Src regulates insulin secretion and glucose metabolism by influencing subcellular localization of glucokinase in pancreatic β-cells

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INTRODUCTION
In pancreatic β-cells, glucose metabolism regulates exocytosis of insulin granules through metabolism-secretion coupling, in which glucose-induced adenosine triphosphate (ATP) production in mitochondria plays an essential role1. Oxidative stress is one of the most significant factors that impairs glucose metabolism in β-cells2. We previously reported that Src plays an important role in the production of reactive oxygen species (ROS)3. In particular, endogenous overproduction of ROS in pancreatic islets of diabetic Goto-Kakizaki rats and ouabain-exposed islets of normoglycemic Wistar rats is reduced by treatment of 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3, 4-d]pyrimidine (PP2), a specific Src inhibitor4, while transient exenatide treatment suppresses Src hyperactivity and reduces ROS overproduction in Goto-Kakizaki rat islets5. In addition, exposure of palmitate increases ROS production with an increase in the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a pathological ROS source in plasma mem-

ABSTRACT
Aims/Introduction: Src, a non-receptor tyrosine kinase, regulates a wide range of cellular functions, and hyperactivity of Src is involved in impaired glucose metabolism in pancreatic β-cells. However, the physiological role of Src in glucose metabolism in normal, unstressed β-cells remains unclear. In the present study, we investigated the role of Src in insulin secretion and glucose metabolism.

Materials and Methods: Src was downregulated using small interfering ribonucleic acid in INS-1 cells, and glucose-induced insulin secretion, adenosine triphosphate content, intracellular calcium concentration, glucose utilization and glucokinase activity were measured. Expression levels of messenger ribonucleic acid and protein of glucokinase were examined by semiquantitative real-time polymerase chain reaction and immunoblotting, respectively. Cells were fractionated by digitonin treatment, and subcellular localization of glucokinase was examined by immunoblotting. Interaction between glucokinase and neuronal nitric oxide synthase was facilitated by Src downregulation.

Results: In Src downregulated INS-1 cells, glucose-induced insulin secretion was impaired, whereas insulin secretion induced by high K+ was not affected. Intracellular adenosine triphosphate content and elevation of intracellular calcium concentration by glucose stimulation were suppressed by Src downregulation. Src downregulation reduced glucose utilization in the presence of high glucose, which was accompanied by a reduction in glucokinase activity without affecting its expression. However, Src downregulation reduced glucokinase in soluble, cytoplasmic fraction, and increased it in pellet containing intracellular organelles. In addition, interaction between glucokinase and neuronal nitric oxide synthase was facilitated by Src downregulation.

Conclusions: Src plays an important role in glucose-induced insulin secretion in pancreatic β-cells through maintaining subcellular localization and activity of glucokinase.
brane, and reduces glucose-induced insulin secretion (GIIS) in INS-1 rat insulinoma cells, whereas Src inhibition reduces ROS production by decreasing its activity and restores insulin secretion6. Src is a non-receptor tyrosine kinase and a member of Src family kinases (SFKs), which are expressed in various types of animal cells7. The activity of Src is regulated by tyrosine phosphorylation at two sites with opposite effect. Briefly, phosphorylation of Tyr527 at the COOH-terminal regulatory domain results in the inactivation of Src, and phosphorylation of Tyr418 at the catalytic domain results in the activation of Src8. Src regulates a wide range of cellular events including cell growth, division, differentiation, survival and programed death, as well as specialized functions, such as immune responses, cell adhesion, migration and endocytosis, whereas dysregulation of Src has been implicated in the etiology of human diseases, especially cancers9. In addition, evidence that Src is related to pancreatic β-cell function has also been reported10–12. However, the physiological role of Src in glucose metabolism in normal, unstressed β-cells remains unclear.

In the present study, we investigated the effects of Src down-regulation in glucose metabolism in intact INS-1 cells to characterize its role more precisely. We show here that Src regulates glucokinase activity to maintain glucose metabolism in pancreatic β-cells.

MATERIALS AND METHODS

Chemicals, cell culture and small interfering ribonucleic acid transfection

ATP, adenosine diphosphate, poly-L-ornithine and diadenosine pentaphosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). PP2 was purchased from Tocris (Ellisville, MO, USA). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise noted. INS-1 cells were cultured in RPMI 1640 medium as previously described14. Stealth™ small interfering ribonucleic acids (siRNAs) were synthesized by Life Technologies (Carlsbad, CA, USA). The sequences of siRNA specific for rat Src, called Src siRNA, and a control siRNA with the nonsense sequence are shown in Table S1. Transfection of siRNAs was carried out as previously reported14, except that the reacted cell number was 5 × 105 cells/mL in the present study. All experiments using siRNA-transfected INS-1 cells were carried out 48 h after transfection, unless otherwise noted.

Isolation of total RNA and semiquantitative real-time polymerase chain reaction

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary deoxyribonucleic acid was synthesized as previously described14. Semiquantitative real-time polymerase chain reaction (PCR) was carried out using SYBR Green PCR Master Mix (Life Technologies) as previously reported14, and results were normalized by the expression of β-actin. The rat sequences of forward and reverse primers to detect Src, glucokinase, glucose transporter 2 (Glut2) and β-actin are shown in Table S2.

Immunoprecipitation and immunoblotting

Immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting was carried out as previously described14. Primary antibodies used were mouse monoclonal anti-Src antibody from Merck KGaA (Darmstadt, Germany); mouse monoclonal anti-complex I (39 kDa subunit), anti-complex III (core II), anti-complex IV (subunit I) and anti-complex V (subunit α) of mitochondrial respiratory chain antibody from Invitrogen (Eugene, OR, USA); rabbit anti-glucokinase antibody and anti-GLUT2 antibody from Abcam (Cambridge, UK); rabbit anti-Lyn, anti-Lck, anti-Fgr, anti-Blk, anti-C-terminal Src kinase (Csk), anti-calnexin and goat anti-Hck antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-neuronal nitric oxide synthase (nNOS) antibody from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal anti-β-actin antibody from Sigma-Aldrich; and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody from Merck KGaA. Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit and mouse antibody from GE Healthcare (Buckinghamshire, UK), and anti-goat antibody from Merck KGaA. Band intensities were quantified with Multi Gauge software (Fuji Film, Tokyo, Japan).

Insulin secretion, ATP content and intracellular calcium concentration

Insulin secretion, ATP content and intracellular calcium concentration ([Ca2+]i) were measured as previously described14. Krebs-Ringer bicarbonate HEPES (KRBH) buffer was composed of 140 mmol/L NaCl, 3.6 mmol/L KCl, 0.5 mmol/L MgSO4, 0.5 mmol/L NaH2PO4, 1.5 mmol/L CaCl2, 2 mmol/L NaHCO3, 0.1% bovine serum albumin and 10 mmol/L HEPES (pH 7.4).

Mitochondrial ATP production

Measurement of ATP production in the mitochondrial fraction was carried out as previously described14, except 3 mmol/L KH2PO4 was included in the solution used for mitochondria suspension and reaction. ATP production was corrected by mitochondrial protein content.

Glucose utilization

Glucose utilization was measured using the previously described method with slight modifications15. Briefly, INS-1 cells cultured on 24-well plates were incubated in KRBH buffer with 2 mmol/L glucose at 37°C for 30 min, followed by incubation in 500 μL of KRBH buffer containing 2 or 10 mmol/L glucose and 1.5 mCi [5-3H] glucose (GE Healthcare) for 60 min. Subsequently, 150 μL of supernatant of each well was transferred into a micro tube containing 50 μL of HCl to completely stop the reaction. The tube was then incubated in the capped vial containing 500 μL of distilled water overnight at 34°C to allow 3H2O (GE Healthcare) in the tube to equilibrate with the water in the vial. During the overnight incubation period, the micro
Measurement of glucokinase activity

Glucokinase activity was measured as previously described with slight modifications. Briefly, after preincubation in KRBH buffer with 2 mmol/L glucose for 60 min, INS-1 cells were lysed and subjected to glucose-6-phosphate dehydrogenase-coupled reaction at two (0.5 and 50 mmol/L) concentrations of glucose for 60 min at 37°C. The glucose phosphorylation rate was estimated as the increase of NADH concentration, which was measured by fluorometric plate reader with excitation 360 nm and emission 460 nm. Glucokinase activity was determined by subtracting hexokinase activity measured at 0.5 mmol/L glucose subtracted from the activity measured at 50 mmol/L glucose.

Subcellular localization of glucokinase

Fractionation of digitonin-permiabilized INS-1 cells was carried out as described previously. Briefly, INS-1 cells were collected and incubated in 50 mmol/L HEPES buffer (pH 7.2) containing 125 mmol/L KCl, 20 mmol/L NaCl, 0.5 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, along with 20 μg/mL digitonin for 10 min at 4°C. After centrifugation in a microcentrifuge (20 min at 12,000 g), both supernatants, the soluble fraction containing cytoplasmic proteins, and the pellet, the insoluble fraction containing any organelles including membrane or nuclei, were collected and used for immunoblotting. The intensities of glucokinase were measured and normalized by those of GAPDH in soluble fraction, and by those of calnexin in pellet.

Statistical analysis

The data are expressed as means ± standard error of the mean. Statistical significance was determined by unpaired Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

Silencing effects of Src in INS-1 cells

Src is a member of SFKs, and several SFKs are expressed in rat pancreatic islets. We first confirmed which SFKs are expressed in INS-1 cells, and Src, Lyn, Blk, Hck, Lck and Fgr were detected, but Fyn and Yes were not detected by immunoblotting (data not shown). Src siRNA was used to achieve highly specific Src downregulation and avoid unexpected cross-reactivity or chemical effects. A total of 48 h after transfection of siRNAs, semiquantitative real-time PCR assays and immunoblotting analysis showed 67.0% reduction of Src messenger RNA (Figure 1a) and 55.7% reduction of Src protein by Src siRNA (Figure 1b) compared with negative control siRNA. Expression levels of other SFKs or Csk, a negative regulator of Src, were not affected by Src downregulation (Figure 1c).

Insulin secretion, intracellular ATP content and [Ca²⁺]ᵢ

At the beginning of the analysis, we investigated the role of Src in insulin secretion. Downregulation of Src decreased GIIS, whereas insulin secretion induced by high K⁺ was not affected (Figure 2a). Insulin content was not affected by Src downregulation (Figure 2b). In addition, exposure of PP2 for 48 h reduced GIIS in INS-1 cells (Figure 2c), whereas transient PP2 treatment for 30 min did not change GIIS in INS-1 cells, as previously reported.
We then evaluated the glucose-induced increase of intracellular ATP content and \([\text{Ca}^{2+}]_i\). Downregulation of Src reduced approximately 30% of intracellular ATP content at a stimulating level of 10 mmol/L glucose (Figure 2d). The elevation of \([\text{Ca}^{2+}]_i\) in response to 10 mmol/L glucose was suppressed and delayed by downregulation of Src. In addition, the rate and amplitude of glucose-induced \([\text{Ca}^{2+}]_i\) oscillation were reduced. When the stimulus was changed from 10 mmol/L glucose to 30 mmol/L K+, \([\text{Ca}^{2+}]_i\) in Src downregulated INS-1 cells increased as quickly as that in control. \([\text{Ca}^{2+}]_i\), evoked by high K+ was also reduced by Src downregulation, whereas the suppressive effect was milder than that evoked by high glucose (Figure 2e,f).

Glucose utilization, glucokinase activity, GLUT2 expression and mitochondrial ATP production

We then evaluated the functions proximal to membrane depolarization. Src downregulation reduced glucose utilization, which reflects the velocity of glucose metabolism in glycolysis, in the presence of 10 mmol/L glucose (Figure 3a), as well as the activity of glucokinase, a rate-limiting enzyme in glycolysis in β-cells (Figure 3b).

Downregulation of Src did not significantly affect expression levels of GLUT2 mRNA or GLUT2 protein (Figure S1). ATP production from mitochondria fraction in the presence of mitochondrial substrates (Figure 3c) or expression levels of mitochondrial respiratory chain complex proteins (Figure 3d), by which oxidative phosphorylation is carried out to produce ATPs, were not altered by Src downregulation.

Expression levels and subcellular localization of glucokinase

We then attempted to determine the mechanism of reduction of glucokinase activity by Src downregulation. The expression levels of glucokinase messenger RNA and protein were not significantly affected by Src downregulation (Figure 4a,b). Therefore, subcellular localization of glucokinase, which affects enzyme activity independent of expression level, was investigated. INS-1 cells were separated to soluble fraction containing cytoplasmic proteins and pellet containing any organelles including membrane or nuclei, and immunoblotting analysis was carried out. Src downregulation was shown to reduce the expression level of glucokinase in soluble fraction and increase that in pellet (Figure 4c). This finding suggests that Src downregulation suppresses glucokinase activity by shifting its subcellular localization.

Interaction of glucokinase with nNOS

It has been reported that glucokinase localization and activity is regulated by nNOS19. Therefore, we examined whether Src regulates the association of glucokinase with nNOS by immunoprecipitation using anti-nNOS antibody, followed by immunoblotting using anti-glucokinase and anti-nNOS antibody. Interaction between glucokinase and nNOS was facilitated by Src downregulation without affecting the protein expression level of nNOS (Figure 5a,b).

**Figure 2** | Effects of Src downregulation on insulin secretion, insulin content, adenosine triphosphate (ATP) content and intracellular calcium concentration. (a) Effects of Src downregulation on insulin secretion. Insulin secretion was measured after 30 min incubation in Krebs-Ringer bicarbonate HEPES (KRBH) buffer with 2 mmol/L glucose (G2), 10 mmol/L glucose (G10) or 2 mmol/L glucose and 30 mmol/L KCl. Data were normalized by protein content (n = 4). Values are expressed as mean ± standard error of the mean. **P < 0.01 compared with control small interfering ribonucleic acid (siRNA) at the corresponding condition. (b) Effects of Src downregulation on insulin content. INS-1 cells were separated to soluble fraction containing any organelles including membrane or nuclei, and immunoblotting analysis was carried out. Src downregulation was shown to reduce the expression level of glucokinase in soluble fraction and increase that in pellet (Figure 4c). This finding suggests that Src downregulation suppresses glucokinase activity by shifting its subcellular localization.
DISCUSSION

We previously reported that Src inhibition by short exposure to PP2 for 30–60 min reduced overproduction of ROS, and restored impaired glucose metabolism and GIIS in pathophysiological conditions, such as that in diabetic Goto-Kakizaki rat islets and INS-1 cells exposed to palmitate, but showed little effect on these parameters in physiological conditions. However, the more prolonged effects of Src inhibition on
pancreatic β-cells in physiological conditions are unknown. The aim of the present study was to clarify the physiological role of Src in pancreatic β-cells in an unstressed condition.

To estimate the role of Src in β-cell function, we first examined insulin secretion. Src downregulation reduced GIIS, but had no effect on high K⁺-induced insulin secretion (Figure 2a, c), and the suppressive effect of Src downregulation on [Ca²⁺]ᵢ elevation during high K⁺ exposure was milder than that during glucose exposure (Figure 2e,f). Glucose-induced increase in intracellular ATP content was also decreased by Src downregulation (Figure 2d). These results show that Src has some effects on glucose metabolism.

To narrow down the candidate site for Src in β-cells, we examined the activity of glycolysis and mitochondrial function. In other cells, Src is reported to be involved in the glycolysis pathway and in the expression of glucose transporters. Src downregulation suppressed glucose utilization in the medium containing 10 mmol/L glucose significantly, and reduced the activity of glucokinase, a rate-limiting enzyme in glycolysis, which determines glucose metabolism in β-cells (Figure 3a,b), but Src downregulation did not affect GLUT2 expression (Figure S1). Src also plays an important role in mitochondrial function in other cells, and mitochondrial oxidative phosphorylation is essential in ATP production, resulting in inactivation of ATP sensitive potassium channels and membrane depolarization. Mitochondrial ATP production and protein expression levels of mitochondrial respiratory chain complex were therefore examined. However, Src downregulation did not affect ATP production significantly when mitochondrial fraction was incubated in the presence of succinate plus rotenone or N,N,N',N'-tetramethyl-p-phenylenediamine plus ascorbate, which renders electrons at complex II or complex IV, respectively (Figure 3c). Protein expression levels of several mitochondrial respiratory chain complex proteins were not altered prominently by Src downregulation in the present study, which implies that mitochondrial mass was not affected (Figure 3d). Apparently, the suppressive effects of Src downregulation on glucose metabolism are derived from decrease in glycolysis as a result of reduced glucokinase activity and not from a decrease in capacity to produce ATP in mitochondria.

In β-cells, glucose metabolism is regulated mainly by reaction at glucokinase in glycolysis, which has far less velocity than those of reactions at other enzymes in glycolysis other than glucokinase and glucose transport. Glucokinase activity is often regulated by its expression level, but it was not affected by Src downregulation in the present study. Glucokinase activity is modified by various post-translational mechanisms including translocation, S-nitrosylation, and interaction with other molecules. In β-cells, glucokinase is localized to insulin granules; it has been reported that glucokinase translocates from the membrane of insulin granules to the cytosol in response to glucose or insulin stimuli, and that glucokinase activity is enhanced by translocation. A fractionation study was therefore carried out, and it was found that Src downregulation reduced and increased protein levels of glucokinase in soluble, cytoplasmic fraction and pellet containing intracellular organelles, respectively (Figure 4c), which is consistent with the results that suppression of glucokinase activity in whole cell extracts is accompanied with a decrease in cytosolic glucokinase. It is possible that Src downregulation shifted glucokinase from cytosol to membrane of insulin granules, which resulted in suppression of its activity.

As mentioned, Src is a non-receptor tyrosine kinase. Glucokinase is reported to contain serine phosphorylation sites, but not tyrosine, and glucokinase has not been considered as a substrate of Src. Because interaction of Src with glucokinase was not observed by immunoprecipitation (data not shown), Src is not likely to interact with glucokinase directly. Therefore, glucokinase would be regulated by Src through an indirect mechanism.

Regarding the mechanism of glucokinase translocation and activation, Rizzo et al. elucidated detail. Subcellular localization of glucokinase is determined by nNOS localization, and insulin treatment increases glucokinase S-nitrosylation nNOS dependently, resulting in dissociation from insulin granules. Naturally occurring mutations of glucokinase cause defects in S-nitrosylation and block glucokinase activation in β-cells. Glucagon-like peptide-1, an incretin, nitrosylates and activates
Src regulates glucokinase activity

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article:

Figure S1| Expression levels of glucose transporter 2.
Table S1| Small interfering ribonucleic acid (siRNA) sequences.
Table S2| Primer sequences used in semiquantitative real-time polymerase chain reaction (PCR).