The effects of Eushearilide, a novel macrolide antifungal antibiotic, on rat mitochondrial respiratory function

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Abstract

Eushearilide, a newly isolated macrolide antibiotic isolated from Eupenicillium shearii, has been examined for its toxic effects on mitochondrial respiration and structure to gain insight into the molecular mechanism for the antifungal activity, by using freshly isolated rat liver mitochondria. Eushearilide inhibited the respiration oxidizing both L-glutamate and succinate. The inhibition was not reversed by N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride, which generates an electron transport shunt over the inhibition sites of rotenone and antimycin A, and was not restored by ascorbate, an artificial substrate for the cytochrome c oxidase. The all of cytochromes a, b and c were kept reduced in the presence of eushearilide in the reduced-minus oxidized difference spectrum of cytochromes. Eushearilide induced a large amplitude swelling in the mitochondria suspended in an isotonic KCl solution. This amplitude swelling was prevented by cyclosporine A, indicating the generation of the permeability pore in the inner membrane. These results suggested that eushearilide could impair mitochondrial respiratory function by inhibiting the electron transport at the site of complex IV (cytochrome c oxidase) in the respiratory chain and by inducing swelling.

Keywords
eushearilide; macrolide antifungal antibiotic; mitochondria

Introduction

Eushearilide (Fig. 1), a newly isolated macrocyclic antibiotic isolated from Eupenicillium shearii, shows fungicidal effect against various sort of fungi and yeasts, including human pathogens Aspergillus fumigatus, Trichophyton spp, and Candida spp1. The Tonoi et al. were able to determine true structure of eushearilide subsequently2. However, the molecular mechanism is not available yet.

Eushearilide has strongly hydrophobic macrolide moiety, which fatty acid enclosed to the cyclic structure, and hydrophilic choline phosphatide moiety, suggesting a potent affinity to phospholipids portion in biomembranes such as mitochondria. It is also expected that eushearilide exerts a membrane-lytic effect like the detergent. Mitochondria play the important role in the bioenergetics system in fungus and yeast cells as well as in animal cells3. The dysfunction in mitochondria results in the death of fungal cells4.

In the present study, therefore, we examined the effects of eushearilide on the respiratory function of isolated rat liver mitochondria to gain insight into the molecular mechanism for the antifungal activity.

Materials and Methods

Eushearilide (Fig. 1) was isolated from Eupenicillium shearii IFM 54447 and was dissolved in N,N-dimethylformamide (DMFA). Miconazole was kindly supplied by Prof. Y. Nozawa and was dissolved in DMFA. ADP, NADH, Tris (Trizma Base), bovine serum albumin (BSA), and N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMP) were purchased from Sigma.

Other reagents were of the purest grade commercially available. Commercial DMFA was purified by the technique of distillation and was kept below 4°C.

Rat liver mitochondria were prepared from liver homogenate of Wistar albino male rats weighing 200-250 g of body weight by the method of Schneider with modifications in the centrifugation speed and the composition of isolation medium5. Mitochondrial respiration was measured at 30°C by using an oxygen
electrode (Iijima Electronics MFG Co. Ltd). Reaction medium was composed of 0.15M KCl, 20 mM Tris, 5mM inorganic phosphate, 5 mM MgCl₂, 0.5 mM EDTA and mitochondria in a final volume of 2.0 ml at pH 7.4. L-Glutamate, succinate, DHQ, TMPD, and ascorbate were added to the reaction medium at the time point shown in the respiration curves.

Mitochondrial swelling was monitored by following absorbance decrease at 520 nm, using a recording spectrophotometer (DU-70 (Beckman)). Reaction medium contained 0.15 M KCl, 5 mM MgCl₂, 0.5 mM EDTA and 20 mM Tris in a final volume of 3 ml (pH 7.4). The reduced-minus oxidized difference spectrum was measured at room temperature in a spectrophotometer DU-70 (Beckman) and the spectrum were recorded.

Mitochondria (0.3 mg of mitochondrial protein) were mixed with or without eushearilide (150 nmol) in the same reaction medium as used for mitochondrial swelling measurement and then incubated under magnetic stirring at room temperature for 15 min. After incubation, the reaction medium containing mitochondria was centrifuged at 10,000 x g for 20 min and the resultant supernatant was used for the measurement of released cytochrome c. Released cytochrome c in the supernatant was reduced by sodium dithionite. The absorbance of reduced cytochrome c at 550 nm was measured in a spectrophotometer V570 (JASCO, Japan). The absorbance at 550 nm in the supernatant obtained from mitochondria with eushearilide was subtracted from the absorbance at 550 nm in the supernatant obtained from mitochondria without eushearilide. From the subtracted absorbance, the rate of cytochrome c released from mitochondria with eushearilide was calculated using the molecular extinction coefficient of reduced cytochrome c (21 x 10³ M⁻¹ cm⁻¹) at 550 nm.

The reaction medium was the same as that used for the measurement of oxygen uptake. Protein was assayed by the method of Lowry et al.⁶, using BSA as the standard.

Results

The effect of eushearilide on mitochondrial respiration using L-glutamate as a respiration substrate

The results of the oxygen consumption rate in mitochondria with L-glutamate in the presence and absence of eushearilide are shown in Fig. 2. A slight oxygen consumption was observed in mitochondria alone because of the oxygen consumption by the mitochondrial intrinsic reductant. Oxygen consumption by addition of L-glutamate and ADP to mitochondria (state 3 respiration) increased. This oxygen consumption terminated when ADP was consumed in the mitochondria with L-glutamate (state 4 respiration). The ratio of the oxygen consumption rate of state 3 to the oxygen consumption rate of state 4 (RC ratio) was 8.9.
The RC ratio of approximately 8-12 has been reported as an indicator of the intact state of the mitochondrial membrane structure\(^7\). Next, we examined the effect of eushearilide on the respiration in mitochondria with L-glutamate and ADP. Mitochondria without eushearilide showed a highly coupled respiration (curve 1). The respiration was inhibited in mitochondria with 75 nmol eushearilide (curve 2). The respiration was further inhibited in mitochondria with 150 nmol eushearilide (curve 3). Eushearilide at concentrations of 75 and 150 nmol inhibited the respiration in mitochondria with L-glutamate by 38.3 and 89.4% (mean value, n = 3), respectively. Thus, eushearilide showed the inhibitory effect on NAD-linked mitochondrial respiration. However, the inhibition of this respiration in mitochondria with L-glutamate and ADP by eushearilide was not reversed by DHQ, an artificial substrate for the complex III (UHQ-cytochrome c oxidoreductase) (curve 3).

**The effect of eushearilide on mitochondrial respiration using succinate as a respiration substrate**

The results of the oxygen consumption rate in mitochondria with succinate in the presence and absence of eushearilide are shown in Fig. 3. Mitochondria without eushearilide showed a highly coupled respiration (curve 1). The respiration was inhibited in mitochondria with 75 nmol eushearilide (curve 2). The respiration was further inhibited in mitochondria with 150 nmol eushearilide (curve 3). Eushearilide at concentrations of 75 and 150 nmol inhibited the respiration in mitochondria with succinate by 65.5 and 87.7% (mean value, n = 3), respectively. Thus, eushearilide showed the inhibitory effect on FAD-linked mitochondrial respiration. However, the inhibition of this respiration in mitochondria with L-glutamate and ADP by eushearilide was not reversed by TMPD, a reagent to generate an electron transport shunt over the inhibition sites of rotenone and antimycin A (curve 3).

**Spectroscopic study of the inhibition site of eushearilide in the respiratory chain**

Reduced-minus oxidized difference spectrum of mitochondrial respiratory chain enzymes oxidizing NADH were measured in the presence or absence of eushearilide. The results are shown in Fig. 4. All of cytochromes in the respiratory chain were reduced in the presence of eushearilide, displaying absorption peaks at 552 nm (cytochromes c and c\(_1\)) and around 605 nm (cytochrome aa\(_3\)) and an absorption shoulder at around 560 nm (cytochrome b). Thus, eushearilide did inhibit the electron transport in the complex IV but did not the complexes I and III.
**Induction of mitochondrial swelling by eushearilide**

When the swelling of mitochondria was examined in the presence or absence of eushearilide, the results were obtained as shown in Fig. 5. Mitochondria without eushearilide showed no change in the absorbance, indicating no swelling (curve 1). Mitochondria with 75 nmol eushearilide showed a rapid decrease in the absorbance, indicating the induction of mitochondrial swelling (curve 2). Mitochondria with 150 nmol eushearilide showed a further decrease in the absorbance (curve 3). Thus, the swelling of mitochondria was accelerated by eushearilide.

**Release of cytochrome c from mitochondria**

The release of cytochrome c from swollen mitochondria in the presence of eushearilide was spectroscopically examined by measuring the reduced-minus oxidized difference spectrum of cytochrome c in the supernatant obtained by centrifugation of the mitochondrial suspension containing eushearilide. As a result, the release of cytochrome c from mitochondria treated with eushearilide was observed and the rate of cytochrome c released from mitochondria treated with 150 nmol eushearilide was 0.22 ± 0.06 µmol / min / mg protein (mean ± S.D, n = 3).

**Discussion**

The effects of eushearilide on mitochondrial respiration function were examined in isolated rat liver mitochondria. Eushearilide inhibited the oxidative phosphorylation and the rate of respiration in mitochondria using L-glutamate or succinate used as the respiration substrate. Thus, eushearilide was found to have a strong respiration-impairing effect on mitochondria. The inhibition was not reversed by TMPD and DHQ, suggesting that the inhibition site was located at cytochrome c oxidase in the respiratory chain (Fig. 6). The inhibition site was confirmed by measuring reduced minus oxidized absorption spectrum in the presence of eushearilide. We have observed in the spectroscopic experiment that cytochromes in the respiratory chain are not reduced in the presence of rotenone, while cytochromes in the respiratory chain are reduced in the presence of eushearilide (unpublished data). This result supports the conclusion from the experiment using oxygen electrode.

Potent antimycotic agents such as azole, clotrimazole, miconazole and econazole impair mitochondrial respiration and induce mitochondrial swelling, which is prevented by cyclosporine A, an authentic inhibitor to mitochondrial swelling concerning the induction of apoptotic cell death. Like miconazole, eushearilide induced mitochondrial swelling and released cytochrome c from mitochondrial inner membranes, which was prevented by cyclosporine A. This result suggests the generation of the permeability transition pore.

These impairing effects of eushearilide on mitochondrial respiratory function may explain the mechanism for the antimycotic activity, though further detailed experiment using electron transport panicles is requested to identify the exact mode of inhibition by eushearilide of the respiratory chain.

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**Fig. 5** Induction of mitochondrial swelling in isotonic KCl solution by eushearilide

Mitochondrial swelling in the presence and absence of eushearilide was measured by monitoring absorbance at 520 nm, using a recording spectrophotometer (Beckman) as described in Materials and Methods. Curve 1, mitochondria without eushearilide; curve 2, mitochondria with 75 nmol eushearilide; curve 3, mitochondria with 150 nmol eushearilide.

**Fig. 6** Proposed inhibition site of eushearilide in the respiratory chain

DHQ: durohydroquinone
TMPD: N,N′,N″,N‴-tetramethyl-p-phenylenediamine
References


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