Diabetogenic Action of Alloxan Following Temporary Interruption of Arterial Blood Flow to Pancreas in Rats

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SUMMARY

Ligation of the superior mesenteric and the superior pancreatico-duodenal arteries just prior to alloxan treatment and their release ten minutes after treatment did not protect rats against development of alloxan diabetes. Prior incubation of alloxan in vitro for a period of ten minutes at a physiological pH value and 37° C. resulted in loss of diabetogenic activity. Identical blood flow interruption without alloxan treatment did not produce elevated blood glucose levels.

After the intravenous injection of alloxan, a substance giving a fluorescence product with alloxan reagent was detected in blood over four hours. The substance remained at higher levels when animals, prior to alloxan treatment, were injected with reduced glutathione. There was also a prolonged detection of alloxan during incubation in whole blood and in buffer containing reduced glutathione. The production of alloxan diabetes following temporary interruption of arterial blood flow is discussed in the light of the present results. DIABETES *19*:81-84, February, 1970.

Although alloxan has been extensively used for the production of experimental diabetes, the mechanism of its diabetogenic action has so far remained obscure. It is well known that alloxan is unstable in physiological solutions;[1-4] its half-life at pH 7.4 and 37° C. is less than one minute.[2] Furthermore, there are indications that alloxan injected into laboratory animals is rapidly decomposed as well.[1,5] This is illustrated by the findings of Leech and Bailey who showed that alloxan injected intravenously can be detected in blood for less than five minutes.[5] Based mostly on these observations, it is widely held that the diabetogenic action of alloxan takes place within a few minutes after the injection. Also in support of this view are the observations of Gomori and Goldner that clamping of a portion of a dog's pancreas protects the animal against the development of alloxan diabetes.[6]

Preliminary experiments performed on rats in this laboratory have shown that alloxan produces severe diabetes, although a considerable portion of pancreas tissue was excluded from arterial blood flow during, and for ten minutes after, the injection of alloxan. Since these results were not in agreement with those obtained earlier on dogs, it was decided to re-examine this discrepancy in more detail. In order to provide a possible explanation for the prolonged action of alloxan in rats, attempts were made to measure alloxan both in vivo and in vitro under a variety of experimental conditions.

METHODS

Male and female albino rats of Wistar stock were used at weights ranging from 180 to 220 gm. Prior to experiments animals were fasted overnight.

Under ether anesthesia, the right external jugular vein was cannulated, and the abdominal cavity was opened by medial abdominal incision. A splanchnic area, including the duodenal and the ventricular portions of the pancreas, was excluded from arterial blood flow by tying off the superior mesenteric and the superior pancreatico-duodenal arteries. The procedure was as follows: intestinal loops, stomach and spleen were pulled out of the abdominal cavity by digital pressure on lateral abdominal walls. The dislodged tissues were drawn to the left side of the animal. The superior mesenteric artery was exposed after the right abdominal wall was pushed aside. Using a straight round surgical needle, cotton thread was placed around the initial portion of the artery as near as possible. Care was taken to leave other tissue structures in the mesenteric root free and untouched. With these precautions, the ligature was placed around the superior pancreatico-duodenal artery after this was approached through the mesenteric membrane between the superior portion of the choledochal duct and the middle liver lobe. Immediately after the tying of arteries, animals were injected with alloxan through the cannulated vein. Both arteries were released ten minutes after the injection of alloxan. After surgery animals were allowed to eat and drink ad libitum.

Alloxan solution was prepared daily by dissolving alloxan (The British Drug Houses Ltd.) in cold citrate-phosphate buffer pH 4.0 at a concentration of 20

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mg./ml. The solution was kept on ice throughout use. The dose of alloxan used in the present studies was 60 mg./kg. body weight. For treatment of rats with incubated alloxan, alloxan solution was incubated at 37° C. for ten minutes after the pH had been adjusted to a value of about 7.3 by the addition of 0.15 M $NaHCO_3$. Just after the end of incubation, the solution was injected at a dose corresponding to 60 mg./kg. body weight of alloxan.

Blood samples for glucose determinations were obtained from a tail vein under slight ether anesthesia. Glucose determinations were carried out using the Nelson colorimetric procedure for the method of Somogyi.[7]

Alloxan determinations were performed using a modification[8] of the method of Archibald.[1] The alloxan reagent consisted of 80 mM. Na_2SO_4, 7.71 mM. Na_2HPO_4, 6.145 mM. citric acid and 5 mg./100 ml. 1,2-phenylenediamine-dihydrochloride. Briefly the procedure for blood alloxan was as follows: A volume of blood, 10 to 25 μl., was added to 5 ml. of the cold alloxan reagent in a plastic test tube. The content was vigorously mixed by a vibrating mixer and centrifuged to separate cells. Supernatants were stored in the dark at room temperature, and readings of fluorescence were made two hours later.

For studies in vivo, reduced glutathione was made isotonic in cold distilled water. The solution was filtered through a sinter/glass filter and then injected five minutes before alloxan via the cannulated jugular vein. The dose was 650 mg./kg. body weight. For studies in vitro, glutathione solution was prepared at concentrations of 50 and 150 mg./ml., respectively.

RESULTS

Diabetogenic action of alloxan with and without prior incubation in vitro. Figure 1 shows the mean values of blood glucose for each group of six animals over a period of ninety-six hours. When animals were treated with alloxan previously incubated at a physiological pH and 37° C., blood glucose values remained normal throughout. However, using alloxan without prior incubation, severe diabetes was produced in spite of the fact that during, and for ten minutes after the injection of alloxan, blood flow through a wide splanchnic area was interrupted. Without alloxan treatment, identical interruption of arterial blood flow did not produce elevated blood glucose levels.

Detection of alloxan in vivo. Results are shown in figure 2. After treatment of rats with alloxan, a substance giving a fluorescence product with 1,2-phenylenediamine was detected in blood over a period of four hours. When animals were injected with reduced glutathione five minutes prior to alloxan treatment, the substance giving a fluorescence product with alloxan reagent was found at higher levels.

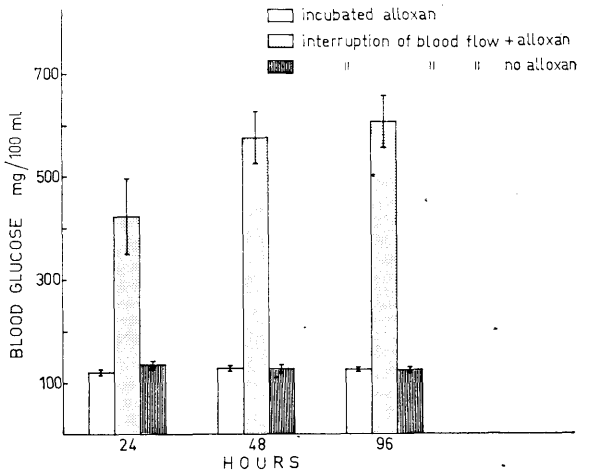

FIG. 1. Effect of alloxan with and without prior incubation on blood glucose levels. Immediately after tying of the superior mesenteric and the superior pancreatico-duodenal arteries, alloxan was injected. In rats not treated with alloxan, the portion of pancreas supplied by the lienal artery was removed together with the spleen during the blood flow interruption.

Detection of alloxan in vitro. Results of incubation of alloxan in 0.1 M phosphate buffer pH 7.4 containing 5 per cent bovine albumin both in the presence and absence of reduced glutathione are presented in figure 3. Experiments with incubation of alloxan in

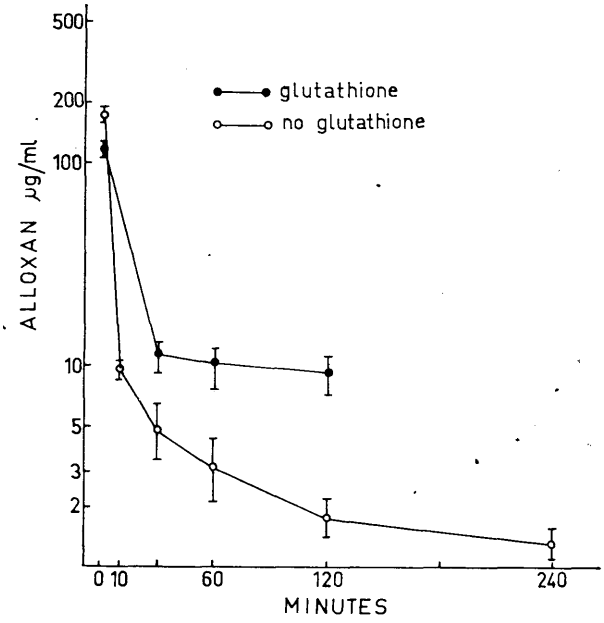

FIG. 2. Detection of blood alloxan in vivo with and without prior treatment with reduced glutathione. Blood alloxan in glutathione-treated rats was followed for two hours.

whole blood and in plasma are given in figure 4. It is seen that there was a prolonged detection of alloxan in whole blood and in the buffer containing reduced glutathione.

DISCUSSION

Incubation of alloxan for ten minutes under conditions employed in the experiment given in figure 1 resulted in loss of diabetogenic activity. This is consistent with earlier observations that in neutral or slightly alkaline media alloxan is rapidly inactivated, mainly through decomposition to alloxanic acid.[3] On the other hand, a period of ten minutes under conditions in vivo was found insufficient for complete inactivation of alloxan. In these experiments, hypoxia induced by the clamping of arteries was presumably an additional factor contributing to beta cell damage. In the control experiment without alloxan treatment, however, hypoxia did not give rise to elevated blood glucose levels. Therefore, this effect of hypoxia was interpreted as being of minor importance in the induction of diabetes. Further support was provided by observations that ischemia lasting for five minutes did not produce any cytological sign of beta cell damage.[9]

No special tests were made to determine an extent

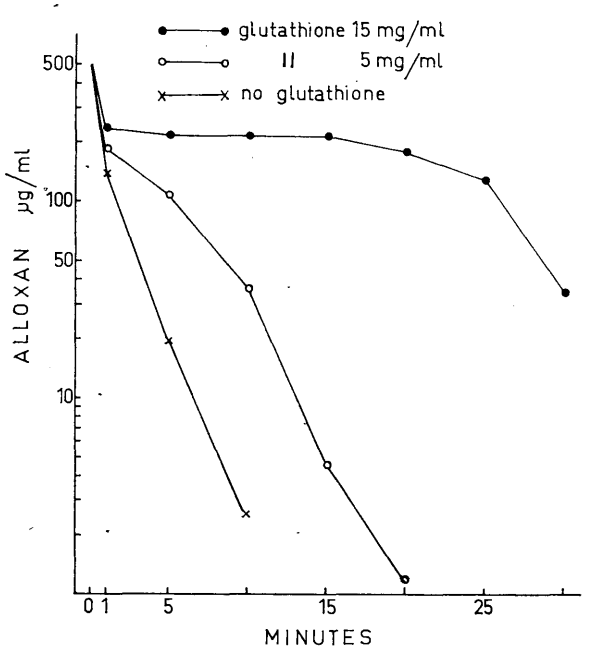

FIG. 3. Incubation of alloxan in 0.1 M phosphate buffer pH 7.4 containing 5 per cent bovine albumin in the presence and absence of reduced glutathione. After prior temperature equilibration to 37° C. with shaking rate of 120 cycles/min., glutathione was added at concentrations as indicated. After subsequent five-minute incubation, an amount of alloxan (500 µg.) was added to 1 ml. of each medium.

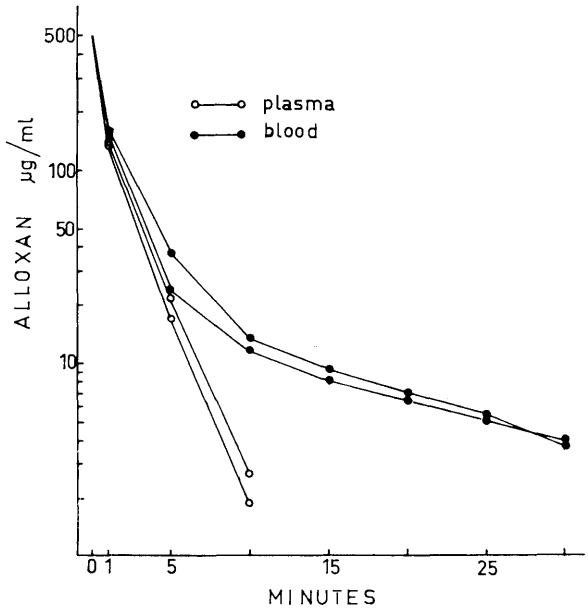

FIG. 4. Incubation of alloxan in whole blood and in plasma. Rat blood was obtained an hour before incubation by the aortal puncture of heparinized animals. Plasma was separated by centrifugation. Conditions of incubation were identical to those given in figure 3.

of collateral blood supply to the area of pancreas tissue affected by tying. However, gross anatomy of rats suggests that neither the inferior mesenteric nor the lienal artery can supply the whole pancreas tissue lying within the duodenal loop and around the choledochal duct. The ventricular portion of pancreas is supplied by a branch of the superior pancreaticoduodenal artery.

At present it is difficult to explain the lack of agreement between the present results and those obtained on dogs[6] and to a smaller extent on rabbits,[16] unless a difference in methodology could account for it. In dogs blood flow was interrupted by placing two rubber-protected clamps along both sides of the duodenal portion of the pancreas. In rabbits the whole mesenteric root was clamped; a protection was afforded but incomplete. It is worthwhile to mention here that both procedures could elicit reflex vasoconstriction in the pancreas, as has been observed by clamping of renal pedicles in dogs.[10] By using the present technic, big nerves and other tissues surrounding arteries were not affected by tying. It is, therefore, believed that interference of reflex vasoconstriction was reduced.

In the present studies, fluorescence in blood of alloxan-treated rats was detected with 1,2-phenylenediamine as alloxan reagent up to four hours after the injection. This obviously is not in agreement either with earlier

results of blood alloxan determinations[1,5] or with the view that alloxan is decomposed in vivo as rapidly as in buffer in vitro. These disagreements are perhaps due to a difference in methodology of alloxan determination as discussed elsewhere.[8]

The initial rate of alloxan decomposition during the course of incubation in phosphate buffer with and without bovine albumin was, as judged from changes in alloxan levels, more or less in agreement with earlier data obtained spectrophotometrically.[2] Similar results were obtained by incubating alloxan in fresh plasma, in contrast to whole blood. It seems, accordingly, that blood cells are of importance for the prolonged detection of alloxan in whole blood in vitro.

In the presence of reduced glutathione both in vivo and in vitro, alloxan remained at higher levels, and its decomposition was delayed. These effects can be explained by the well-known facts that alloxan under certain conditions forms with reduced glutathione and possibly with some other sulfhydryl compounds labile complexes.[11] Since the alloxan reagent is acidic at pH 4.0, it is very likely that at least some of the alloxan complexes are decomposed in the medium as suggested earlier.[14] Also, conditions in the alloxan reagent are very favorable for the conversion of dialuric acid to alloxan.[8] There was no indication that a fluorescent product was formed with alloxanic acid. It is, therefore, assumed that the method for alloxan employed in the present studies measures alloxan, either free or bound in complexes, and also dialuric acid, a reductive product of alloxan. The latter substance is readily oxidized to alloxan by dissolved oxygen.[4,11]

Besides all facts presented, there are still difficulties in understanding the mechanism of induction of alloxan diabetes after temporary interruption of arterial blood flow as employed in the present studies. However, it is possible to speculate that alloxan detected in body fluids ten minutes after its administration is still diabetogenically active. It is assumed that various alloxan complexes are decomposed with time giving dialuric acid, as already discussed by Patterson et al.[11] Evidence was presented that alloxan added to liver homogenates entered into a readily reversible oxidation-reduction system (alloxan-dialuric acid) transferring hydrogen from many sources to molecular oxygen.[4,12] Such a system, inhibiting some enzymes and increasing oxygen requirement, may well operate in the islet tissue in vivo. There is an observation suggesting that rats exposed to hypoxic conditions for four minutes before and two minutes after alloxan treatment are more sensitive to the action of alloxan.[13] This would be in agreement with the interpretation that the diabetogenic action of alloxan is increased in hypoxic tissue, as in the present experiments, after the release of arterial blood flow. A recent demonstration of selective accumulation of radioactivity in the pancreatic islets of mice following administration of labeled alloxan[15] would support a view that alloxan forms complexes with beta cell components. This may be at least a part of the mechanism by which alloxan kills the pancreatic beta cells.

ACKNOWLEDGMENT

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