Inhibition by Secalonic Acid D of Oxidative Phosphorylation and Ca\(^{2+}\)-Induced Swelling in Mitochondria Isolated from Rat Livers

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Received 9 March 1983/Accepted 29 July 1983

The in vitro biological activity of secalonic acid D, a mycotoxin from *Aspergillus ochraceus*, was studied to assess its cytotoxicity for isolated rat liver mitochondria. Secalonic acid D uncoupled the oxidative phosphorylation of mitochondria and caused a mild inhibition of state 3 respiration. Secalonic acid D weakly enhanced latent ATPase activity in mitochondria but suppressed 2,4-dinitrophenol-stimulated ATPase activity. Secalonic acid D did not induce pseudoenergized swelling of mitochondria and markedly inhibited the Ca\(^{2+}\)-induced swelling of mitochondria in KCl isotonic solution.

A xanthone derivative, secalonic acid D (Fig. 1) from *Aspergillus ochraceus* (22) and *Penicillium oxalicum* (15), has been shown to be toxic to rats and mice (11, 13, 14) and to be weakly mutagenic (12). However, its in vitro biological activity has not been investigated. It has been recently demonstrated by us that this compound interacts with bovine serum albumin, phospholipid membrane vesicles (liposomes), and submitochondrial particles and that the molecules bound by them exhibit strong fluorescence when illuminated by UV light (excitation, 380 nm; emission, 535 nm) (9), suggesting the interaction of this compound with hydrophobic biomembranes such as mitochondria. A spectrophotometric study (9) has revealed that secalonic acid D has a pK value of around pH 6.0. A lipophilic phenol compound dissociating protons at around neutral pH can uncouple oxidative phosphorylation in isolated mitochondria (8 and references cited therein). These results imply that secalonic acid D might also show an uncoupling effect on mitochondrial respiration. This possibility prompted us to investigate the uncoupling effect of secalonic acid D on the respiratory system of isolated rat liver mitochondria.

**MATERIALS AND METHODS**

**Reagents.** ADP, ATP, and Tris were purchased from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Kankakee, Ill. Secalonic acid D was isolated from dried mycelia of *A. ochraceus* by a procedure previously described (22). Other reagents were of the purest grade commercially available.

Preparation of rat liver mitochondria and measurement of mitochondrial functions. Rat liver mitochondria were prepared essentially by the method of Schneider (14), using a 0.25 M sucrose solution which contained 0.5 mM EDTA and 10 mM Tris-chloride (pH 7.4). Mitochondrial respiration was measured by a Galvani-type oxygen electrode (Sensanics Japan Co., Tokyo). The basal reaction mixture contained 675 μmol of sucrose, 30 μmol of KCl, 15 μmol of MgCl\(_2\), 1.5 μmol of EDTA, 60 μmol of Tris-chloride (pH 7.4), and 3 μg of mitochondrial protein in a final volume of 3 ml. Mitochondrial ATPase activity was determined by measuring inorganic phosphate (Pi) enzymatically released from ATP. The basal reaction mixture contained 100 μmol of sucrose, 25 μmol of KCl, 2.5 μmol of MgCl\(_2\), 0.25 μmol of EDTA, 2 μmol of ATP, 25 μmol of Tris-chloride (pH 7.4), and 0.2 mg of mitochondrial protein in a final volume of 0.5 ml. The reaction was carried out at 30°C for 5 min and was terminated by adding 0.5 ml of 10% cold trichloroacetic acid. Protein was removed by centrifugation at 3,000 rpm for 5 min, and 0.5 ml of the supernatant fluid was used for Pi determination. The Pi was determined by the method of Chen et al. (3), using ascorbic acid as a reducing agent. Mitochondrial swelling was measured by monitoring the decrease of light absorption (16) at 550 nm with a Hitachi 320S recording spectrophotometer. The reaction mixture contained 300 μmol of KCl, 40 μmol of Tris-chloride (pH 7.4), and 0.8 mg of mitochondrial protein in a final volume of 2.0 ml. The reaction was carried out at 24°C in a glass cuvette set in the recording spectrophotometer. Mitochondrial protein was determined by the method of Lowry et al. (7), using bovine serum albumin as a standard protein.

**RESULTS**

Effect of secalonic acid D on mitochondrial respiration. The oxygram of mitochondrial respiration oxidizing succinate is shown in Fig. 2.
Freshly prepared mitochondria showed phosphorylation tightly coupled to the respiratory chain as indicated by a high respiratory control index (state 3 or state 4 respiration) and by high phosphorylation efficiency (P/O ratio) (Fig. 2, curve 1). The addition of secalonic acid D caused a marked increase of state 4 respiration (oxygen uptake without added ADP) accompanying a decrease in respiratory control index and P/O ratio (Fig. 2, curves 2 and 3), which indicates that this compound uncouples oxidative phosphorylation in isolated mitochondria. Curves 2 and 3 in Fig. 3 show the effect of secalonic acid D on respiratory control index and P/O ratio in mitochondrial respiration, respectively. Mitochondrial respiration was completely uncoupled by this compound at ca. 70 \( \mu \text{M} \). Curve 1 in Fig. 3 shows the inhibition of mitochondrial respiration as a function of secalonic acid D concentration. A mild inhibition of state 3 respiration (ADP-induced respiration) was produced by secalonic acid D similar to many other kinds of uncouplers (10, 19, 20).

**Effect of secalonic acid D on mitochondrial ATPase activity.** Figure 4 shows the effect of secalonic acid D on the latent ATPase activity of mitochondria. Freshly prepared intact mitochondria exhibited very low ATPase activity which was significantly enhanced when mitochondrial respiration was uncoupled by 2,4-dinitrophenol (DNP). It was found that secalonic acid D suppressed DNP-stimulated ATPase activity in a dose-related fashion (Fig. 4, curve 1). Unexpectedly, latent ATPase activity was merely weakly enhanced by this compound, exhibiting a maximum activity at around 20 \( \mu \text{M} \) secalonic acid D (Fig. 4, curve 1). The mechanism for the inhibition of DNP-stimulated ATPase activity by secalonic acid D is not known.

**Effect of secalonic acid D on Ca\(^{2+}\)-induced swelling of mitochondria.** Figure 5 shows the effect of secalonic acid D on Ca\(^{2+}\)-induced swelling of isolated rat liver mitochondria in 0.15 M KCl solution. Secalonic acid D in the absence of Ca\(^{2+}\) did not induce any swelling of mitochondria (Fig. 5, curve 1). The other control (curve 5) shows a marked swelling of mitochondria induced by Ca\(^{2+}\) (1 mM) in the absence of secalonic acid D, as measured by a marked decrease in light absorption at 550 nm. Increasing the concentration of secalonic acid D caused a progressive decrease in the velocity of Ca\(^{2+}\)-induced swelling without a marked effect on the extent of swelling (Fig. 5, curves 2, 3, and 4). The inhibition rate was not severe at high concentrations (>40 \( \mu \text{M} \)) (Fig. 6).

![Structural formula of secalonic acid D](image)

**FIG. 1.** Structural formula of secalonic acid D.

![Effect of secalonic acid D on oxidative phosphorylation in isolated rat liver mitochondria.](image)

**FIG. 2.** Effect of secalonic acid D on oxidative phosphorylation in isolated rat liver mitochondria. The reaction was performed at 24°C. RC, Respiratory control; SA-D, secalonic acid D.

![Effects of various concentrations of secalonic acid D on mitochondrial respiration.](image)

**FIG. 3.** Effects of various concentrations of secalonic acid D on mitochondrial respiration. The reaction conditions were the same as those for the experiment shown in Fig. 2. Curves 1, 2, and 3, effect of secalonic acid D on state 3 respiration (inhibition rate), respiratory control (RC) index, and P/O ratio, respectively.
DISCUSSION

Secalonic acid D inhibited ATP synthesis in isolated rat liver mitochondria mainly by uncoupling oxidative phosphorylation. The molecular mechanism for the uncoupling effect of chemicals on oxidative phosphorylation in mitochondria is not known; however, the importance of lipophilicity and the acid dissociation properties of the chemicals to the uncoupling effect has been well documented (17). A spectrophotometric study has revealed that secalonic acid D possesses a pK value of 6.0 due to ionization of a phenolic group in the xanthone ring (9), indicating that the 1- or 8-hydroxyl group or both in the xanthone ring could release protons at physiological pH and cause the uncoupling activity of secalonic acid D (9). Besides secalonic acid D, secalonic acids A, B(E), C, F, and G have been isolated by the method reviewed by Cole and Cox (4); they are a group of closely related fungal metabolites. Chemically, they are xanthone dimers, having identical molecular weights and elemental formulas. Therefore, it may be expected that all of these secalonic acids exhibit an uncoupling effect on isolated mitochondria.

There is good correlation between uncoupling and the appearance of ATPase activity in mitochondria (17). However, secalonic acid D showed a slight stimulation of the latent ATPase activity and, in addition, suppressed the DNP-stimulated ATPase activity of mitochondria. These observations suggest that secalonic acid D is an uncoupler which inhibits the energy transfer system of mitochondria.

Various kinds of compounds are known to induce pseudoenergized swelling of mitochondria in an isotonic solution of KCl (2, 6), including several quinone pigments from fungi which uncouple mitochondrial respiration (1, 5). However, secalonic acid D did not cause swelling of mitochondria and did not inhibit Ca\(^{2+}\)-induced swelling of mitochondria. A strong hepatotoxic mycotoxin, luteoskyrin, also inhibits Ca\(^{2+}\)-induced swelling in mitochondria. The detailed molecular mechanism for these biological activities is under investigation.

LITERATURE CITED


