Partial protection against streptozotocin-induced hyperglycaemia by superoxide dismutase linked to polyethylene glycol

Kjell Asplund, Kjell Grankvist, Stefan Marklund and Inge-Bert Talljedal

Department of Histology and Cell Biology, Department of Internal Medicine, and Department of Clinical Chemistry, University of Umeå, S-901 87 Umeå, Sweden

Abstract. To test the possible role of superoxide radicals in the diabetogenic action of streptozotocin, blood glucose levels were measured in mice after a single high-dose (150 mg/kg body weight) or multiple low-dose (40 mg/kg for 5 days) injections of streptozotocin. Pre-treatment 6 h before streptozotocin with 250–300 mg/kg superoxide dismutase coupled to polyethylene glycol reduced the hyperglycaemic response in mice injected with a single dose of streptozotocin. The blood glucose levels after multiple low doses of streptozotocin were not affected by superoxide dismutase-polyethylene glycol. Enzymatically inactive superoxide dismutase did not affect the development of hyperglycaemia. The results suggest that superoxide radicals may play a role in the diabetogenic action of streptozotocin injected as a high-dose single bolus.

Streptozotocin and alloxan are commonly used to induce diabetes in experimental animals (Rerup 1970). Highly reactive and noxious oxy-radicals has been implicated in the action of both drugs (Heikkila et al. 1976; Tibaldi et al. 1979; Grankvist et al. 1979; Gandy et al. 1982). Streptozotocin diabetes may be induced either by a single high-dose injection (with ensuing islet necrosis without inflammatory signs), or by multiple low-dose injections leading to insulitis and slow destruction of the islets (Like & Rossini 1976). In keeping with the possibility of oxy-radicals playing a role in streptozotocin diabetes, native superoxide dismutase has previously been shown to protect against single-dose induced glucose intolerance (Robbins et al. 1980) and low-dose streptozotocin diabetes (Gandy et al. 1982) in rats. No protection against low-dose multiple injections of streptozotocin was found by Gold et al. (1981).

Recently we protected mice against alloxan diabetes by injecting a long-time circulating, high molecular weight superoxide dismutase coupled to polyethylene glycol chains (SOD-PEG) 12 h before alloxan (Grankvist et al. 1981a); a substituted superoxide dismutase was employed because the uncomplexed native enzyme has a half-life in plasma of only 6 min (McCord & Wong 1979). In the present study we tested whether long-time circulating SOD-PEG could protect against any of the two diabetes models if injected several hours before streptozotocin.

Materials and Methods

SOD-PEG was obtained, by courtesy of K.-E. Arfors, from Pharmacia Pharmaceutical Co., Uppsala, Sweden. The purified yeast Cu-Zn-superoxide dismutase had been covalently coupled to polyethylene glycol chains to yield a complex molecular weight of about 100 000. Enzymatically inactive SOD-PEG was produced by controlled exposure of the enzyme complex to H₂O₂ (Grankvist et al. 1979). Streptozotocin was a gift from the Upjohn Co., Kalamazoo, MI, USA.

Address for proofs:
Dr. Kjell Grankvist, Department of Histology and Cell Biology, University of Umeå, S-901 87 Umeå, Sweden.
Single-injection high-dose experiments

Male C57BL/KsJ mice, 3 months old, weighing 22–29 g were used. The appropriate diabetogenic dose of streptozotocin was determined by injecting 100, 150 or 200 mg/kg body weight into a tail vein of mice fasted for 15 h. The drug was dissolved in a buffer of the same composition as Krebs-Ringer bicarbonate (DeLuca & Cohen 1964), except that bicarbonate was replaced by 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and that pH was 4.0. Blood (25 μl) was sampled from the tail tips for glucose determination at 24, 48, 72, 96 and 120 h after the streptozotocin injection.

To study elimination of SOD-PEG from circulation, the lyophilized enzyme complex (or an inactivated enzyme complex) was dissolved in distilled water and injected (200 mg/kg body weight) into 4 mice via a tail vein. Blood samples (25 μl each) were obtained from the tail for determination of plasma superoxide dismutase activity 1–72 h later.

To study protection by SOD-PEG, 250 mg of the enzyme complex per kg body weight was given iv to mice fasted for 9 h. After a further 6 h of fasting, streptozotocin was dissolved in buffer as above and immediately injected into a tail vein at a dose of 150 mg/kg body weight. Blood glucose was determined daily for 5 days. The experimental groups consisted of mice injected with: a) buffer without streptozotocin, b) 150 mg streptozotocin/kg body weight without prior administration of SOD-PEG, c) 150 mg streptozotocin/kg body weight 6 h after 250 mg/kg of inactivated SOD-PEG, and d) 150 mg streptozotocin/kg 6 h after 250 mg/kg of active SOD-PEG.

Multiple-injections low-dose experiments

Two series of experiments were performed. In the first series male 4 month old C57BL/KsJ mice were used. In the second series the mice were 8 months old. The appropriate diabetogenic dose of streptozotocin was determined by injecting 30, 40 or 50 mg/kg body weight ip after 6 h of fasting. The injections continued for 5 days. The drug was dissolved in the same buffer as above. Blood was sampled from the tail tip for glucose determination on day 1, 3, 5, 8, 10 and 12.

To study protection by SOD-PEG, 250 mg of enzyme complex per kg was given daily for 5 days only in the first series. In the second series 300 mg/kg SOD-PEG was given ip daily until the 12th day. The experimental groups consisted of mice injected with: a) buffer without streptozotocin, b) 40 mg streptozotocin/kg body weight without prior administration of SOD-PEG, c) 40 mg streptozotocin/kg 6 h after 250 (or 300) mg/kg of active SOD-PEG. In the second series of experiments blood was sampled for superoxide dismutase assay on days 5 and 10, 6 h after SOD-PEG injection.

To study elimination of ip injected SOD-PEG from circulation, the active or inactivated enzyme complex was dissolved in distilled water and injected (200 mg/kg body weight) into 3 mice ip. Blood samples were taken from the tail tip and determined for plasma superoxide dismutase activity 1–72 h later.

Analyses

Blood glucose was determined by an automated glucose oxidase/peroxidase technique (Glox, Kabi AB, Stockholm, Sweden). Superoxide dismutase was assayed in 25 μl blood samples mixed with 500 μl of a solution containing 150 mM NaCl and 3 mM EDTA. After centrifugation, the supernatant was analyzed by a spectrophotometric procedure employing K02 (Marklund 1976). The erythrocyte blood volume fraction was assumed to be 40% in calculation of plasma enzyme activity. The two-tailed Student's t-test was used in the statistical comparison of different test groups.

Results

SOD-PEG circulatory activity

As shown in Fig. 1 the elimination of the injected activity from circulation did not obey first-order kinetics. Six h after the injection of 200 mg SOD-PEG, the reaction had not ended. Blood samples were taken at various time-points, and plasma superoxide dismutase activity was measured. The results were plotted graphically (Fig. 1).
PEG per kg body weight the SOD activity in plasma was approximately 10000 units/ml in both iv and ip injected animals. This is about 20 times higher than the endogenous activity of superoxide dismutase in mouse plasma. Inactivated SOD-PEG was not reactivated in vivo since injection of SOD-PEG did not cause an increase in plasma activity during the 72 h period.

**Single-injection high-dose experiments**

Blood glucose levels in 5 saline-injected mice ranged from 4.1 to 6.0 mM. In 5 animals injected with 100 mg streptozotocin/kg body weight there was a small increase in blood glucose to 6.2–7.2 mM on day 5 after the injection. After 150 or 200 mg streptozotocin/kg body weight all animals developed a manifest hyperglycaemia. Three of 5 mice injected with 200 mg streptozotocin/kg died within 5 days; all receiving 150 mg/kg survived. A streptozotocin dose of 150 mg/kg was therefore selected for the subsequent experiments.

As shown in Table 1, pretreatment with SOD-PEG did not appear to affect the time of onset of streptozotocin-induced hyperglycaemia; definite hyperglycaemia was first recorded on the second day after streptozotocin whether or not SOD-PEG had been given. However, SOD-PEG administered 6 h before streptozotocin significantly decreased the hyperglycaemic response on the 4th and 5th days after streptozotocin. Pretreatment with enzymatically inactive SOD-PEG did not diminish the hyperglycaemia induced by streptozotocin. Blood glucose levels in mice not injected with streptozotocin were unaffected by prior administration of SOD-PEG or inactivated SOD-PEG (data not shown).

**Multiple-injections low-dose experiments**

In animals daily injected with 40 or 60 mg streptozotocin/kg a hyperglycaemic response was noted. Animals injected with 30 mg/kg streptozotocin for 5 days did not develop hyperglycaemia. The 40 mg/kg dose of streptozotocin was used in the experimental procedure. In the first series of experiments, with animals receiving 40 mg streptozotocin/kg alone, the blood glucose level rose to 19.0 ± 4.2 mM (mean ± SE of 4 mice) on the 12th day. Animals receiving active SOD-PEG + streptozotocin on inactive SOD-PEG + streptozotocin had blood glucose levels of 14.5 ± 1.3 (n = 5) and 13.2 ± 3.2 (n = 4) mM, respectively. Controls receiving saline injections alone did not exhibit a hyperglycaemic response and had a mean blood glucose level of 7.5 ± 0.2 mM (n = 5) on the 12th day. The differences between groups that had received streptozotocin with or without active or inactive SOD-PEG are not statistically significant.

In the second series of experiments SOD-PEG was increased to 300 mg/kg body weight and injected once every day for all 12 days. Neither in this case was there any significant difference between the groups receiving streptozotocin alone, or active SOD-PEG + streptozotocin, or inactive SOD-PEG + streptozotocin (data not shown).

The activity of superoxide dismutase in blood after 5 and 10 days is shown in Table 2.

### Table 1.

Effects of prior administration of 250 mg/kg SOD-PEG on the development of hyperglycaemia after a single bolus of streptozotocin (150 mg/kg) iv into mice. The figures are pooled from two separate experiments in each of which a significant protective effect of SOD-PEG was seen.

<table>
<thead>
<tr>
<th>Agents injected</th>
<th>Time after STZ or control injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>A. STZ only (n = 10)</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>B. Active SOD-PEG + STZ (n = 10)</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>C. Inactive SOD-PEG + STZ (n = 8)</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>D. Buffer only (n = 5)</td>
<td>5.4 ± 0.1</td>
</tr>
</tbody>
</table>

* P < 0.025 when tested against group A.
** P < 0.02 when tested against group A, and P < 0.05 when tested against group C.
Table 2.
Plasma superoxide dismutase activity determined on day 5 and 10, 6 h after the daily ip injection of 300 mg/kg of active or inactive SOD-PEG, or buffer only. Streptozotocin (40 mg/kg) was given ip for the first 5 days. Mean ± SE of 4 mice each.

<table>
<thead>
<tr>
<th>Agents injected</th>
<th>Days after first injection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Superoxide dismutase activity (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. STZ only</td>
<td>380 ± 21</td>
<td>321 ± 38</td>
</tr>
<tr>
<td>B. Active SOD-PEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ STZ</td>
<td>13 888 ± 1754</td>
<td>11 808 ± 1496</td>
</tr>
<tr>
<td>C. Inactive SOD-PEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ STZ</td>
<td>771 ± 113</td>
<td>590 ± 7</td>
</tr>
<tr>
<td>D. Buffer only</td>
<td>354 ± 32</td>
<td>325 ± 38</td>
</tr>
</tbody>
</table>

Discussion

Streptozotocin-induced hyperglycaemia in mice was protected against by prior injection of SOD-PEG in the single-injection high-dose model, but not in the multiple-injections low-dose streptozotocin model. Inactivated SOD-PEG had no effect in either of the models, implying superoxide radical involvement in at least the single injection streptozotocin model.

SOD-PEG afforded almost complete protection against alloxan diabetes in mice (Grankvist et al. 1981a). The protection against streptozotocin was however, rather small and might indicate a difference in action of the two drugs. The protection against the single high-dose streptozotocin model may be due to scavenging of superoxide radicals leaking out of the cells. With the low-dose streptozotocin model maybe lesser amounts of radicals are generated, leaking out and being scavenged by SOD-PEG. Protection by extracellular superoxide dismutase against intracellularly generated superoxide radicals has been demonstrated (Lynch & Fridovich 1978).

The mouse is a species with a very high extracellular superoxide dismutase activity; yet, the extracellular activity is only about 1% of that found intracellularly (Grankvist et al. 1981b). Although SOD-PEG injections caused significant increases in the plasma superoxide dismutase activity, the levels attained are only about 10–20% of the intracellular activity. Any physiological effect of injections of SOD-PEG or other superoxide dismutase preparations are therefore probably caused by increases in the extracellular enzyme activity. Significant increases in the intracellular superoxide dismutase activity following enzyme injections would seem to require efficient mechanisms for superoxide dismutase uptake and accumulation; such mechanisms have yet to be demonstrated.

The failure of SOD-PEG to protect against the multiple low-dose streptozotocin diabetes model is not due to immunisation against the complex or its accelerated removal. The superoxide dismutase activities achieved in plasma 6 h after the SOD-PEG injection on days 5 and 10 do not differ significantly (Table 2) and are similar to the level attained after a single injection of SOD-PEG (Fig. 1).

There are several discrepancies between various studies on the protective effect of superoxide dismutase against streptozotocin diabetes. However, with some degree of certainty it may be proposed that, a) streptozotocin exerts its cytotoxic effect primarily on intracellular targets, b) iv injected superoxide dismutase is hardly likely to significantly increase the intracellular enzyme activity, and c) the diabetogenic action of high-dose streptozotocin might in part be mediated by the generation of superoxide radicals.

Acknowledgments

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References


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