Effects of Alloxan on Glucose-Stimulated Insulin Secretion, Glucose Metabolism, and Cyclic Adenosine 3', 5'-Monophosphate Levels in Rat Isolated Islets of Langerhans

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Summary. Insulin secretion was stimulated and cyclic adenosine 3', 5'-monophosphate (cAMP) levels were elevated in isolated rat islets by 27.5 mmol/l glucose. Alloxan caused a dose-dependent decrease in both variables with complete obliteration of insulin release at a concentration of 1.25 mmol/l. D-glucose, in the presence or absence of extracellular calcium, or 3-0-methyl-D-glucose (both at 27.5 mmol/l) protected completely against the effects of alloxan on both glucose-induced insulin release and cAMP levels. 3-0-Methylglucose did not stimulate insulin secretion or elevate cAMP and did not interfere with glucose-stimulated secretion or elevation of cAMP. When glucose-stimulated insulin release was abolished by alloxan, the metabolism of glucose, determined by the rate of ^3H_2O formation from [5-^{3}H] glucose, was depressed by 20%. It is concluded that alloxan altered the adenylate cyclase system such that it could no longer be stimulated by glucose. Glucose-stimulated insulin secretion or elevation of cAMP did not appear essential for glucose to protect against alloxan. Protection by 3-0-methylglucose did not appear to be mediated through an alteration of cAMP metabolism. Alloxan did not inhibit glucose-induced insulin secretion by grossly altering glycolysis.

Key words: Alloxan, cyclic AMP, isolated islets, insulin secretion, glucose metabolism, 3-0-methyl-glucose, glyceraldehyde.

Alloxan has been widely used to induce permanent diabetes in laboratory animals [1, 2]. It has also been shown to abolish acutely the ability of glucose to stimulate insulin secretion from pancreatic tissue [3–6]. Most attention in the last few years has focused on the ability of various compounds, particularly glucose and 3-0-methylglucose, to prevent the effects of alloxan on islet tissue [7, 8]. Research was spurred on by the observation that protection showed relative anomeric specificity for both α-D-glucose and 3-0-methyl-α-D-glucose. It was speculated that alloxan might prove to be a useful probe in determining the characteristics of the glucoreceptor system which is said to exist in islet tissue [9–11].

Since cyclic adenosine 3', 5'-monophosphate (cAMP) has been considered a possible second messenger in glucose-stimulated insulin secretion [12–16], the present experiments were undertaken to establish the possible involvement of cAMP in the altered insulin release patterns seen after alloxan. The role of glucose and 3-0-methylglucose, and their protective effects against alloxan poisoning were also studied.

Methods

Animals

Male Holtzman rats (Madison, Wisc), weighing 300–400 g, had free access to food and water prior to collagenase isolation of the islets.

Insulin Secretion

The composition of the basic perifusion medium was: NaCl, 115 mmol/l; KCl, 5 mmol/l; $CaCl_2$, 2.2 mmol/l; $MgCl_2$, 1 mmol/l; $NaHCO_3$, 24 mmol/l; and 1.7 g/l crystalline bovine serum albumin. The solution was continually gassed with 95% O_2:5% CO_2 and maintained at 37° and pH 7.4. The flow rate was 1 ml/min. One hundred islets were used in each experiment. Each experiment was divided into 5 parts: 1) After loading the islets in the perifusion chambers, a 30 min equilibration period in the basic medium ensued. There were no additional substrates during this
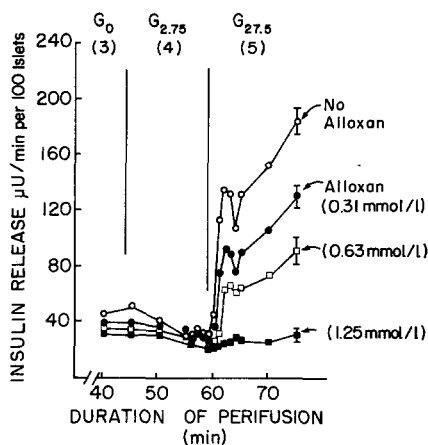
Fig. 1. Effects of various alloxan concentrations on glucose-induced insulin secretion. Batches of 100 islets were perifused as explained in detail in Methods. During period 2 (not shown in the figure) the islets were exposed to 0 (n=5), 0.31 (n=3), 0.62 (n=3), 1.25 (n=7) mmol/l alloxan. During period 5 the ability of the islets to respond to glucose (27.5 mmol/l) was examined. G_0, no glucose; $G_{2.75}$, 2.75 mmol/l glucose; $G_{27.5}$, 27.5 mmol/l glucose. Mean ± SEM of the last perifusate sample is shown. Other points represent mean values

time. 2) The equilibration period was followed by a 5 min exposure to either alloxan alone, alloxan and the sugar tested for protective effect, the sugar alone, or basic medium. 3) The basic medium for an additional 5 min. 4) Glucose (2.75 mmol/l) for 15 min. 5) Glucose (2.75 mmol/l) with or without 3-0-methylglucose (27.5 mmol/l); glucose (27.5 mmol/l) with or without 3-0-methylglucose (27.5 mmol/l) for 20 min. Basal glucose was omitted from the perifusion medium during Periods 1 and 3 to avoid any possible interaction with alloxan. The omission of exogenous fuel for this period of time did not have a detrimental effect on islet responsiveness (unpublished observations). All sugars were dissolved at least 30 min before use to allow mutarotation to occur. After termination of the perifusion, the filter used to support the islets in the perifusion chamber, was removed, quickly frozen, and cAMP determined as previously reported [14]. It should be noted that the initial concentrations of alloxan are presented. However, because the half life of alloxan is only 1 min in this system [4] and the dead space in the perifusion system is approximately 2 ml the effective alloxan concentration reaching the islets was considerably less than that given. (Since the flow rate was 1 ml/min it takes 2 min for the alloxan medium to reach the islets.) In experiments where calcium was omitted from the medium, this cation was absent during period 1 (30 min), period 2 (5 min), and period 3 (5 min). Calcium, 2.2 mmol/l, was readded in period 4. Insulin release was measured by radioimmunossay [17] using human insulin as standard.

Metabolic Experiments

In selected perifusion experiments in the presence or absence of alloxan the ability of islets to utilise glucose was determined by the rate of tritiated water formation from [5-³H] glucose. In these experiments, the filter with attached islets was removed from the perifusion chamber after the 20 min stimulation (period 5) with glucose (27.5 mmol/l). The medium used to incubate the islets was identical to the perifusion medium except for the presence of [5-³H] glucose, 4 to 5 µl, and was prepared in the following man-

ner. Stock [5-³H] glucose was placed in a 10 × 75 mm glass tube and desiccated overnight. To this was added 300 µl of the warmed and oxygenated perifusion medium at the desired glucose concentration; 125 µl of this medium was added to the vial which contained the islets and another 125 µl to a vial without islets. This was necessary to control for the small ³H₂O blank in the [5-³H] glucose. The vials were subsequently placed in a metabolic shaker (100 strokes/min) at 37° for 1 hour. At the end of this time 2 × 50 µl of incubation medium was transferred to a 6 × 30 mm glass tube to which had been added 5 µl of 1 mol/l HCl. The acid tended to minimise in some unknown fashion the tritiated water formed from the unused tritiated glucose. The small glass tubes were then placed in a scintillation vial to which had been added 0.5 ml distilled water. The vials were stoppered and allowed to equilibrate at 37° overnight in an air-circulated incubation room. ³H₂O standards were treated in the same manner to control for incomplete equilibration. At the end of the period the vials were removed from the incubation room and allowed to reach room temperature. The small inner tube was removed and placed in another scintillation vial. This tube contained the unused [5-³H] glucose. Scintillation fluid (10 ml) was added to all vials and the radioactivity determined. After the appropriate corrections for incomplete ³H₂O equilibration, usage was calculated as previously reported [18–20].

Statistics

Significance of difference between experimental groups were tested by Student's t-test.

Chemicals

Alloxan monohydrate, D-glucose, and 3-0-methyl-D-glucose were all purchased from Sigma Chemical Co, St. Louis, Mo. Pure D-glyceraldehyde was obtained from Koch-Light Laboratories Ltd, Colnbrook, England. Collagenase (Type IV) was from Worthington Biochemical, Freehold, N. J. [5-³H] glucose was obtained from Amersham-Searle, Arlington, Il. and the ³H₂O from New England Nuclear, Boston, MA. Scintillation fluid (3a 70) was purchased from Research Products International, Elk Grove, Il. Bovine serum albumin was obtained from Armour Pharmaceuticals, Kankakee, Ill.

Results

Insulin Release

A 5 min exposure to various concentrations of alloxan during period 2 produced a dose-dependent decrease of glucose-stimulated insulin secretion (Fig. 1). Complete blockade was observed at 1.25 mmol/l. If 27.5 mmol/l glucose or 3-0-methylglucose were present during the 5 min exposure to 1.25 mmol/l alloxan, complete protection of glucose-induced secretion was observed (Fig. 2). In the absence of normal extracellular calcium (2.2 mmol/l), which prevents the elevation of insulin secretion [21], glucose still protected against the deleterious effects of alloxan (results not shown). D-Glyceraldehyde, 27.5 mmol/l, provided only marginal protection against alloxan (Fig. 2). Taking into account basal
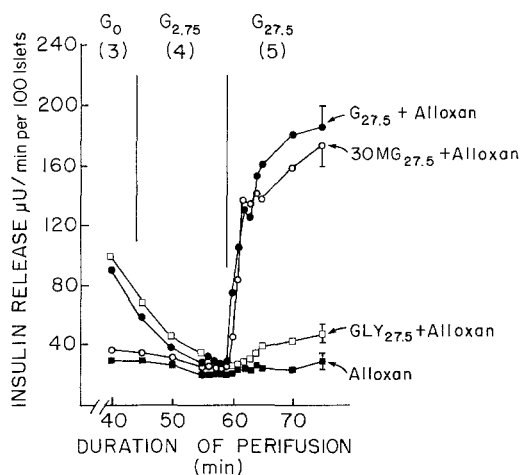
Fig. 2. Protection against alloxan poisoning of glucose-induced insulin secretion by various compounds. Islets were perifused as in Figure 1 except that in period 2 (not shown) the islets were exposed to alloxan (1.25 mmol/l) alone or with glucose (G) (n=5), 3-0-methylglucose (3OMG) (n=4), or glyceraldehyde (GLY) (n=4), all at 27.5 mmol/l. Subsequently, the ability of the islets to respond to 27.5 mmol/l glucose was examined. Other comments as for Figure 1

Fig. 3. Lack of effect of 3-0-methylglucose on insulin secretion. Islets were perifused as previously noted (See legend to Figure 1). During period 5, the islets were exposed to 27.5 mmol/l 3-0-methylglucose in the presence of 2.75 or 27.5 mmol/l glucose (n=4 for each condition). Other comments as for Figure 1

insulin secretion (20 μU/min per 100 islets), the response was 16% of that seen in non-poisoned islets (see Fig. 1). 3-0-methylglucose, 27.5 mmol/l, neither elevated basal release nor interfered with the stimulant action of glucose (Fig. 3).

cAMP in Islets

In Table 1 the effects of various doses of glucose and alloxan on islet cAMP are shown. With 2.75 mmol/l glucose present during the 20 min stimulatory period (line 1) cAMP levels averaged 1.59 ± 0.19 (mean $\pm$ SEM) pmol/100 islets. Pretreatment with 0.31 or 1.25 mmol/l alloxan during period 2 did not alter these basal levels (Table 1, lines 2 and 3). With 27.5 mmol/l glucose present in the final period, cAMP increased 2.3-fold (line 4). Pretreatment with various alloxan doses resulted in a dose-dependent decrease of the cAMP response to 27.5 mmol/l glucose. After exposure to 1.25 mmol/l alloxan in period 2, cAMP values in the presence of 27.5 mmol/l glucose were not different from those observed with 2.75 mmol/l glucose (compare lines 1 and 7). 3-0-methylglucose (27.5 mmol/l) neither interfered with the ability of 27.5 mmol/l glucose to elevate cAMP nor was 3-0-methylglucose capable of elevating cAMP in the presence of basal glucose (lines 8 and 9).

In Table 2 the results of experiments which tested the ability of various compounds to protect against alloxan are shown. Glucose (27.5 mmol/l) or 3-0-
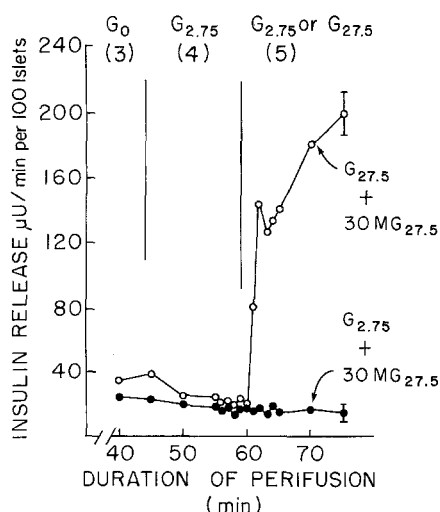
Table 1. Effects of alloxan and sugars on cAMP in isolated islets

Line	Period		Period		cAMP levels
	2 Addition	Concn. mmol/l	5 Addition	Concn. mmol/l	(pmol/100 islets)
1	Glucose	0	Glucose	2.75	1.59 ± 0.19[a] [4]
2	Alloxan	0.31	Glucose	2.75	1.53 ± 0.15 [3]
3	Alloxan	1.25	Glucose	2.75	1.48 ± 0.20 [4]
4	Glucose	0	Glucose	27.5	3.58 ± 0.20 [5]
5	Alloxan	0.31	Glucose	27.5	2.64 ± 0.10 [3]
6	Alloxan	0.63	Glucose	27.5	2.09 ± 0.16 [3]
7	Alloxan	1.25	Glucose	27.5	1.64 ± 0.19 [7]
8	Glucose	0	Glucose + 3-0-methyl- glucose	27.5 27.5	3.68 ± 0.21 [4]
9	Glucose	0	Glucose + 3-0-methyl- glucose	2.75 27.5	1.39 ± 0.18 [4]

Batches of 100 islets were perifused as follows. Period 1, 30 minutes with zero glucose; Period 2, 5 min with zero glucose and different alloxan doses; Period 3, 5 min with zero glucose; Period 4, 15 min with glucose, 2.75 mmol/l; Period 5, 20 min with glucose at 2.75 mmol/l, glucose at 27.5 mmol/l, glucose at 27.5 mmol/l and 3-0-methylglucose at 27.5 mmol/l, or glucose at 2.75 mmol/l plus 3-0-methylglucose at 27.5 mmol/l

[a] Mean $\pm$ SEM for the numbers of experiments stated in parentheses

Table 2. Effects of glucose, 3-0-methylglucose and glyceraldehyde against alloxan depression of cAMP levels

Line	Period 2 Addition	Concn. mmol/l	Period 5 Addition	Concn. mmol/l	cAMP levels (pmol/100 islets)	p values
1	Alloxan	1.25	Glucose	27.5	1.64 ± 0.19^a [7]	
2	Glucose	27.5	Glucose	2.75	1.69 ± 0.21 [3]	2 vs 1: N.S.*
3	Alloxan + Glucose	1.25 27.5	Glucose	27.5	3.31 ± 0.30 [5]	3 vs 1: p < 0.05
4	Alloxan + 3-0-methylglucose	1.25 27.5	Glucose	27.5	3.25 ± 0.17 [4]	4 vs 1: p < 0.05
5	Alloxan + Glyceraldehyde	1.25 27.5	Glucose	27.5	1.90 ± 0.16 [4]	5 vs 1: N.S.

All islets were perifused as explained in the legend of Table 1. In Period 2, in addition to 1.25 mmol/l alloxan, 27.5 mmol/l glucose, 27.5 mmol/l 3-0-methylglucose, or 27.5 mmol/l glyceraldehyde was present
[a] Results are presented as means $\pm$ SEM for the numbers of experiments stated in parentheses
* N.S. = Not significant (p > 0.05) using Student's t-test

methylglucose (27.5 mmol/l) protected the islet cAMP responsiveness to 27.5 mmol/l glucose (lines 3 and 4). In contrast 27.5 mmol/l D-glyceraldehyde had a slight but insignificant protective effect (line 5).

Exposure of islet tissue to 27.5 mmol/l glucose, 27.5 mmol/l 3-0-methylglucose, or 27.5 mmol/l glyceraldehyde alone during period 2 did not affect insulin release measured during exposure to 27.5 mmol/l glucose in period 5. Levels of cAMP measured after the perifusion were also comparable and averaged 3.72 ± 0.25 (n=3), 3.47 ± 0.32 (n=3), and 3.89 ± 0.30 (n=3) under these conditions respectively.

In a further series of experiments, the ability of alloxan poisoned islets to produce ^3H_2O from [5-^3H] glucose was determined. Glucose usage in control, untreated islets averaged 104.9 ± 6.5 pmol/islet/ hour (mean ± SEM, n=4) measured at a glucose concentration of 27.5 mmol/l while alloxan-treated islets utilised 83.7 ± 5.0 pmol of glucose/islet/hour at the same glucose concentration (p < 0.05).

Discussion

Several points emerge from the present study. 1) The ability of glucose to elevate cAMP levels in alloxan poisoned islets was reduced and was parallelled by an inhibition of insulin secretion. 2) Compounds (glucose and 3-0-methylglucose) which completely protect against alloxan poisoning of glucose-induced insulin release, maintained the ability of glucose to elevate cAMP levels while glyceraldehyde, which only marginally protected glucose-induced secretion,

barely preserved the response of the cAMP system. 3) Protection by glucose did not appear to be related to its ability to elevate cAMP or promote insulin secretion since removal of extracellular calcium, which blocked both these responses [14, 21, 22] did not alter its protective ability. 4) While alloxan significantly impaired the ability of islets to form ^3H_2O from [5-^3H] glucose, the effect was small in comparison to the complete blockade of insulin release.

That glucose elevates cAMP levels in isolated islets has been amply documented [12–16]. How this elevation is mediated, either directly through a glucose-receptor mechanism involving the intact glucose molecule or indirectly via a metabolite or cofactor of glucose metabolism is not fully understood. Recently however, Capito and Hedeskov [23] have demonstrated a direct effect of glucose on cAMP accumulation in mouse islet homogenates. This elevation was not blocked by mannoheptulose, a 7-carbon sugar which blocks glucose phosphorylation [24]. The exact interpretation of these results is not clear however, since it is known that mannoheptulose blocks glucose-stimulated cAMP accumulation in intact islet tissue [14, 25]. Since alloxan caused a dose-dependent decrease in both stimulated secretion and cAMP levels it might be postulated that the pronounced inhibition of glucose-induced secretion after alloxan is the result of a rapid inactivation of the adenylate cyclase system. This inhibition of adenylate cyclase might occur by direct alteration of the enzyme itself or through changes in the cofactors necessary for enzyme activation. For example, it has recently been shown [26, 27] that alloxan alters the ionic properties of B-cell membranes and since extracellular calcium

appears essential for glucose-induced accumulation of cAMP in islets [14, 22] such ionic changes may result in a decreased enzymatic response.

The mechanism(s) through which glucose or 3-0-methylglucose protect against alloxan poisoning is not known. Perhaps the simplest explanation for hexose-protection is that various compounds might prevent the transport of alloxan into the cell [28, 29] and its ability to interact and alter the adenylate cyclase system. Glucose, mannose, and 3-0-methylglucose, compounds which presumably utilise the same transport system [30, 31] all protect against alloxan. However, it has recently been demonstrated that while 3-0-methylglucose prevents alloxan accumulation in islets, glucose and mannose actually increase the uptake of the compound [32].

It might also be suggested that these protective compounds interfere with the ability of alloxan to interact directly with the adenylate cyclase system. However, it should be noted that 3-0-methylglucose did not elevate cAMP levels or interfere with the ability of high glucose to do so. Therefore, while a direct protective effect of glucose on adenylate cyclase cannot be ruled out, a direct interaction of 3-0-methylglucose with adenylate cyclase at least at the glucose-sensitive site appears unlikely. Other possibilities might include endogenously generated cAMP or hexose metabolites as protecting agents. However, these possibilities are apparently excluded because 3-0-methylglucose is not metabolised by islets and does not elevate cAMP, the protective effect of glucose occurs in the absence of elevated cAMP (by omitting extracellular Ca^{++}), and glyceraldehyde increases cAMP in islet tissue [15] but does not significantly protect against alloxan. Of course, the possibility that these protecting compounds work by different mechanisms cannot be excluded.

From the present results, it does not appear that a primary blockage of total glucose utilisation can account for the pronounced reduction in glucose-stimulated secretion. However, the results on total glucose usage must be interpreted with caution. The use of the $[5-^3H]$ glucose derivative appears to measure accurately total glucose flux via glycolysis and the pentose phosphate shunt [20] but yields no information on the ratio of glucose metabolised by each pathway. Alloxan might cause a preferential augmentation or reduction of either route of metabolism. In addition, usage rates yield no information on glucose oxidation rates. Recently, it has been shown that alloxan profoundly inhibits glucose oxidation in mouse islets and it was suggested that the mitochondria may be a primary target for alloxan action [33]. While it is possible that glucose or man-

nose derived metabolites or cofactors might somehow spare the mitochondria from alloxan the mechanism of 3-0-methylglucose protection remains puzzling unless alternate mechanisms of protection are postulated.

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