IN VITRO EFFECT OF RIFAMPICIN AND ITS DERIVATIVES ON ENERGY TRANSFER REACTIONS IN MITOCHONDRIA

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In order to clarify the biological effects of rifampicin and its derivatives on liver cells, the actions of these antibiotics on the energy transfer reactions were observed in rat liver mitochondria. Rifampicin, 25-desacetyl rifampicin and rifampicin quinone at concentrations of $2.8\times10^{-5}\sim2.7\times10^{-4}$ M had little effect on the respiration and several energy transfer reactions in rat liver mitochondria, while similar concentrations of 3-formyl rifamycin SV caused a marked decrease in respiratory control and ADP : O ratio with enhancing state 4 respiration, activated Mg^{++}-dependent ATPase and also inhibited ATP-^{32}Pi exchange reaction. These results suggest that 3-formyl rifamycin SV, though not rifampicin itself, acts as a true uncoupler *in vitro* on rat liver mitochondria and its uncoupling action is fairly powerful.

The antibiotic rifampicin,[1] a semisynthetic derivative of rifamycins[2] isolated from *Streptomyces mediterranei*, possesses a broad antibacterial activity against both gram-positive and gram-negative organisms.[2~4] Rifampicin is also very active both *in vitro* and *in vivo* against *Mycobacterium tuberculosis*[2,5~8] and used routinely as an effective antituberculous drug. Its primary action on bacteria is the inhibition of microbial cell growth by binding to the DNA-dependent RNA polymerase and preventing the stabilization of DNA-enzyme complex.[9~11] Thus rifampicin inhibits both *in vitro*[9~12] and *in vivo*[13] the bacterial DNA-dependent RNA synthesis, whereas no similar inhibition occurs in the mammalian DNA-dependent RNA polymerase.[12,14,15]

In accordance with an increase in application of rifampicin to pulmonary tuberculosis, on the other hand, side effects caused by the administration of this antibiotic, such as liver functional disorders,[16~18] leukopenia[19] or thrombopenia,[19~21] allergy-like symptoms,[21~24] *etc.* have been reported. It has therefore become necessary to investigate the mechanism by which these side effects occur. Since drugs are metabolized chiefly in liver when they are introduced into the body, experiments were planned to clarify a relationship between liver functional disorders after the administration of rifampicin and the mitochondrial functions. The present paper describes the actions of rifampicin and its metabolites on the respiration and several energy transfer reactions in isolated liver mitochondria.

Materials and Methods

Animals and Mitochondrial Preparation

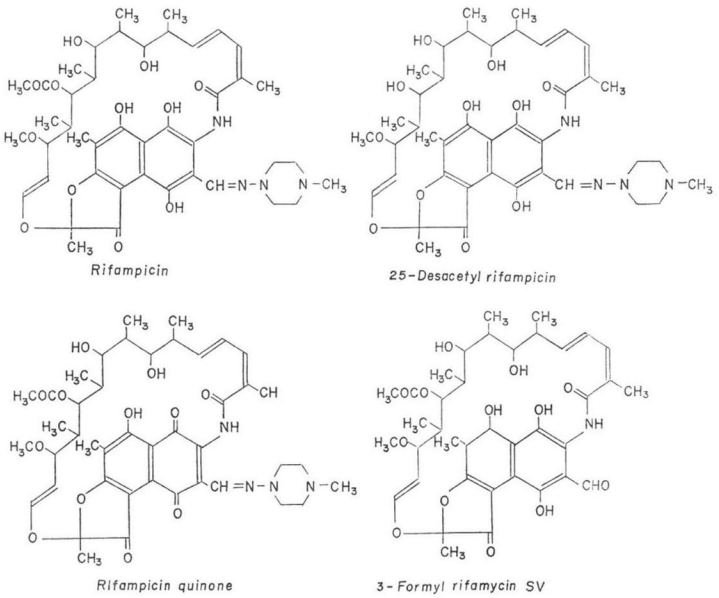
Male Donryu-strain rats weighing 150~200 g fed on a laboratory stock diet (General MR-1, Experimental Animals Kansai Kenkyusho Co. Ltd., Osaka) were fasted overnight. The rats were sacrificed by decapitation and liver mitochondria were isolated according to the method of UTSUMI *et al.*[25] in a medium containing 0.33 M sucrose, 1 mM EDTA and 5 mM Tris-HCl

(pH 7.4). The isolated mitochondria were finally resuspended in the isolation medium and stored in an icebox until use.

Experimental Procedure

Oxygen consumption, oxidative phosphorylation and respiratory control of mitochondria were assayed by bioxygraph connected to a Galvani electrode[26] (Kyusui Kagaku Kenkyusho Co. Ltd., Tokyo). Mitochondria (1.5~2.5 mg protein/ml) were added to a chamber containing 2.5 ml of a medium consisting of 10 mM KH_2PO_4, 80 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.2). At appropriate intervals during the incubation, respiratory substrate, rifampicin or its derivative and ADP were added to the incubation mixture and the oxygen uptake was traced by an autorecorder. The incubation was carried out at 25°C with continuous stirring with a magnetic stirrer. ADP : O ratio and respiratory control of mitochondria were calculated by the method of HAGIHARA.[27]

Fig. 1. Structural formulas of rifampicin and its derivatives.



ATPase activities were determined by the method of TAKAHASHI[28] by measuring Pi liberated from the added ATP at 25°C in a medium consisting of 0.05 M sucrose, 0.02 M KCl, 5 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1 mM EDTA and mitochondria in final volume of 2.0 ml. The ATP-^{32}Pi exchange reaction was performed by using 1 μCi (^{32}P)-orthophosphoric acid in 3 mM phosphate buffer (pH 7.4) and 3 mM ATP at 25°C in the medum described above, and the formation of (^{32}P)-ATP was determined.[29,30] The other details of the experiments are described in the legends of the figures or in the tables.

Chemicals

Rifampicin, 25-desacetyl rifampicin (DA-RFP), rifampicin quinone (RFP-Q) and 3-formyl rifamycin SV (3F-RFM SV) used in the experiments were donated by Daiichi Seiyaku Co. Ltd., Tokyo. Their structural formulas are shown in Fig. 1. (^{32}P)-Orthophosphoric acid (specific activity carrier free) was purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo. ADP and ATP were obtained from the Sigma Chemical Company and the other chemicals were from the commercial products of the highest purity.

Results and Discussion

Several investigations[31,32~34] have been reported concerning the metabolism of rifampicin in the human body, confirming that DA-RFP is a main metabolic product from rifampicin. SUNAHARA *et al.*[35] and SANO *et al.*,[36] having examined urine from tuberculous patients treated with rifampicin, found RFP-Q and some quantity of 3F-RFM SV besides DA-RFP and rifampicin itself. It is necessary, therefore, to examine the effects of these metabolites as well as rifampicin itself. Thus actions of rifampicin and its metabolites on mitochondrial functions,

Table 1. Effect of rifampicin and its derivatives on the respiration and oxidative phosphorylation in mitochondria isolated from livers of normal rats

Mitochondria (4.1 mg protein) were added to a reaction mixture consisting of 10 mM KH_2PO_4, 80 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.2) in a final volume of 2.5 ml. Rifampicin or its derivative was added, respectively, after the addition of 10 μmoles sodium succinate as a respiratory substrate. Then 500 nmoles sodium ADP was added, changes in the rate of oxygen uptake were traced by an autorecorder. Experiments were carried out at 25°C. Each value represents the mean from three trials. Oxygen uptake shows the substrate-level respiration. The abbreviations used are: RFP, rifampicin; DA-RFP, 25-desacetyl rifampicin; RFP-Q, rifampicin quinone; 3F-RFM SV, 3-formyl rifamycin SV.

Additions (M)		Oxygen uptake (natoms/min. per mg protein)	Respiratory control index	ADP : O ratio
None		21.0	4.9	2.0
RFP	2.8×10^{-5}	21.2	5.0	1.9
DA-RFP	2.8×10^{-5}	20.9	4.7	1.8
RFP-Q	2.8×10^{-5}	22.1	4.7	1.7
3F-RFM SV	7×10^{-6}	39.2	2.3	1.6
	1.4×10^{-5}	51.3	1.9	1.4
	2.8×10^{-5}	67.8	1.5	1.1

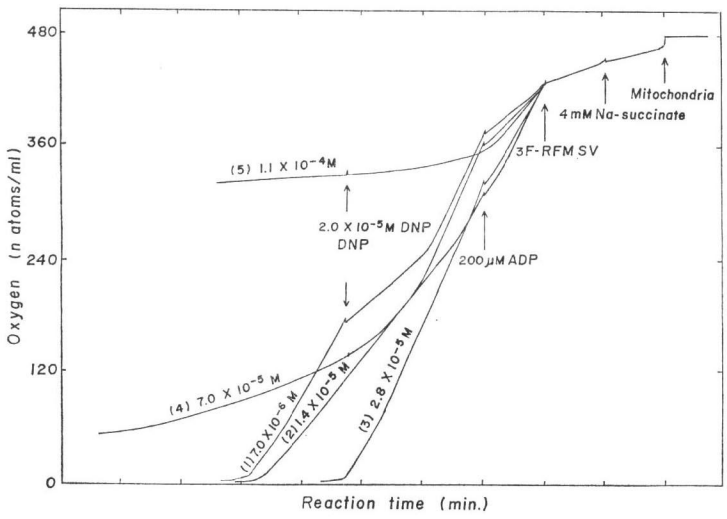
Table 2. Effect of rifampicin and its derivatives on the respiration and oxidative phosphorylation in mitochondria isolated from livers of normal rats

6.0 mg protein of mitochondria and 10 μmoles sodium isocitrate as a respiratory substrate were used. Other conditions were as in Table 1.

Additions (M)		Oxygen uptake (natoms/min. per mg protein)	Respiratory control index	ADP : O ratio
None		15.5	4.6	2.4
RFP	2.8×10^{-5}	17.5	4.6	2.6
DA-RFP	2.8×10^{-5}	16.6	5.8	2.7
RFP-Q	2.8×10^{-5}	16.5	4.7	2.8
3F-RFM SV	7×10^{-6}	25.5	3.6	2.6
	1.4×10^{-5}	33.9	2.4	2.2
	2.8×10^{-5}	43.1	1.4	1.5

Fig. 2. Effect of 3-formyl rifamycin SV on the respiratory activity of rat liver mitochondria.

4.1 mg protein of mitochondria were used.
Other conditions were as in Table 1.

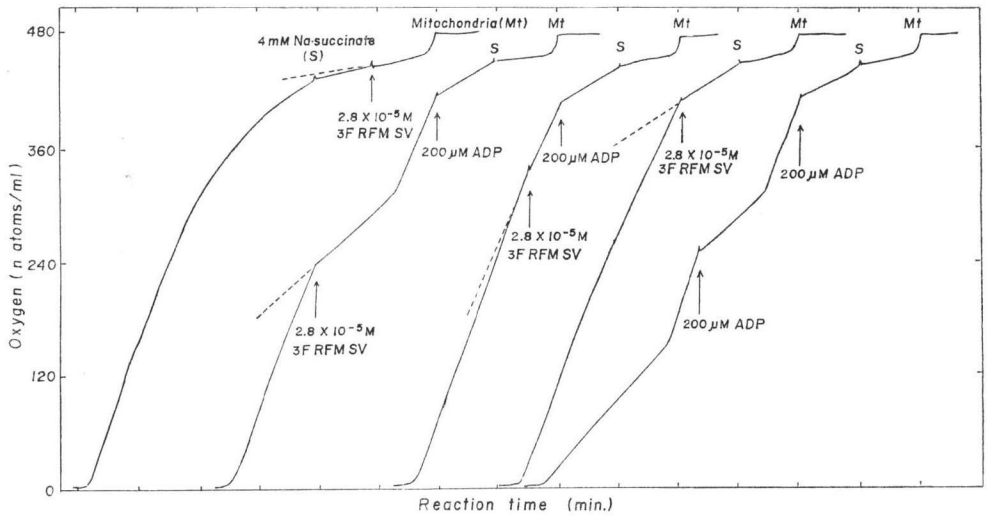


such as respiratory control, oxidative phosphorylation, ATPase activity and ATP-^{32}Pi exchange reaction were examined in a higher animal.

Effect on Respiration and Oxidative Phosphorylation

The effects of rifampicin and its derivatives on the respiration and oxidative phosphorylation of mitochondria isolated from livers of normal rats are summarized in Table 1

Fig. 3. Effect of 3-formyl rifamycin SV on various states of the respiration in rat liver mitochondria. 6.0 mg protein of mitochondria were used. Other conditions were as in Table 1.



and Table 2. As shown in these tables, rifampicin, DA-RFP and RFP-Q did not cause any appreciable effect on the succinate-linked or isocitrate-linked respiration. 3F-RFM SV in a similar concentration, however, caused a marked decrease in respiratory control with enhancing state 4 respiration. This rifampicin derivative also caused a decrease in ADP : O ratio.

These effects of 3F-RFM SV resemble those observed with 2,4-dinitrophenol (DNP) which is a well known uncoupler.[37,38] So it seemed important to study whether these effects were dose-dependent. As shown in Fig. 2, the enhancement of state 4 respiration is proportional to the concentration of the antibiotic in the ranges of $7\times10^{-6}\sim7\times10^{-5}$ M. It may be also noted that 7×10^{-5} M 3F-RFM SV causes a gradual suppression in oxygen consumption after the rapid respiratory release. These effects are clearly enhanced by increasing 3F-RFM SV concentration and the suppressed state 4 respiration cannot be released by DNP. Furthermore, as shown in Fig. 3, 3F-RFM SV caused a clear respiratory release of all the states of respiration.

Effect on Latent ATPase Activity

The enhanced respiration by 3F-RFM SV was completely stopped by an addition of 10^{-4} M KCN. This, together with the decreases in ADP : O ratio and respirtory control, suggests that 3F-RFM SV may uncouple oxidative phsphorylation in mitochondria. These data also suggest that 3F-RFM SV may cause other effects similar to those induced by DNP. Thus the effect of rifampicin and its derivatives on ATPase activities was investigated (Table 3). Rifampicin, DA-RFP and RFP-Q at the concentration of 3.4×10^{-5} M or 2.7×10^{-4} M had little effect on ATPase activities both in the absence and in the presence of DNP, whereas 3F-RFM SV 3.4×10^{-5} M gave a 140 % stimulation of the ATPase activity of mitochondria in the absence of DNP. More remarkable stimulation of ATP hydrolysis was observed with a higher concentration of 3F-RFM SV (Fig. 4). But it did not cause any effects on the DNP-depenent latent ATPase activity.

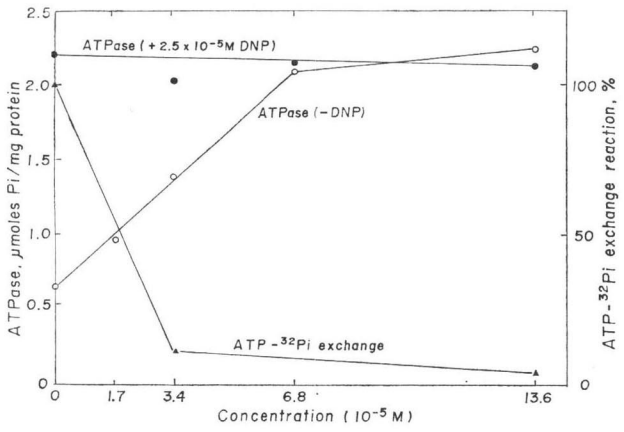
Table 3. Effect of rifampicin and its derivatives on ATPase activity

Rifampicin or its derivative (68 nmoles and 540 nmoles, respectively), 6 μmoles sodium ATP and mitochondria (3.4 mg protein) were added to 1.5 ml of a medium consisting of 0.05 M sucrose, 0.02 M KCl, 5 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$ and 0.1 mM EDTA to give the total volume 2.0 ml. After incubation for 15 min. at 25°C, 1 ml of ice-cold PCA (24 %) was added, well mixed, left standing for 10 min. and then the supernatant was separated by a centrifugation. One ml of the supernatant was removed to a test tube with a stopper containing 1 ml of 1.5 N H_2SO_4, 1 ml of 2 % ammonium molybdate and 4 ml of isobutanol, shaken vigorously for 10 seconds and left standing. Two ml of the upper isobutanol phase was removed into another test tube, 2 ml of 0.5 % ascorbic acid (prepared with 0.05 % sodium bicarbonate solution when used) and 1 ml of ethanol were added, well mixed and incubated for 45 min. at 37°C. After cooled in running water, inorganic phosphate liberated was measured spectrophotometrically at the wave length 700 nm.[28]

Additions (M)		Pi liberated (μmoles Pi/mg protein per 15 min.)	
		−DNP	+DNP (2.5×10^{-5} M)
None		0.60	1.75
Azide	5×10^{-5}	0.28	1.04
RFP	3.4×10^{-5}	0.65	1.63
	2.7×10^{-4}	0.70	1.57
DA-RFP	3.4×10^{-5}	0.63	1.68
	2.7×10^{-4}	0.64	1.70
RFP-Q	3.4×10^{-5}	0.63	1.66
	2.7×10^{-4}	0.67	1.57
3F-RFM SV	3.4×10^{-5}	1.45	1.77
	2.7×10^{-4}	1.68	1.72

Fig. 4. Effect of 3-formyl rifamycin SV on latent ATPase and ATP-^{32}Pi exchange reaction.

Conditions were as in Tables 3 and 4.



Effect on ATP-^{32}Pi Exchange Reaction

Since the action of 3F-RFM SV on the mitochondrial ATPase activity is quite similar to that of DNP, 3F-RFM SV could be a potent inhibitor of ATP-^{32}Pi exchange reaction.

As shown in Table 4, 3F-RFM SV strongly inhibited the ATP-^{32}Pi exchange reaction but rifampicin, DA-RFP and RFP-Q did not cause any practical inhibition. In Fig. 4 the inhibitory effect of different concentration of 3F-RFM SV on the ATP-^{32}Pi exchange reaction and ATPase activities are summarized: the product enhances latent ATPase activity and inhibits ATP-^{32}Pi exchange reaction; these actions are quite similar to those observed with DNP.

Altogether the results show that rifampicin itself has little influence on the phosphorylating respiration of isolated rat liver mitochondria. However, 3F-RFM SV obviously stimulates all the states of respiration and latent ATPase activity, and inhibits respiratory control, oxidative phosphorylation and ATP-^{32}Pi exchange reaction.

It may be concluded, therefore, that 3F-RFM SV, though not rifampicin itself, acts as a true uncoupler on rat liver mitochondria. Furthermore its effects *in vitro* on mitochondria is fairly powerful, since the uncoupling action is as effective as that of DNP at similar low levels of concentration. 3F-RFM SV has been reported to have

Table 4. Effect of rifampicin and its derivatives on the ATP-^{32}Pi exchange reaction

Rifampicin or its derivative (68 nmoles), 6 μmoles sodium ATP, 6 μmoles phosphate buffer (pH 7.4) containing 1 μCi of ^{32}Pi, 100 nmoles azide and mitochondria (4.6 mg protein) were added to 1.3 ml of the medium described in Table 3 to give the total volume 2.0 ml. After incubation for 10 min. at 25°C, treatment with PCA was done and aqueous phase containing organic phosphorus compounds was separated as described before. After washed three times with an equal volume of isobutanol, 1 ml of the aqueous solution was dried on a sample plate and radioactivity was assayed by a gas flow counter (Aloka FC-1E; Japan Radiation and Medical Electronics, Inc.).

Additions (M)		cpm	%
None		79,118	100
DNP	2.5×10^{-4}	2,414	3.1
RFP	3.4×10^{-5}	72,288	91.4
DA-RFP	3.4×10^{-5}	76,945	97.3
RFP-Q	3.4×10^{-5}	75,590	96.0
3F-RFM SV	3.4×10^{-5}	8,229	10.4

a high affinity with protein,[39] and so it is of interest to examine its other biological actions as well. Further researceh should be performed in order to investigate how 3F-RFM SV is formed from rifampicin *in vivo*, which are its effects *in vivo* and whether there is a relationship between the *in vitro* observations and the side effects noticed after the administration of rifampicin to patients with pulmonary tuberculosis.

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