Mitochondria toxicity of blepharismin, a defense toxin produced by ciliated protozoan *Blepharisma japonicum* against predatory protists

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Abstract

The effect of blepharismin, a defense toxin of a ciliate protozoan *Blepharisma japonicum*, on mitochondrial function was studied by using *Tetrahymena thermophila* cells, freshly isolated rat liver mitochondria, and submitochondrial particles (SMP). To consider the chemical structure relevant to the mitochondria toxicity of blepharismin, the effect of hypericin, a structurally similar pigment to blepharismin, on mitochondrial respiration was compared with that of blepharismin.

Blepharismin repressed oxygen uptake activity of *Tetrahymena* cells. To mitochondrial respiration, blepharismin exerted the uncoupling and respiration repressive effects. Hypericin exhibited similar, but a little weaker, impairing effect to those of blepharismin on mitochondrial respiration.

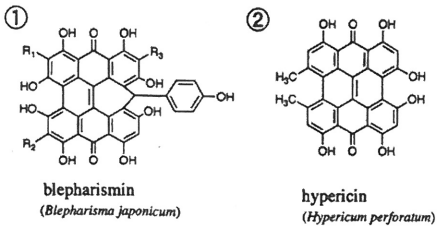
Blepharismin did not lessen NADH and succinate oxidases of SMP, indicating no interference with the electron transport in mitochondria at all.

Blepharismin induced an acute and drastic swelling of mitochondria in the isotonic solutions of alkali metal chlorides (Li, Na, K and Rb chlorides), implying induction of the ion permeability transition in the inner membranes. These results indicate that blepharismin is strongly toxic to mitochondrial functions.

The relationship between the impairing effect on mitochondrial function and defensive effect of blepharismin to predatory protists was discussed.

Introduction

Blepharisma japonicum is a red heterotrichous ciliate. Pigment granules of *B. japonicum* are membrane-bound spherical organelles of 0.3-0.6 μm in diameter mostly localized in the cortex and attached to the cell membrane[1-3]. The granules contain the red photodynamic pigment, blepharismin (Fig.1). *B. japonicum* discharges pigment granules when attacked by *Dileptus margaritifer* at and near the attacked site[4]. The albino mutant and bleached cells of *B. japonicum* are much more vulnerable to *D. margaritifer*[5] and *Amoeba proteus*[6]. Purified blepharismin is highly toxic to seven species of ciliate, in the light as well as in the dark, while the pigment is almost non-toxic to *B. japonicum*[4]. These findings strongly suggested that the pigment granules of *B. japonicum* had a major role in the defense activity against the predatory protists.



	R_1	R_2	R_3
A	Et	Et	H
B	Et	iPr	H
C	iPr	iPr	H
D	Et	iPr	Me
E	iPr	iPr	Me

Fig.1 Chemical structures of blepharismins and hypericin.

Blepharisma japonicum possesses five blepharismins (A-E) that have different alkylgroup substituents (R_1, R_2, R_3)(Checcucci et al. 1997).Blepharismin used here is a mixture of them.

Blepharismin is a photodynamic pigment and even its diluted solution photosensitizes colorless cells[1,4,7]. The toxicity of blepharismin to *D. margaritifer* is evidently elevated up under illuminated conditions[4]. The toxicity of blepharismin is thought to be potentiated by the photo-illumination in consequence of the generation of short-lived oxygen species, such as singlet oxygen ($^1O_2$)[9,10] and hydroxyl radical (OH·)[11]. Notwithstanding the strong intrinsic toxicity (toxicity in the dark) of blepharismin to the defensive ability[8], dearth of knowledge is available at present with regard to the molecular mechanism.

Climacostol, another type of defense toxin of a ciliate protozoa *Climacostomum virens*, has been demonstrated to inhibit motility and mitochondrial respiration of *T. thermophila*[12]. The present study was therefore performed to know the injurious effect of blepharismin on mitochondrial respiratory system, using ciliated *T. thermophila* cells, isolated rat liver mitochondria and submitochondrial particles (SMP). Instead of *T. thermophila* mitochondria, isolated rat liver mitochondria were utilized in the present study because of the technical difficulty in the isolation of tightly coupled mitochondria from *Tetrahymena* cells. SMP were used to study the effect of blepharismin on the electron transport in mitochondria.

Materials and Methods

Reagents: Blepharismin was isolated from *B. japonicum* by the procedure previously described[8] and its ethanol solution was kept in light-shield sample tube on ice. Blepharismin used in the present study is a mixture of blepharismin isomers[13] that have different alkyl group substituents (ethyl and/or isopropyl groups). Hypericin, Tris(Trizma Base), ADP-2Na and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Co. Other reagents were of the purest grade commercially available.

Procedures: Mitochondrial fraction was prepared from rat liver homogenate basically according to the method of Schneider[14] with slight modifications in the centrifugation condition and the composition of the isolation medium. The isolation medium was composed of 0.25 M sucrose, 10 mM Tris, 0.5 mM EDTA (pH 7.4). SMP, which are the reverted membrane vesicles of mitochondrial inner membranes to inside-out direction, were prepared by the method of Ruzicka and Crane[15] from freeze-thaw destructed rat liver mitochondria. The oxygen uptake of mitochondria and SMP were measured at 30°C using oxygen electrode (Iijima Electronics MFG Co. Ltd, Japan). Reaction medium contained 0.15 M KCl, 5 mM $MgCl_2$, 5mM inorganic phosphate, 1.0 mM EDTA and ca. 1.2mg of mitochondrial protein in a final volume of 2.0 ml (pH 7.4). L-glutamate, a substrate to the NAD-linked respiratory chain, contained 20 % of L-malate. State 3 respiration (ADP-driven respiration), state 4 respiration (restricted respiration due to the lack of ADP), RC ratio (the ratio of state 3 respiration to state 4 respiration) were calculated by the method of Chance and Williams[16]. Mitochondrial swelling was monitored by measuring absorbance decrease of mitochondrial suspension in the isotonic medium at 700 nm using recording spectrophotometer UVIDEC 610 (JASCO) according to the finding by Tedeschi and Harris[17] in the correlation between mitochondrial swelling and absorbance decrease. The absorbance decrease at 520 nm is widely employed for monitoring mitochondrial swelling process. In the present study, the absorbance decrease at 700 nm was followed to minimize the artificial influence of light absorbance by blepharismin (maximum absorption wavelengths 490 and 574 nm at pH 7.4). The reaction medium contained 0.15 M alkali metal chloride, 20 mM Tris, 0.5 mM EDTA (pH 7.4). Protein was assayed by the method of Lowry et al[18] using BSA as a standard. All experiments using blepharismin or hypericin were conducted with the dark condition.

Results

The effect of blepharismin on mitochondrial respiration in *Tetrahymena* cells.

The effect of blepharismin on oxygen uptake of *T. thermophila* cells was examined (Fig.2). The oxygen uptake was inhibited by blepharismin after a short lag time, indicating that the mitochondrial respiratory activity of *T. thermophila* was arrested by blepharismin. The oxygen uptake during the lag time after the addition of blepharismin might be the uncoupled respiration of

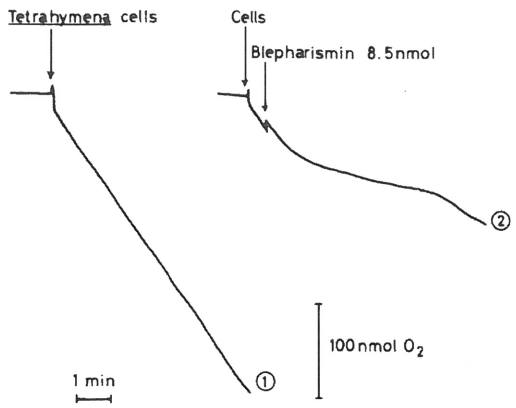


Fig.2 The effect of blepharismin on oxygen uptake by *Tetrahymena* cells

Tetrahymena cells were suspended in the fresh culture medium and oxygen uptake was measured by oxygen electrode at 30°C. Cell number was not counted, but was adjusted to the concentration for the measurement of oxygen uptake.

mitochondria, in which ATP is synthesized no longer.

The effect of blepharismin on the respiration of isolated rat liver mitochondria

The respiration impairing effects of blepharismin on NAD- and succinate-linked respirations were studied by using freshly isolated rat liver mitochondria. In Fig. 3, oxygraph data of mitochondrial respiration oxidizing L-glutamate were depicted. Curve 4 is the control experiment without blepharismin. Freshly prepared mitochondria showed a tightly coupled respiration displaying distinct state 3 and 4 respirations. The oxygen uptake activities of state 3 and 4 respirations were 88 and 6.6 nmol oxygen/mg protein/min, respectively, giving a RC ratio of 13.3. Blepharismin slightly accelerated the state 4 respiration (respiration without ADP) and repressed the state 3 respiration (ADP-driven respiration), indicating the uncoupling effect on oxidative phosphorylation and the respiration repressing effect (curves 2 and 3). At 15 nmol (7.5 μM) of blepharismin, the state 3 respiration was not induced by ADP at all, indicating the complete abolishment of the ATP synthesis in mitochondria, though oxygen utilization was not completely inhibited. Oxygen uptake was strongly inhibited by blepharismin at higher concentration than 30 nmol (15 μM) (data were not shown because of the apparent aggregation of mitochondria, probably by the formation of insoluble conjugates of blepharismin

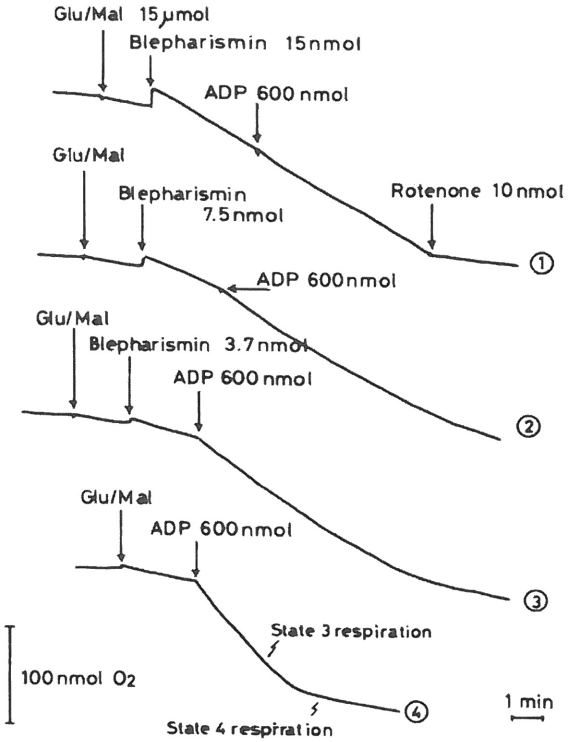


Fig.3 The effect of blepharismin on NAD-linked respiration of isolated rat liver mitochondria

The reaction condition was described in the **Materials and Methods**. Curve 4 shows the control without blepharismin.

with mitochondria at such high concentrations). Blepharismin was thus found to exhibit dual impairing effects on mitochondrial respiration; uncoupling effect and respiration-repressing effect. Similar results were obtained for the succinate-linked respiration (data not shown).

The effects of hypericin on NAD- and FAD-linked respirations

Blepharismin has a *p*-hydroxybenzyl residue on the anthraquinone nucleus, giving an image for the active residue of blepharismin in the mitochondria toxicity. In order to know the role of phenolic structure in the respiration-impairing effect of blepharismin, the effect of hypericin, which has no such hydroxybenzyl structure, on mitochondrial respiration was studied. As shown in Fig. 4, hypericin exhibited similar impairing effect on mitochondrial respiration, displaying the uncoupling and respiration repressive effects, implying little or no contribution of the phenolic hydroxyl group in the respiration-impairing effects.

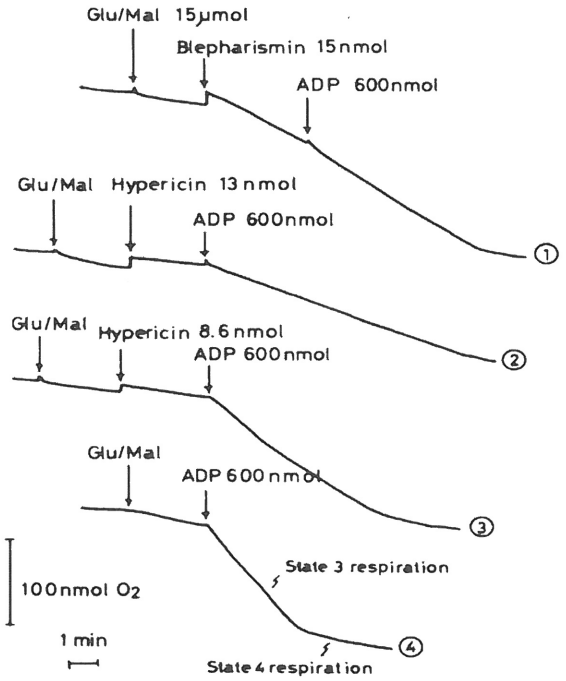


Fig.4 The effect of hypericin on NAD-linked respiration of mitochondria

The reaction condition was same as in Fig. 3

The effect of blepharismin on NADH and succinate oxidases in SMP

Blepharismin showed respiration-repressive effect on the intact mitochondria. The effects of blepharismin on NADH and succinate oxidases of SMP were examined to know the mechanism of respiration-repressive effect. SMP are reverted membrane vesicles to inside-out direction, and NADH and succinate are therefore directly oxidized without regulation by the coupling phenomena between electron transport and energy transfer systems. Fig. 5 shows a sample of oxygraph data of NADH and succinate oxidases of SMP. NADH oxidase was not retarded by blepharismin at the concentrations tested (curve 2), indicating no interference with the activity of respiratory chain enzymes. Hypericin also showed no inhibitory effect on the oxygen uptake activity in SMP (curve 3).

The induction of mitochondrial swelling

Blepharismin has been demonstrated to increase ion permeability of the phospholipid bilayer membrane by forming cation permeable channels[19]. The effect of blepharismin on the ion-permeation across mitochondrial inner membranes was tested by measuring the blepharismin-induced mitochondrial swelling in the isotonic alkali metal chloride solutions. Fig. 6 shows the current alteration of absorbance of mitochondrial suspension in KCl isotonic solution at 700 nm. The addition of blepharismin caused a rapid decrease in the absorbance (curves 1 and 2), indicating the induction of mitochondrial swelling probably by eliciting the ion permeability transition in the inner membranes. The swelling rate and amplitude elevated corresponding to the increase of blepharismin concentrations. Blepharismin-induced swelling was arrested by BSA (curve 3) in consequence of the binding to the hydrophobic anion binding site of BSA molecule. In the presence of BSA, the swelling was not induced by blepharismin (curve 4). Blepharismin

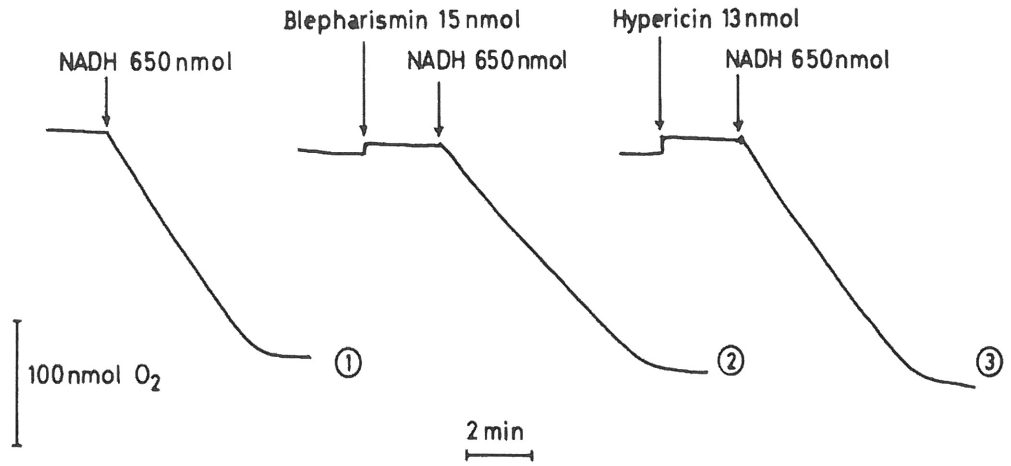


Fig.5 The effect of blepharismin on NADH and succinate oxidases in SMP

The reaction medium was same as in Fig. 3. Protein was 0.6mg in the reaction medium.

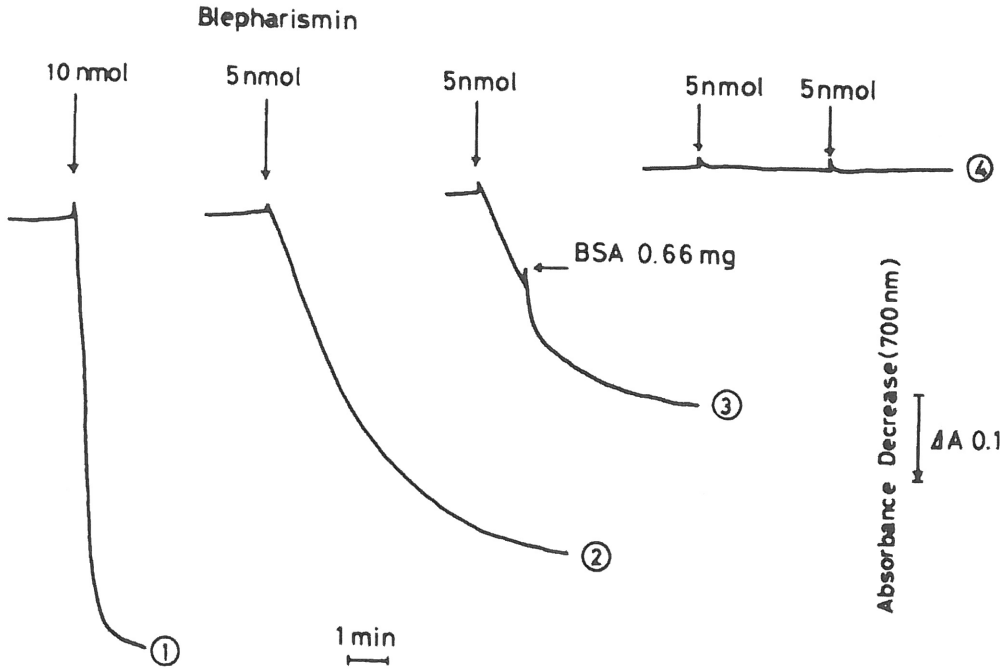


Fig.6 The induction of mitochondrial swelling

The reaction condition was mentioned in Materials and Methods. Curve shows the result of experiment in which reaction was carried out in the presence of BSA (0.66mg). Mitochondrial protein was 0.46mg in the reaction medium (2.0ml).

induced the swelling of mitochondria in the isotonic LiCl, NaCl, RbCl solutions as well as in KCl solution (data not shown), indicating no strict ion selectivity among alkali metal ions in the swelling induction.

Discussion

The effect of blepharismin, which is thought to be a defense toxin of a ciliate protozoan *B. japonicum* against the predatory protists, on mitochondrial reactions was studied using *Tetrahymena* cells, isolated rat liver mitochondria and SMP. The retreating behavior of *D. margaritifer* was observed when it hit *B. japonicum* and purified blepharismin was highly toxic to predator protists[4]. But the molecular mechanism of the toxicity is not available at present.

Blepharismin exhibited the uncoupling effect on oxidative phosphorylation and respiration-repressive effect. The uncoupling effect of blepharismin was not explicitly observed in the experiment with oxygen electrode, the uncoupling effect, which releases the state 4 respiration in the absence of ADP, seemed to be masked by the respiration-repressing effect.

According to the chemi-osmotic theory for the mechanism of oxidative phosphorylation[20], lipophilicity and weak acidity of chemicals are requested as the essential factors for the strong uncoupling effect on oxidative phosphorylation[21,22]. Lipophilic weak acids are able to non-vectorially conduct protons through mitochondrial inner membranes, quenching the transmembrane pH difference and thereby canceling the electro-chemical potential across the inner membranes[23,24]. Blepharismin, a lipophilic natural polyhydroxyquinone, may non-vectorially conduct protons across the inner membranes. Blepharismin showed a pH-dependent spectral alteration exhibiting eminent red-shift of absorption spectrum (data not shown). The absorption maximum wavelengths (484 and 566 nm at pH 4.0) were shifted to longer wavelengths (490 and 574 nm, respectively, at pH 7.4), strongly suggesting the ability of proton conductivity of blepharismin in the inner membranes. The β-positioned hydroxyl group on the anthraquinone

nucleus has been demonstrated to contribute in the potent uncoupling effect on the oxidative phosphorylation[25,26] and in the proton conductivity across phospholipid bilayer membranes[19]. The phenolic hydroxyl group protruded beyond the bis-anthraquinone nucleus shows proton dissociation at higher pH than physiological pH range, indicating no sharing in the uncoupling effect. In fact, hypericin, which has no the protruded *p*-hydroxybenzyl residue, exhibited similar but a little less potent probably due to the less hydrophobicity than that of blepharismin, injurious effect on mitochondrial respiration to that of blepharismin. The α-positioned hydroxyl groups, which are neighboring to carbonyl group in the anthraquinone nucleus, do not contribute in the uncoupling effect[27]. These considerations suggest that the β-positioned hydroxyl groups of blepharismin may be exclusively responsible for the proton conductivity in mitochondrial inner membranes, which is in agreement with the result of experiment using artificial phospholipids planar membrane[19].

Blepharismin induced mitochondrial swelling by eliciting ion-permeability transition in the inner membranes at the similar concentrations to those of uncoupling effect, by which oxidative phosphorylation in mitochondria might be uncoupled.

By the present investigation, blepharismin was substantiated to impair the ATP synthesis system in mitochondria, probably by cancelling membrane electrochemical potential. However, it may be not reasonable to consider that the deterioration of the ATP-generating ability in mitochondria is the very mechanism for the rapid defense reaction against the predatory ciliates, because intracellular ATP does not instantly disappear. The blepharismin-induced moment alteration of membrane electrochemical potential by the instant elicit of ion permeability transition and/or cancel of the proton gradient across biomembranes may be reasonable to consider the swift retreating behavior of the predatory ciliates, for example, *D. margritifer* from *B. japonicum*.

The blepharismin-induced swelling of mitochondria was impeded and protected by BSA, indicating the hydrophobic and ionic interactions of blepharismin with BSA to which variety of hydrophobic phenols bind[28]. Mitochondrion has also membrane proteins, which strongly interact with phenol compounds [29,30]. *p*-Hydroxybenzyl residue may be involved in the protein binding.

From these results it was proposed that the blepharismin-induced the swift cancel of the electro-chemical potential in membranes in consequences of the increment of the proton conductivity and the induction of ion-permeability transition might be mainly contributed in the chemical defense activity of blepharismin against the predatory protozoan.

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