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UNCOUPLING EFFECT OF TOLBUTAMIDE ON OXIDATIVE PHOSPHORYLATION OF LIVER MITOCHONDRIA

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ABSTRACT

1. The effect of tolbutamide on the oxidation rate, respiratory control index and P/O ratio of intact rat liver mitochondria was studied.

2. Addition of more than 30 mg% tolbutamide apparently increased the state 4 respiration rate and decreased RCI, whereas, 80 mg% tolbutamide decreased markedly the state 3 respiration rate than that of control. State 4 respiration rate was accelerated by adding 100 mg% (3.8 mM) tolbutamide, while state 3 respiration rate was decreased and P/O ratio was reduced to zero showing complete uncoupling.

3. Uncoupling of oxidative phosphorylation by 100 mg% tolbutamide was similar to that of DNP, *i.e.*, sensitive to the addition of rutamyin, 100 mg% tolbutamide can induce ATPase activity of intact rat liver mitochondria, and induced ATPase activity was reduced to zero by the addition of rutamyin just like DNP. Therefore, uncoupling site by tolbutamide may be close to the DNP sensitive site.

INTRODUCTION

Tolbutamide* is now widely used for the treatment of diabetes mellitus for its hypoglycemic effect and is well known to stimulate the release of insulin from the pancreas. As for the side-effect of this drug, gastric intolerance and liver damage have been reported[1]. Further, the cardiovascular mortality rate was reported to be 75% higher in the tolbutamide treated when compared with placebo treatment[2]. Therefore it was necessary to clarify the action upon cell metabolism and pharmacological effect of this drug. Recently, Beer and Schepper have reported that 5 mM tolbutamide uncoupled the oxidative phosphorylation of the isolated rat liver mitochondria[3].

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* Tolbutamide: N-(4-Methyl-benzensulfonyl)N'-butylurea.

In this paper, we report an examination of the uncoupling effect and uncoupling site of tolbutamide in isolated and sonicated rat liver mitochondria.

MATERIALS AND METHODS

Preparation of Mitochondria: Rat liver mitochondria were freshly prepared as described by Hogeboom[4] using 0.21 M mannitol, 0.07 M sucrose, and 0.1 mM EDTA in 5 mM Tris-HCl at pH 7.4.

Measurement of Respiration: Oxygen consumption was measured polarographically using the Clark-type oxygen electrode (Beckman Co., Ltd.) at 25°C. The reaction mixture, unless otherwise indicated, contained 0.3 M mannitol, 10 mM Pi, 10 M KCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA in 20 mM Tris-HCl at pH 7.4, 10 mM succinate and 12 mg mitochondrial protein in a final volume of 5 ml. Mitochondrial protein concentration was determined by the Biuret method[5]. The effect of tolbutamide or ethanol on the state 3, state 4 respiration rate and respiratory control index (RCI) was tested by adding them to the medium before the addition of mitochondrial protein, sodium succinate and ADP. In another series of experiment tolbutamide or ethanol was added to the reaction medium approximately two minutes after the addition of ADP. 0.2 mM ADP was used for the determination of RCI. RCI was calculated by the method of Chance and Williams[6].

In some experiments the reaction medium without Pi was used as indicated in the legends of Table 2.

Measurement of P/O: For the determination of P/O, the reaction system contained 7.6 mg mitochondrial protein, 1 mM Pi, 10 mM sodium succinate, 0.2 mM ADP, 0.15 mg yeast hexokinase, type III, 10 mM glucose with or without 3.8 mM tolbutamide in a total volume of 5 ml.

Reaction mixtures were incubated for 5 minutes with continuous stirring with oxygen scensor. After 5 minutes incubation oxygen uptake by liver mitochondria in the reaction system was determined using an oxygen electrode. Pi in the reaction system was also determined by the method of Lindberg and Ernster[7]. pH was adjusted to 7.4 using 20 mM tris-HCl.

Sonication of Mitochondria: The mitochondrial suspension was sonicated for 2 minutes in a 5 A supersonic vibrator (20 K cycle Model UR-150 P, Tominaga Works, Ltd.).

The effects of uncouplers: To study the uncoupling effect of tolbutamide, 20 μM DNP, 400 μM Ca^{++} and rutamycin (2 μg/mg protein) were added respectively to the reaction system with 100 mg% tolbutamide, followed by intact liver mitochondria and 10 mM sodium succinate in this order, and oxygen uptake was measured using an oxygen electrode. The effect of these uncouplers upon the oxygen uptake of intact liver mitochondria without 100 mg% tolbutamide was also examined, and compared with those having 100 mg%

tolbutamide. In a control experiment, 2% ethanol was added to the medium.

ATPase Activity: ATPase activity induced by 50 μM DNP and 3.8 mM tolbutamide in the presence of 2.5 mM Mg was examined according to the method described by Martin and Dotty[8] as follows. The reaction system containing 7.6 mg mitochondrial protein of liver mitochondria, 2.5 mM Mg, 10 mM ATP, 0.3 M mannitol in 10 mM Tris-HCl at pH 7.4 in a total volume of 1 ml was incubated at 37°C for 5 minutes. It was further incubated for the following 3 minutes with 3.8 mM tolbutamide or 50 μM DNP in the presence or absence of rutamycin.

After incubation, liberated Pi was determined by the method of Lindberg and Ernster[7].

ATPase activity was expressed as liberated mμ moles Pi per minute per mitochondrial mg protein.

Reagents: Tolbutamide was purchased from Hochst Co., Ltd., Germany, and was dissolved in absolute ethanol. Each concentration of tolbutamide added before the addition of ADP was 10, 30, 50, 80, and 100 mg%. 100 mg% tolbutamide was equivalent to 3.8 mM tolbutamide.

The effect of various concentrations of ethanol (0.2, 0.6, 1.0, 1.6, and 2.0%) which were used in solubilizing tolbutamide on the respiration rate of liver mitochondria was tested as a control. In the case of adding tolbutamide and ethanol after the addition of ADP, 100 mg% tolbutamide or 2% ethanol was added to the reaction system approximately two minutes after the addition of ADP. Hexokinase (16 unit/mg solid) was purchased from Sigma, Co. Ltd.

RESULTS

Effect of Tolbutamide on Respiration Rate of Isolated Intact Liver Mitochondria

Table 1 shows the effect of various concentrations of tolbutamide and ethanol on the respiratory rate and RCI of intact rat liver mitochondria.

Tolbutamide and ethanol were added to the reaction medium before the addition of mitochondria, succinate and ADP. State 3, state 4 respiration rates and RCI were calculated.

As shown in Table 1 addition of more than 30 mg% tolbutamide apparently increased the state 4 respiration rate and decreased RCI, whereas 80 mg% tolbutamide markedly decreased the state 3 respiration rate as compared with those of control. By the addition of 100 mg% tolbutamide state 4 respiration rate was apparently accelerated and state 3 respiration rate was decreased showing complete uncoupling. On the contrary, ethanol addition has no apparent effect on the respiration rate and RCI, except the addition of 2% ethanol which marginally increased state 4 respiration rate and decreased RCI. In another series of experiments 100 mg% tolbutamide and 2% ethanol were added to the reaction system approximately two minutes after the addition

TABLE 1. Effect of Tolbutamide on Respiration Rate of Isolated Intact Rat Liver Mitochondria

Additions		Respiration rate (mμ atoms 0/min./ml)		
Ethanol (%)	Tolbutamide (mg%)	State 4	State 3	RCI
None	None	15.1	84.9	5.64
0.2	None	17.0	86.5	5.10
0.2	10	16.2	89.9	5.56
0.6	None	17.0	89.9	5.30
0.6	30	22.5	82.4	3.66
1.0	None	15.1	87.3	5.80
1.0	50	26.3	84.9	3.22
1.6	None	21.6	82.4	3.82
1.6	80	37.4	65.0	1.73
2.0	None	22.5	81.0	3.59
2.0	100	45.0	45.0	1.00

Oxygen uptake was measured polarographically with a Clark type oxygen electrode (Beckman Co., Ltd.) at 25°C. The reaction medium contained 0.3 M mannitol, 10 mM Pi, 10 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA, in 20 mM Tris-HCl at pH 7.4, 12 mg of mitochondrial protein, 10 mM succinate, 0.2 mM ADP, various concentration of tolbutamide (10–100 mg%) and/or ethanol (0.2–2%). Total volume was 5 ml. The amount of ethanol and tolbutamide added was tabulated in the table. The state 3 respiration rate, the state 4 respiration rate and RCI were measured according to the method reported by Chance and Williams.

of ADP as shown in Fig. 1. In A of Fig. 1, respiratory control and oxygen uptake of intact liver mitochondria were shown. As shown in B of Fig. 1, the adding of 2% ethanol to the reaction medium two minutes after the addition of ADP increased the state 4 respiration rate compared with those before the addition of ADP. The addition of 100 mg% tolbutamide to the reaction medium increased more apparently the state 4 respiration rate as indicated in C of Fig. 1. The addition of 20 μM DNP to the medium apparently increased the respiration rate of intact liver mitochondria with or without 2% ethanol. But, as shown in C of Fig. 1, DNP release of respiration was apparently decreased by 100 mg% tolbutamide. P/O ratio for normal liver mitochondria in this study was 2.0. P/O ratio was reduced to zero by 3.8 mM tolbutamide.

Effect of Various Uncouplers and Pi on the Reaction Medium on the Oxygen Uptake of the Intact or Sonicated Mitochondria

Table 2 shows the effect of sonication of liver mitochondria, various uncouplers and the effect of tolbutamide.

In the absence of 100 mg% tolbutamide, the oxygen consumption of the intact liver mitochondria was apparently increased by sonic oscillation and the oxygen consumption by the intact or sonicated liver mitochondria was

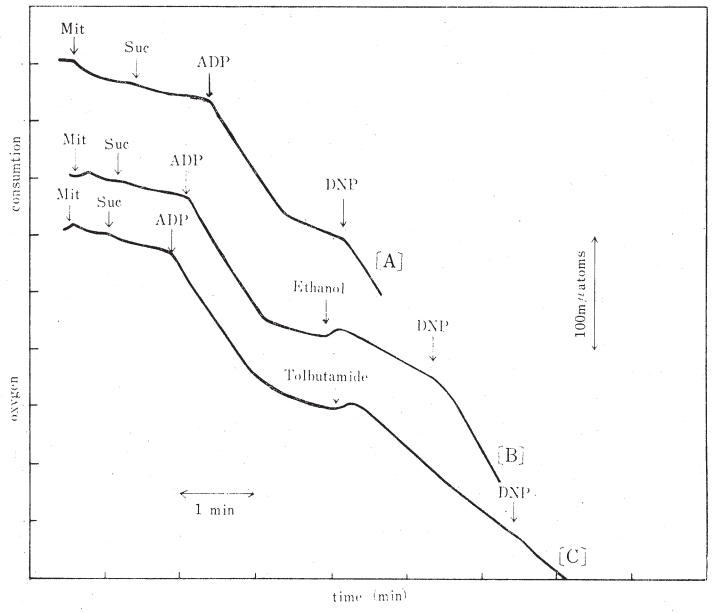

FIG. 1. The effect of tolbutamide and ethanol on the respiration rate and RCI of intact liver mitochondria.

Oxygen uptake of intact rat liver mitochondria was measured with the Clark type oxygen electrode. The original reaction medium contained 0.3 M mannitol, 10 mM Pi, 10 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA in 20 mM Tris-HCl at pH 7.4.

12 mg mitochondrial protein, 10 mM succinate and 0.2 mM ADP were added in this order, and state 4 respiration, state 3 respiration rate and RCI were calculated as described by Chance and Williams and then 100 mg% tolbutamide or 2% ethanol was added to the medium. 20 μM DNP was also added as indicated in the figure.

(A) shows the chart when intact liver mitochondria was used. In (B), 2% ethanol was added to the reaction medium two minutes after the addition of ADP and thereafter, 20 μM DNP was added. In (C), 100 mg% tolbutamide dissolved in ethanol was added two minutes after the addition of ADP as indicated in the figure. 20 μM DNP was also added.

not changed either by the absence or presence of Pi in the reaction medium. In this experiment, tolbutamide and various uncouplers were added to the reaction medium before the addition of mitochondrial protein and succinate. ADP was not added. In the intact mitochondria in the absence of uncouplers, 100 mg% tolbutamide increased the state 4 respiration rate regardless of the presence or absence of Pi in the medium. On the contrary, in the presence of 10 mM Pi, 100 mg% tolbutamide did not accentuate the oxidation rate of the sonicated liver mitochondria but decreased it. In the absence of 10 mM

TABLE 2. Effect of Various Uncouplers and Pi on the Oxygen Uptake of the Intact or Sonicated Rat Liver Mitochondria

Conditions	Uncouplers	Pi in the medium	Tolbutamide	
			−	+
			(mμ atoms 0/min./ml)	
Intact mitochondria	None	+	25	50
	None	−	26	32.5
	Rutamycin (2 μg/mg Protein)	+	27.5	52
	DNP (20 μM)	+	71	52.5
	Ca^{++} (400 μM)	+	71	52.5
Sonicated mitochondria	None	+	57.5	46.5
		−	56.5	24.5

The oxygen uptake of intact or sonicated liver mitochondria with or without various uncouplers was measured in the presence or absence of 100 mg% tolbutamide. Reaction mixtures with or without various uncouplers in the absence or presence of 100 mg% tolbutamide, 12 mg mitochondrial protein were added followed by 10 mM succinate.

The composition of the original reaction medium was the same as described in the legends of Table 1. In the reaction medium without Pi, 10 mM Pi in the medium was ommitted.

In another control experiment 2% ethanol was added to the system instead of 100 mg% tolbutamide. Oxygen uptake of intact or sonicated liver mitochondria was expressed as mμ atoms 0 consumed per minute per mg mitochondrial protein.

Pi, 100 mg% tolbutamide apparently decreased the respiration rate of sonicated liver mitochondria. It was of interest to note that the increase of state 4 respiration rate of intact mitochondria by 100 mg% tolbutamide was more apparent in the presence of Pi than in its absence.

The effect of various uncouplers upon the oxygen uptake of intact liver mitochondria and the uncoupled respiration by 100 mg% tolbutamide are listed in Table 2. In this experiment, various uncouplers with 100 mg% tolbutamide or 2% ethanol were added to the reaction medium with 10 mM Pi before the addition of intact liver mitochondria and succinate.

Rutamycin (2 μg per mg protein) had no effect upon the respiration uncoupled by 100 mg% tolbutamide. In the presence of 10 mM Pi, the oxygen uptake of intact mitochondria uncoupled by 100 mg% tolbutamide was 50 mμ atom, 0/min./ml. In the presence of both rutamycin and 100 mg% tolbutamide the oxygen uptake was 52 mμ atom 0/min./ml. This rate of oxygen uptake was almost the same as the data with only 100 mg% tolbutamide. The release of respiration by 20 μM DNP was apparently decreased by the addition of 100 mg% tolbutamide. The addition of 20 μM DNP increased the respiration rate to 71 mμ atom 0/min./ml. But the addition of both 100 mg% tolbutamide and 20 μM DNP decreased respiration rate to 52.5 mμ atom 0/min./ml. The uncoupled respiration of intact mitochondria by 400 μM Ca^{++} was decreased

by addition of 100 mg% tolbutamide. In conclusion, the uncoupling effect of tolbutamide was insensitive to rutamycin. The respiratory release by DNP or Ca^{++} was apparently decreased by 100 mg% tolbutamide.

ATPase Activity

Table 3 shows the ATPase activity induced by 50 μM DNP and 3.8 mM tolbutamide.

The effect of rutamycin on the ATPase activity induced by 50 μM DNP and 3.8 mM tolbutamide is also shown. 3.8 mM tolbutamide was found to increase ATPase activity of intact rat liver mitochondria just like 50 μM DNP. The addition of rutamycin apparently decreased ATPase activity of liver mitochondria induced by 50 μM DNP and 3.8 mM tolbutamide as shown in Table 3.

TABLE 3. ATPase Activity of Intact Rat Liver Mitochondria Uncoupled by Tolbutamide and DNP

Additions	ATPase (Pi mμ moles/min./mg protein)
None	0.2
50 μM DNP	87.5
50 μM DNP+Rutamycin	0
3.8 mM tolbutamide	53.6
3.8 mM tolbutamide+rutamycin	0

The reaction system in a total volume of 1.0 ml contained 2.5 mM Mg, 7.6 mg mitochondrial protein, 10 mM ATP, and 0.3 M mannitol in 10 mM Tris-HCl at pH 7.4. This system was incubated for 5 minutes, and then 3.8 mM tolbutamide, 50 μM DNP and rutamycin (2 μg/mg protein) were added to the system. After 3 minutes further incubation, liberated Pi was determined by the method of Lindberg and Ernster[7].

ATPase activity was expressed as liberated Pi mμ moles per minutes per mitochondrial mg protein.

DISCUSSION

Beer and Schepper have reported that uncoupling of oxidative phosphorylation of isolated intact liver mitochondria was induced by 5 mM tolbutamide and that uncoupling may explain in part the hypoglycemic action of tolbutamide[1]. They have reported that RCI of liver mitochondria was reduced to 1.0, but the effect of tolbutamide upon P/O ratio was not reported. In the present communication we have demonstrated that 3.8 mM tolbutamide reduced P/O ratio of liver mitochondria to zero and that RCI of liver mitochondria was reduced to 1.0 in agreement with the results of Beer and Scheppers.

ATPase activity of liver mitochondria was induced by 3.8 mM tolbutamide and it was completely inhibited by rutamycin.

These experimental facts suggest that 3.8 mM tolbutamide can be classified as uncoupler. It is probable that the decrease of cellular level of ATP induced by uncoupling may cause a reduction of gluconeogenesis resulting in hypoglycemia. It can not be concluded that the uncoupling effect of tolbutamide was partly responsible for the hypoglycemic action of tolbutamide, because the uncoupling effect of tolbutamide *in vivo* was not tested. It is interesting to mention that the uncoupling effect of tolbutamide was also demonstrated in the beef heart mitochondria (K. Katsumata and M. Hagihara).

The possibility that this uncoupling effect of tolbutamide on the liver and heart muscle mitochondria may have some relation to the side effect of tolbutamide such as liver damage and cardiovascular mortality rate. In the present paper, the effect of tolbutamide on the oxidation rate of liver mitochondria and the uncoupling site of tolbutamide were studied. As shown in Fig. 1, 100 mg% tolbutamide induced complete uncoupling regardless of the presence of ATP in the medium.

The observations that the uncoupling action of tolbutamide was similar to that of DNP, *i.e.*, sensitive to the addition of rutamycin, and induction of ATPase activity of intact rat liver mitochondria, suggest that the uncoupling site must be near the DNP sensitive site. The fact that respiratory release by 20 μM DNP or 400 μM Ca^{++} was decreased by the addition of 3.8 mM tolbutamide also supports this view. It was interesting that the increased oxidation rate by tolbutamide was decreased by the reaction medium without Pi even if mitochondria were disintegrated by sonic irradiation. But the pathogenesis of the effect of Pi upon the uncoupling action of tolbutamide is unknown. Further studies on the mitochondrial metabolism in relation to adverse effects and hypoglycemia caused by this drug are urgently needed.

SUMMARY

In vitro experiments 100 mg% tolbutamide is shown to be an uncoupler for mitochondrial oxidative phosphorylation. It can induce ATPase activity of liver mitochondria, whereas induced ATPase activity is reduced to zero by the addition of rutamycin. It is speculated that the uncoupling site may be close to the DNP sensitive site.

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