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Inhibitory effects of KHG26377 on glutamate dehydrogenase activity in cultured islets

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GDH has been known to be related with hyperinsulinism-hyperammonemia syndrome. We have screened new drugs with a view to developing effective drugs modulating GDH activity. In the present work, we investigated the effects of a new drug, KHG26377 on glutamate formation and GDH activity in cultured rat islets. When KHG26377 was added to the culture medium for 24 h prior to kinetic analysis, the V_{max} of GDH was decreased by 59% whereas K_m is not significantly changed. The concentration of glutamate decreased by 50% and perfusion of islets with KHG26377 reduced insulin release by up to 55%. Our results show that KHG26377 regulates insulin release by inhibiting GDH activity in primary cultured islets and support the previous studies for the connection between GDH activity and insulin release. Further studies are required to determine in vivo effects and pharmacokinetics of the drug. [BMB reports 2010; 43(4): 245-249]

INTRODUCTION

Glutamate dehydrogenase (GDH) is a member of a family of enzymes that catalyze the reversible deamination of L-glutamate to 2-oxoglutarate, found in all living organism (1). Therefore, GDH serves as the major link between carbohydrate and amino acid metabolism. Although there are some debates as to the directionality of the reaction, the prevailing direction for the enzyme reaction is towards glutamate formation in most tissues (2, 3). It has been reported that activation of GDH does induces insulin secretion, suggesting that it may play a role in the pathophysiology of pancreatic β cells (4-6). Specifically, GDH might play a role in glucose stimulated insulin secretion through generation of glutamate (7, 8). Moreover, the importance of GDH in insulin secretion was demonstrated by the discovery that SIRT4 (sirtuin 4) inhibit GDH activity (9, 10). Also the transgenic mice lacking GDH specifically in β cells has shown the quantitative contribution of GDH in normal glucose-stimulated insulin secretion (6). However, the role of GDH in β cell function remains unclear.

The hyperinsulinism-hyperammonemia syndrome has been known to be caused by loss of GTP regulation of GDH (11-13). Therefore, factors that regulate GDH may affect insulin secretion. Previous studies showed that wild-type and hyperinsulinemia/hyperammonemia forms of GDH are inhibited by the green tea polyphenols, epigallocatechin gallate and epicatechin gallate GDH (14). We have demonstrated that perfusion of islets with 5'-deoxypyridoxal phosphate, an inhibitor of GDH, reduces islet GDH activity in parallel with a reduction in insulin release, strongly suggesting a close relationship between GDH activity and insulin secretion (15). *Cimicifuga heracleifolia* extract also regulated insulin release by altering GDH activity in primary cultured islets (16). Recent study has reported three novel GDH inhibitors such as hexachlorophene, GW 5074, and bithionol (17).

We have screened new drugs with a view to developing effective drugs regulating GDH activity related with the hyperinsulinism-hyperammonemia syndrome. The purpose of this study was to determine the effects of a new drug, KHG26377 (2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride) on GDH activity and insulin secretion in pancreatic islets in order to investigate insulinotropic effects of GDH. To our knowledge, this is the first report showing inhibitory effects of KHG 26377 on islet GDH activity and correlation of the effects with its actions on insulin secretion.

RESULTS AND DISCUSSION

Effects of KHG26377 on glutamate content and kinetic parameters of GDH in cultured islets

The importance of GDH in glucose homeostasis was demonstrated by the discovery that a genetic hypoglycemic disorder is caused by loss of GTP regulation of GDH (11, 12). Li *et al.* (14) have suggested that GDH functions predominantly in the direction of glutamate oxidation rather than glutamate syn-

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thesis in mouse islets in mouse islets expressing the H454Y GTP-insensitive mutation of human GDH. Previous studies have demonstrated that GDH was inhibited by hexachlorophene, GW5074, bithionol, green tea polyphenols, epigallocatechin gallate and epicatechin gallate, and 5′-deoxypyridoxal (14, 15, 17). Palmitoyl-CoA also has been suggested in potent inhibitor (18). These inhibitors have used as a pharmacological tool to examine the complex regulation of insulin secretion and as a targeting drug for the therapy of hyperinsulinism- hyperammonemia syndrome by specifically blocking GDH activity.

Although their mechanisms have not been completely understood, various thiazole analogues have been reported as drugs for the regulation of glutamate concentration (19-21). In addition, structurally distinct classes will be necessary for strengthening our therapeutic concept. We have screened new drugs with a view to developing effective drugs against glutamate and GDH related diseases. In the present study, we show that inhibition of GDH by KHG26377 (2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride) reduces insulin release in rat pancreatic islets. The effects of KHG26377 on levels of glutamate in extracts of islet mitochondria are shown in Table 1. Treatment with 60 μM reduced the concentration of glutamate by about 50% even though total protein did not change (Table 1). Perfusion of islets with KHG26377 reduced islet GDH activity in a concentration-dependent manner (Fig. 1A). As shown in Table 1, V_{max} was markedly reduced in the treated group (0.46 units/mg protein) with no significant change in K_m values for substrate and coenzyme. Analysis of crude cell extracts by Western blotting revealed no change in GDH expression (Fig. 1B). The drug itself showed no effects on cell viability (data not shown).

Effects of KHG26377 on insulin secretion in rat pancreatic islets

When investigating the effects on insulin secretion, perfusion of islets with KHG26377 markedly reduced insulin release (Fig. 2A). Measurement of the amount of insulin released from 50 to 80 min gave a maximum reduction of about 55% with 60 μM, and caused a rightward shift in the concentration dependence of glucose-induced insulin secretion in the islets (Fig. 2B). Although reducing at intermediate glucose levels, insulin secretion was unchanged at basal or optimal glucose concentrations. In our previous studies, *C. heracleifolia* extract inhibited glutamate formation and regulated GDH activity in insulin release (14). However, GDH inhibitor such as extract from *C. heracleifolia* without purified process may have unpredictable biochemical mechanism. To find more stable inhibitor, we have tested various compounds synthesized chemically and KHG26377 has been selected. The results in the present work shows that inhibition of GDH activity by KHG 26377 is more sensitive than that of *C. heracleifolia* extract.

Our results also suggest that the decreased glutamate level may affect the secretary response of insulin. Previously, it has been reported that increase in cellular glutamate level stimulates exocytosis in pancreatic β cells and the stimulation of exocytosis by glutamate in INS-1E cells was associated with a higher expression of GDH (8). However, Li *et al*. (14) have suggested GDH functions predominantly in the direction on glutamate oxidation rather than glutamate synthesis in mouse islets and that this flux is tightly controlled by glucose. Although it is in accordance with that reported in the present study, glutamate might be important intracellular signal in the inhibition process of insulin secretion by KHG26377. In agreement with

Table 1. Effects of KHG26377 on glutamate content and kinetic parameters of GDH in cultured islets

Parameters	Control group	KHG26377 treated group
Glutamate (nmol/mg protein)	71.19 ± 1.43	32.11 ± 1.25[a]
V_{max} (units/mg protein)	0.78 ± 0.07	0.46 ± 0.05[a]
$K_{m\ (NADH)}$ (μM)	34.02 ± 1.02	32.22 ± 0.97
$K_{m\ (Ammonia)}$ (mM)	6.11 ± 0.69	6.24 ± 0.75
$K_{m\ (Glutamate)}$ (mM)	5.33 ± 0.29	5.21 ± 0.40
$K_{m\ (\alpha\text{-Ketoglutarate})}$ (mM)	1.87 ± 0.36	1.93 ± 0.29

Values are means ± S.E.M. of three experiments for each group. Statistical comparisons between control and treated (60 μM) groups were made by ANOVA using Fisher's protected least significant different test at the 0.5 significance level. [a]Significant differences between the two groups, P < 0.05.

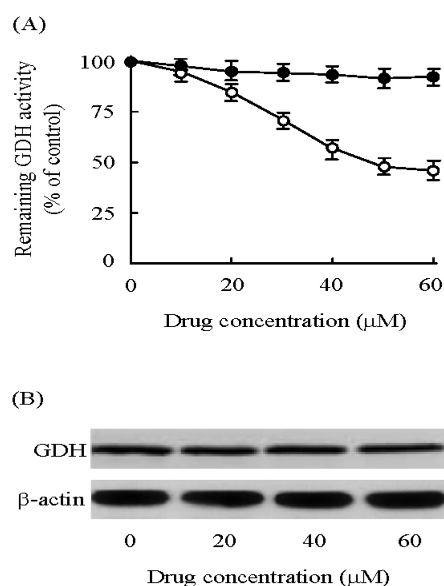

Fig. 1. (A) Dose-dependent inhibition of GDH by KHG26377 in cultured islets. The perfusion medium was supplemented with various concentrations of KHG26377. Samples of the perfusion fluid were withdrawn before and after addition of the agent to measure the remaining activity. Closed circle (control group); open circle (KHG26377 treated group). (B) GDH expression levels examined by Western blotting.

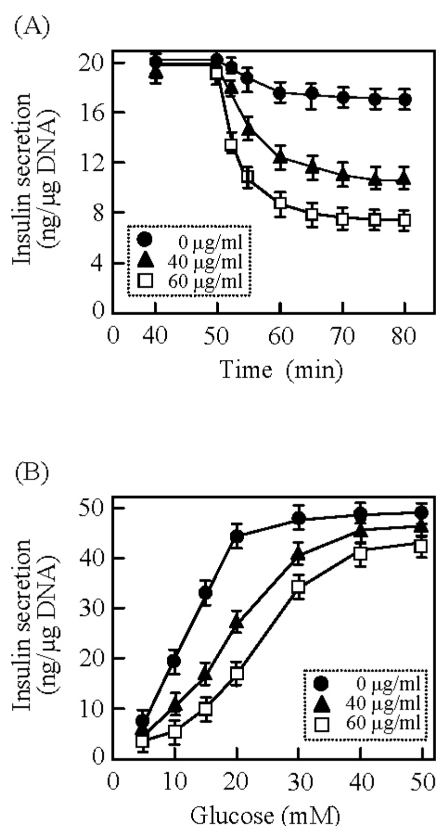

Fig. 2. (A) Effects of KHG26377 on insulin secretion in rat pancreatic islets. Insulin secretion was measured during perfusion of islets with Krebs buffer supplemented with different concentrations of KHG26377 (0-60 μg/ml). Error bars represent S.E.M. of three separate experiments. (B) Effects of KHG26377 on glucose-induced insulin secretion. Cells were perfused with Krebs-Ringer bicarbonate supplemented with various concentrations of glucose in the absence and presence different concentrations of KHG26377 (0-60 μg/ml). Insulin secretion was measured after 1 h. All values represent means ± S.E.M. of three separate experiments.

previous observations (4, 7, 8), our data suggest that regulation of GDH activity and insulin secretion are correlated and KHG26377 may act as more sensitive regulator of islet compared to previous inhibitors. However, the precise mechanism by which glutamate may act as an intracellular signal in insulin secretion remains to be clarified.

In summary, we have shown that KHG26377 regulates insulin release by inhibiting GDH activity in primary cultured islets and support the previous studies for the connection between GDH activity and insulin release. Our results suggest a possibility that this compound may be used to control GDH activity in patients with hyperinsulinism-hyperammonemia syndrome. Further studies are required to determine the in vivo effects and pharmacokinetics of the drug.

MATERIALS AND METHODS

Materials

Insulin, dexamethasone, $NADP^+$, NAD^+, NADH, NADPH, α-ketoglutarate, and L-glutamate were purchased from Sigma. Insulin-free bovine serum albumin fraction, guinea pig anti-porcine insulin serum, and rabbit anti-guinea pig globulin serum were from the Koram Biotech Corp. Fetal calf serum and Dulbecco's modified Eagle's medium was obtained from Gibco. All other chemicals and solvents were of reagent grade or better.

Synthesis of KHG26377

A solution of *N*-methylisothiocyanate (12.43 ml, 0.17 mol) in ethanol (90 ml) was added cyclopropylamine (10 g, 12.28 ml, 0.17 mol) and then the reaction mixture was refluxed over 4 h. The reaction mixture was cooled to room temperature and the ethyl ether was added. The precipitates were collected by filtration to give *N*-methyl-*N*'-cyclopropylthiourea (19.13 g, 86%) as a white solid. A solution of *N*-methyl-*N*'-cyclopropylthiourea (1.3 g, 0.01 mol) in methanol (10 ml) was added an aqueous chloroacetaldehyde solution (40%, 1.96 ml). The reaction mixture was refluxed over 10 h and evaporated to give oily liquid (2.4,976 g). This was separated by column chromatography using silica gel (GF254) and chloroform : methanol (90 : 10, v/v) to afford oily liquid (1.04 g, 55%). Crystallization from methanol and ethyl ether gave 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.559 g) as an white solid. Yield: 55%; mp 236°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.47-0.71 (m, 4H, cyclopropyl CH_2), 2.33 (m, 1H, methine), 3.25 (s, 3H, CH_3), 5.88 (d, ^3J=4.8 Hz, 1H, CH), 6.47 (d, ^3J=4.8 Hz, 1H, CH).

Preparation of mitochondrial extracts and islet perfusion

Mitochondrial extracts were prepared by a modification of a previous method (4). Islets were isolated from the pancreas of Sprague-Dawley rats weighing 200-230 g by standard collagenase digestion and separated from the pancreas by centrifugation on a Ficoll gradient. Only islets that were undamaged and completely free of acinar and connective tissue were used. In experiments involving perfusion, isolated islets were cultured for 1 h in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of humidified 95% air and 5% CO_2 at 37°C. Perfusions were performed in Krebs-Ringer bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM potassium phosphate, 1.16 mM magnesium sulfate, and 25 mM $NaHCO_3$, pH 7.4) containing 5.6 mM glucose and 0.25% insulin-free bovine serum albumin for 1 h at 37°C. When required, the perfusion medium was supplemented with various concentrations of drugs. Samples of the perfusion fluid were withdrawn periodically before and after addition of the agent.

Western blotting

KHG26377-treated cells (1×10^6 cells/10 ml) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, lysates from cells in 0.1 ml of PRO-PREP protein extraction solution were incubated on ice with intermittent vortexing, followed by centrifugation at 14,000 rpm for 20 min at 4°C. Equal amounts of total protein (50 μg) from each treatment group were diluted with loading buffer, boiled and loaded onto 12% SDS-polyacrylamide gels. Samples were electrophoresed and then transferred to a nitrocellulose membrane for Western blotting using monoclonal antibodies against hGDH as described before (22).

Analytical methods

Concentration of glutamate was determined by enzymatic and fluorometric methods (23). Insulin was measured with a single-antibody radioimmunoassay (24) with porcine insulin as standard. DNA from three batches of 30 islets removed at random from each preparation was extracted at room temperature in saline solution (2 M NaCl, 50 mM sodium phosphate, 1.8 mM EDTA, pH 7.4) and assayed fluorometrically with calf thymus DNA (Sigma) as standard.

Enzyme assay and kinetics

GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm (25). All assays were performed in triplicate and initial velocity data were related to a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 25°C. One unit of enzyme is defined as the amount required to oxidize 1 μmol of NADH per min at 25°C. GDH activity was also measured in the direction of oxidative deamination of glutamate (26). To determination K_m and V_{max} values, the assays were carried out in the presence of 1 mM ADP, varying the substrate under investigation while keeping the other substrate and reagents at the optimal concentrations given above. K_m and V_{max} were calculated by linear regression of double-reciprocal plots and are given with standard errors.

Statistical analysis

Statistical analysis was performed either by Student's *t*-test or, where applicable, by ANOVA followed by Student's *t*-test. Unless otherwise mentioned, each experimental point represents the mean of triplicate determinations on separate preparations, and standard deviation are indicated by error bars in each figure.

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