The Protective Effects of *Lycium bararum* Polysaccharides on Alloxan-induced Rat Pancreatic Islets Damage

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Abstract To study the protective effects of *Lycium bararum* polysaccharides (*Lb*-PS) on isolated pancreatic islets which were incubated with 4 mmol/L alloxan (AXN), Insulin released into medium and in islets was detected by radioimmunoassay and glucokinase and SOD activity, NO and malonaldehyde (MDA) production were measured by colorimetric assay. Results indicated that AXN significantly inhibited the insulin release in response to glucose and insulin content in islets. In addition, there was a higher NO and MDA concentration and reduced glucokinase and SOD activity in islets incubated with AXN. *Lb*-PS could restore glucokinase and SOD activity, insulin synthesis and secretion inhibited by AXN, and reduce NO and MDA production in AXN-induced islets. Therefore *Lb*-PS could protect glucose-induced insulin synthesis and insulin secretion through reducing NO production and maintaining glucokinase and SOD activity in pancreatic β cells.

Key words *Lycium bararum* polysaacharides; alloxan; glucokinase; insulin; NO

Lycium bararum polysaccharides (*Lb*-PS) are extracted from Chinese wolfberry *Lycium bararum.* Its fruit is called "Gouqizi" in China and has been regarded as a traditional Chinese medicine used for the prevention and treatment of various human disease. *Lb*-PS has many pharmacological effects[1] such as increasing immunity, antitumor, anti-senility, protecting liver, reducing blood glucose and blood lipid and etc. However, the hypoglycemic mechanism of *Lycium bararum* polysaccharides remains unclear.

Alloxan is a prompt and potent inductor of diabetes in experimental animals because of its damaging effect on insulin-producing β cell of pancreas. The mechanism of alloxan is now generally accepted that free radicals are involved in the initiation of the damage that ultimately leads to β cell death.

In order to test the effects of *Lb*-PS on alloxan-induced rat islets, we investigate the insulin synthesis and secretion, glucokinase and superoxide dismutase (SOD) activity, endogenous nitric oxide (NO) and malonaldehyde (MDA) production. Because SOD plays an important role in the protection from peroxidation by scavenging the superoxide radical, we want to reveal wheather *Lb*-PS has effects against radical reactions induced by alloxan (AXN) through increasing SOD activity and inhibiting MDA production. Pervious experimental data suggest that insulin secretion, glucokinase activity and NO production are relative to each other. It is well known that NO inhibits glucose-induced insulin secretion[2]. Glucokinase is expressed in pancreatic β cells and acts as a glucose sensor for insulin secretion[3]. Patients with glucokinase mutation display reduced insulin secretion, and therefore are at a high risk of developing diabetes[4].

In the present study, we examine the effects of *Lb*-

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PS on alloxan-induced rat islets through the variation of insulin, NO and MDA concentrations, glucokinase and SOD activity in culture medium or isolated pancreatic islets. We want to find the hypoglycemic mechanism of *Lb*-PS.

1 Materials and Methods

1.1 Materials

Lb-PS was obtained from Ninxia Pharmacological Institute, China. Alloxan, Ficoll (type 400-DL), collagenase P and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co.. The nitrite assay kit was obtained from Jiancheng Biological Institute, China. The insulin radioimmunoassay kit was obtained from Shanghai Biological Product Institute, China. Other chemicals were reagent grade.

1.2 Islet isolation and alloxan treatment[5]

Pancreatic islets were isolated from male Spraque-Dawley rats weighing 250–300 g in the morning after an overnight fast. Collagenase P was dissolved in Hanks balanced salt solution (HBSS) at a concentration of 1 mg/ml (pH 7.6). The solution was perfused via the duct of whole pancreas and warmed at 37 ℃ for 40 min for collagenase digestion. Then HBSS with 10%fetal calf serum (FCS) was injected to stop the digestion. The tissue was dissociated by tearing, and passed over filters with pore sizes of 200 μm. Aliquots of 4 ml were transferred to 50 ml tubes, and suspended in 4.3 ml of 5 × RPMI-1640 medium and 5 ml each of Ficoll with densities 1.085, 1.075 and 1.045. The tubes were centrifuged at 550 *g* for 25 min at 22 ℃. Islet-enriched layers were removed, washed and resuspended in RPMI-1640 medium with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37 ℃ in 5%CO_2 for up to 7 days.

Studies were performed 2 days after pancreatic islets harvest. 250–300 cultured islets were transferred to each pore of millipore chambers. AXN at the final concentration 4 mmol/L was added to RPMI-1640 medium with 10%FCS to make pancreatic islets damaged. 10^{-5},10^{-4},10^{-3},10^{-2} mg/ml *Lb*-PS was added to the medium respectively with AXN to study the protective effect of LBP.

1.3 Measurement of insulin

Insulin released from islets into culture medium and synthesized in the islets was monitored by radioimmunoassay (RIA). Test mediums were RPIM-1640 with 10% FCS and supplemented with (1) 2.7mmol/L glucose alone or with 10^{-5}–10^{-2} mg/ml *Lb*-PS, (2) 16.7 mmol/L glucose alone or with 10^{-5}–10^{-2} mg/ml *Lb*-PS, (3) 4 mmol/L AXN and 2.7 mmol/L glucose or with 10^{-5}–10^{-2} mg/ml *Lb*-PS, (4) 4 mmol/L AXN and 16.7 mmol/L glucose or with 10^{-5}–10^{-2} mg/ml *Lb*-PS. The pancreatic islets were incubated with different mediums for 24 h, then test mediums were removed and stored at −20 ℃ for subsequent insulin measurements. After test mediums were removed, the pancreatic islets was washed 3 times with Hanks and broken by freeze thawing 3 times. The freeze thawing liquid was stored at −20 ℃ for subsequent insulin measurements.

1.4 Measurement of glucokinase activity *in vitro*

The activity of glucokinase was spectrophotometrically measured. Control and treated islets were broken by freezing and thawing. The protein content in freeze thawing liquid was tested by the method reported by Lowry *et al.*[6]. The enzyme reaction was performed in solution consisting of (in mmol/L) 50 trisaminomethane, 6.0 $MgCl_2$, 0.5 or 100 glucose and 5 ATP (adjusted to pH 7.5 with HCl) at room temperature. Glucokinase activity was estimated through the variation of concentration of NADH based on the following reaction: glucose-6-phosphate + NAD → 6-phosphoglucono-lactone + NADH catalyzed by NAD-dependent glucose-6-phosphate dehydrogenase. Correction for hexokinase activity was applied by subtracting the activity measured at 0.5 mmol/L glucose from the activity measured at 100 mmol/L glucose. One unit was defined as the amount of enzyme which catalyzes the formation of 1 μmol/L NADH in a minute.

1.5 Measurement of nitrite

The nitrite production was measured by colorimetric assay. Nitrate in the culture medium was reduced to nitrite by adding sulfanilamide, and afterward, nitrite was converted to azo product by mixture with N-(1-Naphthyl)-ethylenediamine. The amount of nitrite was determined by photometric measurement of

the absorbance of emission from azo products (at 546 nm).

1.6 Measurement of superoxide dismutase activity

The assay of superoxide dismutase activity was based on the inhibition of nitroblue tetrazolium (NBT) photochemical reduction by superoxide dismutase[7]. The reaction mixture contained 0.013 mol/L methione, 1.3 $\times 10^{-6}$ mol/L riboflavin, 7.5 $\times 10^{-5}$ mol/L NBT, and 0.05 mol/L phosphate buffer (pH 7.8). 50 μl of freeze thawing liquid was added to a final 3 ml of reaction mixture. The reaction was initiated and lasted 15 min (37 ℃). The optical density at 560 nm was recorded.

1.7 Lipid peroxidation measurement

Lipid peroxidation levels were measured by the thiobarbituric acid method and expressed in terms of MDA formed[8].

1.8 Statistical analysis

The statistical analysis of results and the significance of the differences were evaluated by unpaired Student's *t* test, and $P < 0.05$ was considered significant. Results were expressed as $\bar{x} \pm s$ with the retorted *n* being the number of experiments from different islets isolations.

2 Results

2.1 The effect of *Lb*-PS on insulin secretion in AXN-induced islets

As illustrated in Table 1, 10^{-5}–10^{-2} mg/ml *Lb*-PS did not affect the insulin release at the basal level and insulin content in islets. But 16.7 mmol/L glucose enhanced the insulin release.

AXN inhibited the insulin secretion in the presence of 2.7 mmol/L and 16.7 mmol/L glucose, and also reduced the insulin content in islets obviously (Table 2).

In the 10^{-5}–10^{-4} mg/ml *Lb*-PS-treated groups, insulin secretions stimulated by 16.7 mmol/L glucose were partly inhibited by exposure to 4 mmol/L AXN. In the 10^{-3}–10^{-2}mg/ml LBP-treated groups, insulin secretions remained at levels similar to control. LBP could greatly increase the insulin contents in the AXN-induced islets (Table 2).

2.2 The effect of *Lb*-PS on glucokinase activity in AXN-induced islets

The glucokinase activity in islets exposed to AXN was significantly reduced, compared with the activity in control islets. In the 10^{-2} mg/ml *Lb*-PS -treated group, the glucokinase activity in islets exposed to AXN showed level similar to that in control (Table 3). The glucokinase activity in *Lb*-PS-treated groups, were all significantly higher than AXN-treated group, and the higher the *Lb*-PS concentration was, the more significant the effect on increasing glucokinase activity was.

2.3 The effect of LBP on NO production in AXN-induced islets

Table 1 The influence of LBP on insulin secretion and synthesis in normal islets (*n*=6)

Group	Insulin secretion at different concentration glucose (μU/ml)		Insulin content in cell (μU/ml)
	2.7 mmol/L glucose	16.7 mmol/L glucose	
Control	12.31±2.86	360.52±216.13	194.19±28.58
10^{-5} mg/ml *Lb*-PS-treated	9.95±1.44	321.16±116.52	200.31±29.61
10^{-4} mg/ml *Lb*-PS-treated	10.18±0.60	396.12±140.14	200.42±32.63
10^{-3} mg/ml *Lb*-PS-treated	10.62±0.19	340.46±77.56	215.03±28.28
10^{-2} mg/ml *Lb*-PS-treated	11.74±1.83	294.77±176.72	192.33±35.38

Table 2 The influence of LBP on insulin secretion and synthesis in control and AXN-induced islets (*n*=6)

Group	Insulin secretion at different concentration glucose (μU/ml)		Insulin content in cell (μU/ml)
	2.7 mmol/L glucose	16.7 mmol/L glucose	
Control	8.01±1.12	240.50±43.21	520.62±45.07
AXN-treated	5.93±0.45$^{\triangle}$	111.06±40.79$^{\triangle\triangle}$	270.32±80.62$^{\triangle\triangle}$
AXN and 10^{-5} mg/ml *Lb*-PS-treated	6.20±5.43$^{\triangle}$	143.00±48.03$^{*\triangle}$	380.65±65.23$^{*\triangle}$
AXN and 10^{-4} mg/ml *Lb*-PS-treated	6.53±2.62$^{\triangle}$	175.19±118.38$^{*\triangle}$	370.24±30.08$^{*\triangle}$
AXN and 10^{-3} mg/ml *Lb*-PS-treated	6.82±1.19$^{\triangle}$	232.61±162.82*	400.09±49.97$^{*\triangle}$
AXN and 10^{-2} mg/ml *Lb*-PS-treated	7.06±0.51$^{\triangle}$	210.36±70.20*	370.01±38.43$^{*\triangle}$

$^{*}P<0.05$ vs. AXN group; $^{\triangle\triangle}P<0.01$, $^{\triangle}P<0.05$, vs. control.

Table 3 The influence of *Lb*-PS on glucokinase activity in control and AXN-induced islets (n=6)

Group	Hexokinase activity (U/mg protein)	Low-K_m hexokinaseactivity (U/mg protein)	Glucokinase activity (U/mg protein)
Control	22.96±2.63	15.88±2.35	7.08±2.32
AXN-treated	18.09±1.08	14.36±1.02	3.73±1.06$^{\triangle}$
AXN and 10^{-5} mg/ml *Lb*-PS-treated	20.79±7.03	16.13±7.01	4.65±3.00$^{*\triangle}$
AXN and 10^{-4} mg/ml *Lb*-PS-treated	21.93±3.41	16.13±3.21	5.79±3.56$^{**\triangle}$
AXN and 10^{-3} mg/ml *Lb*-PS-treated	22.77±6.37	17.13±5.68	5.63±5.37$^{**\triangle}$
AXN and 10^{-2} mg/ml *Lb*-PS-treated	21.65±6.14	14.69±6.32	6.96±5.32**

$^{**}P$<0.01, $^{*}P$<0.05 vs. AXN group; $^{\triangle}P$<0.05 vs. control.

Table 4 The influence of *Lb*-PS on NO production in control and AXN- induced islets (n=6)

Group	NO concentration in culture medium (μmol/L)
Control	50.76±1.65
AXN-treated	68.50±1.22$^{\triangle\triangle}$
AXN and 10^{-5} mg/ml *Lb*-PS-treated	52.43±2.92**
AXN and 10^{-4} mg/ml *Lb*-PS-treated	51.74±0.48**
AXN and 10^{-3} mg/ml *Lb*-PS-treated	51.30±0.93**
AXN and 10^{-2} mg/ml *Lb*-PS-treated	49.12±1.45**

$^{**}P$<0.01 vs. AXN group; $^{\triangle\triangle}P$<0.01 vs. control.

Table 5 The influence of *Lb*-PS on SOD activity and MDA content in control and AXN-induced islets (n=6)

Group	SOD activity (NU/ml)	MDA content (nmol/10^5cells)
Control	10.02±0.56	8.76±0.17
AXN-treated	2.45±0.62$^{\triangle\triangle}$	10.49±0.36$^{\triangle}$
AXN and 10^{-5} mg/ml *Lb*-PS-treated	5.07±0.38$^{**\triangle}$	10.01±0.21$^{\triangle}$
AXN and 10^{-4} mg/ml *Lb*-PS-treated	6.40±0.16$^{**\triangle}$	9.45±0.26*
AXN and 10^{-3} mg/ml *Lb*-PS-treated	8.01±0.16**	9.22±0.18*
AXN and 10^{-2} mg/ml *Lb*-PS-treated	9.21±0.72**	8.92±0.04*

$^{**}P$<0.01 vs. AXN group; $^{\triangle\triangle}P$<0.01, $^{\triangle}P$<0.05 vs. control.

In the presence of AXN, the NO concentration in culture medium was 34.8% higher than in control. After treatment of rat islets with 10^{-5}–10^{-2} mg/ml *Lb*-PS, NO productions were decreased to the level similar to that in control (Table 4).

2.4 The effect of LBP on SOD activity and MDA content in AXN- induced islets

As illustrated in Table 5, AXN inhibited SOD activity in pancreatic islets significantly. The degree of increase in SOD activity was nearly proportional to *Lb*-PS concentration. MDA content was significantly higher in AXN-induced islets. Pretreatment with 10^{-4}–10^{-2} mg/ml *Lb*-PS decreased MDA in the pancreatic islets.

3 Discussion

AXN can especially damage pancreatic β cells because it is very active and ready to react with SH groups which are abundant in pancreatic β cells. AXN causes the free radical reactions in islets making mitochondria swollen and augmenting of superoxide anions (O_2^-) and hydroxyl radicals (OH·)[9]. O_2^- reacts with NO to produce $ONOO^-$. $ONOO^-$ has a stronger toxicity than O_2^- and OH·. In the previous studies, $ONOO^-$ has been detected in most pancreatic β cells from diabetes rats. We found there was a higher NO concentration in culture medium of AXN-treated islets. NO probably results in other chemical reactions to damage the structure and function of pancreatic β cells.

In rat islets incubated with 4 mmol/L AXN, the insulin release in response to glucose and insulin content in islets were selectively inhibited. In addition, glucokinase activity in the islets was reduced. It has been reported that NO inhibits insulin secretion, and the mechanism for the phenomenon is thought to be that NO inhibits phospholipase C activity[10], cAMP synthesis[11] and insulin mRNA synthesis and increase the production of free radicals[12]. Other studies show that glucokinase activity is suppressed by exposure to a low dose of NO. Glucokinase is a rate-limiting enzyme in glycolysis and its inhibition is known to result in impaired glucose-induced insulin secretion. Accordingly, our findings about the increased NO concentration stimulated by AXN implicate that AXN can disturbe glucokinase activity and glucose-induced insulin release in islets. This suggests that glucokinase is conformationally transformed in presence of NO, leading to the suppressive glucokinase activity.

Several different mechanisms could be hypothesized

to explain the *Lb*-PS's restoration of glucokinase activity, insulin synthesis and secretion which were disturbed by AXN. First, *Lb*-PS might scavenge NO or $ONOO^-$ from islets, or might scavenge O_2^- and OH· to reduce the $ONOO^-$. Zhang *et al.*[13] have showed that *Lb*-PS could eliminate OH· and protect cells from the damage of free radicals. Our experiment also revealed that *Lb*-PS increased SOD activity in pancreatic islets to scavenge the superoxide radical and inhibit lipid peroxidation. Second, *Lb*-PS might activate many of the sites responsible for augmentation of insulin release. Third, *Lb*-PS might facilitate repair of sites injured by AXN. The findings that glucose-induced insulin secretion and glucokinase activity in *Lb*-PS-treated islets exposed to AXN are similar to control islets strongly suggest that *Lb*-PS protects glucokinase activity from AXN attack, promotes glucose metabolism and insulin release in response to glucose. Finally, *Lb*-PS has a protective function on structural transformation of the enzyme. *Lb*-PS might protect glucose-induced insulin secretion from NO attack not only by reducing NO production, but also by maintaining glucokinase and SOD activity in pancreatic β cells. Analysis of variance showed that differences between the *Lb*-PS-treated groups and AXN-induced group were significant. But the results of *Lb*-PS-treated groups were similar to that in control. And protective effects of *Lb*-PS were more obvious as pretreatment with *Lb*-PS of higher concentration (10^{-3}–10^{-2} mg/ml). In this paper we proved the hypoglycemic effect of *Lb*-PS.

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枸杞多糖对四氧嘧啶损伤的大鼠胰岛细胞的保护作用

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摘要 报道了枸杞多糖（*Lb*-PS）对 4 mmol/L 四氧嘧啶（AXN）损伤的离体培养的大鼠胰岛细胞的保护作用。实验分为正常对照素、AXN 损伤组和 *Lb*-PS 保护组。采用放射免疫分析法测定胰岛细胞内胰岛素水平以及葡萄糖刺激的胰岛素释放水平。分光光度比色法测定细胞内 SOD 和葡萄糖激酶的活性，以及培养基中 NO 和 MDA 的含量。结果表明，AXN 显著抑制细胞内的胰岛素合成和葡萄糖刺激的胰岛素释放，以及 SOD 和葡萄糖激酶的活性。AXN 促使培养基中 NO 和 MDA 浓度的显著增加。在同时加入 AXN 和 10^{-5}~10^{-2} mg/ml *Lb*-PS 的实验组中，均发现能不同程度地保护胰岛细胞免受 AXN 的损伤。*Lb*-PS 能恢复 AXN 损伤的胰岛细胞的胰岛素合成和释放水平，以及 SOD 和葡萄糖激酶的活性，使其基本达到正常对照组的水平。*Lb*-PS 还能降低培养基中 NO 和 MDA 的浓度。因此，*Lb*-PS 可能通过减少胰岛 β 细胞的 NO 产量和维持 SOD 和葡萄糖激酶的活性，最终起到保护胰岛素合成和释放功能的作用。

关键词 枸杞多糖；四氧嘧啶；葡萄糖激酶；胰岛素；一氧化氮

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