THE EFFECTS OF ECHINATIN AND ITS RELATED COMPOUNDS ON THE MITOCHONDRIAL ENERGY TRANSFER REACTION

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Abstract.....To investigate the mechanism by which various biological action of licorice root are brought about, the effects of echinatin as a small constituent of *Glycyrrhiza echinata* and several related compounds on mitochondrial energy transfer reactions were examined. The results obtained were as follows: 1) Echinatin, 4'-hydroxychalcone, chalcone and 3,4'-dihydroxychalcone at a low concentration cause deterioration of respiratory control and oxidative phosphorylation of isolated rat liver mitochondria. 2) Chalcone and 4'-hydroxychalcone stimulate both latent and DNP-ATPase activity of mitochondria. Echinatin inhibits DNP-ATPase activity while stimulating range latent ATPase activity in the low concentration. 3) Chalcone and 4'-hydroxychalcone induce a rapid potassium release from mitochondrial vesicles, while echinatin and 3,4'-dihydroxychalcone have lesser effect than the former two substances. From these results, it can be concluded that echinatin and several related compounds disturb the mitochondrial energy transfer reactions and membrane permeability.

Key words: Licorice, echinatin, chalcone, mitochondria, oxidative phosphorylation, potassium release

INTRODUCTION

As one of many traditional Japanese and Chinese drugs, the licorice root has been used for various purposes from ancient times. As can be seen by its anti-inflammatory effect (Finney and Somers, 1958), anti-ulcer effect (Desmarez, 1953; Takagi et al., 1965; Ishii, 1970), various physiological actions due to its hormone-like actions (Yano, 1958; VAN Hulle, 1970; Kumagai, 1973) and its anti-tumor effect (Watanabe, 1973), its clinical application based on these actions covers a wide area. On the other hand, suppressive actions on the central nervous system, such as respiratory paralysis (Finney et al., 1958), have been reported as evidence of the toxicity of the licorice constituents. Yet few investigations can be found on the mechanism by which the above biological actions of licorice are brought about. Therefore we examined the *in vitro* effect of echinatin as a small constituent of *Glycyrrhiza echinata* and several related compounds on the biological membrane. Present data indicate that echinatin and other compounds examined influence oxidative phosphorylation and other energy-linked functions of isolated mitochondria from rat liver.

MATERIALS AND METHODS

Animals and preparation of mitochondria: Male Donryu rats weighing 250–300 g were sacrificed by decapitation after overnight fasting. Liver mitochondria were prepared according to a modification (Utsumi, 1963) of the method of Hogeboom and Schneider (1948) with a medium containing 0.25 M sucrose, 0.2 mM EDTA and 3 mM Tris-HCl buffer (pH 7.4). The isolated mitochondria were resuspended and washed twice with the above EDTA-free medium. The mitochondrial protein concentration was determined by the biuret reaction using bovine serum albumin as a standard.

Measurement of oxygen uptake and oxidative phosphorylation: Oxygen uptake by isolated mitochondria was measured in a medium containing 0.3 M mannitol, 10 mM KCl, 3 mM MgCl₂ and 3 mM phosphate buffer (pH 7.4) with a Galvanic oxygen electrode connected to an autorecorder. The ADP: O ratio and respiratory control index (RCI) were calculated from the traces of oxygen uptake recorded according to the method of Hagihara (1961).

Measurement of mitochondrial ATPase activity: Mitochondrial latent and DNP-induced ATPase activities were measured as follows: A reaction medium containing 0.2 M sucrose, 20 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA and 5 mM Tris-HCl buffer (pH 7.4) was mixed with 3 mM disodium ATP and mitochondria (approximately 2–3 mg protein) in a final volume of 2.0 ml. Incubation was carried out at 25°C for 15 min. One milliliter of ice-cold TCA (24 %) was added, mixed, left standing for 10 min and the supernatant solution was separated by centrifugation. An aliquot of the solution was used as material for the determination of inorganic phosphate liberated according to the method of Takahashi (1955).

Measurement of K⁺ release: The K⁺ concentration change of the reaction medium

during incubation of mitochondria was measured at 25°C with a K⁺ electrode (Beckman 39137) connected to a pH meter. The reaction medium contained 0.3 M mannitol and 10 mM Tris-HCl buffer (pH 7.4).

Reagents: Echinatin and the related compounds shown in Fig. 1 were donated by the Japanese Research Institute for Photosensitizing Dyes Co. Ltd. (Okayama). These compounds were dissolved in dimethylsulfoxide (DMSO) and added to the medium at a final concentration of 0.5-1.0% DMSO in volume. ATP and ADP were from Boehringer-Mannheim GmbH. Other chemicals used were commercial products of reagent grade.

$R_1 = R_2 = R_3 = H$: Chalcone

$R_1 = OH, R_2 = R_3 = H$: 4'-Hydroxychalcone

$R_1 = R_3 = OH, R_2 = H$: 3,4'-Dihydroxychalcone

$R_1 = R_2 = OH, R_3 = COOH$: 3-Carboxy-2,4'-dihydroxy-chalcone

$R_1 = R_2 = H$: Echinatin

$R_1 = H, R_2 = \text{glucosyl}$: Echinatin-4-glucoside

$R_1 = \text{glucosyl}, R_2 = H$: Echinatin-4'-glucoside

$R_1 = R_2 = \text{glucosyl}$: Echinatin -4,4'-diglucoside

Fig. 1. Structural formulas of echinatin and related compounds.

RESULTS

Effect on respiratory activity of isolated mitochondria: The effects of echinatin and related compounds on the respiratory activity of isolated rat liver mitochondria were summarized in Table 1. No recognizable effect of 0.5-1.0% DMSO as a solvent was seen on respiratory control and the ADP : O ratio of intact mitochondria. In the presence of chalcone, 4'-hydroxychalcone, 3,4'-dihydroxychalcone, 3-carboxy-2,4'-dihydroxychalcone or echinatin at a final concentration of 0.24 mM or 0.18 mM, a considerable change in the respiratory activity of mitochondria appeared; a deterioration in RCI due to the inhibition of state 3 respiration and/or acceleration of state 4 respira-

Table 1. Effect of Echinatin and Related Compounds on Mitochondrial Respiratory Activity

Additions	Oxygen uptake	(natoms/min.)	ADP/O	RCI
	state 4 (5 mM Succinate)	state 3 (0.25 mM ADP)		
a) None	46.6	213.2	2.09	4.58
Dimethylsulfoxide (0.5 %)	45.1	203.4	2.04	4.51
Chalcone (0.24 mM)	105.4	112.7	1.46	1.07
4'-Hydroxychalcone (0.24 mM)	181.3	181.3	—	1.00
3,4'-Dihydroxychalcone (0.24 mM)	64.7	115.6	1.41	1.79
3-Carboxy-2,4' dihydroxy-chalcone (0.18 mM)	68.6	161.7	1.65	2.36
Echinatin (0.18 mM)	134.8	134.8	—	1.00
b) None	39.7	183.8	2.08	4.63
Dimethylsulfoxide (1 %)	39.2	183.8	2.16	4.69
Echinatin 4-glucoside (2.3 mM)	39.2	125.0	2.03	3.19
Echinatin-4'-glucoside (2.3 mM)	40.2	63.7	1.31	1.56
Echinatin-4,4'-glucoside (1.7 mM)	36.3	159.7	2.11	4.40

Experiment a) and b) are different in their mitochondrial source. Mitochondrial protein was 1.8 mg per ml in experiment a) and 1.4 mg per ml in experiment b). Mitochondria were incubated in 3.0 ml of a reaction medium containing 0.3 M mannitol, 10 mM KCl, 3 mM MgCl₂ and 3mM phosphate buffer (pH 7.4). Echinatin and related compounds dissolved in dimethylsulfoxide were added to the medium, followed by 5mM sodium succinate and 250 μ M ADP. The incubation was carried out at 25°C with continuous stirring on a magnetic stirrer.

tion, and corresponding deterioration in the ADP : O ratio. This effect was remarkable in the cases of chalcone, 4'-hydroxychalcone and echinatin.

Equal concentration of echinatin-4-glucoside, echinatin-4'-glucoside or echinatin-4,4'-diglucoside had no effect. Only at a high concentration of 2.3 mM, did echinatin-4'-glucoside lower both RCI and the ADP : O ratio without enhancing state 4 respiration.

Fig. 2 represents the effect of chalcone and echinatin on the state 4 and state 3 respiration. On state 4 respiration, 0.48 mM chalcone and 0.25 mM echinatin resulted in increases of 130% and approximately 300% of the control, respectively. On the other hand, state 3 respiration was decreased to 65% and 50% of the control by the same concentration of echinatin and chalcone, respectively,

The effects of chalcone and echinatin on the various states of respiration were represented in Fig. 3. Namely, these two compounds increased state 4 respiration, and decreased DNP-stimulated respiration as well as state 3 respiration. Consequently, they seemed to uncouple oxidative phosphorylation. Nevertheless, some differences may be pointed out between the actions of these two compounds ; e.g. the uncoupling

Echinatin on mitochondria

Fig. 2. Effect of echinatin and chalcone on state 4 and state 3 respiration of isolated rat liver mitochondria. 2.5 mg protein of mitochondria per milliliter was used. Other conditions were the same as in Table 1.

action of echinatin is stronger than that of chalcone and increased oxygen uptake with Ca^{2+} incorporation can be recognized even in the presence of echinatin, whereas it can not be recognized in the presence of chalcone.

Effect on mitochondrial ATPase activity : In intact mitochondria, Mg^{2+} -dependent ATPase activity is negligible. However, it is stimulated by uncouplers or by damage to membranes. This stimulated ATPase activity is inhibited by energy transfer inhibitors such as oligomycin (Lardy et al., 1958). For further clarification of the action of echinatin and related compounds on mitochondria, the effect on mitochondrial ATPase activity was examined. As shown in Table 2, chalcone and 4'-hydroxychalcone increased both Mg^{2+} -dependent latent ATPase and DNP-stimulated ATPase activities at a low concentration (0.24 mM). Of the echinatin glucosides examined, echinatin-4'-glucoside considerably affected mitochondrial ATPase activities only at a high concentration which also affects respiratory activity.

Fig.4 indicates the concentration dependency of chalcone-stimulated ATPase activity. Echinatin also stimulated latent ATPase activity in the range of its low concentration, and inhibited DNP-stimulated ATPase activity depending on its concentration.

Effect on the membrane permeability of mitochondria : The physiological functions of biological membranes are closely related to their structure, and both are easily influenced by various external conditions. To explain the mode of action of these compounds on mitochondrial membrane, the K^+ compartmentation of mitochondria, which sensitively reflects the structural and functional changes, was examined.

As shown in Fig. 5, chalcone and 4'-hydroxychalcone induced a remarkable release of intramitochondrial K^+ at the same concentration that affected oxidative phosphory-

Fig. 3. Effect of echinatin (A) and chalcone (B) on the respiratory activity of rat liver mitochondria. Experimental conditions were fundamentally the same as in Table 1. Abbreviations Mt; mitochondria, S; succinate, ADP; sodium adenosinediphosphate, Echi; echinatin, Chal; chalcone, DNP; 2,4-dinitrophenol.

lation and the ATPase activities of mitochondria. Echinatin and 3,4'-dihydroxychalcone also induced K^+ release from mitochondria.

Echinatin on mitochondria

Table 2. Effect of Echinatin and Related Compounds on Mitochondrial ATPase Activity

Additions		Pi liberated (μ moles/mg protein/15min.)	
		-DNP	+DNP (2.5×10^{-5} M)
a)None		0.14	1.65
Dimethylsulfoxide	(1 %)	0.08	1.76
Chalcone	(0.24 mM)	2.84	2.74
4'-Hydroxychalcone	(0.24 mM)	2.85	2.57
3,4'-Dihydroxychalcone	(0.24 mM)	0.34	1.64
3-Carboxy-2,4'-dihydroxychalcone	(0.18 mM)	0.75	0.63
Echinatin	(0.18 mM)	0.13	0.11
b)None		0.15	1.73
Dimethylsulfoxide	(1 %)	0.23	1.96
Echinatin-4-glucoside	(2.3 mM)	0.22	1.65
Echinatin-4'-gucoside	(2.3 mM)	0.33	0.47
Echinatin-4,4'-digluconide	(1.7 mM)	0.19	2.06

Echinatin or related compounds dissolved in dimethylsulfoxide and mitochondria (2.08 mg protein in experiment a) and 3.07 mg protein in experiment b) were added to a 1.5 ml of medium containing 0.2 M sucrose, 0.02 M KCl, 5 mM Tris-HCl buffer (pH 7.4), 3 mM MgCl₂ 0.1 mM EDTA and 3 mM ATP. Other conditions are detailed in the text.

Fig. 4. Effect of echinatin and chalcone on the latent and DNP-stimulated ATPase activity of rat liver mitochondria. Experimental conditions were the same as in Table 2.

Fig. 5. Effect of echinatin and related compounds on the K^+ compartmentation of rat liver mitochondria. Mitochondria (0.28 mg protein/ml) were incubated in a 5.0 ml of the reaction medium containing 0.3 M mannitol, 10 mM Tris-HCl buffer (pH 7.4) at 25°C with continuous stirring. Intramitochondrial K^+ content was estimated by measuring the K^+ depleted by adding a surfactant, Triton $\times 100$. Measurement was carried out with a glass K^+ electrode connected to a pH-meter.

DISCUSSION

It is not easy to explain the action of echinatin and related compounds on mitochondria only from their effect on respiratory activity. However, when the effect on mitochondrial ATPase activities is taken into consideration, their mode of action seems clearer. Namely, in addition to the marked increase in state 4 respiration and the decrease in RCI and the ADP : O ratio, stimulation of latent ATPase activity by chalcone or 4'-hydroxychalcone strongly suggests their uncoupling action. On the other hand, echinatin-4'-glucoside (2.3 mM), which suppresses state 3 respiration, RCI, the ADP : O ratio and DNP-stimulated ATPase activity, seems to inhibit energy transfer reactions.

There are certain differences in the mode of action between echinatin and chalcone. Similarly to chalcone, an increase in state 4 respiration and decrease in RCI and the ADP : O ratio by echinatin suggest uncoupling of oxidative phosphorylation. Indeed a low concentration of echinatin (45 μ M or 90 μ M) accelerates the Mg^{2+} -dependent latent ATPase activity of untreated intact mitochondria (Fig. 4). However, DNP-stimulated ATPase activity is inhibited by echinatin. This means there is a possibility that echinatin acts also as an inhibitor of energy transfer reactions.

According to the chemiosmotic hypothesis (Mitchell, 1961), the inner membrane of mitochondria is impermeable to H^+ and H^+ is distributed outside of the membrane

depending on the energy of respiration or ATP hydrolysis, resulting in a H^+ concentration gradient between the inside and outside of the mitochondrial membrane. It is generally proposed that uncouplers transfer H^+ from outside of the inner membrane as proton conductor, diminishing the H^+ concentration gradient. Most uncouplers dissociate into the anion type, and therefore, the radicals dissociated or the electron attractiveness of the molecule seem important for examination of uncoupling action. From this point of view, chalcone as the simplest of these compounds used cannot be expected to possess such properties as described above. Hanstein (1976) has pointed out that protonophoretic property is not always indispensable for uncoupling of oxidative phosphorylation. It has been reported that some organic compounds which do not seem to have acid dissociation radicals have uncoupling action (Terada, 1979). The present data suggest that the uncoupling action of chalcone is probably due to another mechanism which is different in manner as has been seen in the case of picric acid (Hanstein, 1976).

The relationship between the uncoupling action of these compounds and their hydrophobicity may be explained to a certain extent by uncoupling and K^+ releasing action. For example, 4'-hydroxychalcone (0.25 mM) uncouples oxidative phosphorylation and accelerates intramitochondrial K^+ release, whereas chalcone-4'-glucoside of the same concentration shows no effect. Such a difference is attributable to the difference in hydrophobicity probably due to 4'-hydroxy and 4'-glucosyl groups or other physiological properties of these two compounds. Suolinna et al. (1975) examined the effect of flavonoids and found that 2,4',5'6'-tetrahydroxychalcone (14.7 μM) inhibits $Na^+ - K^+ - ATPase$ but has little or no effect on mitochondrial ATPase, though 2',4'-dihydroxychalcone and 4,4'-dihydroxychalcone (83 μM) have no effect on either. They pointed out that the inhibitory activity of this compound with intact cells is only marginal, perhaps again due to its permeability.

Both 4'-hydroxychalcone and echinatin uncoupled oxidative phosphorylation. However, the former was stronger in K^+ releasing action than the latter, the former activating the mitochondrial latent ATPase but the latter inhibiting it. This difference was probably due to $-OH$ or $-OCH_3$ groups of echination. A high concentration of chalcone-4'-glucoside (2.3 mM) also uncoupled oxidative phosphorylation, whereas echinatin-4'-glucoside of the same concentration acted as an inhibitor similar to the mode of action of oligomycin (data not shown). Furthermore, as seen between echinatin-4'-glucoside and echinatin-4'-glucoside (Table 1. and Table 2.), the position of glucoside seemed to cause considerable differences in biological action.

In conclusion, the present study made it clear that echinatin and several related compounds affect oxidative phosphorylation and the membrane permeability of mitochondria. However, further consideration is necessary as to the relationship between these experimental results obtained and the therapeutic effects as well as side effects of licorice constituents.

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