Inhibitory Effect of Diphenylhydantoin on the Diabetogenic Action of Alloxan in the Mouse

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SUMMARY

The effect of the pretreatment of mice with diphenylhydantoin (DPH) on the development of alloxan diabetes was studied. DPH, in intraperitoneal doses of 20 to 45 mg./kg., administered one hour prior to alloxan, was found to prevent the development of alloxan (75 mg./kg., intravenously) diabetes. Administration of DPH after alloxan had no effect. The initial hyperglycemic response to alloxan (measured forty-five minutes post-alloxan) was potentiated by DPH pretreatment. Intravenous glutathione, administered one minute prior to alloxan, inhibited both the initial and chronic hyperglycemia produced by alloxan. A structural similarity between DPH and alloxan suggests that DPH may be protecting pancreatic beta cell binding sites from alloxan. DIABETES *21*:80-83, February, 1972.

Alloxan is the most widely used agent for the induction of experimental diabetes. In spite of the extensive work conducted with this chemical, the exact mechanism through which it produces chronic diabetes mellitus remains obscure. Several hypotheses on the mechanism of action of alloxan have been put forward; however, most have been shown to be either untenable or insufficient evidence has been generated to support them.[1]

One possible mechanism of action of alloxan concerns the effect of the chemical on the integrity of the pancreatic beta cell membrane. Hughes et al.[2] have suggested that the hypoglycemic phase of the action of alloxan is the result of an uncontrolled leakage of insulin from the damaged beta cell. It is well documented that alloxan destroys the integrity of the pancreatic beta cell permeability barrier. Several reports have demonstrated that alloxan increases the penetration of mannitol into beta cells [3-5] and promotes the leakage of insulin and other protein from the cells.[6,7] Further, this disruption of the cell membrane by alloxan appears to be specific for pancreatic beta cells.[8]

Levin et al.[9] have recently reported that the anticonvulsant drug, diphenylhydantoin (DPH), inhibits insulin secretion in the isolated, perfused pancreas. The mechanism of this inhibitory effect of DPH is not known. The authors suggested, however, that an effect on intracellular sodium ion concentrations might be involved. DPH is known to reduce intracellular concentrations of sodium in brain cells as well as cardiac and skeletal muscles.[10] Further, it has been reported[11] that treatments which result in depletion of intracellular sodium also inhibit insulin secretion.

A structural similarity exists between alloxan and DPH, both chemicals possessing the ureido group. Since both agents exert effects on the pancreas and they both contain the ureido group, it is possible that they may be acting at the same site but producing opposing effects. For this reason, experiments were conducted to assess the interaction between the two chemicals and the effects of this interaction on the development of chronic diabetes mellitus.

METHODS

Male Swiss albino mice (Laboratory Supply Co., Indianapolis) weighing 18 to 24 gm. were used in all experiments. The animals where allowed at least three days to acclimate to laboratory conditions before experimentation. The mice were housed in groups of twenty with free access to food and water at all times.

All drug solutions were prepared in distilled water prior to use. In the case of alloxan, all injections were made within twenty minutes after preparation of the solutions. Solutions were prepared at concentrations which allowed for the administration of volume doses

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of 10 ml./kg. DPH sodium was administered intraperitoneally while glutathione and alloxan were administered intravenously.

Two technics were used for the collection of blood samples for glucose analysis. When repeated measures on the same animal were made, blood was obtained from the orbital sinus. In some experiments, single blood samples were taken and in these cases the animals were decapitated and the free flowing blood was collected in oxalated beakers. During each experiment only one technic was employed for the collection of blood.

Blood glucose levels were determined enzymatically by the glucose oxidase method.* For the purposes of designating percentage of animals with diabetes mellitus, animals were judged diabetic when blood glucose values were in excess of 300 mg./100 ml. Differences between treatment groups were assessed for statistical significance by the Student's *t* test.

RESULTS

The results shown in table 1 summarize the effects of a one-hour pretreatment of mice with 40 mg./kg., intraperitoneally of DPH on the blood glucose responses to alloxan (75 mg./kg., intravenously). It may be seen from these data that alloxan alone produced an unequivocal hyperglycemic response forty-five minutes after administration. This hyperglycemic response was also present in the DPH pretreated mice; in fact, the hyperglycemia in these animals was significantly greater ($p < 0.01$) than that in the animals receiving alloxan alone. Twenty-four hours after the administration of alloxan all of the animals receiving alloxan alone were diabetic (blood glucose in excess of 300 mg./100 ml.). None of the animals receiving DPH prior to alloxan however, developed diabetes; in fact, the mean blood glucose value for this group was slightly below the control value ($p < 0.01$).

Since alloxan-induced diabetes may take as long as forty-eight hours to be completely manifested, it was decided to determine blood glucose levels one week after treatment with alloxan or the combined treatment of DPH and alloxan. The results of this experiment are also shown in table 1. Again, all of the animals receiving alloxan alone were diabetic and the mean blood glucose value was 357 mg./100 ml. as compared to 113 mg./100 ml. in the control group. The animals receiving DPH prior to alloxan all exhibited blood glucose values within normal limits. Gross examination of these animals revealed that the mice treated with DPH and alloxan appeared to be completely normal, while those receiving only alloxan were emaciated and obviously in poor health. The pancreases from these animals were prepared with Gomori's chrom alum hematoxylin phloxine stain and examined histologically for changes in islet tissue. The islet tissue from the animals treated with DPH plus alloxan were identical to those obtained from control animals, whereas those from mice receiving only alloxan exhibited marked necrosis of the beta cells and a complete absence of insulin granules.

Since it has been shown that elevation of blood glutathione levels protects against the development of alloxan-induced diabetes, it was decided to compare glutathione protection to DPH protection. The results of this experiment are shown in table 2. Forty-five minutes after the administration of alloxan alone the animals exhibited

* Worthington Biochemicals, Freehold, N. J.

TABLE 1

Effect of DPH pretreatment on blood glucose responses to alloxan in mice

Treatment	Mean blood glucose (mg./100 ml.) Hours after alloxan* 0.75	24	168
Control	129 ± 4	162 ± 6	113 ± 3
DPH alone	144 ± 10†	148 ± 8	
Alloxan alone	348 ± 23‡	484 ± 53‡	357 ± 20‡
DPH plus alloxan	451 ± 45§	123 ± 4‖	95 ± 5

* DPH (40 mg./kg., intraperitoneally) was administered sixty minutes prior to alloxan (75 mg./kg., intravenously). Blood glucose values were determined 0.75, 24 and 168 hr. after alloxan administration. Values represent mean ± SEM for groups of five mice.
† Significantly greater than control ($p<0.05$)
‡ Significantly greater than control ($p<0.001$)
§ Significantly greater than alloxan alone ($p<0.01$)
‖ Significantly less than control and DPH alone ($p<0.05$)

TABLE 2

Effect of glutathione (GSH) pretreatment on blood glucose responses to alloxan in mice

Treatment	Mean blood glucose (mg./100 ml.) Hours after alloxan* 0.75	24
Control	120 ± 4	110 ± 6
GSH alone	126 ± 7	141 ± 6
Alloxan alone	374 ± 29†	525 ± 17†
GSH plus alloxan	151 ± 5‡	127 ± 5

* GSH (800 mg./kg., intravenously) was administered 1.0 min. prior to alloxan (75 mg./kg., intravenously). Blood glucose values were determined 0.75 and 24 hr. after alloxan administration. Values represent mean ± SEM for groups of five mice.
† Significantly greater than control and GSH plus alloxan ($p<0.001$).
‡ Significantly greater than control ($p<0.05$).

TABLE 3

Effect of time of administration of DPH on development of alloxan diabetes in mice

Treatment	Mean blood glucose (mg./100 ml.)	No. diabetic* / No. tested
Control	151 ± 4	0/5
Alloxan	405 ± 71†	4/5
DPH prior to alloxan		
5 min. pre-alloxan	264 ± 29‡	1/5
DPH after alloxan		
10 min. post-alloxan	470 ± 51†	6/7
20 min. post-alloxan	378 ± 84†	4/6
40 min. post-alloxan	449 ± 23†	5/6

* DPH (40 mg./kg., intraperitoneally) was administered at various time intervals prior to and after alloxan (75 mg./kg., intravenously) administration. Blood glucose values were determined twenty-four hours after alloxan administration. Values represent mean ± SEM. Mice were considered to be diabetic if blood glucose levels exceeded 300 mg./100 ml.

† Significantly greater than control ($p<0.001$).

‡ Significantly greater than control but less than alloxan alone ($p<0.05$).

the typical hyperglycemic response. Animals which had received an intravenous dose of 800 mg./kg. of glutathione one minute prior to the injection of alloxan, exhibited a slight hyperglycemic response. The magnitude of this hyperglycemia was significantly smaller than that observed after alloxan alone, however. Similarly, at the twenty-four-hour interval, animals treated with alloxan alone exhibited diabetes while those pretreated with glutathione did not.

The results shown in table 3 summarize experiments to determine the effectiveness of DPH in blocking the development of chronic diabetes when administered at various time intervals before and after the administration of alloxan. No protection was afforded by the administration of DPH at any of the intervals tested after the injection of alloxan. When DPH was administered five minutes prior to alloxan however, some degree of protection was noted. Blood glucose values for these animals were still significantly higher than control values, however only one of these animals exhibited a blood glucose value in excess of 300 mg./100 ml.

The results in table 4 show the effect of different dosage levels of DPH, administered one hour prior to alloxan, on the development of chronic diabetes. Doses of 20 and 45 mg./kg. of DPH protected all animals against the development of diabetes while a dose of 10 mg./kg. was without effect.

DISCUSSION

The results of the present experiments clearly demonstrate that DPH is capable of protecting mice against alloxan-induced diabetes. This protective effect of DPH could be mediated through a reduction of the amount of alloxan reaching the pancreas. Gomori and Goldner[12] have demonstrated that mechanically reducing the flow of blood to portions of the pancreas for less than five minutes after alloxan administration results in protection of those portions of the pancreas which have been isolated from the normal circulation. Pharmacologically, epinephrine, which causes constriction of the splanchnic vessels, is known to protect against alloxan-induced diabetes.[13] We know of no evidence which indicates that DPH alters pancreatic blood flow.

TABLE 4

Effect of various doses of DPH on development of alloxan diabetes in mice

Dosage level (mg./kg., i.p.)	Mean blood glucose (mg./100 ml.)	No. diabetic* / No. tested
Control	136 ± 9	0/5
45	133 ± 7	0/5
20	113 ± 3	0/5
10	358 ± 10†	5/5

* DPH was administered intraperitoneally one hour prior to alloxan (75 mg./kg., intravenously). Blood glucose values were determined twenty-four hours after alloxan administration. Values represent mean ± SEM. Mice were considered to be diabetic if blood glucose levels exceeded 300 mg./100 ml.

† Significantly greater than control ($p<0.05$).

A second means of reducing the amount of alloxan reaching the pancreas is through a direct reaction between DPH and alloxan in the circulation. A direct reaction of this type has been suggested as the mechanism of protection against alloxan which is afforded by reducing agents,[14] and sulfhydryl-containing chemicals.[15-19] These agents react with alloxan to form nondiabetogenic products. This explanation of the action of DPH also seems to be only a remote possibility. When the sulfhydryl-containing glutathione was administered prior to alloxan, the animals were protected against the initial hyperglycemic effect as well as the development of chronic diabetes. Identical results have been reported by Nath and Sahu[20] in their experiments with glucose, acetoacetate and their condensation product which also reacts directly with alloxan in the circulation. In our experiments with DPH, pretreatment with the anticonvulsant did not block the early hyperglycemia; in fact, the initial hyperglycemia was potentiated by the pretreatment. This increased initial hyperglycemia could be a reflection of the known inhibitory effect of DPH on insulin secretion.[9] This finding of potentiation of the initial alloxan-induced hyperglycemia does not seem to be consistent with the possibility that alloxan is inacti-

vated by a direct reaction with DPH.

Our experiments do not completely rule out the possibility that DPH reduces the amount of alloxan reaching the pancreas. Additional experiments will be required to clarify this point.

The possibility that sulfhydryl-containing compounds are involved in the protective effect of DPH still exists. It is possible that the pretreatment with DPH results in an increase in pancreatic levels of some naturally occurring protective compound, such as glutathione, and an inactivating reaction could occur within the pancreas. This possibility remains to be explored.

The structural similarities between DPH and alloxan offer an inviting possibility for the explanation of the protective effect of DPH. Both agents possess the ureido grouping and interestingly, this configuration is also found in the hypoglycemic sulfonylureas. In the case of alloxan, the nitrogen atoms appear to be essential for diabetogenic activity. Substitutions on these nitrogen atoms yield agents with less diabetogenic specificity.[1] Levin et al.[9] have demonstrated that DPH inhibits glucose-stimulated insulin release in the isolated, perfused pancreas. It is possible that the ureido group is responsible for the binding of both DPH and alloxan to the beta cell membranes. In this case, pretreatment with DPH could protect the receptor site from binding with alloxan, thus preventing the beta cytotoxic action of alloxan. Studies are currently in progress to assess this possibility.

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REFERENCES

[1] Rerup, C. C.: Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev. *22*:485-518, 1970.

[2] Hughes, H., Ware, L. L., and Young, F. G.: Diabetogenic action of alloxan. Lancet *246*:148-50, 1944.

[3] Watkins, D.: Studies on the site of action of alloxan. The effect of alloxan on islet cell membranes. Anat. Rec. *142*:290, 1962.

[4] Watkins, D., Cooperstein, S. J., and Lazarow, A.: Studies on the site of action of alloxan. Effect of alloxan on the permeability of the toadfish islet cell membrane to mannitol. Biol. Bull. *121*:412-13, 1961.

[5] Watkins, D., Cooperstein, S. J., and Lazarow, A.: Effect of sulfhydryl-binding reagents on islet tissue permeability: Protection and reversal by thiol compounds. J. Pharmacol. Exp. Ther. *176*:42-51, 1971.

[6] Cooperstein, S. J., Watkins, D., and Lazarow, A.: The effect of alloxan on islet tissue permeability. *In* The Structure and Metabolism of the Pancreatic Islets. Brolin, S. E., Hellman, B., and Knutson, H., Eds. New York, Pergamon, 1964, pp. 389-410.

[7] Watkins, D., Cooperstein, S. J., and Lazarow, A.: The effect of alloxan on the permeability of pancreatic islet tissue in vitro. Amer. J. Physiol. *207*:436-40, 1964.

[8] Watkins, D., Cooperstein, S. J., and Lazarow, A.: Effect of sulfhydryl reagents on permeability of toadfish islet tissue. Amer. J. Physiol. *219*:503-09, 1970.

[9] Levin, S. R., Booker, J., Smith, D. F., and Grodsky, G.M.: Inhibition of insulin secretion by diphenylhydantoin in the isolated perfused pancreas. J. Clin. Endocr. *30*:400-01, 1970.

[10] Woodbury, D. M.: Effect of diphenylhydantoin on electrolytes and radiosodium turnover in brain and other tissues of normal, hyponatremic and postictal rats. J. Pharmacol. Exp. Ther. *115*:74-95, 1955.

[11] Hales, C. N., and Milner, R. D. G.: The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. (London) *194*:725-43, 1968.

[12] Gomori, G., and Goldner, M. G.: Acute nature of alloxan damage. Proc. Soc. Exp. Biol. Med. *58*:323-33, 1945.

[13] Carrasco-Formiguerra, R.: Suppressive action of epinephrine on the diabetogenic effect of alloxan. Diabetes *16*:130-32, 1967.

[14] Weinglass, A. R., Frame, E. G., and Williams, R. H.: Inhibition of diabetogenic action of alloxan. Proc. Soc. Exp. Biol. Med. *58*:216-19, 1945.

[15] Chesler, A., and Tislowitz, R.: Oral efficacy of BAL in protecting rats against alloxan diabetes. Science *106*:345, 1947.

[16] Lazarow, A.: Protective effect of glutathione and cysteine against alloxan diabetes in the rat. Proc. Soc. Exp. Biol. Med. *61*:441-47, 1946.

[17] Lazarow, A.: Further studies of effect of sulphur compounds on production of diabetes with alloxan. Proc. Soc. Exp. Biol. Med. *66*:4-7, 1947.

[18] Lazarow, A.: Protection against alloxan diabetes. Anat. Rec. *97*:353, 1947.

[19] Lazarow, A., and Patterson, J. W.: The mechanism of cysteine and glutathione protection against alloxan diabetes. Science *108*:308-09, 1948.

[20] Nath, M. C., and Sahu, V. K.: Prevention of alloxan diabetes by a condensation product of glucose and acetoacetate. I. Metabolism *5*:3-10, 1956.