Attenuation of Streptozotocin Diabetes with Superoxide Dismutase-like Copper(II)(3,5-Diisopropylsalicylate)₂ in the Rat

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Summary. Experimental diabetes can be produced by agents with specific toxicity for pancreatic islet B cells. This effect has been reported to be modified both in vitro and in vivo by various radical scavengers including the enzyme superoxide dismutase. Copper(II)(3,5-diisopropylsalicylate)₂ is lipophilic and possesses superoxide dismutase bioactivity. Prior administration of this compound to male rats appeared to attenuate the severity of streptozotocin-induced diabetes as assessed by glycosuria and glucose tolerance. Diisopropylsalicylate, which has no superoxide dismutase activity, did not alter the

severity of streptozotocin-induced diabetes. Rats treated with the copper complex, with streptozotocin or with a combination of the two agents gained 50% less weight than untreated controls, or rats treated with diisopropylsalicylate. The attenuation of diabetes by the copper-complex may represent partial protection of the B cells against streptozotocin damage, although an extrapancreatic, toxic effect cannot be ruled out.

Key words: Copper, superoxide dismutase, rats, streptozotocin.

The enzyme superoxide dismutase (EC.1.15.1.1.) is a scavenger of superoxide radicals and protects the integrity of cell components. Streptozotocin is a glucosamine derivative which exhibits relatively selective toxicity for pancreatic islet B cells and is widely used experimentally to produce diabetes mellitus in animals. Streptozotocin and other B cell toxins, such as alloxan and Vacor [1], have been reported to inhibit superoxide dismutase enzymatic activity [2], possibly leading to an impairment of endogenous detoxification of the superoxide radical. This highly reactive oxygen radical may react directly with cell components or combine with hydrogen peroxide to yield the more toxic hydroxyl radical [3]. There is evidence that the deleterious effects of chemical diabetogenic agents on B cells are mediated by such oxygen radicals [4, 5].

The use of radical scavenger therapy before the administration of alloxan was reported first by Heikkila et al. [5]. A variety of chemical and enzymatic oxygen radical scavengers have now been used for protection against both alloxan and streptozotocin effects in vitro and in vivo. Intravenous injection of copper-zinc superoxide dismutase before streptozotocin prevents diabetes in rats [6, 7]. Partial protection against alloxan was obtained in mice using a polyethylene glycol derivative of superoxide dismutase [8]. Likewise, isolated islets are protected in vitro by superoxide dismutase from the ef-

fects of alloxan [9] or streptozotocin [7] as measured by insulin secretion in response to a glucose challenge. Superoxide dismutase is reported also to protect isolated islets from islet DNA strand breaks and inhibition of proinsulin synthesis induced by alloxan but gave no protection against similar effects induced by streptozotocin [10]. These findings suggest that similar agents may be worth testing for the prophylaxis of different experimental models of diabetes. However, injection of a large molecular weight protein over an extended course may induce immunopathological sequelae [11]. In addition isolation and purification of the enzyme are costly. Copper(II)salicylate₂ and other copper compounds have been shown to have superoxide dismutase-like activity [12] and possess anti-inflammatory [13], anti-ulcer [14], anticonvulsant [15] and anti-tumour [16] activity. The possible practical advantages of such compounds as potential models of prophylactic agents led us to study their efficacy in the attenuation of streptozotocin diabetes.

Methods

Streptozotocin was kindly supplied by Dr. W. Dulín (Upjohn, Kalamazoo, Michigan, USA) and dissolved in 100 mmol/l sodium acetate/acetic acid buffer (pH 5.2) immediately before IV injection (50 mg/kg body weight) via the tail of fasted animals.

Table 1. Protection against streptozotocin diabetes by superoxide dismutase-like copper salicylate derivatives

Preinjection agent	Dose (mg/kg)	Streptozotocin	Plasma glucose (mmol/l)		Glycosuria range
			0 min	60 min	
Diluent	–	–	3.4 ± 0.4	7.8 ± 0.3 (4)	0–1 +
3,5-Diisopropylsalicylic acid	41	–	3.4 ± 1.3	14.0 ± 1.6 (4)	0–1 +
Copper(II)(3,5-diisopropylsalicylate) ₂	48	–	6.3 ± 0.3	8.7 ± 1.4 (4)	0–1 +
Diluent	–	+	16.9 ± 2.0	24.9 ± 1.6 (5)	2–3 +
Salicylic acid	32	+	8.7 ± 0.6	29.8 ± 1.0 (4)	2–3 +
3,5-Diisopropylsalicylic acid	11	+	14.4 ± 4.8	27.4 ± 2.1 (4)	3–4 +
3,5-Diisopropylsalicylic acid	41	+	8.3 ± 1.9	20.2 ± 2.5 (5)	3–4 +
Copper(II)(salicylate) ₂	38	+	6.3 ± 0.3	21.6 ± 1.8 (7)	0–2 +
Copper(II)(3,5-diisopropylsalicylate) ₂	12	+	6.3 ± 0.8	21.7 ± 4.1 (4)	0–1 +
Copper(II)(3,5-diisopropylsalicylate) ₂	48	+	3.4 ± 0.3	13.7 ± 0.6 ^a (5)	0–1 +
Copper(II)(3,5-diisopropylsalicylate) ₂	120	+	1.7 ± 0.2	13.6 ± 0.6 ^a (5)	0–1 +

Plasma glucose concentrations are expressed as mean ± SEM; the number of rats is shown in parentheses; ^a $p < 0.0001$: significantly different from streptozotocin-injected control rats

Salicylic acid was obtained from Fisher Scientific, Norcross, Georgia, USA. 3,5-Diisopropylsalicylic acid, copper(II)(salicylate)₂, copper(II)(3,5-diisopropylsalicylate)₂ and copper(II)(aspartate) were synthesized and characterized as previously described [11]. All copper derivatives demonstrated superoxide dismutase-like activity in the assay based upon superoxide dismutase-mediated inhibition of the reduction of nitro blue tetrazolium in the hypoxanthine/xanthine oxidase system [17].

The salicylates and copper derivatives were administered by two routes. For IP injection, salicylates and copper (II) derivatives were dissolved in 1% ETOH/H₂O and injected 15 min before streptozotocin at the following doses/kg body weight: copper(II)(salicylate)₂: 38 mg; copper(II)(3,5-diisopropylsalicylate)₂: 12, 48 or 120 mg; copper(II)(aspartate): 20 mg; salicylic acid: 32 mg; 3,5-diisopropylsalicylic acid: 11 or 41 mg. For SC injection salicylates emulsified in 0.25% polyoxyethylenesorbitan mono-oleate/H₂O were administered 2 and 12 h before streptozotocin at the following doses (per injection/kg body weight): salicylic acid: 32 mg; 3,5-diisopropylsalicylic acid: 11 mg; copper(II)(salicylate)₂: 38 mg; copper(II)(3,5-diisopropylsalicylate)₂: 12 mg. Control rats were injected with the diluents alone. Each group consisted of at least four animals.

Male rats of the Wistar strain (175–225 g) were fasted 12 h before the experiments. Diabetes was assessed by IP glucose tolerance tests [6], following an overnight fast, 3 days after the injection of streptozotocin (50 mg/kg). Rats were maintained under light methoxyflurane anaesthesia throughout the glucose tolerance test. The animals were bled from freshly cut tails. After a baseline sample (0 min), the animals were given an IP bolus of glucose (1 g/kg body weight in 25% solution). Blood samples were taken 15, 30 and 60 min after the glucose injection and analyzed for plasma glucose concentration by the Beckman glucose oxidase system. Animals were monitored daily after injection for glycosuria by TesTape (Eli Lilly, Indianapolis, Indiana, USA) and day 3 values are presented. In the initial series (Table 1), all animals were evaluated by both glucose tolerance test and glycosuria. Diabetic animals were defined as those having a plasma glucose level > 20 mmol/l at 60 min after glucose and glycosuria of > 2+. Differences between mean plasma glucose concentrations among the groups were greatest 60 min after the IP injection of glucose. These measurements were used to assess the effectiveness of treatments by one way analysis of variance [18].

In some studies, random non-fasting plasma glucose concentration was determined on blood samples obtained by cardiac puncture. In one series, anaesthetized rats fasted overnight were injected IP with salicylates or copper complexes or their diluent and plasma glucose measured 15 and 30 min later, using blood from cut tails. This was done to ascertain that any protective effect against streptozotocin diabetes was not mediated by hyperglycaemia induced by these agents at the time of the streptozotocin injections. The plasma glucose of rats injected with the highest doses of salicylate, 3,5-diisopropylsa-

licylate or their copper complexes were not significantly different from those observed in diluent-treated controls (range 3–8 mmol/l; $n = 3$ each group).

Serum calcium, phosphate, blood urea nitrogen, uric acid, cholesterol, total protein, albumin, alkaline phosphatase, and serum glutamate-oxalacetate-transaminase were measured on day 3 by the clinical chemistry laboratory (SMA 12/60 profile, Technicon Instruments) on rats treated with diisopropylsalicylate (41 mg/kg) and copper(II)(3,5-diisopropylsalicylate)₂ (120 mg/kg) for comparison with controls to assess liver and renal function ($n = 6$ each group).

Results

Copper(II)(3,5-diisopropylsalicylate)₂ caused attenuation of diabetes when administered IP 15 min before streptozotocin (Table 1). Salicylic acid and 3,5-diisopropylsalicylic acid, which lack superoxide dismutase-like activity, did not attenuate diabetes significantly (Table 1). Copper(II)(salicylate)₂ was also ineffective. Copper(II)(aspartate) showed no significant protection as measured by a random non-fasting blood glucose value (21.1 ± 1.9 mmol/l for copper(II)(aspartate) versus 22.2 ± 1.6 mmol/l for controls, $n = 5$ each group) in two separate experiments. Glycosuria measurements also showed no difference between the two groups as both were in the range of 3–4+. Thus further experimentation with this compound was abandoned.

The glucose tolerance of rats treated with copper(II)(3,5-diisopropylsalicylate)₂ at doses of 48 or 120 mg/kg before streptozotocin injection was significantly better than those receiving streptozotocin alone ($p < 0.0001$) or those pretreated with copper free 3,5-diisopropylsalicylic acid ($p < 0.01$). Plasma glucose levels 15 or 30 min after copper(II)(3,5-diisopropylsalicylate)₂ injection were normal, eliminating the possibility of protection via hyperglycaemia at the time of streptozotocin injection. Of the agents tested (Table 1), only copper(II)(salicylate)₂ caused clinical signs of toxicity when administered in conjunction with streptozotocin; 30% died within 24 h and the remaining rats appeared sick. Mild elevation of blood urea nitrogen was seen in rats

receiving copper(II)(3,5-diisopropylsalicylate)₂ (0.15–0.20 versus 0.1–0.15 mmol/l). However the uric acid level (0.1–0.15 mmol/l for treated animals versus 0.1–0.15 mmol/l for control animals) and total serum protein (53–55 g/l for treated animals versus 53–54 g/l for control animals) were not significantly different. Glycosuria of rats treated with streptozotocin alone was 3–4+ while that of copper(II)(3,5-diisopropylsalicylate)₂ pretreated rats was 0–1+. Serum calcium and phosphate were not affected by treatment with copper(II)(3,5-diisopropylsalicylate)₂ or by streptozotocin or a combination of the two agents (2.6–2.7 mmol/l for calcium, 5.6–5.8 mmol/l for phosphate in all groups). Tests which may reflect acute hepatic damage, e.g. serum albumin (range: 2.0–2.2 mmol/l), cholesterol (range: 1.2–1.4 mmol/l), alkaline phosphatase (range: 136–198 IU), and serum glutamate-oxalacetate transaminase (range: 99–115 IU) were not affected by any of the treatments.

In one study, groups of five to six animals were weighed daily for 5 days after injection of the different drugs. The cumulative 5-day-weight gain of control rats was 22.4 ± 1.03 g, and that of rats injected with diisopropylsalicylate (41 mg) was 22.2 ± 1.31 g. By contrast, rats which had received streptozotocin (50 mg/kg) gained 10.5 ± 1.31 g, and this rate was not significantly altered by pretreatment with 41 mg diisopropylsalicylate (8.2 ± 2.2 g) or 48 mg copper(II)(3,5-diisopropylsalicylate)₂ (9.2 ± 1.65 g). Rats injected with 48 mg copper(II)(3,5-diisopropylsalicylate)₂ alone, gained significantly less weight than the controls (9.0 ± 2.68 g, $p < 0.01$) and were not significantly different from any of the groups treated with streptozotocin. Although food intake was not quantitated, it appears evident that the copper derivative exerted some toxic effects, presumably causing reduced food intake.

In rats treated with salicylates or copper derivatives by SC injection before streptozotocin, random non-fasting plasma glucose concentrations were not different from those receiving streptozotocin alone (> 15 mmol/l). The effectiveness of this route of administration was not evaluated further.

Discussion

The purpose of these experiments was to assess the efficacy of superoxide dismutase-like copper compounds as prophylactic agents against streptozotocin diabetes. The lipophilic copper(II)(3,5-diisopropylsalicylate)₂ partially protected against streptozotocin-induced diabetes. The two other copper compounds tested did not afford any protection, possibly due to their lack of lipophilicity. As the diisopropylsalicylate itself was not effective, the action of the copper(II)(3,5-diisopropylsalicylate)₂ was most likely due to the presence of copper.

There is considerable evidence that oxygen radicals play a role in mediating the toxicity of chemical diabe-

togenic agents to islet B cells. Thus, it is possible that the selective vulnerability of B cells to these toxins represents at least in part their exquisite sensitivity to oxygen radical damage [19, 20]. The apparent efficacy of copper(II)(3,5-diisopropylsalicylate)₂ in protecting rats against streptozotocin is similar to that reported for the polyethylene glycol-superoxide dismutase derivative against alloxan in mice [8]. Although it is possible that the attenuation of streptozotocin-induced diabetes by treatment with the copper-derivative represented a protective effect on B cells, we cannot rule out the possibility that extrapancreatic, toxic effects of copper may have caused the improvement in glucose tolerance and glycosuria. There is a large array of other non-peptide organic compounds with the capacity to scavenge oxygen radicals [19]. Screening such compounds for their ability to prevent or attenuate experimental diabetes may be worthwhile since it is possible that oxygen radicals are involved in B cell damage by a number of apparently unrelated noxious agents and may play some role in the aetiology of insulin-dependent diabetes. In screening such compounds, caution is recommended to differentiate protective effects on the B cells from extrapancreatic effects.

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