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Inhibition of Rat Liver Glucokinase by Alloxan and Ninhydrin

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In a recent paper, we reported that glucokinase in pancreatic islets might be a critical site which mediates the inhibition of glucose-stimulated insulin secretion by alloxan and ninhydrin. The action mechanisms of the two agents on rat liver glucokinase (instead of islet glucokinase) were studied here.

Both alloxan and ninhydrin irreversibly inhibited rat liver glucokinase in concentration-dependent manners. The inhibitory effects of alloxan and ninhydrin on glucokinase were blocked by the presence of hexoses (D-glucose and D-mannose) that serve as substrates of the enzyme. The blockade provided by D-glucose showed α-anomeric preference, as was also observed in the phosphorylation of D-glucose by glucokinase. Protection against the inhibition of glucokinase by alloxan or ninhydrin was also afforded by D-mannoheptulose, a competitive inhibitor of the enzyme with respect to D-glucose. These results suggest that the inhibitory sites of alloxan and ninhydrin are at or near the substrate-binding site of glucokinase.

Keywords——liver glucokinase; alloxan; ninhydrin; glucokinase inhibition; sugar-mediated protection; glucose anomer

Alloxan[1,2] and ninhydrin[3,4] are known to abolish acutely the ability of glucose to stimulate insulin secretion from pancreatic islets. Glucokinase (adenosine triphosphate (ATP): D-glucose 6-phosphotransferase, EC 2.7.1.2) has been suggested to play a key role in the glucose-stimulated insulin secretory process.[5-7] Recently, we[8] have demonstrated that a brief exposure of isolated pancreatic islets to alloxan or ninhydrin inhibits the subsequently assayed glucokinase activity, and that the inhibition is blocked by hexoses which protect against the inhibitory effects of these agents on glucose-stimulated insulin secretion. These findings suggested that the inhibitory effects of alloxan and ninhydrin on insulin secretion are mediated by inhibiting islet glucokinase.

In this paper, we deal with the action mechanisms of alloxan and ninhydrin on glucokinase in a cell-free system. The use of islet glucokinase, rather than liver glucokinase, is unquestionably desirable for the purpose of this study. However, it is difficult to obtain enough islet glucokinase for the present purpose. Since liver glucokinase is quite similar to islet glucokinase in various properties,[9] and the liver contains high activity of glucokinase, we used liver glucokinase instead of islet glucokinase in this study.

Materials and Methods

Materials——Alloxan monohydrate, phenylmethylsulfonyl fluoride, *tert*-butyl hydroperoxide, α-D-glucose, β-D-glucose, D-mannoheptulose, dithiothreitol, glutathione (GSH), bovine serum albumin (fraction V), superoxide dismutase (from bovine blood), and catalase (from bovine liver) were purchased from Sigma Chemical Co. Glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) was obtained from Oriental Yeast Co., Ltd. and 6-aminocaproic acid from Nakarai Chemicals, Ltd. Ninhydrin and polyethylene glycol 4000 (average molecular weight: 3000—3700) were purchased from Wako Pure Chemical Industries, Ltd. Diethyl aminoethyl (DEAE)-cellulose (DE-52) was supplied by Whatman Biochemicals.

Glucokinase Assay——Glucokinase activity was measured by following the production of glucose 6-phosphate in an assay coupled to the reduction of nicotinamide adenine dinucleotide (NAD) in the presence of excess glucose 6-phosphate dehydrogenase. The assay mixture contained 200 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM NAD, 5 mM ATP, 50 mM glucose, 100 μg/ml bovine serum albumin, 1 unit/ml glucose 6-phosphate dehydrogenase, and an enzyme sample in a final volume of 1 ml. For the assay of hexokinases (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), the final glucose concentration in the assay mixture was arranged to be in the range of 0.5—1.0 mM. The reaction mixture was preincubated for 1 min at 37 °C and the reaction was started by the addition of sample solution. The reaction velocity was measured as the rate of increase in absorbance monitored at 340 nm during 2—3 min after initiation of the reaction. One unit is the amount of enzyme that catalyzes the phosphorylation of 1 μmol of glucose per min.

Protein Determination——Protein was assayed by the method of Bradford[10] using Bio-Rad Protein Assay Dye Reagent and bovine serum albumin as a standard.

Preparation of Rat Liver Glucokinase——All purification procedures were carried out at 4 °C.

Extraction: Livers (90 g) removed from fed male Sprague-Dawley rats weighing 200—250 g were minced and then homogenized with a Polytron homogenizer in 2 vol. of buffer A consisting of 20 mM potassium phosphate buffer (pH 7.0), 100 mM KCl, 1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 50 mM glucose, 1 mM 6-aminocaproic acid, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $15000 \times g$ for 30 min. The supernatant was further centrifuged at $90000 \times g$ for 60 min and the resulting supernatant was saved as the crude extract.

Polyethylene Glycol Fractionation: Forty-eight percent (w/v) polyethylene glycol in buffer A was added with stirring to the crude extract to give a final concentration of 12%. After stirring of the mixture for 30 min, precipitated proteins were removed by centrifugation at $15000 \times g$ for 30 min. The supernatant fraction was adjusted to pH 6.5 with 5% phosphoric acid.

Batchwise Chromatography on DEAE-Cellulose: To the supernatant fraction (150 ml) was added with stirring 10 g of DEAE-cellulose, previously equilibrated with buffer B (consisting of 20 mM potassium phosphate buffer (pH 6.5), 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 mM glucose, 5% (v/v) glycerol, 1 mM 6-aminocaproic acid, and 0.2 mM phenylmethylsulfonyl fluoride). Stirring was continued till completion of the adsorption of glucokinase. DEAE-cellulose was collected on a glass filter and washed with 500 ml of buffer B under suction. The washed DEAE-cellulose was packed into a column (2.2×8 cm) and buffer B was passed through the column until the absorbance at 280 nm reached the base-line. Glucokinase was eluted by raising the KCl concentration in buffer B to 200 mM. All fractions containing glucokinase activity were pooled and concentrated by ultrafiltration in an Amicon Diaflo cell with a YM-5 membrane.

Gel Filtration on Sephadex G-75: The concentrate (15 ml) was applied to a Sephadex G-75 column (3.6×50 cm) operated in buffer C consisting of 20 mM potassium phosphate buffer (pH 7.0), 200 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 mM glucose, and 5% (v/v) glycerol at 20 ml/h. The fractions containing glucokinase were pooled, concentrated by ultrafiltration, and stored at 4 °C. The partially purified enzyme had a specific activity of 17 units/mg protein and contained no detectable activity of hexokinase.

Incubation of Glucokinase with Alloxan, Ninhydrin, or *tert*-Butyl Hydroperoxide——Prior to experiments, glucose was substantially removed from a glucokinase stock solution by repeating two consecutive operations, a 10-fold concentration by ultrafiltration in an Amicon Centricon-10 and a 10-fold dilution with glucose-free buffer C, three times. The glucose-free enzyme solution was prepared to give an enzyme concentration of about 4 units/ml and used within 12 h. The enzyme solution (5 μl) was added to a test tube containing 75 μl of incubation buffer (20 mM Hepes–KOH buffer, pH 7.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 50 μg/ml bovine serum albumin), which was supplemented, if necessary, with one or more of such chemicals as sugar, GSH, superoxide dismutase, and catalase. In experiments with α-or β-D-glucose, the anomer was dissolved just before use. The mixture (80 μl) was preincubated for 1 min at 37 °C and the incubation was started by the addition of 20 μl of a test compound (alloxan, ninhydrin, or *tert*-butyl hydroperoxide) solution in incubation buffer. At the indicated intervals, the incubation was terminated by the addition of 1 ml of ice-cold stopping buffer (100 mM Tris–HCl buffer, pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 50 mM glucose, 50 μg/ml bovine serum albumin, and 5 mM GSH). The enzyme activity was then measured with 100 μl aliquots. Control experiments were conducted in parallel with inhibition experiments by replacing the test compound solution with incubation buffer.

Results

Effect of Alloxan and Ninhydrin on Glucokinase Activity

Incubation of glucokinase with alloxan for 5 min at 37 °C caused concentration-dependent inhibition of the subsequently assayed glucokinase activity (Fig. 1). Half-maximal inhibition was attained with about 8 μM alloxan. No inhibition of glucokinase activity

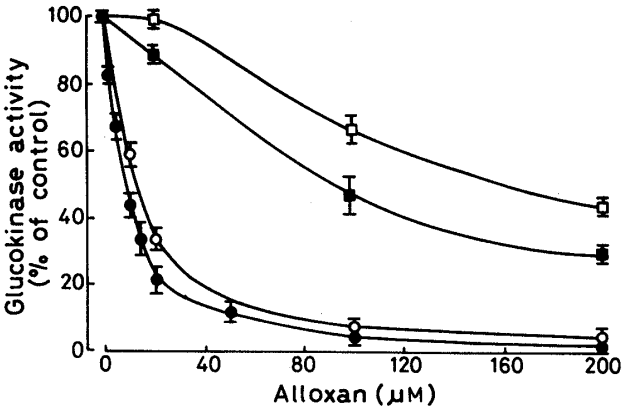

Fig. 1. Glucokinase Activity as a Function of Alloxan Concentration

Glucokinase was incubated for 5 min at 37 °C with various concentrations of alloxan (<200 μM) in the absence (●) or presence of 5 mM glucose (○), 5 mM GSH (■), or 5 mM glucose plus 5 mM GSH (□). Data represent the means±S.D. of four experiments.

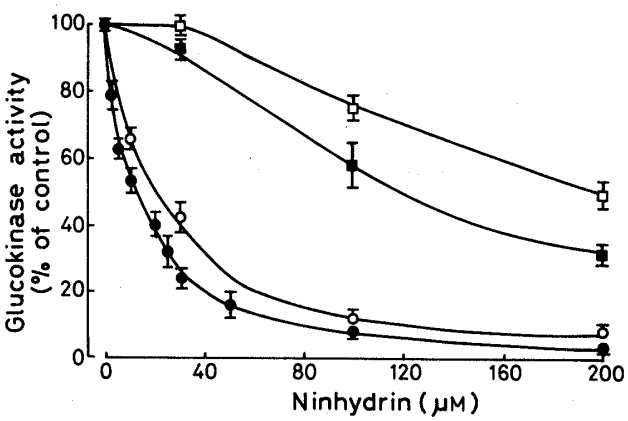

Fig. 2. Glucokinase Activity as a Function of Ninhydrin Concentration

Glucokinase was incubated for 5 min at 37 °C with various concentrations of ninhydrin (<200 μM) in the absence (●) or presence of 5 mM glucose (○), 5 mM GSH (■), or 5 mM glucose plus 5 mM GSH (□). Data represent the means±S.D. of four experiments.

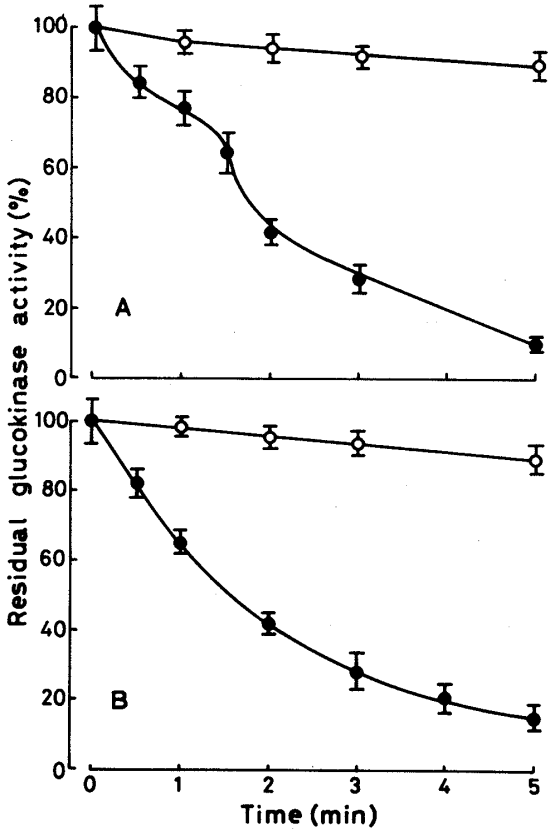

Fig. 3. Time Courses of the Inhibition of Glucokinase by Alloxan and Ninhydrin

(A) Glucokinase was incubated at 37 °C for various periods of time in the absence (○) or presence (●) of 50 μM alloxan. (B) Glucokinase was incubated at 37 °C for various periods of time in the absence (○) or presence (●) of 50 μM ninhydrin.

Data represent the means±S.D. of four experiments.

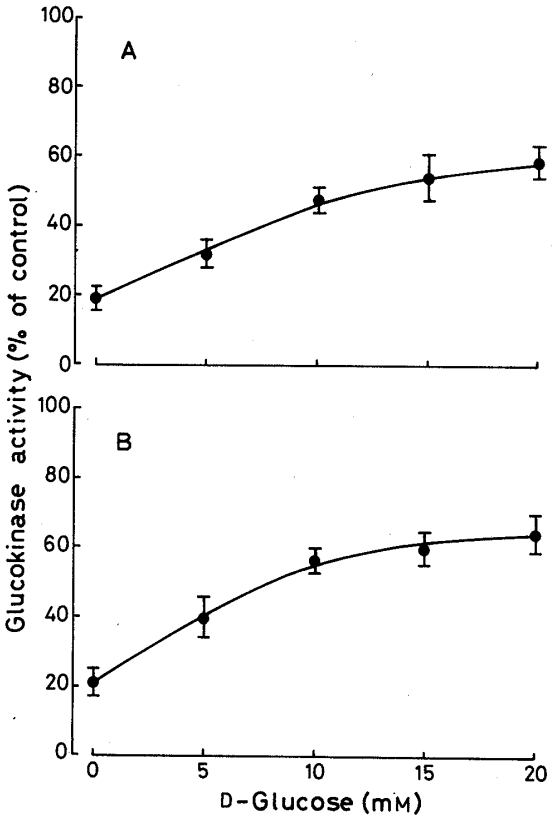

Fig. 4. Effect of D-Glucose Concentration on Alloxan- and Ninhydrin-Induced Inhibition of Glucokinase

Glucokinase was incubated for 5 min at 37 °C with 20 μM alloxan (A) or 30 μM ninhydrin (B) in the presence of various concentrations of D-glucose. Data represent the means±S.D. of four experiments.

occurred when alloxan (200 μM) was allowed to decompose to alloxanic acid before exposure to the enzyme (data not shown). Incubation of the enzyme with ninhydrin for 5 min at 37 °C also produced concentration-dependent inhibition of the subsequently assayed glucokinase activity with half-maximal inhibition at about 13 μM ninhydrin (Fig. 2). As illustrated in Fig. 3, glucokinase was inhibited time-dependently by alloxan and ninhydrin, whereas the enzyme activity was very slowly lost in the absence of alloxan and ninhydrin.

Further studies on the effect of alloxan and ninhydrin on glucokinase activity were performed in the presence of 5 mM glucose and 5 mM GSH, separately or in combination (Figs. 1 and 2). The inhibitory actions of alloxan (<20 μM) and ninhydrin (<30 μM) on glucokinase activity were significantly prevented by the presence of 5 mM glucose. Glucose (5 mM), however, did not appreciably protect glucokinase against the inhibition by alloxan and ninhydrin at higher concentrations than 50 μM. The presence of 5 mM GSH with or without 5 mM glucose showed marked protection against the inhibition of glucokinase activity by alloxan and ninhydrin at concentrations up to 200 μM.

The following result showed that the inhibition of glucokinase by alloxan and ninhydrin was irreversible. The reaction mixture containing glucokinase and alloxan or ninhydrin was incubated for 5 min at 37 °C, mixed with stopping buffer, concentrated 10 times by ultrafiltration in an Amicon Centricon-10, diluted 10 times with stopping buffer, and then subjected to the assay of glucokinase activity; there was no significant difference between the glucokinase activities observed after such a procedure and after a routine procedure described

TABLE I. Effect of Sugars on Alloxan Inhibition of Glucokinase Activity

Treatment[a]	% of control[b]	p[c]
Control	100±1.6 (5)	
Alloxan	21±1.5 (5)	
Alloxan + α-D-glucose	67±1.0 (4)	<0.001
Alloxan + equilibrated D-glucose	55±1.2 (4)	<0.001
Alloxan + β-D-glucose	38±0.9 (4)	<0.001
Alloxan + D-mannose	47±1.1 (4)	<0.001
Alloxan + D-mannoheptulose	46±1.6 (4)	<0.001
Alloxan + 3-*O*-methyl-D-glucose	23±1.2 (4)	>0.1
Alloxan + D-galactose	22±0.7 (4)	>0.1

a) Glucokinase was treated for 5 min at 37 °C with 20 μM alloxan with or without the indicated sugars (15 mM). *b*) Data represent the mean±S.D. with the number of experiments in parentheses. *c*) The p values refer to the statistical significance using Student's t-test as compared with alloxan alone.

TABLE II. Effect of Sugars on Ninhydrin Inhibition of Glucokinase Activity

Treatment[a]	% of control[b]	p[c]
Control	100±1.9 (6)	
Ninhydrin	24±1.4 (5)	
Ninhydrin + α-D-glucose	63±1.8 (4)	<0.001
Ninhydrin + equilibrated D-glucose	60±1.1 (4)	<0.001
Ninhydrin + β-D-glucose	42±1.3 (5)	<0.001
Ninhydrin + D-mannose	50±2.0 (4)	<0.001
Ninhydrin + D-mannoheptulose	48±5.5 (4)	<0.01
Ninhydrin + 3-*O*-methyl-D-glucose	24±1.0 (4)	>0.1
Ninhydrin + D-galactose	24±0.9 (4)	>0.1

a) Glucokinase was treated for 5 min at 37 °C with 30 μM ninhydrin with or without the indicated sugars (15 mM). *b*) Data represent the mean±S.D. with the number of experiments in parentheses. *c*) The p values refer to the statistical significance using Student's t-test as compared with ninhydrin alone.

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Effect of Sugars on Alloxan- or Ninhydrin-Induced Inhibition of Glucokinase

The abilities of various sugars to prevent the inhibitory effects of alloxan and ninhydrin on glucokinase activity were examined by incubating the enzyme with alloxan (20 μM) or ninhydrin (30 μM) in the presence of sugar (15 mM). Of the sugars used, D-glucose and D-mannose are substrates of glucokinase, D-mannoheptulose is a competitive inhibitor, and 3-*O*-methyl-D-glucose and D-galactose are neither substrates nor inhibitors. The inhibition of glucokinase activity by alloxan and ninhydrin was significantly prevented by D-glucose, D-mannose, and D-mannoheptulose, but not by 3-*O*-methyl-D-glucose and D-galactose (Tables I and II). The order of ability of the three effective sugars to protect against the inhibition of glucokinase activity by alloxan and ninhydrin was as follows: D-glucose > D-mannose $\geq$D-mannoheptulose. α-D-Glucose showed significantly greater protection against the inhibitory effects of alloxan and ninhydrin than β-D-glucose.

The protection by glucose against alloxan- and ninhydrin-induced inhibition of glucokinase was concentration-dependent up to at least 15 mM (Fig. 4).

Effect of Superoxide Dismutase and Catalase on Alloxan-Induced Inhibition of Glucokinase Activity

To evaluate the possibility that the inhibition of glucokinase by alloxan may be mediated through the generation of reactive oxygen-containing radicals, the abilities of superoxide dismutase and catalase to protect against alloxan-induced inhibition of glucokinase activity were tested. Neither superoxide dismutase (1000 units/ml) nor catalase (200 units/ml) attenuated the inhibition of glucokinase activity caused by incubation with 20 μM alloxan for 5 min at 37 °C.

Discussion

In the present study, we demonstrated that partially purified glucokinase was effectively inhibited by alloxan and ninhydrin. The alloxan concentration (about 8 μM) necessary for half-maximal inhibition of glucokinase activity in a cell-free system was much lower than those (about 2.3 mM and 310 μM) in the experiments with hepatocytes[11] and pancreatic islets,[8] respectively. The ninhydrin concentration (about 13 μM) necessary for half-maximal inhibition of glucokinase activity in cell-free experiments was also lower than that (about 40 μM) in the islet experiments.[8] Such low vulnerability of glucokinase to alloxan and ninhydrin in the experiments with cells would be due to the presence in cells of physiological protector(s) (*e. g.*, D-glucose and GSH) against the inhibitory effects of alloxan and ninhydrin, since the inhibition of partially purified glucokinase by alloxan and ninhydrin was prevented by such intracellular substances (Figs. 1 and 2).

Alloxan can be reduced to dialuric acid by reducing agents such as ascorbic acid[12] and NADH.[13] Dialuric acid can then spontaneously reduce oxygen to form superoxide radical, followed by formation of hydrogen peroxide and hydroxyl radical.[14] All of these oxygen species are highly reactive with known cytotoxicities.[15] In this study, incubation of glucokinase with alloxan was carried out in the presence of a reducing agent, dithiothreitol (50 μM). Therefore, alloxan might exert its inhibitory action on glucokinase through the formation of activated oxygen species. However, two lines of evidence exclude this possibility. First, neither superoxide dismutase nor catalase provided protection against the inhibitory effect of alloxan on glucokinase activity. Second, incubation of glucokinase with *tert*-butyl hydroperoxide (1 mM), which can mimic the cytotoxic effect of peroxide,[16] did not affect the enzyme activity at all (data not shown).

Alloxan is known to react with GSH[17,18] and sulfhydryl (SH) groups of protein[17,19] to

form addition products. Ninhydrin can also form addition products by reaction with cysteine[20,21] and SH groups of protein.[22] Alloxan and ninhydrin were found to irreversibly inhibit glucokinase. Substrates (D-glucose and D-mannose) and a competitive inhibitor (D-mannoheptulose) of glucokinase act as protectors against the inhibition of the enzyme by alloxan and ninhydrin. Furthermore, the two agents are sterically similar to substrates (D-glucose and D-mannose).[23] These facts suggest that both alloxan and ninhydrin may react with the essential SH group(s) at or near the substrate-binding site of glucokinase, which is known to be a thiol enzyme.[24] However, this remains to be proven with a glucokinase preparation which is homogeneous.

The relative potency of D-glucose and D-mannose in protecting glucokinase from the inhibition by alloxan and ninhydrin paralleled both that[8] in protecting against the inhibitory effects of the two agents on insulin secretion, and the relative affinity of the two hexoses for glucokinase.[5] In addition, the protective effect of D-glucose against the inhibition of glucokinase activity by alloxan and ninhydrin showed α-anomeric preference, as was observed in the phosphorylation of D-glucose by glucokinase[5] and in the protection by the hexose against the inhibition of insulin secretion by alloxan[2,8] and ninhydrin.[3,8] These results suggest that D-glucose and D-mannose exert their protective actions against the alloxan- and ninhydrin-induced inhibition of insulin secretion by blocking the interaction between glucokinase and these agents. The protective effect of 3-*O*-methyl-D-glucose on the inhibition of insulin secretion by alloxan is known to result from the inhibition of alloxan uptake by the pancreatic islet cells.[25] Indeed, 3-*O*-methyl-D-glucose showed no protection against the inhibition of glucokinase activity by alloxan in a cell-free system, whereas the sugar provided substantial protection against the inhibition of glucokinase activity in islets caused by exposure of the islets to alloxan.[8] On the other hand, 3-*O*-methyl-D-glucose was ineffective not only in preventing ninhydrin-induced inhibition of insulin secretion, but also in protecting glucokinase from inhibition by the agent both in experiments with islets[8] and in a cell-free system. Therefore, 3-*O*-methyl-D-glucose may not inhibit the entry of ninhydrin into islets.

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