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EFFECT OF INSULIN ON MITOCHONDRIAL RESPIRATORY CONTROL AND OXIDATIVE PHOSPHORYLATION OF ALLOXAN DIABETIC RATS

A POSSIBLE NEW ACTION OF INSULIN ON LIVER MITOCHONDRIA

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ABSTRACT

Respiratory control index and ADP/O ratio of the liver mitochondria from a group of alloxan diabetic rats were significantly decreased compared to a normal group.

Insulin treatment on alloxan diabetic rats improved both indexes up to the normal levels. However, insulin addition *in vitro* into mitochondria suspension had no effect on the both indexes.

It was found that insulin addition *in vitro* blocks the uncoupled respiration induced by 2, 4-dinitrophenol.

One of insulin actions, a disulfide hormone, *in vivo* was proposed to protect mitochondria from uncoupling factor in connection with thioldisulfide interchange to produce the conformational in mitochodria membrane.

The reason of reduced efficiency of oxidative phosphorylation in alloxan diabetic rats might be partly due to lack of protective action of insulin.

Recently it has become apparent that insulin has multiple physiological actions besides its hormonal action on carbohydrate metabolism. However, the mechanism of insulin actions has not been yet elucidated. There are many studies concerning the relationship between insulin and mitochondria. For example, Lee and Williams[1] demonstrated the binding of insulin with rat liver mitochondria, and Kaplan and Greenberg[2] claimed that insulin increased the formation of energy rich phsphate bonds. Hall *et al.*[3], also reported a decrease in P/O ratio of depancreatized rat and cat liver mitochondria. On

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the other hand, there are contradictory reports of no significant difference of oxidative phosphorylation in depancreatized cat[4], rat[4], alloxan diabetic rat[5] and rabbit[6]. The major reasons of contradiction seems to be that there were difficulties in preparating intact mitochondria in measuring accurately the efficiency of oxidative phosphoryration. Recent advances makes it possible to determine the intactness of mitochondria by accurate ADP/O ratio measurement. Chance and Williams[8] documented the respiratory control index (R. C. I.) as a parameter of intactness and efficiency of oxidative phosphorylation. The precise mechanism of mitochondrial respipatory control has not yet been established.

In this communication, it is intended to clarify the mechanism of insulin action *in vivo* on mitochondrial oxidative phosphorylation by measuring R. C. I. as well as ADP/O ratio of liver mitochondria of alloxan diabetic rats with and without insulin treatment. Furthermore, insulin action *in vitro* was studied by measuring its interaction with the mitochondrial membrane in the presence of an unconpler of oxidative phosphorylation.

EXPERIMENTAL

Alloxan Diabetic Rats—Wistar male rats (body weight around 200 g) were fed Oriental laboratory chew (Type M) with free water supply for at least 10 days in a temperature controlled room. Then, alloxan hexahydrate (5% w/v) dissolved in physiological salt solution was injected subcutaneously to rat. Doses of alloxan was 200 mg/kg weight of rat. One week after the injection, 0.1 ml of blood sample was taken from the tail vein after 4 hrs fasting. Eleven rats whose blood sugar level was over 200 mg% were specified as diabetic

TABLE 1. Preparation of mitochondria

3 g of rat liver + 27 ml of a mixture, 0.21 M in mannitol, 0.07 M in sucrose and 0.1 mM in EDTA

↓

homegenized with Polytoron homogenizer

↓

add 30 ml of the mixture

↓

centrifuge 5 min, 700 g at 5°C

↓

supernatant was centrifuged 10 min, 8000 g at 5°C

↓

sediments were homegenized with 30 ml of the mixture

↓

centrifuge 10 min, 8000 g at 5°C

↓

sediments were homogenized with appropriate volume of the mixture

group. The group was divided into 6 rats of the insulin treated group and 5 rats of untreated group. Insulin treatment was carried out for 2 weeks by subcutaneous injection of Lente insulin 2 Units per rat per day. Six rats of the non-diabetic control group was treated for 2 weeks by injecting only physiological salt solution. Alloxan diabetic rats were killed 3 weeks after alloxan injection. Rats were killed by decapitation after taking blood samples. The liver was homogenized by a Polytron homogenizer type 20 ST in a medium containing 0.21 M sucrose, 0.07 M mannitol and 0.1 M EDTA. The mitochondria were prepared by the procedure listed in Table I.

R. C. I. and ADP/O ratio measurement—For the measurement of respiration and phosphorylation of mitochondria, succinate was used as a substrate with a mixture, 0.25 M mannitol, 20 mM KCl, 10 mM phosphate, 2 mM Mg^{++}, and 0.2 mM EDTA, according to Chance and Hagihara[7]. The rate of oxygen uptake was measured by polarographic oxygen electrode (Beckman's oxygen sensor). The R. C. I. and ADP/O ratio were calculated by the method of Chance and Williams[8], viz.,

$$\text{ADP/O} = \mu\text{moles of ADP added}/\mu\text{atoms of oxygen consumpted}$$

$$\text{R. C. I.} = \frac{\text{rate of oxygen consumption with ADP}}{\text{rate of oxygen consumption without ADP}} = \frac{\text{rate of State 3 respiration}}{\text{rate of State 4 respiration}}$$

The protein content of mitochondria suspension was measured by Biuret method[10]. GIucagon free crystalline bovine insulin (24.8 U/mg) was supplied by Shimizu Co., oxytocin and vasopresin were purchased from Eli Lilly Company. The R. C. I. and ADP/O ratio with and without insulin were recorded on a single chart.

RESULTS

R. C. I. and ADP/O of alloxan diabetic and normal rats.—Mean values of R. C. I. and ADP/O of alloxan diabetic group were 3.82 ± 0.48 and 1.55 ± 0.12, respectively, and were significantly lower ($P \leqq 0.05$) than those of the normal group, 5.9 ± 0.67 and 1.80 ± 0.01, respectively, as shown in Table 2. These ratios were significantly restored ($P \leqq 0.05$) after insulin treatment for 2 weeks. Typical charts of R. C. I. and ADP/O measurement of normal and alloxan diabetic groups are shown in Fig. 1 and 2, respectively. Both R. C. I. and ADP/O were improved by insulin treatment correlating with improved fasting blood sugar level, as listed in Table 2.

TABLE 2. Respiratory control index and ADP/O ratio of liver mitochondria of the normal and the alloxan didbetic rats

	Normal rats						Alloxan diabetic rats: untreated group					Alloxan diabetic rats: insulin-treated group					
Rat No.	1	2	3	4	5	6	1	2	3	4	5	1	2	3	4	5	6
RCI	5.0	5.5	7.0	6.5	6.0	5.5	3.0	4.0	4.3	4.2	3.6	5.0	5.4	5.2	5.4	5.0	6.5
Means ± S. D.	5.92±0.67						3.82±0.48					5.42±9.51					
ADP/O	1.80	1.89	1.77	1.78	1.80	1.76	1.33	1.60	1.68	1.58	1.56	1.75	1.82	1.68	1.76	1.80	1.78
Means ± S. D.	1.80±0.01						1.55±0.12					1.77±0.01					
Fasting blood sugar	76	59	62	72	94	72	312	282	284	303	203	132	142	102	101	113	108
Means ± S. D.	72.5±11.3						276.8±12.2					116.3±15.6					

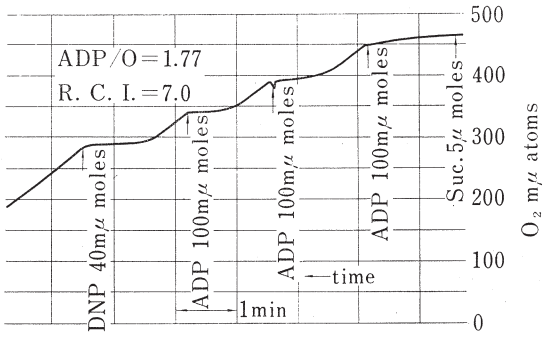

FIG. 1. Respiratory control and ADP/O of liver mitochondria of the normal rat.

The reaction mixture was 0.25 M in mannitol, 20 mM in KCl, 5 mM in phosphate, 2 mM in $MgCl_2$ and 0.2 mM in EDTA, it contained 1 mg protein of mitochondrria per ml. The total volume was 10 ml, the temperature, 30°C. The amounts of added substances per ml of the mixture are given on the figure. Changes in oxygen content in the mixture were recorded by a Beckman polarographic oxygen electrode (Clark type) with continuous stirring. The oxygen electrode was calibrated against air saturated distilled water at the appropriate temperature. pH of the whole reaction mixture was 7.4.

Effect of insulin in vitro. Insulin was added to mitochondria suspension at a final concentration of 1.2 m Units/ml of medium. However, no improvement of either R. C. I. or ADP/O was observed using mitochondria from alloxan treated diabetic rats. With insulin in the medium, the group not treated with insulin showed a mean R. C. I. value of 3.79 ± 0.64 and an ADP/O ratio of 1.55 ± 0.15. No significant difference was found with those values listed in Table 1, obtained without insulin in the medium. Insulin addition *in vitro* also showed no significant influence on the R. C. I. and ADP/O of mitochon-

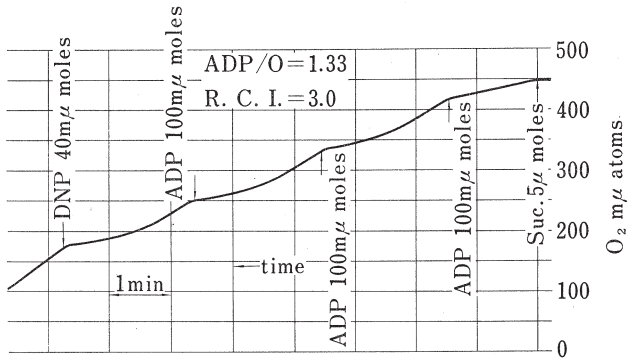

FIG. 2. Respiratory control and ADP/O of liver mitochondria of alloxan diabetic rat.

The experimental conditions were those described in Fig. 1, except the mitochondria were prepared from alloxan diabetic rat liver.

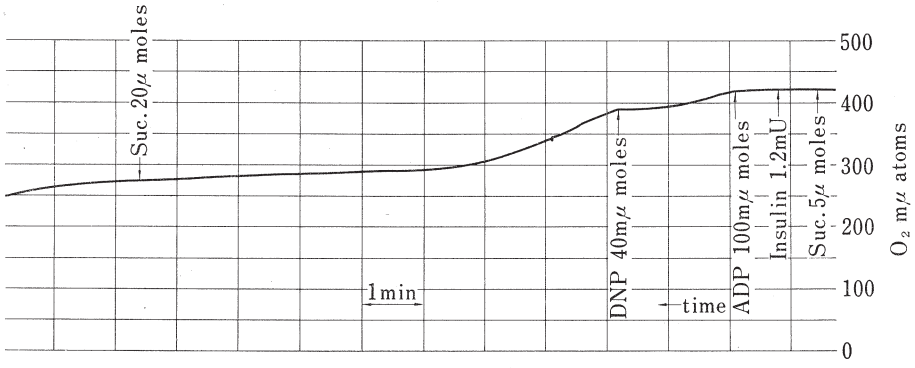

FIG. 3. Effect of insulin on the uncoupled respiration induced by 2, 4 dinitrophenol.

The experimental conditions were those described in Fig. 1. Insulin was added before 2, 4 dinitrophenol.

dria from the normal group. The mean values in this case were calculated to be 5.86 ± 0.67 and 1.83 ± 0.02, respectively.

On the other hand, it was found that insulin added *in vitro* (1.2 mUnits/ml of medium) prevented the uncoupling action of 2, 4-dinitrophenol (DNP). As shown in Fig. 3, insulin added to the medium did not influence either R. C. I. and ADP/O. However, respiration uncoupled by dinitrophenol was blocked after a one minute lag. This blocking action was found especially when insulin was added to the medium before uncoupled respiration. As shown in Fig. 4 and 5, tremendous amount of insulin (120 mUnits/ml of medium) was required to reveal the blocking of uncoupled respiration when insulin was added after uncoupled respiration started.

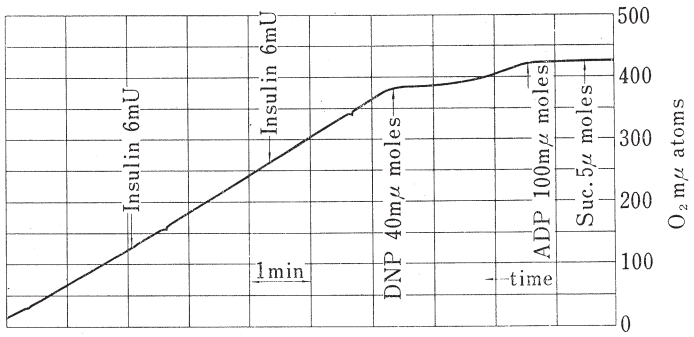

FIG. 4. Effect of insulin on the uncoupled respiration

The experimental conditions were those described in Fig. 1. Insulin was added after 2, 4 dinitrophenol.

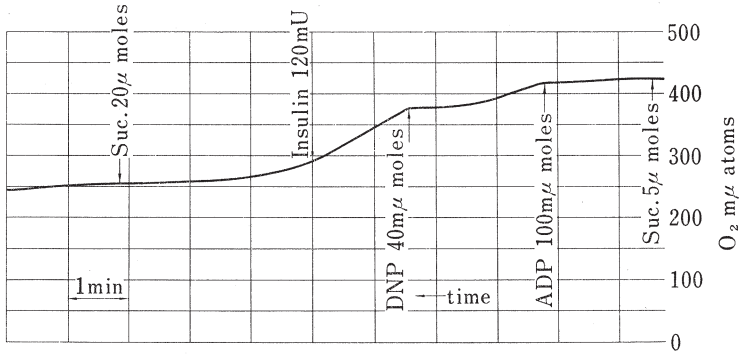

FIG. 5. Effect of insulin on the uncoupled respiration

The experimental conditions were those described in Fig. 1. A large amount (120 mU/ml) of insulin was added after 2, 4 dinitrophenol.

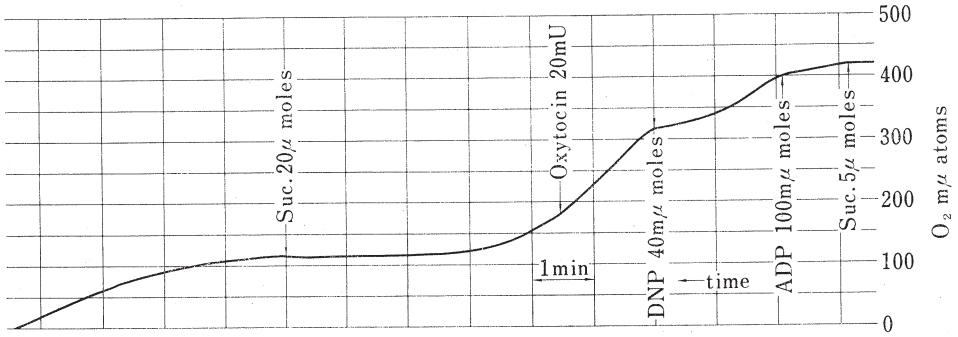

FIG. 6. Effect of oxytocin on the uncoupled respiration

The experimental conditions were those described in Fig. 1.

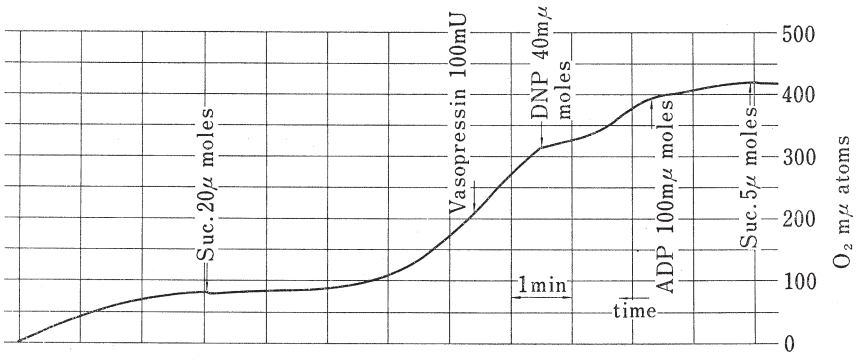

FIG. 7. Effect of vasopressin on the uncoupled respiration
The experimental conditions were those described in Fig. 1.

Effect of other polypetide hormones—With regard to structural similarity, other polypeptide hormones having disulfide linkage were examined for their possible blocking action on uncoupled respiration. As shown in Fig. 6 and 7, oxytocin and vasopressin acted on uncoupled respiration just like insulin. In the presence of these polypeptide hormones, uncoupled respiration induced by 2, 4-dinitrophenol was almost completely blocked. However, their blocking activities per unit were different.

DISCUSSION

There are many contradictory reports[3) 5)] on the action of insulin to oxidative phosphorylation, but little work has been reported on the respiratory control of alloxan diabetic rat mitochondria. As commented by Lardy and Wellman[11)], then by Chance and Williams[8)], respiratory control of mitochondria represents the efficiency of mitochondrial respiration to couple high energy phosphate bond formation. Low value of R. C. I. means that a certain amount of respiration was wasted without high energy bond formation, in other words, R. C. I. is a good indicator to show the intactness of mitochondria.

In this paper, it wss found that alloxan diabetic rat liver mitochondria had low R. C. I. and ADP/O. Some reports claim[5)] that liver mitochondria from alloxan treated diabetic rats exhibit normal oxidative phosphorylation. However, this discrepancy may be attributed to the difference of methodology in determining the efficieney of oxidative phosphorylation. As it is well known, R. C. I. is more sensitive with regards to intactness of mitochondria than P/O ratio. It was also found that addition of insulin *in vitro* to alloxan diabetic mitochondria does not improve both R. C. I. and ADP/O. However, as shown in Fig. 3, insulin even at physiological concentration does block the uncoupled respiration of normal mitochondria induced by an uncoupler, 2, 4-dinitrophenol.

These facts imply that insulin has some action *in vitro* on mitochondria to antagonize to uncoupling factor. As insulin could enter into liver cell, protective effect of insulin upon the mitochondrial oxidative phosphorylation from uncoupling factor might be one of important physiological action of insulin *in vivo*. The lowered R. C. I. and ADP/O of alloxan diabetic rat may be partly due to loss of protective effect of insulin, and partly due to increased susceptability of mitochondria to metabolic disorders caused by insulin deficiency.

Blocking action of insulin and other disufide hormones on uncoupled respiretion may indicate an interaction between insulin and sulfhydryl groups of the mitochondrial membrane that may be essential for conformational changes of mitochondria, such as swelling and shrinking. Even if the mitochondria are not the primary physiological target for all the disulfide hormones, it is remarkable that three different disulfide hormones having intense swelling activity were demonstrated to be blocking agents for uncoupled respiration. Schwarz *et al.*[12], have shown that these hormones bring about permeability changes in certain membranes, presumably by disulfide thiol interchange reactions between hormone and membrane protein to cause alternations in tertiary or quaternary structure of membrane proteins and thus in membrane permeability. Lehninger and Neubert[13] have suggested that this action of disulfide hormone may also occur in the mitochondrial membrane. Our results may indicate in turn that the mechanism of uncoupling and coupling of mitochondrial respiration is closely related to the membrane conformational changes in which -SH groups and to -S-S- groups in the membrane are critical, and also that one of the insulin action is to protect oxidative phosphoryation system from some uncoupling factor.

SUMMARY

1) Three week after alloxan injection, the liver mitochondria from a group of alloxan diabetic rats were examined for their respiratory control index and ADP/O ratio and compared with a normal group. Both indexes were significantly decreased in the case of alloxan diabetic rats.

2) Insulin treatment (2 Units per rat per day) on alloxan diabetic rats over two weeks improved both R. C. I. and ADP/O up to the normal values. However, insulin addition to mitochondrial suspension *in vitro* had no effect on both indexes.

3) It was found that insulin added *in vitro* blocked the uncoupled respiration induced by 2, 4-dinitrophenol. The same effects were observed when other polypeptide hormones having disulfide linkage, oxytocin and vasopressin, were added *in vitro*. One of insulin action *in vivo* was proposed to protect mitochondria from uncoupling factor in connection with -SH groups in eddition to -S-S- groups in mitochondrial membrane.

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