ANTIOXIDANT PROBUCOL PREVENTS DEVELOPMENT OF ALLOXAN DIABETES AND HYPOACTIVITY OF ANTIOXIDANT ENZYMES IN RAT TISSUES

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Much information has now been collected on the important role of free-radical oxidation in the etiology and pathogenesis of many different pathological states such as atherosclerosis, myocardial ischemia, etc. [6]. In recent years evidence has been published to show that diabetes mellitus can also be included under the heading of free-radical pathology [1, 15]. One of the most widely used experimental models of diabetes in animals is that induced by alloxan [9]. The damaging effect of alloxan is attributable to its rapid reduction to dialuric acid, autooxidation of which leads to the formation of H_2O_2 and active forms of oxygen, such as $O_2^{\cdot}$ and $HO^{\cdot}$ [15]. Considering the abundance of experimental evidence in support of a free-radical mechanism of action of alloxan [12, 15]. in our opinion alloxan diabetes can be regarded as a simple and convenient model of preradical pathology and it can be used to evaluate the efficacy of antioxidant drugs. In view of the important function of antioxidant enzymes such as superoxide dismutase and glutathione-dependent lipoperoxidases, in the utilization of active forms of oxygen and of lipoperoxides in the tissues [4], one hypothesis to explain the selective toxicity of alloxan for pancreatic beta-cells is the low level of enzymic protection of these cells against the action of active forms of oxygen and of lipoperoxides [11].

The aim of this investigation was to study the protective action of probucol, a widely used antioxidant drug, on a model of alloxan diabetes and its effect on activity of antioxidant enzymes.

EXPERIMENTAL METHOD

Experiments were carried out on 42 August rats weighing 190 ± 10 g. The animals were divided into 5 groups (the number of animals in each group is given in Table 1): 1) animals receiving a single intravenous injection of a diabetogenic dose of alloxan (40 mg/kg); 2) intact rats; 3) rats receiving a single dose of 50 mg/kg probucol perorally in 2% starch solution 24 h before injection of alloxan; 4 and 5) rats receiving probucol in a dose of 50 mg/kg daily for 3 and 7 days respectively before receiving the injection of alloxan. The blood glucose level was determined by the standard orthotoluidine method; the serum immunoreactive insulin (IRI) level was determined with the aid of "Rio-INS-PG^{125}I" test kits (USSR/CIS); glucose and ketone bodies in the urine were detected qualitatively by "Gluketur-Test" strips (Boehringer Mannheim GmbH). Activity of superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione-S-transferase (GT), and glutathione reductase (GR) in supernatants of the liver and pancreas (8000*g*, 1 min) was determined as described previously [3, 5], using a "Hitachi 220A" spectrophotometer and an FP-901 chemical analyzer (Labsystems Oy). Enzyme activity was measured in the tissues of rats of the

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TABLE 1. Effect of Preliminary Injection of Probucol on Development of Alloxan Diabetes in Rats

Group of animals	Blood glucose concentration, mmoles/liter		Serum IRI level, pmoles/liter	
	before injection of alloxan	after injection of alloxan	before injection of alloxan	after injection of alloxan
1 (18)	4,3±0,6	20,8±1,8	110,1±11,6	3,8±0,8
2 (6)	4,0±0,4	—	113,3±10,2	—
3 (6)	4,1±0,6	5,5±0,4*	107,5±7,7	99,2±7,0*
4 (6)	4,2±0,3	4,9±0,6*	115,2±15,3	105,6±15,3*
5 (6)	4,5±0,7	4,5±0,4*	122,2±8,3	116,7±10,3*

Legend. Determinations carried out 3, 7, and 10 days after a single intravenous injection of alloxan (40 mg/kg); number of animals in groups shown between parentheses; Asterisk indicates significance of differences at $p \leq 0.05$ level compared with group 1.

experimental groups (1, 3, and 5) 10 days after injection of alloxan. The protein concentration was determined by the microbiuret method. Small pieces of pancreas for histologic investigation were fixed in Bouin's fluid and dehydrated in alcohols; paraffin sections were cut and stained for differentiation of islet cells by aldehyde-fuchsine [10] or pseudoisocyanin [8] to reveal the intracellular insulin depot in UV light.

Probucol (fenbutol) was synthesized at the All-Union Scientific Center for Biologically Active Substances, Kupavna.

EXPERIMENTAL RESULTS

A single injection of alloxan into the rats led to the appearance of glucosuria and of ketone bodies in the urine (detected qualitatively), to a raised blood glucose level and a lowered serum insulin content, evidence of the development of experimental diabetes in the animals (Table 1).

Morphologic investigation of the pancreas of intact animals (group 2) revealed numerous large and medium-sized islets, which were clearly demarcated from exocrine tissue and contained beta-cells, abundantly packed with aldehyde-fuchsine granules. Alpha-cells were distributed around the periphery of the islet in the form of a brush border (Fig. 1a). The depot form of insulin (Fig. 1) was detected by the histochemical luminescence reaction with pseudoisocyanin in the pancreatic beta-cells (Fig. 1b). After injection of alloxan the rats of group 1 showed characteristic destructive changes in the endocrine tissue of the pancreas: the number and size of the islets were sharply reduced, they were greatly deformed, and pycnotic nuclei and shrunken cytoplasm were seen in them. On staining with aldehyde-fuchsine many islets were found to be completely degranulated (Fig. 1c), and only in a few of them were single beta-cells with a small quantity of specific granular material visible; alpha-cells were distributed not only around the periphery of the islet, but also in its center. The metachromatic reaction for insulin with pseudoisocyanin was not present in the beta-cells (Fig. 1d).

Preliminary injection of probucol for 1, 3, and 7 days in a dose of 50 mg/kg completely prevented the manifestation of the diabetogenic action alloxan (Table 1), evidence of the high efficacy of the therapeutic use of antioxidants and of probucol in particular, in the treatment of free-radical diseases caused by hyperproduction of active forms of oxygen and lipoperoxides.

In the experiments rats (groups 3-5), which received alloxan preceded by the antioxidant probucol for 1, 3, and 7 days, the histologic structure of the pancreas showed no appreciable changes: the pancreatic islets contained beta-cells, more less filled with aldehyde-fuchsine granules (Fig. 1e), but beta-cells without granules also were found in the field of vision; these latter had no granules, but their cytoplasm appeared clear and translucent, indicating absence of the characteristic morphoiogic features of diabetes. The highly sensitive reaction to insulin with pseudoisocyanin was positive (Fig. 1f) and did not differ significantly from that of intact animals (Fig. 1b).

Activity of the antioxidant enzymes SOD and GP fell considerably in the pancreas and liver of rats with alloxan diabetes (Table 2). Considering the possibility of inactivation of SOD and GP through the action of H_2O_2 and of other active forms of oxygen in vitro, it can be postulated that the sharp decline in activity of these enzymes in the liver and, in particular, in the pancreas of rats with alloxan diabetes reflects more intensive generation of ac-

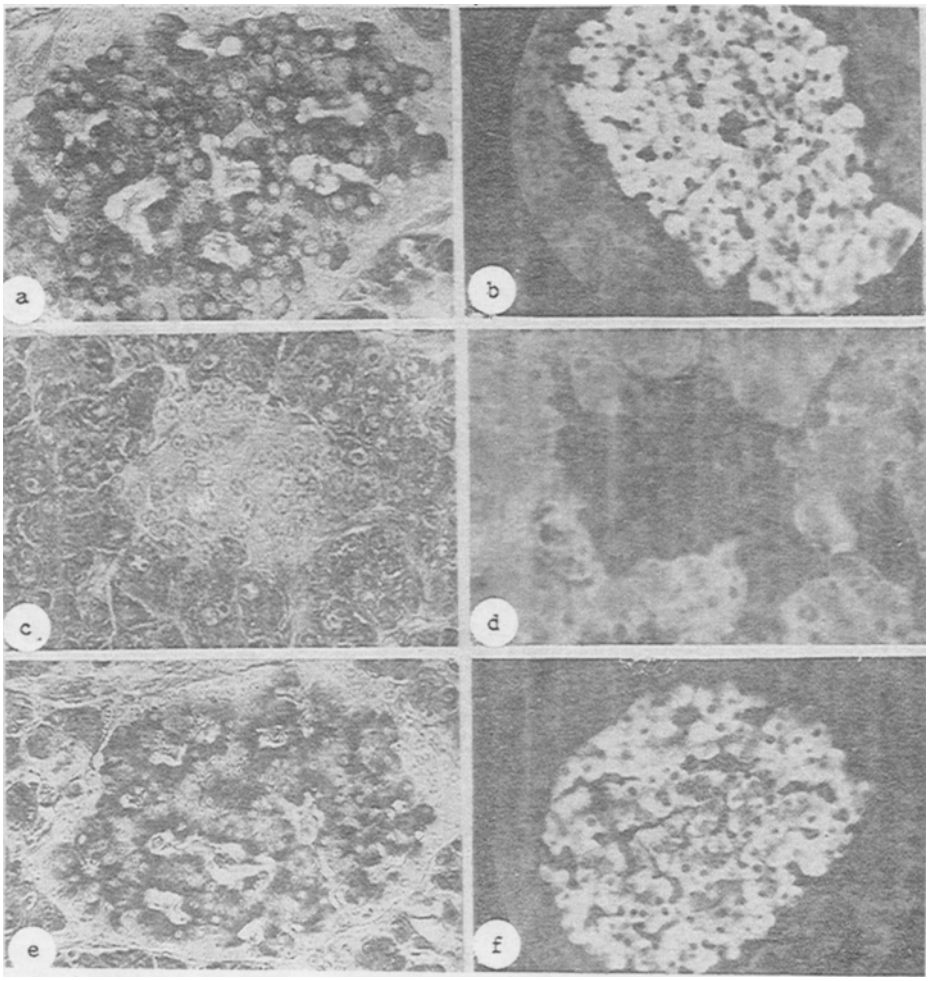

Fig. 1. Effect of preliminary injection of probucol on development of resistance of pancreatic beta-cells to toxic action of alloxan: a) pancreatic islet of intact rat. Abundance of specific granules in beta-cells. Stained with aldehyde-fuchsine, 280×; b) histochemical reaction with pseudoisocyanin for insulin in beta-cells of intact rats. Luminescence microscopy. 18×; c) pancreatic islet of rat after injection of alloxan. Destructive changes in beta-cells with foci of necrosis. Aldehyde-fuchsine. 280×; d) metachromatic reaction with pseudoisocyanin for insulin absent in pancreatic islets of rat after injection of alloxan. Luminescence microscopy. 180×; e) pancreatic islet of rat receiving preliminary injections (7 days) of probucol before injection of alloxan. Specific granules preserved in beta-cells. Aldehyde-fuchsine. 280×; f) positive histochemical reaction with pseudoisocyanin for insulin in beta-cells of a rat receiving preliminary injections of probucol (7 days) before alloxan. Luminescence microscopy. 180×.

tive forms of oxygen and of other radical products during reduction of alloxan into dialuric acid, and its subsequent oxidation. Total GT activity (using 1-chloro-2,4-dinitrobenzene as the substrate) and activity of GR, an enzyme involved in glutathione bioregeneration, also were significantly reduced in these same tissues three days after administration of alloxan to the animals (Table 2). Meanwhile, after preliminary administration of a single or multiple doses of probucol into the rats before alloxan was given, activity of the antioxidant enzymes (SOD and, in particular, GP, in the pancreas) increased significantly (Table 2) compared with levels of activity of these enzymes in intact animals and, more especially, compared with their levels in animals with alloxan diabetes (Table 2). Activity of these enzymes in the liver after alloxan injection, preceded by a single dose of probucol, was somewhat lower than their level in intact animals, but after repeated administration of probucol before the creation of alloxan diabetes, it was

TABLE 2. Effect of Preliminary Injection of 50 mg/kg Probucol on Activity of Antioxidant Enzymes in Pancreas and Liver of Rats after Injection of Alloxan

Group of animals	Activity of antioxidant enzymes, U/mg protein							
	SOD		GP		GT		GR	
	pancreas	liver	pancreas	liver	pancreas	liver	pancreas	liver
1-	2,6±0,52	28,8±1,6	0,03±0,006	0,28±0,01	0,03±0,003	0,32±0,005	0,03±0,007	0,12±0,002
2-	5,9±1,1*	62,2±1,8*	0,08±0,001*	0,7±0,05*	0,09±0,005*	0,73 ±0,001*	0,08±0,002*	0,56±0,001*
3-	8,2±1,0*	48,2±1,2*	0,17±0,002*	0,50 ±0,001*	0,07±0,004*	0,88±0,02*	0,03±0,001	0,16±0,004*
4-	8,7±1,1*	67,2±1,6*	0,23±0,02*	0,67 ±0,006*	0,07±0,004*	2,7±0,03*	0,04±0,003	0,22±0,003*
5-	10,5±2,0*	74,7±1,8*	0,23±0,003*	0,65±0,05*	0,1±0,002*	3,1±0,1*	0,05±0,001*	0,40±0,002*

significantly higher than enzyme activity in the liver of animals with alloxan diabetes (Table 2). Administration of probucol to rats before injection of alloxan prevented the decrease in GT activity in the pancreas and liver (Table 2), but activity of this enzyme in the pancreas after injection of probucol did not differ significantly from its activity in the same tissues of intact animals; however, repeated injections of probucol before alloxan led to a marked increase in GT activity (by 3.7-4.2 times) in the rat liver (Table 2). This fact can be explained by the known effect of induction of hepatic glutathione-S-transferases by xenobiotics [14] and, in particular, by hydrophobic synthetic antioxidants, such as ionol [2].

Incidentally, the method we used makes it possible to determine activity, not of glutathione peroxidase, but of glutathionyl lipoperoxidase, i.e., total activity of splenic GP and of isozymes of GT, which can reduce organic hydroperoxides (isozymes 1,1; 1,2; 2,2). Table 2 shows that glutathionyl lipoperoxidase activity in the liver of rats which received probucol before the creation of the model of alloxan diabetes (groups 3 and 5) did not differ significantly from the level of activity of this enzyme in intact animals. Accordingly, it can be concluded that the increase in glutathione-S-transferase activity in groups 4 and 5 of our experiments was not due to preferential induction of GT isozymes capable of reducing lipoperoxides. GR activity in the liver and pancreas of rats receiving probucol before injection of alloxan, was significantly lower than activity of this enzyme in the tissues of intact animals, but significantly higher than GR activity in rats with alloxan diabetes, except its activity in the pancreas of the rats of group 3. Thus activity of SOD, GP, GR, and GR is significantly lower during the development of alloxan diabetes in the pancreas and liver compared with the level of its activity in the tissues of intact animals. Even a single injection of probucol before alloxan prevented the decrease in activity of these enzymes in the same tissues in virtually every case. Injection of probucol for 3-7 days before the beginning of alloxan diabetes, however, led to a significant increase in activity of the antioxidant enzymes SOD and GP in the pancreas, but not in the liver of the experimental rats.

Since the primary lesions in alloxan diabetes undoubtedly are caused by active forms of oxygen, whose inactivation by phenolic antioxidants of the probucol type is impossible, it must be pointed out that the protective action of probucol, revealed in our experiments (Table 1), is evidence of the important role of lipid peroxidation processes in damage to the pancreatic beta-cells. This hypothesis is supported by the sharp increase in activity of GP, an enzyme utilizing lipoperoxides, in our experiments in the pancreas of rats receiving probucol before injection of alloxan; it is instructive, moreover, to consider that injection of probucol into the same animals did not increase activity of SOD, but prevented its decrease, which is observed in rats with alloxan diabetes (Table 2).

It can thus be tentatively suggested that the protective action of probucol in alloxan diabetes (Table 1) is realized not only through direct interaction of the antioxidant with lipid radicals, but also to a large extent to the protective effect against antioxidant enzymes in the pancreas. Consequently, the results of our investigations indicate a marked protective effect of probucol during the development of this free-radical pathology, in agreement with its successful use in another pathological state connected with intensification of free-radical processes in atherosclerosis

[7]. From our point of view the possibility of increasing activity of antioxidant enzymes in the tissues by repeated doses of probucol, which we found previously and confirmed in the present investigation (Table 2), is extremely important. In this connection it is interesting to note that the action of streptozocin, another powerful diabetogenic agent, may also be connected to some extent with damage to the enzyme systems utilizing lipoperoxides in cells of the pancreas [15]. It must be noted that the structure of streptozocin is very similar to that of the known antitumor agent 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), and that, unlike BCNU, streptozocin accumulates selectively in the beta-cells, probably due to the fact that it contains a glucose residue [15]. Meanwhile, BCNU has been found to have a powerful inhibitory action in vitro and in vivo on GR, but not on GP [13]. Naturally inhibition of GR is in fact equivalent to inhibition of GP, for reduction of lipoperoxides is impossible without bioregeneration of glutathione. It can thus be tentatively suggested that streptozocin, a structural analog of BCNU, exhibits its diabetogenic action, in particular, through inhibition of GR and, consequently, of the enzyme system utilizing lipoperoxides in the beta-cells of the pancreas. Experimental verification of this hypothesis is currently in progress at the All-Union Cardiologic Scientific Center, Russian Academy of Medical Sciences. Our results given in this publication indicate that the use of probucol is a convenient tool with which to influence enzymic utilization of active forms of oxygen and lipoperoxides in the tissues in free-radical diseases.

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