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Effects of Alloxan on the Islets of Langerhans: Why does Alloxan not Stimulate Insulin Release?

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INTRODUCTION

Ever since the discovery that alloxan causes a selective B-cell necrosis in the islets of Langerhans (7,8), this drug has been of great interest in diabetologic research. Firstly, alloxan has been videly used to induce diabetes in laboratory animals in order to investigate the complications of the disease. Secondly, much work has been performed on the effects of the drug on the islets themselves. The aim of these efforts has been to advance our knowledge on the mode of action of alloxan on the islet B-cells and elucidate further the problem of insulin production. The latter type of study assumes a direct effect of the drug on the islets ultimately leading to necrosis. In vivo, such a direct effect was demonstrated elegantly by Bo Hellman in his very first work on the islets of Langerhans (22). In vitro, isolated islets of Langerhans, exposed to alloxan and subsequently maintained in tissue culture, show conspicuous structural signs of damage in addition to a decrease in metabolic capacity (10). These structural derangements depend on the glucose concentration of the culture medium (Borg, unpublished observations). However, it is far from clear that the effects of alloxan on the structure of the islets precede the functional deterioration caused by the drug. The primary action of alloxan on the islets of Langerhans may rather be on their functional machinery. In experiments with isolated perfused rat pancreas (35) and perifused isolated islets (30,40) it has been shown that alloxan produces an early monophasic insulin response and induces an irreversible inhibition of secretion. These findings have led to a hypothesis suggesting that alloxan could be recognized by a B-cell glucoreceptor, which initiates insulin release (38,40). Against this background it seems justified to ask why alloxan does not stimulate islet insulin secretion continuously.

To make possible a detailed investigation of the acute B-cytotoxic action of alloxan we have established an in vitro model system based on isolated islets of Langerhans from normal mice. Using this system we have studied insulin secretion and certain aspects of plasma membrane function and cell metabolism in islets treated with the drug.

METHODS

Isolated islets of Langerhans were prepared from adult male NMRI-mice by a collagenase (E.C. 3.4.24.3) digestion method (25). The islets were exposed to alloxan at 4 °C. The incubation medium consisted of 2 mmol/l alloxan monohydrate in a phosphate buffer (28), pH 7.4, which was supplemented with 2.8 mmol/l D-glucose. After alloxan exposure for 30 min, the islets were washed twice in alloxan-free incubation medium. Control islets were treated similarly, except that alloxan was omitted from the medium. A careful check was made to ensure that pH changes due to alloxan did not influence the results.

Islet insulin release in response to glucose or leucine was studied after alloxan treatment. Duplicate samples of islets were incubated for 60 min at 37 °C in 250 µl of a bicarbonate-buffered medium (29) with 25 mmol/l HEPES and 2 mg/ml albumin and D-glucose or L-leucine as appropriate. Insulin was determined by a radioimmunological assay (17) with crystalline mouse insulin as a standard.

After exposure to alloxan, the islet activity of the enzyme adenylate cyclase (E.C. 4.6.1.1) was estimated by the method of Howell and Montague (24). 200 - 300 islets were homogenized in 500 µl of ice-cold buffer, and samples (50 µl) of the homogenate were mixed with 50 µl of the assay medium containing 1 mmol/l $[\alpha\text{-}^{32}P]$ ATP with a specific radioactivity of 620 MBq/mmol. To achieve maximal stimulation of the enzyme activity some media were supplemented with 10 mmol/l KF. The mixture was incubated at 37 °C for 30 min and the reaction was stopped by boiling for 3 min. Cyclic $[^{32}P]$ AMP formed during the reaction was separated from the reaction mixture on columns of neutral alumina and measured by counting the Čerenkov radiation in a liquid-scintillation spectrometer. Protein content of tissue homogenates was determined by the method of Lowry et al. (32).

The glucose utilization by alloxan-treated islets was determined essentially as described by Ashcroft et al. (1). Duplicate batches of islets were incubated for 60 min at 37 °C in a bicarbonate buffer (29) containing 2.8 or 28 mmol/l D-$[5\text{-}^3H]$ glucose with a specific radioactivity of 2200 and 220 MBq/mmol, respectively. The tritiated water formed from the radioactive glucose was measured by liquid-scintillation counting. The recovery of tritium from known amounts of ^3H_2O was 65 % under these experimental conditions.

The oxidation of glucose or leucine in islets, which had been exposed to alloxan, was estimated by measurements of $^{14}CO_2$ evolved from radioactive substrates. Duplicate groups of islets were incubated at 37 °C for 60 min in a bicarbonate-buffered medium (12) with either 28 mmol/l D-$[U\text{-}^{14}C]$ glucose (44.4 MBq/mmol) or 10 mmol/l L-$[U\text{-}^{14}C]$ leucine (18.5 MBq/mmol). The details of the incubation technique were essentially as described by Edwards et al. (9).

RESULTS AND DISCUSSION

From studies of islet uptake of $[2\text{-}^{14}C]$ alloxan (39) it seems clear that alloxan diffuses freely across the B-cell plasma membrane, and, furthermore, it was concluded that the plasma membrane was impermeable to the decomposition product of the drug. These findings could explain the accumulation of alloxan in the islets of Langerhans, which has been demonstrated by autoradiography (15,16). Probably alloxan taken up by the B-cells is rapidly decomposed intracellularly at a physiologic temperature, and the decomposition product is entrapped. However, in our experimental system rapid decomposition of the drug is avoided by the low temperature during alloxan treatment of the isolated islets. Equilibration with all components of the islet cells is thus facilitated, and the washing out of alloxan from the islets should also be very effective providing binding of the drug does not prevent it.

Despite the washings performed it was found that the alloxan treatment inhibited subsequent islet insulin release at 37 °C (Fig. 1). Control islets increased their insulin secretion three times over basal level at a maximal glucose stimulation ($P < 0.05$) and one and a half times over basal level at a maximal leucine challenge ($P < 0.01$), whereas islets exposed to alloxan did not show any response to glucose or leucine whatsoever. These results confirm earlier findings of a complete alloxan-dependent inhibition of the islet secretory response to glucose (6,14,23,26,37,43) or leucine (36). Moreover, they imply that even after a careful washing of the islets some alloxan remains intracellularly, probably firmly bound to some cell constituent.

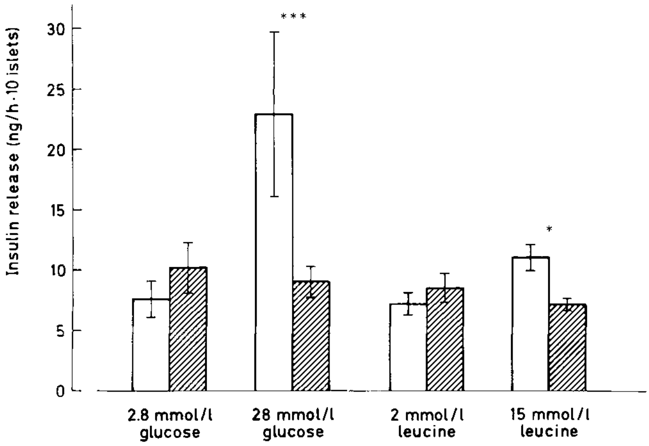

Fig. 1. Effects of alloxan on insulin release by mouse islets of Langerhans. Isolated islets were incubated at 4 °C for 30 min either in absence (□) or presence (▨) of 2 mmol/l alloxan. Subsequently the insulin release was measured at 37 °C in the presence of glucose or leucine as indicated. The probabilities of chance differences between control and alloxan-treated islets are: $^{*}P < 0.05$ and $^{***}P < 0.001$.

From studies on alloxan reactivity in solution it has been proposed that the drug is reduced to dialuric acid (5), which by spontaneous reaction with oxygen generates superoxide radicals, $O_2^{-}\bullet$, hydrogen peroxide, H_2O_2, and hydroxyl radicals, •OH (4). All three of these species are very reactive and might be responsible for the B-cytotoxicity of alloxan. The fact that superoxide dismutase (E.C. 1.15.1.1), catalase (E.C. 1.11.1.6) and several hydroxyl radical scavengers protect against the effects of alloxan on the islets of Langerhans (11,13,18,19, 20,21) is consistent with the idea that the hydroxyl radical in particular may be the cytotoxic intermediate (11,21). This radical could interfere with a variety of cellular processes and thus explain the whole spectrum of effects, which alloxan exerts on the islet B-cells. However, due to its high reactivity the range of action of the hydroxyl radical is probably very short, and it may be argued that, if the drug binds specifically to some cell constituent, only one or a few cellular events may be directly affected be the drug action and other effects would be secondary phenomena.

The hypothesis that alloxan can be recognized by a B-cell glucoreceptor (38, 40) presumably implies that the drug interacts with the B-cell plasma membrane. Such an interpretation may be supported by the finding of alloxan-induced alterations in plasma membrane morphology as revealed by ultrastructural studies of freeze-fractured rat islets (34). Furthermore, it has been shown that glucose-stimulated increase of islet cyclic AMP content is abolished in islets exposed to alloxan (43) and an interaction with the membrane bound (27,31) enzyme adenylate cyclase (E.C. 4.6.1.1) is possible. However, in our experiments alloxan apparently had no effects on islet adenylate cyclase (E.C. 4.6.1.1) activity (Fig. 2). Considering the present finding, it may be argued that the cytotoxicity of alloxan depends on interaction with cellular targets other than the plasma membrane. Moreover, it is known that other plasma membrane-associated phenomena such as islet cell glucose uptake (33) or leucine uptake (Borg, unpublished ob-

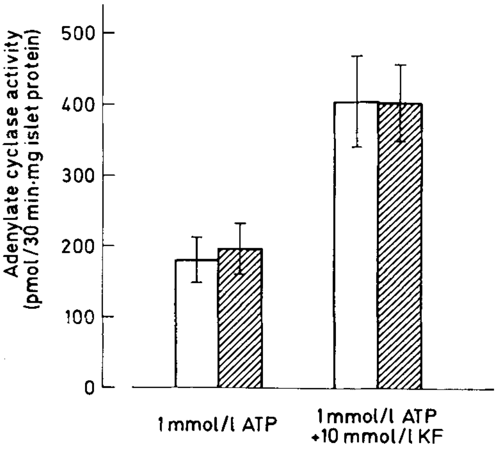

Fig. 2. Effects of alloxan on adenylate cyclase activity in mouse islets of Langerhans. Isolated islets were incubated at 4 °C for 30 min either in absence (□) or presence (▨) of 2 mmol/l alloxan and the enzyme activity was subsequently measured in homogenates of the islets. The basal activity was determined without fluoride in the assay medium, whereas the stimulated activity was determined with 10 mmol/l potassium fluoride in the medium.

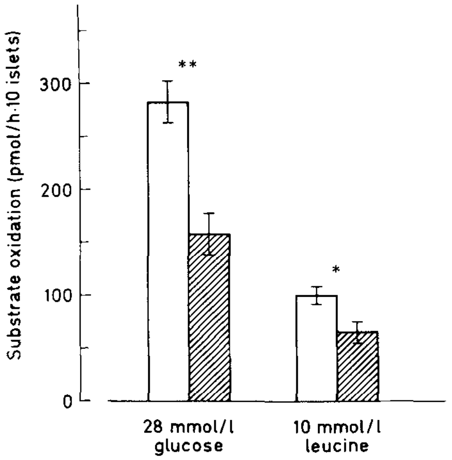

Fig. 3. Effects of alloxan on substrate oxidation by mouse islets of Langerhans. Isolated islets were incubated at 4 °C for 30 min either in absence (□) or presence (▨) of 2 mmol/l alloxan and the oxidation of glucose or leucine was subsequently measured at 37 °C. The probabilities of chance differences between control and alloxan-treated islets are: $*P < 0.05$ and $**P < 0.01$.

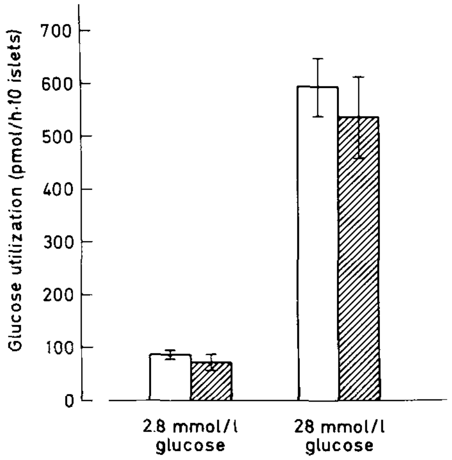

Fig. 4. Effect of alloxan on glucose utilization by mouse islets of Langerhans. Isolated islets were incubated at 4 °C for 30 min either in absence (□) or presence (▨) of 2 mmol/l alloxan and the glucose utilization at 2.8 mmol/l and 28 mmol/l was subsequently measured at 37 °C.

servations) are not affected by alloxan.

In the present investigation, the islet oxidation-rate of both glucose and leucine was, however, decreased by 40 % after alloxan treatment of the islets (Fig. 3). On the other hand, the drug had no effect on islet glucose utilization (Fig. 4). These results and the fact that alloxan does not affect islet anaerobic glycolysis (14) make it tempting to suppose that the primary cytotoxic action of alloxan is exerted on the B-cell mitochondria. This assumption is further supported by the very early degenerative changes found in these mitochondria after alloxan treatment in vivo (2,41,42). Indeed, it has been proposed that alloxan affects primarly sulfhydryl-dependent mitochondrial transport systems for inorganic phosphate (3) and certain metabolites (Boquist, this volume), which in turn leads to impaired cellular function and viability.

The answer to why alloxan does not stimulate islet insulin secretion continuously may lie in the toxic action of drug decomposition, whereas the initiation of insulin release promoted by alloxan in glucose-free media (30,35,40) may be effected by a transitory interaction between the intact drug molecule and a putative B-cell glucose receptor. It seems likely that the acute B-cytotoxicity, ultimately leading to cell necrosis, involves derangements of sites responsible for the oxidative metabolism of the B-cell.

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REFERENCES

1. Ashcroft, S.J.H., Weerasinghe, L.C.C., Bassett, J.M. & Randle, P.J.: The pentose cycle and insulin release in mouse pancreatic islets. Biochem J 126: 525-532, 1972.
2. Boquist, L.: The endocrine pancreas in early alloxan diabetes. Including study of the alloxan inhibitory effect of feeding and some hexoses. Acta pathol microbiol scand Sect A 85:219-229, 1977.
3. Boquist, L.: A new hypothesis for alloxan diabetes. Acta pathol microbiol scand Sect A 88:201-209, 1980.
4. Cohen, G. & Heikkila, R.E.: The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J biol Chem 249:2447-2452, 1974.
5. Deamer, D.W., Heikkila, R.E., Panganamala, R.V., Cohen, G. & Cornwell, D.G.: The alloxan-dialuric acid cycle and the generation of hydrogen peroxide. Physiol Chem Phys 3:426-430, 1971.
6. Dixit, P.K., Bui, B.T. & Hernandez, R.E.: Comparison of the effect of diabetogenic agents on the microdissected pancreatic islet tissue of the rat. Proc Soc exp Biol Med 140:1418-1423, 1972.
7. Dunn, J.S. & McLetchie, N.G.B.: Experimental alloxan diabetes in the rat. Lancet ii:384-387, 1943.
8. Dunn, J.S., Sheehan, H.L. & McLetchie, N.G.B.: Necrosis of islets of Langerhans produced experimentally. Lancet i:484-487, 1943.
9. Edwards, J.C., Hellerström, C., Petersson, B. & Taylor, K.W.: Oxidation of glucose and fatty acids in normal and in A_2-cell rich pancreatic islets isolated from guinea-pigs. Diabetologia 8:93-98, 1972.
10. Eide, S.J., Borg, L.A.H. & Hellerström, C.: An in vitro model for studies of the B-cytotoxic effects of alloxan. Diabetologia 12:388, 1976.
11. Fischer, L.J. & Hamburger, S.A.: Inhibition of alloxan action in isolated pancreatic islets by superoxide dismutase, catalase, and a metal chelator. Diabetes 29:213-216, 1980.
12. Gey, G.O. & Gey, M.K.: The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am J Cancer 27: 45-76, 1936.
13. Grankvist, K., Marklund, S., Sehlin, J. & Täljedal, I.-B.: Superoxide dismutase, catalase and scavengers of hydroxyl radical protect against the toxic action of alloxan on pancreatic islet cells in vitro. Biochem J 182:17-25, 1979.
14. Gunnarsson, R. & Hellerström, C.: Acute effects of alloxan on the metabolism and insulin secretion of the pancreatic B-cell. Horm metab Res 5:404-409, 1973.
15. Hammarström, L., Hellman, B. & Ullberg, S.: On the accumulation of alloxan in the pancreatic β-cells. Diabetologia 3:340-345, 1967.
16. Hammarström, L. & Ullberg, S.: Specific uptake of labelled alloxan in the pancreatic islets. Nature(Lond) 212:708-709, 1966.
17. Heding, L.G.: Determination of total serum insulin (IRI) in insulin-treated diabetic patients. Diabetologia 8:260-266, 1972.
18. Heikkila, R.E.: The prevention of alloxan-induced diabetes in mice by dimethyl sulfoxide. Eur J Pharmacol 44:191-193, 1977.
19. Heikkila, R.E., Barden, H. & Cohen, G.: Prevention of alloxan-induced diabetes by ethanol administration. J Pharmacol exp Ther 190:501-506, 1974.

20. Heikkila, R.E. & Cabbat, F.S.: Protection against alloxan-induced diabetes in mice by the hydroxyl radical scavenger dimethylurea. Eur J Pharmacol 52: 57-60, 1978.
21. Heikkila, R.E., Winston, B. & Cohen, G.: Alloxan-induced diabetes - Evidence for hydroxyl radical as a cytotoxic intermediate. Biochem Pharmacol 25:1085-1092, 1976.
22. Hellman, B. & Diderholm, H.: The diabetogenic effect of alloxan after elimination of extra-pancreatic factors. Acta endocrinol(Kbh) 20:81-87, 1955.
23. Henquin, J.C., Malvaux, P. & Lambert, A.E.: Alloxan-induced alteration of insulin release, rubidium efflux and glucose metabolism in rat islets stimulated by various secretagogues. Diabetologia 16:253-260, 1979.
24. Howell, S.L. & Montague, W.: Adenylate cyclase activity in isolated rat islets of Langerhans. Effects of agents which alter rates of insulin secretion. Biochim biophys Acta 320:44-52, 1973.
25. Howell, S.L. & Taylor, K.W.: Potassium ions and the secretion of insulin by islets of Langerhans incubated in vitro. Biochem J 108:17-24, 1968.
26. Howell, S.L. & Taylor, K.W.: The acute pancreatic effect of alloxan in the rabbit. J Endocrinol 37:421-427, 1967.
27. Howell, S.L. & Whitfield, M.: Cytochemical localization of adenyl cyclase activity in rat islets of Langerhans. J Histochem Cytochem 20:873-879, 1972.
28. Krebs, H.A.: Untersuchungen über den Stoffwechsel der Aminosäuren im Tierkörper. Hoppe-Seyler´s Z physiol Chem 217:191-227, 1933.
29. Krebs, H.A. & Henseleit, K.: Untersuchungen über die Harnstoffbildung im Tierkörper. Hoppe-Seyler´s Z physiol Chem 210:33-66, 1932.
30. Lacy, P.E., McDaniel, M.L., Fink, C.J. & Roth, C.: Effect of methylxanthines on alloxan inhibition of insulin release. Diabetologia 11:501-507, 1975.
31. Lernmark, Å., Nathans, A. & Steiner, D.F.: Preparation and characterization of plasma membrane-enriched fractions from rat pancreatic islets. J Cell Biol 71:606-623, 1976.
32. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J.: Protein measurement with the Folin phenol reagent. J biol Chem 193:265-275, 1951.
33. McDaniel, M.L., Anderson, S., Fink, J., Roth, C. & Lacy, P.E.: Effect of alloxan on permeability and hexose transport in rat pancreatic islets. Endocrinology 97:68-75, 1975.
34. Orci, L., Amherdt, M., Malaisse-Lagae, F., Ravazzola, M., Malaisse, W.J., Perrelet, A. & Renold, A.E.: Islet cell membrane alteration by diabetogenic drugs. Lab Invest 34:451-454, 1976.
35. Pagliara, A.S., Stillings, S., Zawalich, W.S., Williams, A.D. & Matschinsky, F.M.: Glucose and 3-O-methylglucose protection against alloxan poisoning of pancreatic alpha and beta cells. Diabetes 26:973-979, 1977.
36. Tomita, T.: Effect of alloxan on arginine- and leucine-induced insulin secretion in isolated islets. FEBS Lett 72:79-82, 1976.
37. Tomita, T., Lacy, P.E., Matschinsky, F.M. & McDaniel, M.L.: Effect of alloxan on insulin secretion in isolated rat islets perifused in vitro. Diabetes 23:517-524, 1974.
38. Weaver, D.C., Barry, C.D., McDaniel, M.L., Marshall, G.R. & Lacy, P.E.: Molecular requirements for recognition at a glucoreceptor for insulin release. Mol Pharmacol 16:361-368, 1979.
39. Weaver, D.C., McDaniel, M.L. & Lacy, P.E.: Alloxan uptake by isolated rat islets of Langerhans. Endocrinology 102:1847-1855, 1978.
40. Weaver, D.C., McDaniel, M.L., Naber, S.P., Barry, C.D. & Lacy, P.E.: Alloxan stimulation and inhibition of insulin release from isolated rat islets of Langerhans. Diabetes 27:1205-1214, 1978.
41. Wellmann, K.F., Volk, B.W. & Lazarus, S.S.: Ultrastructural pancreatic beta-cell changes in rabbits after small and large doses of alloxan. Diabetes 16: 242-251, 1967.
42. Williamson, J.R. & Lacy, P.E.: Electron microscopy of islet cells in alloxan-treated rabbits. A M A Arch Pathol 67:102-109, 1959.
43. Zawalich, W.S., Karl, R.C. & Matschinsky, F.M.: Effects of alloxan on glucose-stimulated insulin secretion, glucose metabolism, and cyclic adenosine 3´,5´-monophosphate levels in rat isolated islets of Langerhans. Diabetologia 16:115-120, 1979.