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PROTECTIVE MECHANISM OF GLUCOSE AGAINST ALLOXAN-INDUCED PANCREATIC ß-CELL DAMAGE

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Summary: Glucose prevented the alloxan- or H_2O_2-induced inhibition of insulin secretion in rat pancreatic islets. Hydrogen peroxide was detected during the incubation of islets with alloxan, and this generation of hydrogen peroxide was not affected by glucose. Treatment of ß-cells with alloxan or H_2O_2 caused elevation of cytosolic free Ca^{2+} and decrease of cellular NAD^+. Glucose blocked the decrease of cellular NAD^+ level, but did not abolish the increase of cytosolic Ca^{2+}. These results indicate that glucose protected pancreatic ß-cell damage after the H_2O_2 generation and Ca^{2+} influx on a chain of reactions in the diabetogenesis of alloxan.

In pancreatic ß-cells, alloxan causes cytotoxicity and inhibition of glucose-stimulated insulin secretion, both of which are known to be protected by glucose (1-3). Alloxan inhibits glucokinase (4,5), a primary ß-cell glucose sensor for insulin secretion (6), causing the blocking of glucose-stimulated insulin secretion. This inhibition of enzyme activity by alloxan can be abolished by glucose (1,4,5). But the protection mechanism of glucose on alloxan-induced ß-cell cytotoxicity is not clear yet.

Although the precise cytotoxic mechanism of alloxan has not been fully understood, evidences indicate that alloxan-induced ß-cell damage is mediated through the generation of cytotoxic oxygen free radicals (7-9). Okamoto (9) has proposed that reactive oxygen species produced from alloxan causes DNA strand breaks, and the damaged DNA activates nuclear poly(ADP-ribose) synthetase, which depletes the cellular pool of NAD^+, resulting in ß-cell damage. There were some reports that increase of cytosolic Ca^{2+} also plays an important role in the diabetogenesis of alloxan, between the radical generation and DNA fragmentation (10-12).

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In this paper, we studied the protective mechanism of glucose on alloxan-induced pancreatic ß-cell damage, and presented the evidences that protection was exerted after the H_2O_2 generation and Ca^{2+} influx.

MATERIALS AND METHODS

Preparation of islets: Pancreatic islets were isolated from male Sprague-Dawley rats weighing approximately 200 g by collagenase (Type V, Sigma) digestion as described by Lacy and Kostianovsky (13).

Insulin release from islets: Islets were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 25 mM HEPES, 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate and 0.3% bovine serum albumin (modified K-R solution) (12). Batches of 10 islets were incubated at 37°C for 30 min with 1 mM alloxan or 0.5 mM H_2O_2 in the presence or absence of 20 mM glucose. After washing with modified K-R solution, islets were reincubated in 1 ml of same solution containing 20 mM glucose for 60 min at 37°C. The released insulin in supernatant was measured by radioimmunoassay (Coat-A-Count Insulin kit, Diagnostic Products Cooperation).

Measurement of H_2O_2: The amount of H_2O_2 produced by alloxan was determined by chemiluminescence (14). Batches of 150 islets were incubated at 37°C for 10 min in 300 µl of modified K-R solution with or without 20 mM glucose or 15,000 units/ml catalase. After addition of 1 mM alloxan, incubation was continued at 37°C. At the indicated time, an aliquot of supernatant was removed and mixed with 1 mM luminol (Sigma) dissolved in 100 mM glycine buffer (pH 12.6). Chemiluminescence was measured using luminometer (Lumat LB9501, Berthold). Urea hydrogen peroxide (Sigma) was used as a standard.

Determination of islet NAD^+ content: Batches of 150 islets were disrupted by sonication with cold 0.6 M perchloric acid, and the extract was neutralized with 3 N KOH. NAD^+ content in the extract was determined as described by Bernofsky and Swan (15).

Isolation of ß-cells: Islets of Langerhans isolated by collagenase digestion was further dispersed mechanically into single cells in Ca^{2+}-free medium as described by Lernmark et al. (16). Islets washed with RPMI medium (RPMI 1640 culture medium supplemented with 20 mM HEPES, 0.35 mg/ml $NaHCO_3$, 300 µg/ml L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) containing 2 mM EGTA and 1% bovine serum albumin, was gently aspirated five to seven times with a Pasteur pipette. Single cells were cultured on 35 mm culture dish in RPMI medium containing 10% fetal bovine serum (Gibco). After 6 h, unattached cells were transferred to new culture dish and cultured for 3-4 days before experiment.

Measurement of cytosolic free Ca^{2+}: The change of intracellular free Ca^{2+} was analyzed with Fluo 3 using laser scanning confocal microscope (ACAS 570, Meridian) as described previously (12). ß-cells cultured for 3-4 days were treated with 3 µM Fluo 3-AM (Sigma) at 37°C for 1 h, washed twice, and incubated for 10 min with or without 20 mM glucose in modified K-R solution. After mounting the culture dish on the stage of confocal microscope, the change of cytoplasmic fluorescence intensity of selected cells was monitored. Alloxan (final 1 mM) or H_2O_2 (final 0.5 mM) was added at 60 sec after starting the recording.

RESULTS

The preventive effect of glucose on the inhibition of insulin secretion by alloxan was investigated in isolated rat pancreatic islets. As shown in Fig. 1, alloxan severely inhibited

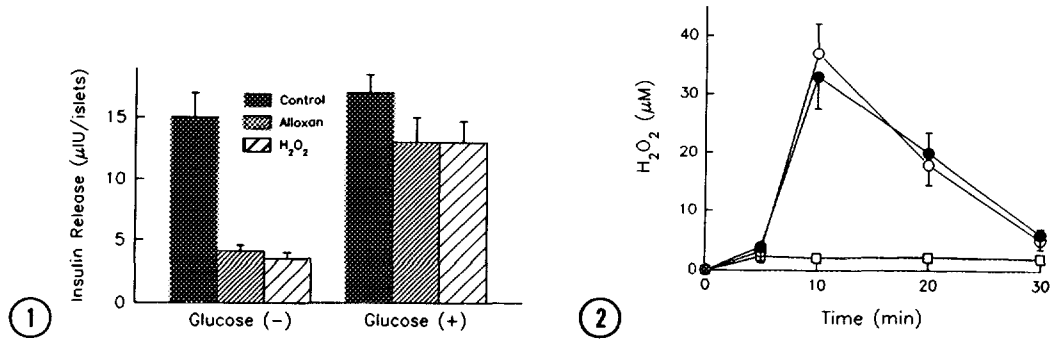

Fig. 1. Effect of glucose on the inhibition of insulin secretion by alloxan or H_2O_2 in rat pancreatic islets. Batches of 10 islets were incubated at 37°C for 30 min with 1 mM alloxan or 0.5 mM H_2O_2 in the presence or absence of 20 mM glucose. After washing with fresh incubation medium, islets were reincubated in 1 ml of same solution containing 20 mM glucose for 60 min at 37°C. The released insulin in supernatant was measured by radioimmunoassay. Data were given as mean ± S.D.

Fig. 2. Effect of glucose on the H_2O_2 generation by alloxan in rat pancreatic islets. Batches of 150 islets were incubated at 37°C for 10 min either alloxan alone (●) or in the presence of 20 mM glucose (○) or 15,000 units/ml catalase (□). After addition of 1 mM alloxan, the amount of H_2O_2 in aliquot of supernatant was determined by chemiluminescence. Each value denotes the mean ± S.D. of three separate experiments.

glucose-stimulated insulin secretion at 1 mM concentration. But islets treated by alloxan in the presence of 20 mM glucose retained the ability to secrete insulin.

Takasu et al. (7) suggested that alloxan-induced ß-cell damage is occurred through H_2O_2. The addition of 1 mM alloxan to islet suspension resulted in the generation of H_2O_2, as evidenced by complete removal with catalase (Fig. 2). H_2O_2 at 0.5 mM concentration inhibited the glucose-induced insulin secretion in isolated pancreatic islets (Fig. 1), and this inhibition was also prevented by glucose. However, glucose did not affect H_2O_2 generation by alloxan (Fig. 2).

Alloxan has been reported to cause the consumption of NAD^+ by islet nuclear poly(ADP-ribose) synthetase (7,9). Evidences suggest that poly(ADP-ribose) synthetase is activated when DNA is fragmented (8,9). Significant reduction of islet NAD^+ content was found when islets were incubated with 1 mM alloxan or 0.5 mM H_2O_2 (Table 1). On the other hand, islets incubated with alloxan or H_2O_2 in the presence of glucose showed no decrease of NAD^+. Presence of glucose alone did not cause significant change in cellular NAD^+ level. These results suggest that glucose can protect the alloxan- or H_2O_2-induced islet cell cytotoxicity caused by DNA fragmentation.

Recently, Kim et al. (12) reported that Ca^{2+}-dependent mechanism contributes to alloxan- and H_2O_2-induced DNA strand break of pancreatic ß-cells, which could be prevented by verapamil, a Ca^{2+}-antagonist. To verify the effect of glucose on Ca^{2+}-influx, change of cytosolic

Table 1. Effect of glucose on NAD^+ content of islets treated with alloxan or H_2O_2

Treatment	Islet NAD^+ content
	(p mol/islet)
None	2.68 ± 0.65
Alloxan	1.22 ± 0.20
Alloxan + Glucose	2.22 ± 0.58
H_2O_2	0.84 ± 0.27
H_2O_2 + Glucose	2.06 ± 0.55

After incubation of 100 islets at 37°C for 30 min with 1 mM alloxan or 0.5 mM H_2O_2 in the presence or absence of 20 mM glucose, NAD^+ content of islets was determined. Data were given as the mean ± S.D. of five separate experiments.

free Ca^{2+} were traced using Fluo 3 in isolated ß-cells. Alloxan caused the increase of cytosolic free Ca^{2+}, and concomitant presence of glucose did not affect the Ca^{2+} influx by alloxan (Fig. 3). There was no fluorescence change in control cell during the recording period. Similar results were obtained with 0.5 mM H_2O_2 instead of alloxan in the absence or presence of glucose (data not shown).

DISCUSSION

Glucose is both fuel and physiologic stimulus for insulin secretion in pancreatic ß-cells. There are reports that alloxan can cause the direct inactivation of glucokinase, a ß-cell glucose

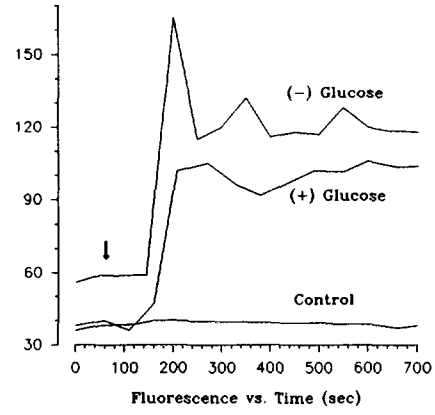

Fig. 3. Effect of glucose on the increase of cytosolic free Ca^{2+} by alloxan in rat pancreatic ß-cells. ß-Cell suspension was prepared in Ca^{2+}-free solution as described in "Materials and Methods". Cells were cultured in RPMI medium for 3-4 days and treated with 3 μM Fluo 3-AM at 37°C for 1 h. After washing out, cells were incubated for 10 min in the presence or absence of 20 mM glucose and the cellular fluorescence intensity was monitored under the confocal microscope. Alloxan (final 1 mM) was added at 60 sec as indicated with arrow after starting the recording. Control denotes the fluorescence change of the cell in the presence of 20 mM glucose.

sensor for insulin secretion (6), which can be protected by glucose (1-3). But only 5 min incubation with 1 mM alloxan is known to be enough to cause a considerable DNA strand break in pancreatic ß-cells (8), suggesting another protection site of glucose in addition to glucokinase.

Researches to establish a chain of cytotoxic effects of alloxan are underway, and evidences for initial damage to plasma membrane (17), mitochondria (18) and nuclei (8) are available. According to Okamoto's model (9), reactive oxygen radicals produced from alloxan induce DNA strand breaks, and poly(ADP-ribose) synthetase acts to repair the DNA breaks, consuming islet NAD^+ (8,19). Our results of H_2O_2 generation and decrease of NAD^+ in alloxan-treated islets support the Okamoto's proposal.

Glucose protected the decrease of NAD^+, but did not affect the production of H_2O_2, indicating that antidiabetogenic step of glucose is distal to H_2O_2 generation in the chain of cytotoxic action of alloxan. Glucose can protect the Chinese hamster ovary cells from the damage by hydrogen peroxide (20), and the absence of glucose can cause endothelial cells to be more susceptible to oxidative damage by H_2O_2 (21,22). They suggested the role of glucose on pentose phosphate pathway for the detoxification of H_2O_2 via the glutathione redox cycle. But under our conditions, the presence of glucose could not cause the significant reduction of H_2O_2 produced from alloxan by islets.

The association of cytotoxicity with a sustained increase of cytosolic Ca^{2+} level was reported in many different cell types including pancreatic ß-cells (23,24). Kim et al. (12) reported the protective action of verapamil, a Ca^{2+}-antagonist, on the cytotoxic effect of alloxan and H_2O_2 in pancreatic ß-cells, and its protective action was exerted between the H_2O_2 generation and DNA fragmentation. Glucose did not affect the Ca^{2+} influx caused by alloxan and H_2O_2 in isolated pancreatic ß-cells.

Environmental agents modulate the incidence of insulin-dependent diabetes mellitus (IDDM) (25), possibly by inducing the initial ß-cell lesions. Numerous immunological and genetic studies including nonobese diabetic mice (26) have established that cellular and humoral autoimmunity against pancreatic ß-cells is important in the pathogenesis of IDDM (27), presumably subsequent to ß-cell injury exerted by viruses and/or chemotoxins (28). Our results indicate that glucose can prevent the alloxan-induced cytotoxicity of ß-cells at the later steps, after H_2O_2 generation and Ca^{2+} influx. The exact protection mechanism of glucose in the sequence of ß-cell cytotoxicity remains to be elucidated.

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