the likelihood ratio were determined and shown in Table 3. CPI is the most relevant of these indices for selecting insulin therapy to achieve good glycemic control, because the likelihood ratio and AUC of CPI is greatest.

The ROC curve of CPI of patients who achieved <7.4% HbA_{1c} within 6 months after discharge is shown in Figure 2. According to ROC curves of CPI in Figure 2, the AUC (0.75), cut-off values (optimal: 1.2; 90% specificity 0.8; 90% sensitivity 1.7 ng/mg), and values at optimal cut-off points including sensitivity (73%), specificity (71%) and the likelihood ratio (2.5) were determined.

DISCUSSION

Medical nutritional therapy (MNT) improves glycemic control in patients with type 2 diabetes regardless of their modes of therapy including diet alone, OHA and insulin[22–24]. Diet therapy is the basis and starting point of treatment of all patients with diabetes[25], and failure of diet therapy alone might predict the inability to attain optimal glycemic control by any of these modes of therapy. To precisely analyze the relationship between endogenous insulin secretion and the appropriate mode of therapy for achieving good glycemic control, we used data of hospitalized patients under optimal therapy including proper MNT. Thus, our results are more likely to be valid in patients with appropriate care behaviors. Although inappropriate care behavior is an obstacle to achieving good glycemic control over a longer duration, our results suggest a basis for beginning insulin therapy in patients who do not achieve good glycemic control with diet alone or OHA despite the practice of appropriate care behavior.

Table 3 | Analysis of indices using serum C-peptide of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge

	FCPR	CPR-6 min	ΔCPR	SUIT	CPI
AUC	0.69	0.71	0.69	0.72	0.75
Cut-off values	(ng/mL)	(ng/mL)	(ng/mL)	(%)	(ng/mg)
Optimal	1.75	3.75	2.25	30	1.1
90% Specificity	1.00	2.25	1.00	20	0.7
90% Sensitivity	2.75	5.25	3.25	55	1.7
Values at optimal cut-off points					
Sensitivity (%)	70	74	82	61	61
Specificity (%)	66	65	49	73	78
Likelihood ratio	2.0	2.1	1.6	2.3	2.8

AUC, area under receiver–operator characteristics curve; CPI, C-peptide index; ΔCPR, increment of C-peptide immunoreactivity; CPR-6 min, C-peptide immunoreactivity 6 min after intravenous injection of glucagon; FCPR, fasting C-peptide immunoreactivity; SUIT, secretory unit of islet in transplantation index

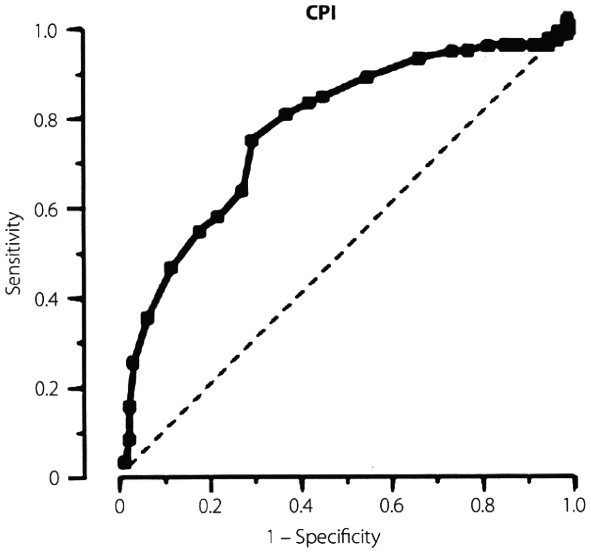

Figure 2 | Receiver–operator characteristic curve of C-peptide index (CPI) of patients who achieved <7.4% HbA_{1c} within 6 months after discharge.

In the present study, just 42% of patients achieved good control during hospital admission, partly because the aim of admission was not necessarily to achieve good control during the period of admission, but to establish a treatment policy for the achievement of good control after discharge. The percentage of patients treated with insulin at discharge was higher in the non-achieved group than in the achieved group (non-achieved group: 67%; achieved group: 41%). Of the patients treated with OHA at admission in the achieved group, 39% had therapy changed to insulin, whereas 73% of the patients treated with OHA at admission in the non-achieved group had therapy changed to insulin. These results might indicate more intensive therapy in the case of the non-achieved group. Of the 136 patients in the non-achieved group at discharge, 76 showed <7.4% HbA_{1c} within 6 months after discharge, showing fair glycemic control in some of the patients of this group over the longer term. As shown in Table 1, the non-achieved group had more progressive diabetic complications and more years from diagnosis compared with the achieved group. These factors might prompt therapy that aims at a more gradual improvement of glycemic control to prevent hypoglycemia. In addition, the non-achieved group showed higher glycemic levels at admission than that of the achieved group, whereas the duration of hospitalization was similar.

Although there have been several reports regarding the utility of indices of endogenous insulin secretion to indicate initiation of insulin therapy to improve glycemic control[11–14], none has compared the utility of the various indices. In the present study, as shown by the likelihood ratio and by AUC, CPI is shown to be the most useful among the five indices.

CPI was used as an index of endogenous insulin secretion in several reports[26–28], but its advantage over other indices and the scientific basis was unclear. The SUIT index (SUIT) was developed using FCPR and plasma glucose level after islet transplantation[19]. The linear relationship between FCPR and FPG in individual subjects shows a plasma glucose level (61.7 mg/dL) assumed to suppress C-peptide to zero. Transplantation of islets from non-diabetic donors increases the slope (FCPR/[FPG – 61.7]), suggesting an index of transplanted β-cell mass. Although a correlation between SUIT and CPR 6 min after intravenous injection of 1 mg glucagon (CPR-6 min) is observed in type 2 diabetes ($r = 0.58$), it is weaker than that in patients after islet transplantation ($r = 0.82$)[19].

Autopsy reveals that β-cell mass is decreased in patients with type 2 diabetes compared with that in healthy subjects[29–31]. Recently, in 33 subjects at various stages of glucose tolerance, a correlation between β-cell areas of a sample obtained during pancreatectomy, and serum levels of CPR and insulin before the operation was analyzed[32]. Interestingly, β-cell areas are positively correlated with fasting insulin/FPG ($r = 0.51$, $P = 0.0024$) and FCPR/FPG ($r = 0.63$, $P < 0.0001$), but are not significantly