Nagoya J. med. Sci. 35: 141-150, 1973

UNCOUPLING EFFECT OF HYPOGLYCEMIC SULPHONYLUREAS ON THE ISOLATED RAT LIVER MITOCHONDRIA

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ABSTRACT

The effect of several hypoglycemic sulphonylureas upon the oxidative phosphorylation of intact rat liver mitochondria was examined. Following results were obtained and discussed in relation to hypoglycemic action and uncoupling site of these drugs.

1. All hypoglycemic sulphonylurea drugs tested, *i.e.*, tolbutamide, carbutamide, chlorpropamide, acetohexamide, glybenclamide, and glyclopyramide were found to be uncouplers to the intact rat liver mitochonbria.

2. They were also found to decrease respiratory release of 20 μM DNP.

3. Sulphonylureas could induce latent ATPase activity of intact rat liver mitochondria. ATPase activity induced by glybenclamide was reduced to zero by the addition of rutamycin just like DNP.

4. Judging from the above data, uncoupling site of sulphonylureas may be near DNP site.

INTRODUCTION

The hypoglycemic sulphonylureas, chlorpropamide, tolbutamide, carbutamide have been recently shown to be potent inhibitors of *in vitro* protein synthesis in rat tissues[1)2)]. Chlorpropamide and tolbutamide but not carbutamide have been also shown to increase oxygen uptake by rat liver mitochondria and to abolish respiratory control[3)].

Uncoupling effect of tolbutamide and chlorpropamide is undoubtedly relevant to many *in vitro* observations with these drugs such as the decrease of cellular level of ATP[3)]. The inhibition of protein synthesis by hypoglycemic sulphonylureas observed in *in vitro* experiment are supposedly the result of this uncoupling effect. But its significance with respect to the hypoglycemic

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Received for publication September 8, 1972.

action of these drug is not clear.

In this paper, the authors undertook to test the action of many hypoglycemic sulphonylureas including these drugs upon the isolated mitochondrial oxidation, phosphorylation and p/o ratio and found that all hypoglycemic sulphonylureas tested are uncouplers on the isolated mitochondrial preparations. In this paper the uncoupling effect of hypoglycemic sulphonylureas was discussed in ralation to the hypoglycemia. Uncoupling site of sulphonylurea was also discussed.

METHODS

Rat liver mitochondria were freshly prepared as described by G. H. Hogeboom[4] using the preparation mixture consisting of 0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA in 5 mM Tris-HCl, pH 7.4. Oxygen consumption was measured polarographically using the Clark type oxygen electrode (Beckman Co., Ltd.) at 25°C. The reaction mixture contained 0.3 M mannitol, 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA and 12 mg mitochondrial protein in a final volume of 5 ml, except where indicated.

Mitochondrial protein concentration was determined by the Biuret method using deoxycholate[5]. Carbutamide, chlorpropamide and tolbutamide were used by dissolving in absolute ethanol.

Glyclopyramide, acetohexamide and glybenclamide were used by dissolving in dimethylsuloxide (DMSO).

Various concentrations of sulphonylureas were added to the reaction medium before the addition of mitochondrial protein, succinate and ADP, and the minimum effective dose of sulphonylureas for complete uncoupling was examined. 10 mM succinate was used as substrate. 0.2 mM ADP was used for the determination of respiratory control index (RCI), which was calculated by the method of Chance and Williams[6]. Finally 20 μM DNP was added and respiratory release by 20 μM DNP and the effect of sulphonylureas upon this release were tested.

As control experiments, in stead of sulphonylureas, 2% and 6% ethanol, 2% and 4% DMSO were used.

The effect of ethanol and DMSO which were used in dissolving sulphonylureas on the respiration rate of liver mitochondria were also tested.

The effect of various hypoglycemic sulphonylureas upon p/o ratio of liver mitochondria was examined as follows.

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 hexokinase from yeast, type III, 10 mM glucose with or without 3.8 mM tolbutamide, 3.61 mM chlorpropamide, 5.52 mM carbutamide, 0.4 mM glybenclaminde, 3.08 mM acetohexamide, and 6.5 mM glyclopyramide.

Total volume was 5 ml, and was incubated for 5 minutes stirring with oxygen scenser. After 5 minutes incubation, oxygen uptake by liver mitochondria in the reaction system was determined by oxygen electrode. Pi in the reaction system was also determined by the method of Lindberg and Ernster[7]. pH of this system was adjusted to 7.4 by 20 mM Tris-HCl.

The effect of 20 μM DNP on the respiration rate of liver mitochondria with ethanol or DMSO was examined.

pH of the reaction medium was adjusted to 7.4.

The effect of various sulphonylureas on the latent ATPase acivity of intact rat liver mitochondria was examined as follows. The reaction mixture had a total volume of 1.0 ml which contained 10 mM Tris-HCl (pH 7.4), 0.3 M Mannitol, 10 mM ATP, and 5 mg protein of mitochondria. After 5 minutes of pre-incubation of the reaction mixture without 10 mM ATP various concentrations of sulphonylureas equivalent to the minimum amount for complete uncoupling and 30 μM DNP were added to the system, and then, the reaction was started by adding 10 mM ATP, at 37°C for 3 minutes and stopped by addition of 2 ml of silicotungstate.

Pi was determined in the supernatant according to the method of Lindberg and Ernster[7]. ATPase activity induced by 50 μM DNP and 0.4 mM glybenclamide in the presence of 2.5 mM Mg and the effect of rutamycin on the ATPase activity was examined as follows.

The reaction system had a total volume of 1.0 ml which contained 7.6 mg mitochondrial protein of liver mitochondria, 2.5 mM $MgCl_2$, 10 mM ATP, and 0.3 M mannitol in 10 mM Tris-HCl at pH 7.4. ATPase activity was determined with 0.4 mM glybenclamide or 50 μM DNP in the presence or absence of rutamycin by the method mentioned above.

After incubation, liberated Pi was determined by the method of Lindberg and Ernster[8].

ATPase activity was expressed as liberated Pi mμ moles per minute per mg mitochondrial protein. Tolbutamide and glybenclamide were kindly donated by HOCHST CO., LTD., Germany, chlorpropamide by PFEIZER CO., LTD., Germany, acetohexamide by SHIONOGI CO., LTD., Japan, carbutamide by ONO CO., LTD., Japan, glyclopyramide by KYORIN CO., LTD., Japan. ADP was obtained from SIGMA CHEMICAL CO., ST. LOUIS, MO. USA.

Hexokinase (16 units/mg solid) was purchased from SIGMA CO., LTD. USA.

RESULTS

The state 3, state 4 respiration rate and RCI of intact rat liver mitochondria are shown in Table 1. The addition of various sulphonylureas *i.e.*, tolbutamide, chlorpropamide, carbutamide, glybenclamide, acetohexamide and glyclopyramide induced complete uncoupling of oxidative phosphorylation of

TABLE 1. The Effect of Various Sulphonylureas upon Mitochondrial Oxidation Rate and RCI

No.	Addition Ethanol (%)	Addition DMSO (%)	Sulphonylurea	Respiration State 4	Respiration State 3	RCI
1	None	None	None	15.7	83.9	5.3
2	2	None	None	20.1	81.4	4.1
3	6	None	None	30.2	75.8	2.5
4	2	None	3.8 mM tolbutamide (100 mg%)	52.5	52.5	1.0
5	2	None	3.61 mM chlorpropamide (100 mg%)	60.3	60.3	1.0
6	6	None	5.52 mM carbutamide (150 mg%)	61.5	61.5	1.0
7	None	2	None	27.5	105.7	3.8
8	None	4	None	27.5	105.8	3.6
9	None	0.4	0.4 mM glybenclamide (20 mg%)	37.6	37.6	1.0
10	None	2	3.08 mM acetohexamide (100 mg%)	73.5	73.5	1.0
11	None	4	6.5 mM glyclopyramide (200 mg%)	61.5	61.5	1.0

Oxygen uptake was measured polarographically with the Clark type oxygen electrode (Beckman Co., Ltd.) at 25°C. The reaction medium contained 0.3 M mannitol, 10 mM Pi, 20 mM Tris-HCl (pH 7.4), 10 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA. Various hypoglycemic sulphonylureas, 12 mg of mitochondrial protein, 10 mM sodium succinate, 0.2 mM ADP. and 20 μM DNP were added to the medium in this order. They were added to the medium as shown in Fig. 1. As control, ethanol or DMSO was added in stead of sulphonylureas. The amount of ethanol, DMSO, added was tabulated in Table 1. The state 3 respiration rate, the state 4 respiration rate and RCI were measured according to the method reported by Chance and Willams[6].

The respiratory release by 20 μM DNP and the effect of various sulphonylureas upon the respiratory release by DNP were also examined.

intact rat liver mitochondria. The minimum effective dose for complete uncoupling of tolbutamide, chlorpamide and carbutamide in ethanolic solution were 3.8 mM, 3.61 mM and 5.52 mM respectively.

The minimum dose for complete uncoupling of glybenclamide, acetohexamide and glyclopyramide dissolved in DMSO was 0.4 mM, 3.08 mM and 6.5 mM. The state 3 respiration was most clearly decreased by 0.4 mM glybenclamide.

The effect of ethanol and DMSO upon state 3, state 4 respiration and RCI were also examined. As shown in Table 1, 2% ethanol addition increased state 4 respiration and decreased RCI to a small extent. 6% ethanol addition increased state 4 respiration rate to 30.2 mμ atoms 0/min/ml and decreased RCI, but not so apparent as those of sulphonylureas.

Addition of 2% and 4% DMSO increased state 4 and state 3 respiration and decreased RCI. Although solvents of sulphonylureas such as ethanol and DMSO increased slightly state 4 respiration and decreased RCI, the effect of

TABLE 2. The Effect of Sulphonylureas on p/o Ratio of Liver Mitochondria

Addition	p/o
None	2.0
3.8 mM tolbutamide	0
3.61 mM chlorpropamide	0
5.52 mM carbutamide	0.54
0.4 mM glybenclamide	0
3.0 mM acetohexamide	0
6.5 mM glyclopyramide	0

The reaction system contained 7.6 mg mitochondrial protein, 1 mM Pi, 10 mM sodium succinate, 0.2 mM ADP, 0.15 mg hexokinase from yeast Type III, 10 mM glucose with or without various sulphonylureas listed in the table. Total volume was made up to 5 ml, and was incubated for 5 minutes stirring with oxygen scenser. After 5 minutes incubation, oxygen uptake by liver mitochondria in the reaction system was determined by oxygen electrode.

Pi in the reaction system was also determined by the method of Lindberg and Ernster[7].

pH of this system was adjusted to 7.4 by 20 mM Tris-HCl.

hypoglycemic sulphonylurea tested upon respiration rate and RCI was more apparent than these solvents and all of the sulphonylureas tested in this experiment showed complete uncoupling.

The effect of glybenclamide on the oxidative phosphorylation of rat liver mitochondria was most apparent, and 0.4 mM glybenclamide increased state 3 respiration. The minimum amount of glybenclamide sufficient for complete uncoupling was 1/5-1/10 those of other hypoglycemic sulphonylureas. The effect of various sulphonylureas on the p/o ratio of liver mitochondria is shown in Table 2.

Table 2 shows that p/o ratio of normal liver mitochondria was 2.0, and it was reduced to zero by hypoglycemic sulphonylureas added, except carbutamide. Addition of 5.52 mM carbutamide reduced p/o ratio to 0.54.

The effect of the various concentrations of glybenclamide added to the medium on the oxidation rate and RCI is tabulated in Table 3. 0.1 mM glybenclamide increased markedly state 4 respiration and decreased RCI. More than 0.2 mM glybenclamide increased state 4 respiration and decreased both state 3 respiration and RCI.

Respiratory release of 20 μM DNP by intact rat liver mitochondria was 115 mμ atoms 0 per min per ml as shown in Table 4. Respiration by 20 μM DNP was not changed by 2% or 6% ethanol and 2% or 4% DMSO.

TABLE 3. The Effsct of Various Concentrations of Glybenclamide on the Mitochondrial Oxidation Rate and RCI

Additions		Respiration		RCI
Glybenclamide	DMSO(%)	State 4	State 3	
None	None	14.1	84.9	5.6
0.1 mM (5 mg%)	0.1	37.5	90.0	2.4
0.2 mM (10 mg%)	0.2	45.0	67.5	1.5
0.4 mM (20 mg%)	0.4	30.0	30.0	1.0
10 mM (50 mg%)	1	15.0	15.0	1.0
15.1 mM (100 mg%)	2	15.1	15.1	1.0

0.1-15.1 mM (5-100 mg%) glybenclamide and 0.1-2% DMSO were added to the reaction medium, and the effect of these substances upon the oxidation rate and RCI of intact rat liver mitochondria was examined.

Experimental condition was same as in Table 1, except that as sulphonylurea, glybenclamide dissolved in DMSO was used and that 0.1-2% DMSO was also used.

TABLE 4. The Effect of DNP on Uncoupled Respiration of Various Sulphonylureas

Additions			DNP Respiration
Ethanol (%)	DMSO (%)	Sulphonylurea	
None	None	None	115
2	None	None	122.5
6	None	None	112.5
2	None	3.8 mM tolbutamide	52.5
2	None	3.61 mM chlorpropamide	50
6	None	5.52 mM carbutamide	85
None	0.4	None	96
None	2.0	None	126
None	4.0	None	106.5
None	0.4	0.4 mM glybenclamide	28.0
None	2.0	3.08 mM acetohexamide	92.5
None	4.0	6.5 mM glyclopyramide	50.4

The effect of 20 μM DNP on the respiration rate of liver mitochondria with ethanol or DMSO was examined.

Various concentrations of sulphonylureas which were minimum amounts sufficient for complete uncoupling were added to the system which contained reaction medium as described in Table 1. Sodium succinate was added and thereafter 20 μM DNP was also added. Oxygen uptake of liver mitochondria after the addition of 20 μM DNP was estimated.

On the contrary, all of the hypoglycemic sulphonylureas tested in this experiment apparently decreased respiratory release by 20 μM DNP as shown in Table 4. The effect of glybenclamide upon the respiratory release of DNP

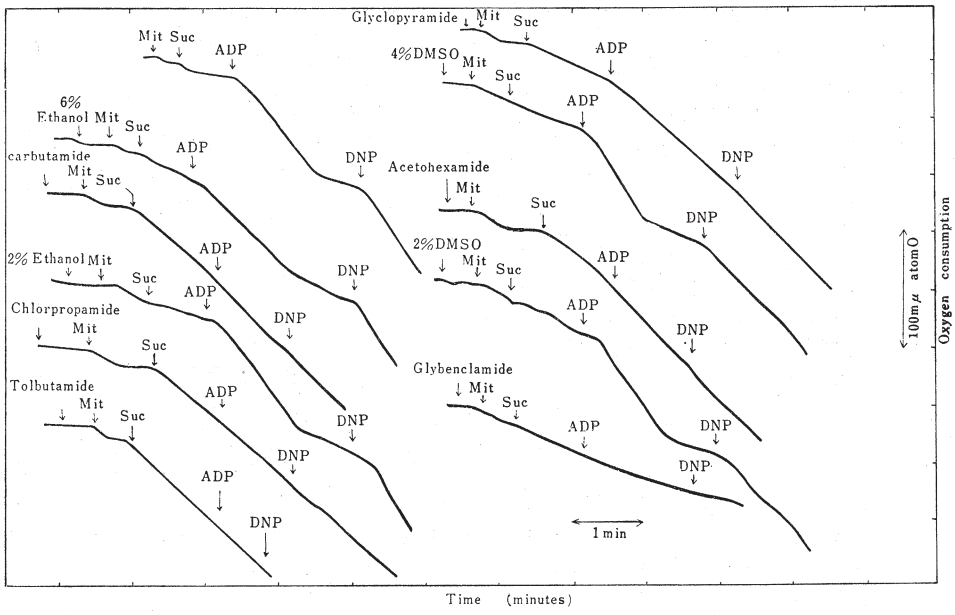

FIG. 1. The effect of various sulphonylurea on the mitochondrial respiration.

was most apparent.

0.4 mM glybenclamide decreased respiration of DNP to 28 mμ atoms 0/min/ml. All other sulphonylurea drugs, especially 3.8 mM tolbutamide, 3.61 mM chlorpropamide, and 6.5 mM glyclopyramide also decreased markedly respiratory release of 20 μM DNP.

The effect of hypoglycemic sulphonylureas upon the oxidative phosphorylation and DNP release of intact rat liver mitochondria are shown in Fig. 1. The effect of various sulphonylureas minimum concentration sufficient for complete uncoupling upon the ATPase activity was examined and tabulated in Table 5. All sulphonylureas tested were found to induce ATPase activity. Compared to the higher concentration of sulphonylureas, DNP could apparently induce ATPase activity at 30 μM. As control, in the absence of DNP and sulphonylureas, ATPase activity was almost zero. Whether ATPase activity induced by sulphonylureas was sensitive to rutamycin is important. ATPase activity induced by glybenclamide and DNP was examined. As shown in Table 6 ATPase activity of liver mitochondria in the presence of Mg was induced by 50 μM DNP or 0.4 mM glybenclamide, the addition of rutamycin to this system apparently reduced ATPase activity induced by DNP or glybenclamide to zero.

DISCUSSION

It was clearly shown that all of the hypoglycemic sulphonylureas includ-

TABLE 5. The Effect of Various Sulphonylureas upon the ATPase Activity of Intact Rat Liver Mitochondria

Sulphonylureas	DNP	ATPase Activity (mμ Pi liberated/mg protein/min)
None	None	0.1
None	30 μM	87.2
3.8 mM tolbutamide	None	81.4
3.61 mM chlorpropamide	None	18.6
5.52 mM carbutamide	None	32.3
0.4 mM glybenclamide	None	18.7
3.08 mM acetohexamide	None	144.5
6.5 mM glyclopyramide	None	114.3

The effect of various sulphonylureas upon the ATPase activity of intact rat liver mitochondria was examined. The reaction mixture had a total volume of 1.0 ml which contained 10 mM Tris-HCl (7.4), 0.3 M Mannitol, 10 mM ATP and 5 mg protein of mitochondria.

After 5 minutes pre-incubation of the system without 10 mM ATP, various concentrations of sulphonylureas and 30 μM DNP were added to the system and the reaction was started by adding 10 mM ATP, at 37°C for 3 minutes and stopped by addition of 2 ml of silicotungstate. Pi was determined in the supernatant according to the method of Lindberg and Ernster[7].

TABLE 6. ATPase activity of Intact Rat Liver Mitochondria Uncoupled by Glybenclamide

Addition	ATPase activity (Pi mμ moles/min/mg protein)
None	0
50 μM DNP	87.5
50 μM DNP+rutamycin	0
0.4 mM glybenclamide	42.6
0.4 mM glybenclamide+rutamycin	0

The reaction system had a total volume of 1.0 ml which contained 7.6 mg mitochondrial protein, 2.5 mM $MgCl_2$, 10 mM ATP, in 10 mM Tris-HCl at pH 7.4. The reaction system was incubated at 37°C for 5 minutes. This system was incubated at 37°C for 3 minutes with 0.4 mM glybenclamide or 50 μM DNP in the absence or the presence of rutamycin.

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

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

ing carbutamide tested in this experiment decreased state 3 respiration, increased state 4 respiration rate, and that p/o ratio of normal liver mitochondria was reduced to zero by sulphonylureas, except carbutamide, showing complete nncoupling. Beer and Schepper have reported that carbutamide

produced no influence on the oxidative phosphorylation of intact rat liver mitochondria. In the present communication carbutamide was found to reduce RCI and p/o ratio. The cause of the difference between Schepper's result and ours is unknown, but may be due to the difference of solvents. Scheppers *et al.* dissolved carbutamide in alkali solution[3].

The decrease of state 3 respiration rate was most pronounced in 20 mg% glybenclamide. In case of the addition of 2% and 6% ethanol, state 4 respiration rate was increased and RCI was decreased. In both 2% and 4% DMSO, state 4 respiration was increased and RCI was decreased, but the effect of ethanol and DMSO used in this experiment on the mitochondria oxidation rate was slight. Hypoglycemic sulphonylureas tested in this study were found to decrease respiratory release of 20 μM DNP. On the contrary ethanol and DMSO did not have any effect on the respiratory release of 20 μM DNP.

Judging from the fact that all of the sulphonylureas were shown to induce ATPase activity of intact rat liver mitochondria, and that ATPase activity induced by glybenclamide was reduced to zero by the addition of rutamycin just like DNP, uncoupling site of sulphonylureas may be near the DNP site.

As for the hypoglycemic action of sulphonylurea and liver, Bornstein described activity inhibition of 30% of alanine transaminase prepared from rat liver by carbutamide and tolbutamide *in vitro*, but only at 100 mg% sulphonylurea concentration[8]. *In vivo* inhibition of liver alanine transaminase can first be proved 24 hours after sulphonylurea administration[9].

As for uncouplers and blood glucose, Reid investigated the effect of DNP on the blood glucose of 8 female maturity-onset diabetes and reported that DNP can decrease blood glucose of maturity-onset diabetes to a small extent[10].

It is possible to suppose that uncoupling by sulphonylureas can cause a decrease of gluconeogenesis through the reduction of ATP in the liver. But the precise relation between the hypoglycemic effect of sulphonylurea and uncoupling is not apparent because *in vivo* effect of drugs on liver mitochondria has not been tested. Although the physiological significance of these uncoupling actions of sulphonylureas is unknown, the fact that all hypoglycemic sulphonylureas tested were uncouplers is interesting in respect to the hypoglycemic action of these drug.

SUMMARY

Chlorpropamide and tolbutamide have been shown to abolish respiratory control. In this paper, the authors clarified that all hypoglycemic sulphonylurees tested, *i.e.*, tolbutamide, carbutamide, chlorpropamide, acetohexamide, glybenclamide, and glyclopyramide were uncouplers to the intact rat liver mitochondria, and that the uncoupling site of sulphonylureas may be near the DNP site. This uncoupling effect was discussed in relation to the hypoglycemia.

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