An Allosteric Activator of Glucokinase Impairs the Interaction of Glucokinase and Glucokinase Regulatory Protein and Regulates Glucose Metabolism*

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Glucokinase (GK) plays a key role in the control of blood glucose homeostasis. We identified a small molecule GK activator, compound A, that increased the glucose affinity and maximal velocity \( V_{\text{max}} \) of GK. Compound A augmented insulin secretion from isolated rat islets and enhanced glucose utilization in primary cultured rat hepatocytes. In rat oral glucose tolerance tests, orally administrated compound A lowered plasma glucose elevation with a concomitant increase in plasma insulin and hepatic glycogen. In liver, GK activity is acutely controlled by its association to the glucokinase regulatory protein (GKRP). In order to decipher the molecular aspects of how GK activator affects the shuttling of GK between nucleus and cytoplasm, the effect of compound A on GK-GKRP interaction was further investigated. Compound A increased the level of cytoplasmic GK in both isolated rat primary hepatocytes and the liver tissues from rats. Experiments in a cell-free system revealed that compound A interacted with glucose-bound free GK, thereby impairing the association of GK and GKRP. On the other hand, compound A did not bind to glucose-unbound GK or GKRP-associated GK. Furthermore, we found that glucose-dependent GK-GKRP interaction also required ATP. Given the combined prominent role of GK on insulin secretion and hepatic glucose metabolism where the GK-GKRP mechanism is involved, activation of GK has a new therapeutic potential in the treatment of type 2 diabetes.

There are three key aspects of type 2 diabetes pathogenesis, which are the focus of current and future therapies: insulin resistance, defective insulin secretion, and increased hepatic glucose production. Glucokinase (GK) is the predominant glucose phosphorylation enzyme in pancreatic \( \beta \)-cells and hepatocytes. GK plays an important role as a glucose sensor for controlling plasma glucose homeostasis by enhancing insulin secretion from pancreatic \( \beta \)-cells and glucose metabolism in the liver (1, 2), which provides rational expectations that enhancement of GK activity would be a novel therapeutic strategy for type 2 diabetes. Consistent with this rationale, recently discovered small molecule allosteric activators of GK have been demonstrated to have antidiabetic efficacy in rodents (3, 4).

To investigate the mechanism of action of GK activators, the interaction between GK and glucokinase regulatory protein (GKRP) is a key aspect. It is well known that hepatic GK activity is modulated by the endogenous inhibitor, glucokinase regulatory protein (GKRP) (5–8). GK is localized in the nucleus as an inactive complex with GKRP at low glucose concentrations and is dissociated from the GK-GKRP complex and translocated to the cytoplasm at high glucose concentrations, which triggers glucose disposal (9). Modulators of the GK-GKRP interaction have been shown to enhance hepatic glucose disposal (7). We recently solved the co-crystal structure of hepatic GK complex with GK activator, in which GK undergoes a large conformational change between the active and inactive forms at different glucose concentrations. According to these findings, we hypothesized that GK activator stabilized the active form of GK (10), which could influence not only the GK enzymatic activity but also the GK-GKRP interaction.

In the present study, we report the mechanism of action of the novel GK activator, compound A, on the interaction of GK and GKRP and on GK subcellular localization in liver cells, the effect of GK activator in pancreatic islets and hepatocytes.

**EXPERIMENTAL PROCEDURES**

**Chemical**—Compound A (2-amino-5-(4-methyl-4H-(1,2,4)-triazole-3-yl-sulfanyl)-N-(4-methyl-thiazole-2-yl)benzamide) was prepared by Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd.

**Animals**—Wistar rats (Charles River Japan, Inc.) were housed in the standard laboratory cages and fed normal rodent chow and water \textit{ad libitum} until use. All animal experiments were conducted according to the Guidelines for Animal Experiments at Tsukuba Research Institutes Banyu Pharmaceutical Co., Ltd.

**Preparation of Recombinant GK and GKRP**—Recombinant N-terminal FLAG-tagged human and rat liver GK were expressed in \textit{Escherichia coli} strain DH5\(\alpha\) as described previously (11). N-terminal His-tagged human liver GK was expressed in \textit{E. coli} strain BL21 (DE3) using pET28a(+) vector (Novagen, Darmstadt, Germany). Human GKRP was expressed in vacculovirus SF9 cells as a C-terminal FLAG-tagged fusion protein using pBacPac 9 vector (Clontech, Mountain View, CA).
(11). FLAG-tagged GK and GKRPs were purified by anti-FLAG M2 affinity gel (Sigma-Aldrich). His-tagged GK was purified using a His-Bind purification kit (Novagen).

**Purification of Antibodies**—The antisera against human and rat liver GK and human GKRPs were generated in rabbits using recombinant FLAG-tagged GK and GKRPs as the antigen, respectively. The IgG components were obtained by protein A-Sepharose chromatography (Pfizer/Pharmacia, Groton, CT). For anti-rat liver GK antibody, the IgG component was further purified as described previously (12).

**Glucokinase Assay**—GK activity was measured by the glucose-6-phosphate dehydrogenase coupled continuous spectrophotometric assay at 30 °C (11). Recombinant FLAG-tagged human hepatic GK was incubated in assay buffer containing 25 mM HEPES, pH 7.2, 1 mM dithiothreitol, 0.5 mM thionicotinamide adenine dinucleotide, 2 mM MgCl₂, 1 mM ATP, 2 units/ml glucose-6-phosphate dehydrogenase, and the indicated concentrations of glucose and compound A.

**Assay of Insulin Secretion**—Islets of Langerhans were isolated from 9-week-old male Wistar rats by collagenase perfusion. After culturing overnight, insulin secretion of the islets was measured as previously reported (13). Briefly, groups of 8–10 islets were incubated at 37 °C for 1.5 h in Krebs-Ringer bicarbonate buffer containing 2.8, 7.4, or 16.7 mM glucose, with or without addition of 10 or 30 μM compound A. Insulin concentration in the assay buffer was measured with an insulin ELISA kit (Morinaga, Yokohama, Japan).

**Glucose Phosphorylation, Glucose Oxidation, Glycogen Synthesis, and Glucose Output Assays in Hepatocytes**—Hepatocytes were isolated from 9-week-old male Wistar rats by collagenase perfusion. The effects of compound A on glucose phosphorylation, glycogen oxidation, and glycogen synthesis were measured as described previously (14). For measurement of glucose output, cells were incubated in glucose-free Dulbecco’s modified Eagle’s medium containing 10 mM dihydroxyacetone and compound A. After incubation, glucose concentration in the medium was determined by using determiner GLE (Kyowa Medex, Tokyo, Japan).

**Oral Glucose Tolerance Test (OGTT)**—9-week-old male Wistar rats were fasted overnight before performing the test. The rats were orally administrated either compound A or vehicle alone (0.5% methylcellulose solution), followed 30 min later by an oral glucose challenge (2 g/kg). Plasma glucose concentrations were measured just prior to and following the glucose challenge (30, 60, 90, and 120 min). The blood insulin level was measured using an insulin ELISA kit (Morinaga). For measurement of glycogen content, livers were removed from the rats treated with compound A and glucose in the same schedule as described above, and hepatic glycogen was analyzed using a standard enzymatic assay (15).

**Immunohistochemistry of GK in Rat Liver Tissue**—9-week-old male Wistar rats were fasted overnight, and on the next morning, the rats were orally administrated either 10 mg/kg of compound A or vehicle alone (0.5% methylcellulose solution). Livers were taken from the rats at 0.5, 1, and 4 h after oral treatment. The liver tissues were excised and immersed overnight at 4 °C in Zamboni's solution (saturated picric acid/4% paraformaldehyde). The liver slices were dehydrated, embedded in paraffin, and sectioned at a thickness of 3 μm. The sections were mounted on silanized slides. Immunohistochemical staining was carried out as described previously (16). The total optical density of GK in the nucleus was quantitated and normalized by the total area of nucleus by computerized image analysis using Image-Pro Plus software (Planetron Inc. Tokyo, Japan).

**Immunofluorescent Staining of GK in Primary Rat Hepatocytes**—Hepatocyte monolayers were washed with PBS and fixed for 40 min at 25 °C with 4% paraformaldehyde in PBS. Next, the monolayers were washed with PBS and permeabilized for 40 min at 25 °C with 0.5% (v/v) Triton X-100 in PBS. The permeabilized monolayers were washed with PBS and incubated with 3% BSA for 40 min at 25 °C. They were then incubated with anti-GK IgG overnight at 4 °C, followed by Alexa Fluor 488 goat anti rabbit IgG (H+L) (Invitrogen/Molecular Probes, Carlsbad, CA) for 40 min at 25 °C. Each incubation step was followed by three washes with PBS.

**Immunoprecipitation Assay of GK and GKRPs**—FLAG-tagged GKRPs immobilized to anti-FLAG M2 affinity gel beads (Sigma-Aldrich) was incubated at 25 °C with His-tagged GK in a buffer of 25 mM HEPES, pH 7.2, 1 mM dithiothreitol, 1 mM MgCl₂, and several concentrations of glucose with or without fructose 6-phosphate. After 30 min, the GKRPs were precipitated. The GKRPs were then eluted with FLAG peptides (Sigma-Aldrich) and separated by SDS-PAGE. Proteins were detected by silver staining (Daichi Pure Chemicals, Tokyo, Japan) and the intensities of the GKRPs were quantified using Imaging Gauge software (Fujifilm, Tokyo, Japan). For determination of the effect of compound A on the interaction between GKRPs, the GKRPs were preincubated with compound A for 10 min at 25 °C before formation of the GKRPs complexes. The method was applied for determination of the effect of glucose or AMP-PNP (ATP analog: Sigma-Aldrich) on the interaction between GKRPs.

**Statistical Analysis**—Statistical analysis was performed using Student’s paired t test.

**RESULTS**

**Effect of Glucokinase Activator, Compound A, on the Enzymatic Activity of Glucokinase**—Compound A at concentrations from 0.05 to 30 μM enhanced the catalytic activity of recombinant human liver GK in a dose-dependent manner (Fig. 1A). Kinetic analysis of GK showed that the shape of the glucose saturation curve changed from sigmoidal to Michaelis-Menten hyperbolic and that compound A increased the Vₘₐₓ (Fig. 1B). In the presence of 10 μM compound A, the Vₘₐₓ of the enzyme increased from 11.7 to 15.3 μmol/min/mg, and the substrate concentration at 0.5 Vₘₐₓ (L[S]ₐ₅₃) for glucose decreased from 7.7 to 0.8 mM. Other hexokinase isozymes of human HK-I, HK-II, and HK-III were unaffected by the compound even at 30 μM (data not shown). Thus, the activating effect of compound A was specific for GK. Compound A also activated rat and mouse liver GK with similar potency observed with human liver GK (data not shown).
GK Activator Impairs GK-GKRP Interaction

**Effect of Compound A on Islet Glucose-Induced Insulin Release**

In the presence of 30 mM compound A, the rate of glucose-stimulated insulin release was evaluated in cultured rat islets at 2.8, 7.4, and 16.7 mM glucose. In the presence of 30 mM compound A, the amount of insulin secreted at 2.8 mM glucose was almost equivalent to that secreted from control islets at 16.7 mM glucose. The amount of insulin was not further increased between 7.4 and 16.7 mM glucose in the islets treated with 30 μM compound A, suggesting that the secretion was at a maximum.

**Effect of Compound A on In Vivo Glucose Metabolism**

In an oral glucose tolerance test (OGTT) using fasted rats, the plasma insulin levels were significantly increased, indicating that enhanced insulin release contributes to the glucose-lowering efficacy of compound A (Fig. 4B). The hepatic glycogen levels were increased in a time-dependent manner by compound A, indicating the direct activation of hepatic GK by compound A (Fig. 4C). Consistent with the results of the cell-based assay, these data demonstrate that the dual pancreatic and hepatic actions of the GK activator contribute to its glucose lowering efficacy in vivo.

**Effect of Glucokinase Activator on the Localization of GK in Rat Liver Tissue**

The effect of compound A on the distribution of GK was further investigated in rat liver tissue at 0.5, 1, and 4 h after oral treatment with the compound A at 10 mg/kg (Fig. 5). The distribution of GK in the liver tissues of 24 h-fasted rats, GK was predominantly detected in the nucleus. As a reference, we also examined the distribution of GK after oral administration of 2 g/kg glucose. As compared with 0 h, at 0.5 h after administration of compound A, the intensity of staining of nuclear GK diminished as the intensity of cytoplasmic staining increased concomitantly in liver cells, indicative of the translocation of nuclear GK to the cytoplasm. This effect was transient, because at 1.0 h, the cytoplasmic signal decreased, and the nuclear signal increased. At 4.0 h, GK immunostaining was found again in the nucleus only. Similar phenomena were observed in the liver of rats treated with 2 g/kg glucose in which the translocation of GK into the cytoplasm reached a peak at 1.0 h followed by GK moving back to the nucleus at 4.0 h. These data indicated that compound A mimicked the glucose effect on the translocation of GK.

**Effect of Glucokinase Activator on the Localization of GK in Rat Primary Hepatocytes**

We next studied the effect of compound A on the localization of GK in rat primary hepatocytes by immunostaining with anti-GK antibody (Fig. 6). In hepatocyte monolayers cultured in 5.5 mM glucose, GK was exclusively stained in the nucleus (Fig. 6A). When the hepatocytes were exposed to 25 mM glucose, the nuclear-distributed GK was translocated to the cytoplasm (Fig. 6B). By treating with 30 μM of compound A in the presence of 5.5 mM glucose, GK was translocated from the nucleus to the cytoplasm within 10 min (Fig. 6E). The cytoplasm-localized GK was redistrib-
GK Activator Impairs GK-GKRP Interaction

Compounds A increased the hepatic glucose phosphorylation apparently by enhancing GK catalytic activity. Because compound A increased the affinity of glucose to GK by changing protein conformation (Fig. 1 and Ref. 10), we investigated the effect of compound A on the GK-GKRP interaction. We established a co-immunoprecipitation assay using recombinant proteins in which we can quantitatively measure the amount of GK bound to GKRP. A mixture of recombinant His-tagged human hepatic GK and FLAG-tagged human GKRP in the ratio of 4:1 (16:4 μg) yielded a nearly 1:1 complex in the presence of 5.5 mM glucose and 0.4 mM fructose 6-phosphate, which is known to enhance the association of GK and GKRP (17). Under this condition, compound A inhibited the interaction between GK and GKRP with an IC50 value of 16.4 μM (Fig. 7B). In order to clarify whether compound A inhibits the association of GK and GKRP or enhances the dissociation of GK from the GK-GKRP complex, compound A was added to the incubation mixture before or after formation of the GK-GKRP complex (Fig. 7C) in the absence of fructose 6-phosphate. When GK was preincubated with compound A before addition of GKRP in the presence of 5.5 or 25 mM glucose, the amount of GK bound to GKRP was reduced to 30% or 20% of the level of the corresponding vehicle control, respectively. On the other hand, when compound A was added to established GK-GKRP complexes in the presence of 5.5 or 25 mM glucose, the amount of GK bound to GKRP was 75% or 63% of the level of the vehicle control, respectively. These observations suggested that compound A preferentially interacts with free GK rather than the established GK-GKRP complex, resulting in inhibition of the association of GK and GKRP.

To investigate whether glucose affects these interactions, the same assay was carried out in the absence of glucose (Fig. 7C). In the absence of glucose, GK preferentially interacts with GKRP. Surprisingly, compound A had no effect on the dissociation or association of GK and GKRP, indicating that compound A only binds to glucokinase in the presence of glucose. These results illustrate that compound A interacts with only the glucose-bound, free GK, but not with glucose-unbound or GKRP-associated GK.

The Effect of Glucose and ATP on GK-GKRP Interaction—In the immunoprecipitation assay, the interaction of GK and GKRP was little affected by 25 mM of glucose in the absence of ATP (Fig. 7C). On the other hand, most GK was dissociated from GKRP in hepatocytes cultured in high concentration of glucose (Ref. 12 and Fig. 6). Therefore, we evaluated the effect of glucose on the interaction of GK and GKRP in the presence of ATP. To prevent the hydrolysis of ATP by GK, ATP analog of AMP-PNP was used. Both ATP and AMP-PNP had little effect on the interaction of GK and GKRP by themselves in the absence of glucose (Fig. 8A). As shown in Fig. 8B, in the presence of 0.5 mM of AMP-PNP, the amount of GK bound to GKRP in the presence 5.5 mM glucose was almost the same as the control level without glucose and AMP-PNP, whereas the amount of GK bound to GKRP was reduced by half in the presence of 25 mM glucose. These results suggested that glucose-responsive inhibition of GK-GKRP interaction was evident only in the presence of ATP. The amount of the bound GK in the presence of 25 mM glucose and 0.5 mM AMP-PNP was further reduced by the treatment.
with compound A, suggesting that GK activator is effective in high glucose condition.

DISCUSSION

We recently reported crystal structures of GK in a complex with and without our GK activator (10). GK activator interacts with GK-specific novel allosteric sites located in the hinge region where several gain-of-functional mutations are clustered (10, 18). Compound A has more metabolic stability than previous compound (10), enabling its use in in vivo studies. In the present study, compound A enhanced the enzymatic activity of GK by decreasing the $S_{0.5}$ for glucose and increasing the $V_{\text{max}}$. Compound A increased the glucose-induced insulin secretion in isolated pancreatic islets. In addition, compound A enhanced the major hepatic glycolytic pathway (glucose phosphorylation, oxidation and glycogen synthesis) in rat primary hepatocytes. Compound A also impaired glucose output induced by dihydroxyacetone challenge, possibly because of the enhancement of the relative activity of glycolytic flow against gluconeogenesis.

In hepatocyte, GK-GKRP interaction is a key mechanism for quick regulation of GK activity in response to changes in intracellular glucose concentrations. We hypothesized that the GK activator could affect the GK-GKRP interaction and measured the amount of GK in the complex with GKRP using a quantitative immunoprecipitation assay. We demonstrated that compound A inhibited the association of GK and GKRP with little effect on their dissociation. Importantly, in the absence of the GK substrate, glucose, compound A had no effect on the GK-GKRP interaction. From these data, we speculated that compound A preferentially associates with glucose-bound free GK but not with glucose-unbound free GK or with GK in a complex with GKRP. In the presence of glucose, GK stays in the active form in which a GK activator-accessible allosteric site exists. However, in the absence of glucose, GK undergoes a conformational change to form a so-called super-open inactive state in which the allosteric site is completely absent, thus preventing the binding of GK activator (10). Taken together, it could be a possible idea that compound A binds only to the glucose-induced activated form of GK and stabilizes the activated state, thereby interfering with the formation of the GK-GKRP complex.

In the immunoprecipitation assay, the amounts of GK-GKRP complex formation reached similar levels when glucose concentrations varied from 0 mM to 25 mM glucose in the absence of ATP, suggesting that GKRP can associate with glucose-bound GK as well as glucose-unbound GK. Glucose-dependent disruption of the complex was observed in the presence of non-hydrolytic ATP analog of AMP-PNP. AMP-PNP did not impair the association of GK and GKRP by itself. When GK was pre-incubated with compound A, the rate of complex formation was reduced by elevation of the glucose concentration (Fig. 7C, 5.5 versus 25 mM), whereas the complex formation was not reduced by elevation of the AMP-PNP concentration (data not shown), indicating that glucose binding is critically important to induce the conformation change to the active form of GK.
GK Activator Impairs GK-GKRP Interaction

Based on this observation and the structural information, it is possible that the glucose and compound A have additive effects in changing GK structure to a more active form that precludes its association with GKRP. It has been reported that inactive GK is localized in the nucleus as a complex with GKRP in hepatocytes and that GK is released from the complex to the cytoplasm when the glucose concentration is elevated (19). We observed that treatment with compound A at a low glucose concentration enhanced the translocation of GK from the nucleus to the cytoplasm in both primary cultured rat hepatocytes and in rat liver tissue. Brocklehurst et al. (20) also reported that translocation of GK to cytoplasm was caused by their synthetic GK activators in hepatocytes. The translocation of GK caused by treatment with compound A was similar to that in the hepatocytes treated with a high dose of glucose, indicating that compound A mimicked high glucose under low glucose conditions. Based on this and the above described data, it is proposed that compound A enhances glucose metabolism by dual mechanisms in hepatocytes by increasing the catalytic activity of GK itself and by stabilizing the activated state of GK, resulting in prevention of the association with GKRP and increasing the amount of free GK in the cytoplasm. It has been suggested that even at low glucose concentrations, a small portion of GK is constantly shuttling between the nucleus and the cytoplasm of the liver cell (21). We speculate that compound A inhibits the shuttling and keeps GK in cytoplasm, consistent with the experimental observation that the level of cytosolic GK was increased in the presence of compound A.

GKRP has been proposed to function not only as an inhibitory protein of GK but also as an anchor protein of GK in the hepatocyte nucleus, where GK is protected from degradation. Profiling of GKRP knock-out mouse shows that GK protein levels in hepatocytes are decreased (22, 23). Therefore, increasing the amount of GK in the cytoplasm by treatment with compound A could raise the potential risk of proteolysis of GK after chronic treatment, resulting in impairment of liver glucose utilization. However, the levels of hepatic GK protein were unchanged or even elevated in mice chronically treated with compound A (data not shown). The reason why hepatic GK protein was not decreased by chronic compound A treatment may be due to the reversible translocation of GK by compound A (Figs. 5 and 6) or enhanced translocation of GK by compound A (Fig. 5). The hepatic glucose metabolism is thought to be enhanced by direct activation of hepatic GK or secondary effect of increased plasma insulin and hepatic glycogen synthesis (Fig. 4). The hepatic glucose metabolism is thought to be enhanced by direct activation of hepatic GK or secondary effect of increased plasma insulin presumably because of beta-cell GK activation. We recently observed that glucokinase activator was able to enhance net hepatic glucose uptake in the absence of insulin secretion by somatostatin infusion (24). From this evidence, both stimulation of insulin secretion and enhancement of hepatic glucose utilization are clearly involved in the mechanism of the glucose lowering efficacy of compound A. In conclusion, we demonstrate that a novel glucokinase activator, compound A, has blood glucose-lowering efficacy due to a dual mechanism of augmenting islet insulin release and enhancing hepatic glucose disposal. In the liver, the GK activator was found to work in two ways, i.e. by increasing GK enzyme catalytic activity and enhancing GK translocation. Both of these effects contribute to augmentation of hepatic glucose disposal. These findings suggest that GK activators have outstanding potentials to provide a new therapeutic approach for the treatment of type 2 diabetes and related disorders.

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