Role of Antioxidant Enzymes and Antioxidant Compound Probucol in Antiradical Protection of Pancreatic β-Cells during Alloxan-Induced Diabetes

V. Z. Lankin, V. I. Korchin*, G. G. Konovalova, M. O. Lisina, A. K. Tikhaze, and I. G. Akmaev*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 137, No. 1, pp. 27-30, January, 2004
Original article submitted July 2, 2003

The severity of disturbances in carbohydrate metabolism in rats with alloxan-induced diabetes depended on activity of antioxidant enzymes in the target organ (pancreas). Damage to the pancreas is related to intensive generation of reactive oxygen species, free radicals, and lipid peroxides. Alloxan-induced diabetes in rats is a free radical disease, which *in vivo* serves as a useful model for the search for pharmacological preparations with antiradical and antioxidant properties. The antioxidant compound probucol indirectly increased activity of antioxidant enzymes in the pancreas and prevented the development of alloxan-induced diabetes in rats. Our results indicate that different sensitivity of laboratory animals of various species (rats and guinea pigs) to the influence of alloxan is associated with abnormal variations in activity of enzymes utilizing reactive oxygen species and lipid peroxides in mammalian pancreatic cells.

Key Words: *alloxan-induced diabetes; antioxidant enzymes; antioxidants; probucol*

An impressive body of evidence indicates that various low-molecular-weight antioxidants, including α-tocopherol, β-carotene, ubiquinone Q_{10} (ubiphenol Q_{10}), ascorbic acid, and glutathione, play a role in the regulation of free radical processes in cells [2,5]. It should be emphasized that high-molecular-weight antioxidants (antioxidant enzymes) also maintain a constant concentration of free radicals harmless to the cell [1,4, 12]. The pathogenesis of alloxan-induced diabetes in rats is associated with free radical processes [13]. After single intravenous injection alloxan is enzymatically reduced into dialuric acid in rat pancreatic cells [14]. Autooxidation of dialuric acid results in the generation of superoxide anion radicals ($O_2^{\bullet-}$) and other reactive oxygen species (ROS) [14].

Laboratory for Biochemistry of Free Radical Processes, A. L. Myasnikov Institute of Cardiology, Russian Research-and-Production Center for Cardiology, Russian Ministry of Health; *Research Center for Endocrinology, Russian Academy of Medical Sciences, Moscow

ROS induce free radical oxidation of unsaturated lipids in biological membranes with the formation of lipid peroxides. Spontaneous homolysis of these compounds leads to the formation of secondary free radicals capable of damaging new molecules of phospholipids and biopolymers (proteins and nucleic acids) [4, 5,12]. Here we evaluated the role of key antioxidant enzymes superoxide dismutase (SOD) detoxifying $O_2^{\bullet-}$, GSH-dependent glutathione peroxidase (GSH-peroxidase), and glutathione-S-transferase (GSH-transferase) in the molecular mechanisms underlying protection of rat pancreatic β-cells synthesizing insulin from the influence of ROS and free radicals induced by alloxan.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats and guinea pigs weighing 180±20 and 330±50 g, respectively. Alloxan (Sigma) in a single dose of 40 mg/kg was injected intravenously to intact rats to model dia-

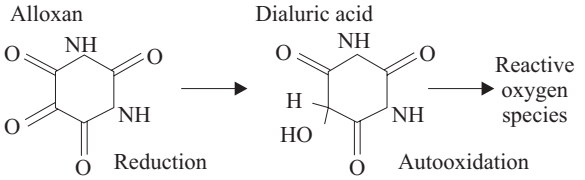
0007-4888/04/1371-0020

betes. Some rats received antioxidant compound probucol (50 mg/kg, Sigma) in 2% starch gel through a tube for 7 days before administration of alloxan.

For biochemical assay the animals were killed under ether anesthesia 14 days after alloxan injection. Plasma glucose level was measured by the glucose oxidase method using Lachema kits. The content of immunoreactive insulin was estimated with Cea-Ire-Sorin kits. Samples of the pancreas were homogenized in 50 mM K-Na-phosphate buffer (pH 7.4) using an Ultra Turrax SDT-1810 Janke & Kunkel IKA-Werk tissue disintegrator. Antioxidant enzyme activity was measured in supernatants obtained after centrifugation of pancreatic homogenates at 8000*g* for 15 min. Cu,Zn-SOD activity was estimated by inhibition of nitroblue tetrazolium reduction with superoxide radical $O_2^{\bullet-}$ generated in the xanthine-xanthine oxidase system. The kinetics of formazan formation was recorded on a Hitachi-557 spectrophotometer at 560 nm [10]. The amount of SOD causing a 50% inhibition of nitroblue tetrazolium reduction was taken as a unit of enzyme activity [10]. Activity of Se-containing GSH-peroxidase was measured in a coupled glutathione reductase system by the rate of NADPH oxidation at 340 nm. A kinetic study was performed with the substrate tert-butyl hydroperoxide on a FP-901 chemical analyzer (Labsystems Oy) [6]. The amount of GSH-peroxidase oxidizing 1 μmol GSH over 1 min was taken as a unit of enzyme activity [6]. Total GSH-transferase activity was estimated by the rate of enzymatic conjugation of reduced GSH with 1-chloro-2,4-dinitrobenzene using a FP-901 chemical analyzer (Labsystems Oy) [6]. The amount of GSH-S-transferase necessary for conjugation of 1 μmol GSH over 1 min was taken as a unit of enzyme activity [6].

RESULTS

In vivo alloxan-induced damage is mediated by the free radical mechanism, which is shown in the following scheme:



Alloxan sharply suppressed insulin synthesis in animals, which is consistent with published data [14]. Plasma insulin level decreased more than by 20 times after alloxan injection (Table 1). These changes were accompanied by the development of hyperglycemia: blood glucose concentration increased by 5 times (Table 1). No changes in carbohydrate metabolism were observed after peroral administration of the antioxidant compound probucol in a dose of 50 mg/kg for 7 days (Table 1). However, injection of alloxan after pretreatment with probucol did not cause hypoinsulinemia and hyperglycemia (Table 1).

Our previous studies showed that probucol therapy increases antioxidant enzyme activity in blood cells from patients with coronary heart disease [9]. Further studies showed that probucol increases the rate of enzymatic GSH-dependent utilization of lipid peroxides in tissues [11]. The present study demonstrated that even short-term treatment with probucol (1 week) increased activity of antioxidant enzymes in rat pancreatic cells (Table 2). SOD activity in rat pancreatic cells increased more than by 45% after probucol administration. It should be emphasized that activity of lipid peroxide-utilizing enzymes GSH-peroxidase and GSH-transferase increased by 3.5 and 1.5 times, respectively (Table 2). The protective effect of probucol on rats with alloxan-induced diabetes (Table 1) is probably related to improvement in enzymatic detoxification of ROS and lipid peroxides. They are formed during autooxidation of alloxan derivative dialuric acid and free radical oxidation of the biological substrate. Our suggestion is confirmed by the data that alloxan decreased activities of SOD, GSH-peroxidase, and GSH-transferase in rat pancreatic cells more than by 2 times (Table 2). These changes are probably associated with a partial inactivation of the corresponding enzymes by ROS, free radicals, and secondary oxidation products in high concentrations [4,5]. It should be emphasized that after pretreatment with probucol increasing antioxidant enzyme activity in the pancreas, activities of SOD, GSH-peroxidase, and GSH-transferase remained high in rats receiving alloxan (Table 2).

Previous studies showed that injection of alloxan is followed by the development of hypoinsulinemia and hyperglycemia in alloxan-sensitive, but not in

TABLE 1. Effects of Single Intravenous Injection of Alloxan and Peroral Pretreatment with the Antioxidant Compound Probucol for 7 Days on Major Indexes of Carbohydrate Metabolism in Rat Plasma (*M*±*m*)

Group	Insulin, pmol/liter	Glucose, mmol/liter
Intact (*n*=40)	110.1±10.2	4.35±0.44
Alloxan (*n*=40)	4.6±0.8*	20.82±2.45*
Probucol without further injection of alloxan (*n*=14)	102.6±9.8	4.52±0.56
Probucol and further injection of alloxan (*n*=14)	104.5±9.2	4.47±0.52

Note. Here and in Tables 2 and 3: *$p<0.05$ compared to intact animals.

TABLE 2. Effects of Single Intravenous Injection of Alloxan and Peroral Pretreatment with Antioxidant Compound Probucol for 7 Days on Antioxidant Enzyme Activity in Rat Pancreas (*M*±*m*, U/mg protein)

Group	SOD	GSH-peroxidase	GSH-transferase
Intact (*n*=40)	5.9±0.3	0.068±0.005	0.087±0.004
Alloxan (*n*=14)	2.7±0.7*	0.032±0.004*	0.037±0.005*
Probucol without further injection of alloxan (*n*=14)	8.6±0.8*	0.234±0.010*	0.129±0.011*
Probucol and further injection of alloxan (*n*=14)	10.5±1.3*	0.230±0.012*	0.105±0.009*

TABLE 3. Activity of Key Antioxidant Enzymes in the Pancreas of Rats and Guinea Pigs before and after Injection of Alloxan in Various Doses for 14 Days (*M*±*m*, U/mg protein)

Group	SOD	GSH-peroxidase	GSH-transferase
Intact rats (*n*=8)	6.1±1.4	0.092±0.003	0.076±0.005
Rats receiving 40 mg/kg alloxan (*n*=12)	2.4±0.7*	0.034±0.007*	0.029±0.006*
Intact guinea pigs (*n*=10)	26.8±1.5	0.34±0.03	0.54±0.04
Guinea pigs receiving 100 mg/kg alloxan (*n*=8)	23.5±1.2	0.27±0.02	0.42±0.02
Guinea pigs receiving 200 mg/kg alloxan (*n*=8)	20.9±1.6*	0.22±0.04*	0.38±0.03*

alloxan-resistant guinea pigs [7,15]. This phenomenon was not explained. In our experiments the initial concentrations of glucose and immunoreactive insulin in the plasma practically did not differ in intact rats (4.42±0.38 mmol/liter and 106.4±6.6 pmol/liter, respectively) and guinea pigs (4.75±0.57 mmol/liter and 115.4±9.6 pmol/liter, respectively). Glucose level in rat plasma increased to 20.46±2.50 mmol/liter, while the amount of immunoreactive insulin decreased to 4.6±1.1 pmol/liter 2 weeks after intravenous injection of alloxan in a single dose of 40 mg/kg (similarly to previous studies, Table 1). At the same time, administration of alloxan in higher doses did not impair carbohydrate metabolism in guinea pigs over 2-week observations. Plasma glucose level in these animals remained practically unchanged after injection of alloxan in a dose of 100 mg/kg (5.85±0.45 mmol/liter, $p>0.05$) and increased less than by 30% after administration of this substance in a dose of 200 mg/kg (6.08±0.36 mmol/liter, $p<0.05$). The amount of immunoreactive insulin in guinea pig plasma decreased after injection of alloxan in doses of 100 and 200 mg/kg (88.5±8.4 and 74.8±5.7 pmol/liter, respectively). However, the degree of changes observed in guinea pigs (23-35%) was incomparable to the alloxan-induced inhibition of insulin synthesis in rats. Injection of alloxan in low dose (40 mg/kg) to rats was followed by a decrease in plasma insulin concentration to 4.6±1.1 pmol/liter (more than by 20 times). Activities of SOD, GSH-peroxidase, and GSH-transferase in pancreatic cells of alloxan-resistant guinea pigs were abnormally high and surpassed those in alloxan-sensitive rats by 4.4, 3.7, and 7.1 times, respectively (Table 3). Moreover, antioxidant enzyme activity in the pancreas of alloxan-sensitive rats decreased after injection of alloxan in a dose of 40 mg/kg (Tables 2, 3). However, alloxan in a higher dose (100 mg/kg) had no effect on activity of antioxidant enzymes in pancreatic cells of alloxan-resistant guinea pigs (Table 3). Only administration of alloxan in a subtoxic dose of 200 mg/kg insignificantly impaired carbohydrate metabolism and decreased antioxidant enzyme activity in the pancreas of guinea pigs (by 12-35%, Table 3).

Our results show that the severity of disturbances in carbohydrate metabolism in rats with alloxan-induced diabetes depends on activity of antioxidant enzymes in the target organ (pancreas). Damage to the pancreas is related to intensive generation of ROS, free radicals, and lipid peroxides. Alloxan-induced diabetes in rats is a free radical disease, which *in vivo* serves as a useful model for the search for pharmacological preparations with antiradical and antioxidant properties. Antioxidant compound probucol indirectly increases activity of antioxidant enzymes in the pancreas and prevents the development of alloxan-induced diabetes in rats. These data indicate that different sensitivity of laboratory animals of various species (rats and guinea pigs) to alloxan is associated with abnormal variations in activity of enzymes utilizing ROS and lipid peroxides in mammalian pancreatic cells. Dysregulation of free radical processes accompanies non-insulin-dependent diabetes mellitus [3,8,14]. Our previous studies showed that the antioxidant compound probucol stabilizes compensation of carbohydrate metabolism in patients without increasing the dose of hypoglycemic preparations [8].

This work was partially supported by the Russian Foundation for Basic Research (grant No. 03-04-48344).

REFERENCES

1. *Biochemistry of Lipids and Their Role in Metabolism* [in Russian], Ed. S. E. Severin, Moscow (1981), pp. 75-95.
2. N. K. Zenkov, N. V. Kandalintseva, V. Z. Lankin, *et al*., *Phenol Antioxidants* [in Russian], Novosibirsk (2003).
3. N. K. Zenkov, V. Z. Lankin, and E. B. Men'shchikova, *Oxidative Stress. Biochemical and Pathophysiological Aspects* [in Russian], Moscow (2001).
4. V. Z. Lankin, A. K. Tikhaze, and Yu. N. Belenkov, *Kardiologiya*, **40**, No. 7, 48-61 (2000).
5. V. Z. Lankin, A. K. Tikhaze, and Yu. N. Belenkov, *Free Radical Processes under Normal and Pathological Conditions (Manual for Physicians)* [in Russian], Moscow (2001).
6. V. Z. Lankin, A. K. Tikhaze, A. L. Kovalevskaya, *et al*., *Dokl. Akad. Nauk SSSR*, **261**, No. 6, 1467-1470 (1981).
7. V. I. Lapin, V. I. Korchin, G. G. Meiramov, *et al*., *Pat. Fiziol*., No. 4, 36-39 (1973).
8. L. V. Nedosugova, V. Z. Lankin, M. I. Balabolkin, *et al*., *Byull. Eksp. Biol. Med*., **136**, No. 8, 152-155 (2003).
9. A. K. Tikhaze, V. Z. Lankin, V. P. Mikhin, *et al*., *Ter. Arkh*., **59**, No. 9, 35-41 (1997).
10. C. Beauchamp and J. Fridovich, *Anal. Biochem*., **44**, 276-278 (1971).
11. D. Kumar, V. Palace, I. Danelisen, *et al*., *J. Mol. Cell. Cardiol*., **33**, A62 (2001).
12. V. Lankin, *Free Radicals, Nitric Oxide, and Inflammation: Molecular, Biochemical, and Clinical Aspects*, Amsterdam (2003), Vol. 344, pp. 8-23.
13. V. Z. Lankin, V. I. Korchin, G. G. Konovalova, *et al*., *Free Radic. Biol. Med*., **16**, 15 (1994).
14. L. W. Oberley, *Ibid*., **5**, 113-124 (1988).
15. R. Schmidt, *Der Alloxandiabetes. Morphologie, Chemismus und Literatur*, Leipzig (1967).