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Effect of insulin on oxygen free radicals and oxidative phosphorylation in liver mitochondria of diabetic rats

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KEY WORDS insulin; experimental diabetes mellitus; mitochondria; free radicals; oxidative phosphorylation

ABSTRACT

AIM: To observe the effects of insulin on liver mitochondrial respiratory function, activity of H^+-ATPase, and superoxide anion free radicals production in diabetic rats. **METHODS**: Rats were injected iv with alloxan 40 mg/kg to induce diabetes. The liver mitochondrial respiratory function was assayed by measurement of oxygen consumption using a Clark oxygen electrode. Superoxide anion production was assayed using chemiluminescence method. Activities of H^+-ATPase were measured by luciferin-luciferase system and inorganic phosphorus's method. **RESULTS**: Insulin 1 U/kg sc daily for 9 weeks improved oxidative phosphorylation, respiratory rate state 3 ($P<0.05$), respiratory control ration ($P<0.01$), and ADP:O ratio ($P<0.01$), but there were no obvious effect on respiratory rate state 4 ($P>0.05$). In the insulin group, synthesis activity of H^+-ATPase was obviously increased ($P<0.05$) and hydrolytic activity of H^+-ATPase was remarkably decreased ($P<0.01$), compared with the diabetes group. Insulin 1 U/kg for 9 weeks apparently decreased the production of $O_2^{\cdot-}$ ($P<0.01$) in liver mitochondria of diabetic rats. **CONCLUSION**: Insulin can prevent the injury from superoxide anion in liver mitochondria, and improve the function of the liver mitochondria oxidative phosphorylation.

INTRODUCTION

Diabetes can induce chronic damage in many organs, such as kidney, liver, and heart. This pathological damage is associated with oxidative stress[1], overload of oxygen free radicals being an important perpetuator of oxidative stress. Mitochondria is not only a main cell organelle to produce free radicals, but is also sensitive to injury caused by overload of free radicals. The liver mitochondria, relative to the cori cycle, glucose-alanine cycle, and urea cycle, plays an important role in the metabolism of an organism.

Conventional insulin therapy has been used in insulin dependent diabetes mellitus to prevent hyperglycemia. It has been reported that insulin can treat the liver disease precipitated by type 1 diabetes mellitus[2,3]. However how insulin cures the liver injury is still not entirely understood. The aim of this study was to observe the effects of insulin on respiratory function and activities of H^+-ATPase in liver mitochondria of diabetic rats, and to assess whether insulin could prevent against the production of superoxide anion.

MATERIALS AND METHODS

Experimental animals Sprague-Dawley rats (male, Grade Ⅱ, Certificate № 006, weighing 200 g ± 20 g) were provided by Experimental Animal Center of Institute of Zoology, Chinese Academy of Sciences. The rats were divided into 3 groups, control, diabetic, and insulin treated group. In diabetic and insulin treated groups, alloxan monohydrate was injected through tail vein at 40 mg/kg. Control group was injected with saline. All the rats were fed normally.

Reagents 2-Methyl-6-(*p*-methoxyl)-3, 7-dihydroimidazol (1,2-a) pyrazin-3-one (MCLA), succinate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethansulfonsaure (HEPES), were purchased from Merck Company. Alloxan, edetic acid, rotenone, adensine diphosphate, bovine serum albumin, and superoxide dismutase were the products of Sigma Company. Insulin was produced by Shanghai Biochemistry Pharmaceutical Factory. Other reagents were of AR grade.

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Measurement of Glu and HbA1c The Glu and HbA1c were determined spectrophotometerically (BECKMAN DU-7000), using glucose kits and microcolumn chromatography, respectively.

Preparation of mitochondria Mitochondria was isolated from rat liver by standard differential centrifugation in a buffer containing sucrose 250 mmol/L, HEPES 2 mmol/L, edetic acid 0.1 mmol/L, pH 7.4, at 4 ℃. Mitochondrial protein content was determined by biuret reaction method using BSA as standard.

Measurement of oxygen consumption By using a Clark oxygen electrode (YSI Model 53 oxygen monitor, USA) the oxygen consumption rates of mitochondria in succinate-stimulated respiratory state 4 and ADP-stimulated respiratory state 3 were monitored at 25 ℃ in 3 mL medium, which contained sucrose 225 mmol/L, $MgCl_2$ 5 mmol/L, KH_2PO_4 10 mmol/L, KCl 20 mmol/L, Tris-HCl 2 mmol/L, and rotenone 4 μmol/L, pH 7.4. The concentration of mitochondria was 1.0 g/L in all the experiments. The oxygen consumption rates of mitochondria under different respiratory states, respiratory control ration (RCR), and ADP:O ratio were calculated[4].

Measurement of H^+-ATPase activity The H^+-ATPase synthesis activity was measured in luciferin-luciferase system. And inorganic phosphorus's method[5] was used to measure the H^+-ATPase hydrolytic activity. The results were described by 1250 Luminometer (LKB, USA).

Superoxide anion assay Using the chemiluminescence method[6], superoxide anions were measured at 25 ℃ in 1 mL buffer, which contained sucrose 300 mmol/L, KH_2PO_4 10 mmol/L, KCl 10 mmol/L, $MgCl_2$ 0.05 mmol/L, Tris-HCl 10 mmol/L, MCLA 2 μmol/L, rotenone 4 μmol/L, pH 7.4. Concentration of mitochondria was 0.1 g/L. Succinate 5 mmol/L was added to start the reaction. The results were described by 1250 Luminometer (LKB, USA).

Statistical analysis All data were shown as $\bar{x} \pm s$. Statistical analysis was performed using t test.

RESULTS

Effect of insulin on Glu and HbA1c in experimental rats The values of Glu and HbA1c in insulin group were obviously lower than those in diabetic group ($P < 0.01$, Tab 1).

Effect of insulin on production of $O_2^{\cdot-}$ in the diabetic rat liver mitochondria The $O_2^{\cdot-}$ values in normal, diabetic, and insulin groups were (0.8 ± 0.3), (1.4 ± 0.4), (0.72 ± 0.24) μmol·min^{-1}·g^{-1} protein, respectively. The production of $O_2^{\cdot-}$ in the diabetic rat were much higher, as compared to normal group ($P < 0.01$). The production of $O_2^{\cdot-}$ was obviously decreased due to insulin 1 U/kg sc for 9 weeks, compared with the diabetic group ($P < 0.01$, Fig 1).

Tab 1. Effect of insulin on Glu and HbA1c in experimental rats. $n = 7$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normal. $^fP < 0.01$ *vs* diabetes.

Group	Dose/ U·kg^{-1}	Glu/ mmol·L^{-1}	HbA1c/%
Normal	–	7.3±1.4	1.7±0.4
Diabetes	–	21.0±1.8^c	3.9±1.0^c
Insulin	1	8.9±1.3^f	1.9±0.6^f

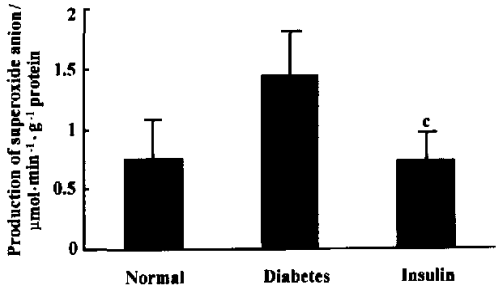

Fig 1. Effect of insulin on the production of superoxide anion. $^cP < 0.01$ *vs* diabetes. $n = 7$. $\bar{x} \pm s$.

Effect of insulin on H^+-ATPase activity in diabetic rat liver mitochondria The synthesis activity of H^+-ATPase in the diabetic group was lower than that in the normal group ($P < 0.05$), but the synthesis activity of H^+-ATPase in the insulin group was markedly increased ($P < 0.05$). The hydrolytic activity of H^+-ATPase was higher in the diabetic group than that in the normal group ($P < 0.01$), after treatment with insulin H^+-ATPase hydrolytic activity was decreased ($P < 0.01$, Tab 2), compared to the diabetic group.

Effect of insulin on the respiratory function of liver mitochondria The values of respiratory state 3, RCR, and ADP:O ratio in the diabetic group were remarkably decreased, compared to the normal group. The values of state 4 decreased slightly. In the insulin group, respiratory state 3, RCR, and ADP:O ra-

tio were obviously higher than those in the diabetic group. However, there was no obvious effect on respiratory state 4 ($P > 0.05$, Tab 3).

Tab 2. Effect of insulin on activity of H^+-ATPase in liver mitochondria in diabetic rats. $n = 7$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **normal.** $^eP < 0.05$, $^fP < 0.01$ *vs* **diabetes.**

Group	Dose/ $U \cdot kg^{-1}$	Synthesis activity of H^+-ATPase/ $U \cdot g^{-1}$ protein	Hydrolytic activity of H^+-ATPase/ $U \cdot g^{-1}$ protein
Normal	–	77 ± 19	1.62 ± 0.10
Diabetes	–	53 ± 14[b]	2.11 ± 0.15[c]
Insulin	1	72 ± 13[e]	1.74 ± 0.09[f]

DISCUSSION

In a long-term hyperglycemia state, the production of $O_2^{\cdot-}$ increases in the liver mitochondria. The increase in free radicals is the most important mechanism leading to mitochondria membrane injury[7,8]. Chronic ROS exposure can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids, and also inactivates the iron-sulfur (Fe-S) centers of ETC complexes Ⅰ, Ⅱ, and Ⅲ and TCA cycle aconitase, resulting in shutdown of the mitochondrial energy production[9]. But the effects of insulin on the production of superoxide anion have not been reported so far. In our experiments, the production of $O_2^{\cdot-}$ in liver mitochondria was apparently decreased after administration of insulin. It proves that insulin may prevent the production of $O_2^{\cdot-}$ and protect mitochondrial membrane structure. Thus, insulin-treatment protects the mitochondrial oxidative phosphorylation function.

RCR and ADP:O ratio, used as the respiratory function indices of mitochondria, are used to reflect the level of oxidative phosphorylation. The present results show that insulin can remarkably increase the RCR, ADP:O ratio. It indicates that insulin can improve the respiratory function of liver mitochondria. The results are similar to those reported by Kazue[2]. In our study. it was indicated that the effects were caused by regulation of respiratory state 3, but not by respiratory state 4.

On the other hand, in the diabetic group, the changes in H^+-ATPase synthesis activity and H^+-ATPase hydrolytic activity proved the damaged mitochondria ETC and the difficulty of ATP synthesis. After treatment with sc insulin, increase in synthesis activity of H^+-ATPase and decrease in hydrolytic activity of H^+-ATPase showed that insulin could enhance mitochondrial respiratory function and the synthesis of ATP. It also demonstrated that oxidative phosphorylation function was improved.

Thus, from the above results, it is indicated that insulin can inhibit the injury from superoxide anions in liver mitochondria, and improve the liver mitochondrial oxidative phosphorylation function. Thus the mechanism of insulin effect on diabetes is not only due to reducing the level of hyperglycemia, but also due to the effects on liver mitochondrial function.

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Tab 3. Effect of insulin on respiratory function in liver mitochondria. $n = 7$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **normal.** $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* **diabetes.**

Group	Dose/ $U \cdot kg^{-1}$	State 3/ $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein	State 4/ $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein	RCR	ADP:O ratio
Normal	–	90 ± 28	29 ± 7	3.0 ± 0.8	1.65 ± 0.14
Diabetes	–	34 ± 6[c]	20 ± 6[b]	1.6 ± 0.6[c]	1.15 ± 0.27[b]
Insulin	1	49 ± 12[e]	18 ± 4[d]	2.8 ± 0.4[f]	1.56 ± 0.18[f]

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胰岛素对糖尿病大鼠肝线粒体氧自由基和氧化磷酸化的影响

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关键词 胰岛素; 实验性糖尿病; 线粒体; 自由基; 氧化磷酸化

目的: 研究胰岛素对糖尿病大鼠肝线粒体功能, 质子 ATP 酶活力及超氧阴离子的影响. **方法**: 大鼠尾静脉注射四氧嘧啶,造成糖尿病动物模型. 离心制备线粒体, 测氧仪测定态 3 呼吸率、态 4 呼吸率、呼吸控制率和磷氧比. 用化学发光法测定超氧阴离子生成量, 质子 ATP 酶合成与水解活力分别采用荧光素-荧光素酶法和无机磷法测定. **结果**: (1)糖尿病大鼠超氧阴离子生成增多,皮下注射胰岛素治疗 9 周后,$O_2^{\cdot-}$生成量减少. (2)在糖尿病状态下,胰岛素治疗能提高质子 ATP 合成酶活力, 降低质子 ATP 水解酶活力. (3)胰岛素通过改善态 3 呼吸率调节呼吸控制率和磷氧比. **结论**: 胰岛素能抑制$O_2^{\cdot-}$生成,提高 ATP 的合成,改善肝线粒体氧化磷酸化功能.

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