Protection Against Alloxan-Induced Diabetes by Various Urea Derivatives: Relationship between Protective Effects and Reactivity with the Hydroxyl Radical

J. TIBALDI, J. BENJAMIN, F. S. CABBAT and R. E. HEIKKILA
Department of Neurology, New York University, New York, New York
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ABSTRACT

Several urea derivatives (monomethylurea, monoethylurea and diethylurea) given i.p. to mice 30 min before alloxan (75 mg/kg i.v.) were able to prevent the diabetogenic actions of alloxan. Protection by these agents correlated reasonably well with their capacity to react with (scavenge) the hydroxyl radical. Protection did not correlate with the capacity of the above agents to cause a transient hyperglycemia at the point of alloxan administration, which might have also been a potential means of protection. These data extend previously published data on the capacity of hydroxyl radical scavengers to protect against alloxan and add evidence to the concept that the hydroxyl radical, generated within the β cells, is the species derived from alloxan responsible for the damage to β cells.

It has previously been shown that dimethylurea, (DMU), a compound which has a very high reactivity with the hydroxyl radical, given to mice before alloxan, was able to prevent the diabetogenic actions of alloxan (Heikkila and Cabbat, 1978). Moreover, several other hydroxyl radical scavengers, some with similarities in structure to dimethylurea, others with very little structural similarities, were able to prevent alloxan-induced diabetes (Heikkila et al., 1974, 1976; Cohen et al., 1975; Heikkila, 1977). In contrast, urea, a compound with low reactivity with the hydroxyl radical, was not protective against alloxan. All of these data tend to implicate the hydroxyl radical as the causative species in alloxan-induced diabetes.

In the present study, we have tested the capacity of several analogs of dimethylurea to protect against alloxan-induced diabetes. If the hydroxyl radical were involved in alloxan-induced diabetes, one would predict that better hydroxyl radical scavengers would be better as protective agents. The data will show that this is indeed the case.

Materials and Methods

Male Swiss-Webster mice weighing 25 to 30 g were used in the experiments. Food was routinely withheld from the mice 3 to 4 hr before the drug treatments described below. At 30 min before alloxan, some mice received an i.p. injection of the urea derivative in isotonic saline; other animals received only the isotonic saline (0.5 ml) before alloxan. For structures of the urea derivatives used see figure 1. Alloxan monohydrate at 75 mg/kg was then injected into a tail vein of the mouse. Just before injection, the alloxan was prepared in cold isotonic saline and then kept on ice to prevent degradation. Each mouse received 0.2 ml of the alloxan solution. Food was returned to the mice approximately 1 hr after the alloxan.

Mice were decapitated 72 hr after alloxan, and blood was collected on pieces of parafilm and then 100-μl aliquots were rapidly transfered to 3.9-ml aliquots of cold-distilled water. The blood was then deproteinized with barium hydroxide and zinc sulfate, and blood sugar was measured by a glucose-oxidase (Trinder) method. In other experiments, blood sugar was measured 30 min after the above-described i.p. injections. This normally would have been the time that the mice received alloxan.

The alloxan was obtained from Calbiochem (Los Angeles, CA), the urea derivatives from the Aldrich Chemical Co. (Milwaukee, WI) or from Eastman Kodak (Rochester, NY), and the glucose oxidase assay kit for the glucose determinations from Boehringer-Mannheim Corp. (Indianapolis, IN). All other chemicals and reagents used were from standard sources.

Results

All mice that received 75 mg/kg of alloxan 30 min after an i.p. dose of saline were hyperglycemic at 72 hr (fig. 2). For example, all 14 mice receiving saline followed by alloxan had blood glucose values greater than 400 mg/100 ml, a value well within the accepted diabetic range. In contrast, the mice receiving saline alone (no alloxan) had 72-hr blood glucose values ranging from 98 to 160 mg/100 ml. Most of the mice that received urea at 6 g/kg before alloxan also exhibited the hyperglycemia normally associated with alloxan (fig. 2). For example, 12 of the 17 mice that were injected with alloxan 30 min after urea had blood glucose values greater than 400 mg/100 ml. Of
compared to animals receiving only the saline vehicle. (Note substituted urea derivatives had blood glucose values administered.) Several of the mice receiving urea or the that 30 mm would normally have been the time of alloxan MEU alone and blood sugar was measured 30 mm later and same range as saline-treated animals (fig. MEU at this dose are considered to be protective alloxan had blood glucose levels greater than 300 mg/100 ml while in contrast only 1 of 15 mice receiving alloxan after 10 g/kg of MMU had a blood glucose level above 300 mg/100 ml. The lower doses of MMU afforded lesser protection, but the protection was still obvious at 6 g/kg. The great difficulties in working with alloxan which often face investigators can be appreciated when one compares figures 2 and 4. Several points are obvious. Firstly, the range of blood sugar values seen in animals receiving saline plus alloxan is greater in figure 4 than in figure 2, even though the mean values (574 and 568 mg/100 ml, respectively) are very close. The two (nondiabetic) saline plus alloxan animals seen in figure 4 are not uncommon in

30 min after urea as after MEU while MEU was clearly superior as a protective agent. Furthermore, MMU was largely protective and yet relatively few animals exhibited a hyperglycemia after MMU alone (compare figs. 2 and 3).

Alloxan at 75 mg/kg was also given to mice 30 min after MMU at either 6, 8 or 10 g/kg (fig. 4). Note the relatively clear dose-response relationship with MMU, namely, the better protection against the diabetogenic actions of alloxan with increasing doses of MMU. MMU at 10 g/kg was strongly protective. For example, note that 13 of 15 mice receiving alloxan after saline had blood glucose levels greater than 300 mg/100 ml while in contrast only 1 of 15 mice receiving alloxan after 10 g/kg of MMU had a blood glucose level above 300 mg/100 ml. The lower doses of MMU afforded lesser protection, but the protection was still obvious at 6 g/kg. The great difficulties in working with alloxan which often face investigators can be appreciated when one compares figures 2 and 4. Several points are obvious. Firstly, the range of blood sugar values seen in animals receiving saline plus alloxan is greater in figure 4 than in figure 2, even though the mean values (574 and 568 mg/100 ml, respectively) are very close. The two (nondiabetic) saline plus alloxan animals seen in figure 4 are not uncommon in

the remaining five mice, three were in the normal range while two were slightly hyperglycemic (blood glucose > 200 mg/100 ml). Thus, 15 of 17 of the urea-pretreated animals may be considered diabetic. In contrast to the results obtained with urea, most of the mice receiving either monomethylurea (MMU) or monoethylurea (MEU) (both 6 g/kg) followed by alloxan had blood glucose values in the normal range. Thus, MMU and MEU at this dose are considered to be protective against alloxan. Note also that mice receiving urea, MMU or MEU alone (no alloxan) had 72-hr blood glucose values in the same range as saline-treated animals (fig. 2).

In separate experiments, mice were given urea, MMU or MEU alone and blood sugar was measured 30 min later and compared to animals receiving receiving only the saline vehicle. (Note that 30 min would normally have been the time of alloxan administration.) Several of the mice receiving urea or the substituted urea derivatives had blood glucose values considerably higher than control (fig. 3), although most animals were in the same range as the saline-treated mice. For example, 4 of 18 mice receiving urea, 2 of 18 mice receiving MMU and 7 of 18 mice receiving MEU had 30-min blood glucose levels greater than 200 mg/100 ml (fig. 3). Thus, MMU and MEU have the capacity to protect against alloxan (fig. 2) under conditions in which they do not cause consistent increases in blood sugar (fig. 3). Moreover, only slightly fewer animals were hyperglycemic
alloxan experiments where often, for no apparent reason alloxan-injected animals do not become diabetic. Moreover, there tends to be a somewhat greater proportion of severely diabetic animals in figure 4 than in figure 2. For example, 8 of 15 mice in figure 4 as compared to 4 of 14 animals in figure 2 have blood glucose values greater than 600 mg/100 ml. Another point of concern is that a protective agent like MMU appears to be “more protective” in figure 2 than in figure 4. All of these findings are not unusual and make experimentation with alloxan difficult within an individual laboratory and certainly make comparisons between different laboratories extremely difficult.

Diethylurea (DEU, 1 g/kg) also protected against alloxan (fig 5A). Note that most mice receiving alloxan after saline exhibited hyperglycemia at 72 hr (15 of 19 mice had blood sugars above 300 mg/100 ml) while most of the mice receiving alloxan after DEU had blood sugars in the normal range (18 of 19 under 200 mg/100 ml only 1 of 19 above 300 mg/100 ml). Again there is no correlation between the blood sugar at the point of alloxan administration and the degree of protection. Most animals receiving DEU alone had blood sugar values well within the range of the mice receiving saline (fig. 5B).

Rate constants for reactivity with the hydroxyl radical (-OH) are given in table 1. It may be seen that MMU is less reactive than MEU which in turn is less reactive than DEU, although the absolute rate constants for all three are in fact very high. Data for DMU are shown for comparative purposes. In contrast to the above substituted urea derivatives, urea itself has a very low rate constant for reactivity with -OH. In fact urea is least reactive with -OH in an extensive series of compounds listed by Dorfman and Adams (1973).

**Discussion**

It was previously shown in vitro that dialuric acid, the two-electron reduction product of alloxan, spontaneously autoxidizes in aqueous media to form several very reactive species (Deamer et al., 1971; Cohen and Heikkila, 1974). Among them are the superoxide radical (O2·−), hydrogen peroxide (H2O2) and the hydroxyl radical (-OH). It is further known that reducing agents such as ascorbic acid can mediate the reduction of alloxan to dialuric acid (Deamer et al., 1971; Cohen and Heikkila, 1974; Richardson, 1982). Moreover H2O2 production can be detected in vivo after alloxan administration to mice (Heikkila et al., 1974). These data indicate that alloxan is reduced to dialuric acid in vivo, perhaps by a tissue reducing agent like ascorbic acid. This follows in that H2O2 production from alloxan itself, without a reduction to an autooxidizable compound like dialuric acid, is theoretically impossible (i.e., alloxan cannot donate two electrons to oxygen to form H2O2). One might speculate that one or a combination of the above species derived from O2·− formed within the β cells after alloxan accumulation, might be responsible for alloxan-induced diabetes. This is not unreasonable in that all three of these agents are extremely reactive and have known cytotoxicities (Pryor, 1973). It should also be emphasized that it has recently been shown that H2O2, O2·− or -OH can produce deleterious effects in isolated rat pancreatic islets (Fischer and Hamburger, 1979).

In the last several years, we have been trying to determine whether one of the above species, namely, the hydroxyl radical, might be the causative agent in alloxan-induced diabetes. The hydroxyl radical is among the most powerful oxidizing species known and is thought to be responsible for the damaging effects of ionizing radiation (Pryor, 1973). Our attention became focused on the hydroxyl radical from early experiments in which ethyl alcohol, a compound with very high reactivity with the hydroxyl radical, was able to prevent alloxan-induced diabetes (Heikkila et al., 1974). This protection by ethanol might theoretically have been through removal of H2O2 by the peroxidatic activity of catalase, but later experiments with a series of aliphatic alcohols (methanol, ethanol, n-propanol, n-butanol) showed a negative correlation between their protective capacity and their capacity to serve as substrates for the peroxidatic activity of catalase (Heikkila et al., 1976). In contrast, there was a positive correlation between reactivity with the hydroxyl radical and degree of protection. It has subsequently been shown that several other agents, all of which shared one feature in common, namely, high reactivity with the hydroxyl radical, were able to protect against alloxan. These agents include thiourea, phenylthiazolylthiourea, dimethylsulfoxide (DMSO) and dimethyurea (Heikkila et al., 1974, 1976; Heikkila and Cabbat, 1978; Cohen et al., 1975; Heikkila, 1977). All of these data suggest that the hydroxyl radical is indeed the causative agent in alloxan-induced diabetes.

In the present study, DEU, MEU and MMU all protected against the diabetogenic actions of alloxan. DEU was the most powerful protective agent, being protective at doses as low as 1 g/kg (fig. 5A). It appeared that MEU was better as a protective agent than MMU (see fig. 2). Additionally, MEU at a dose of 4 g/kg protected against alloxan while at the same dose MMU provided no protection. For example, animals treated with 4 g/kg of MEU before alloxan had a mean 48-hr blood sugar of 205 mg/100 ml.
± 115 (n = 16) while in the same set of experiments animals receiving saline before alloxan had a blood sugar value of 695 ± 81 mg/100 ml (n = 16, mean ± S.D.).

The order of reactivity with -OH was the same as the order of protection against alloxan. For example, compare rate constants in table 1 with extent of protection in figure 2. Thus, in the present study, as in previous studies, there appears to be a reasonably good correlation between reactivity with the hydroxyl radical and protection against alloxan-induced damage. In other studies DMU was also protective against 75 mg/kg of alloxan at a dose of 4 g/kg (Heikkila and Cabbat, 1978).

It has previously been demonstrated that agents which elevate blood glucose can protect against alloxan (Schauberger et al., 1977; Zawalich and Beidler, 1973). In the present study, for most agents there appears to be no positive correlation between the rise in blood sugar caused by the drug alone at the point of alloxan administration and the protection afforded by the compound. For example, DEU at 1 g/kg protects without a consistent rise in sugar, as does MEU. In other experiments (Heikkila and Cabbat, 1978) DMU also protected against alloxan without causing a rise in blood sugar at the point of alloxan administration. However, in the present study, it appeared that a certain number of animals receiving alloxan after 6 g/kg of urea did not develop diabetes (fig. 2). This number failing to develop diabetes is certainly higher in the urea group than in the saline group (fig. 2). Interestingly, about the same proportion of mice had an elevated sugar 30 min after urea (fig. 3) as did not develop a strong diabetes (fig. 2). It is conceivable that a small number of mice were protected against the diabetogenic actions of alloxan by the hyperglycemic effects of 6 g/kg of urea. Although this may be the case, certainly the primary protection afforded by derivatives of urea cannot be due to an increase in blood sugar.

In conclusion, there appears to be a good correlation between the protection against alloxan with the substituted ureas and their reactivity with the hydroxyl radical. This study now extends our earlier observations and increases the number of chemical agents able to protect against alloxan. As stated previously, if reactivity with the hydroxyl radical is not an adequate explanation for the protection seen with the various agents, then an alternative explanation or alternative explanations must be given for all of these protective agents. With the lack of other plausible mechanisms of protection against alloxan, one must at least be forced to conclude tentatively that the means of protection of all of the agents we have studied is their reactivity with the hydroxyl radical and further, that the hydroxyl radical, generated in the β cell after alloxan accumulation, is the causative species in alloxan-induced diabetes.

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References


Send reprint requests to: Dr. R. E. Heikkila, Department of Neurology, 5th Ave. and 100th St., New York, NY 10029.