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Short communication

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Thenoyltrifluoroacetone, a potent inhibitor of carboxylesterase activity

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Abstract

Thenoyltrifluoroacetone (TTFA), a conventional mitochondrial complex II inhibitor, was found to inhibit purified porcine liver carboxylesterase non-competitively with a K_i of 0.61×10^{-6} M and an IC_{50} of 0.54×10^{-6} M. Both rat plasma and liver mitochondrial esterases were inhibited in a concentration-dependent fashion. Results indicate that TTFA is a potent inhibitor of carboxylesterase activity, in addition to its ability to inhibit mitochondrial complex II activity. Therefore, caution is warranted in using TTFA as a mitochondrial complex inhibitor in combination with esterase substrates, such as fluorescence probes or vitamin E esters.

Keywords: Carboxylesterase; Thenoyltrifluoroacetone; Mitochondrial complex II

1. Introduction

Carboxylesterases represent a family of esterases that hydrolyze a wide variety of substrates, including therapeutic drugs and xenobiotics. For example, carboxylesterases are known to act as detoxifying enzymes by decreasing the overall concentration of organophosphate insecticides at the target enzyme site [1]. TTFA (4,4,4-trifluoro-1[2-thienyl]-1,3-butanedione) has been used extensively as a mitochondrial complex II inhibitor in studies examining the role of mitochondria in the production of reactive oxygen species, in cellular function, and in cell death [2–7]. Recent mitochondrial experiments in our laboratory suggested that TTFA may inhibit the hydrolysis of the succinate ester of tocopherol to tocopherol in isolated hepatocytes, and thus we examined the effect of TTFA on carboxylesterase activity. We believe that this is the first report showing that TTFA is a potent carboxylesterase inhibitor.

2. Materials and methods

2.1. Isolation of rat liver mitochondria and rat plasma

Hepatic mitochondria were isolated based on the methods of Hovius *et al.* [8] with modifications. Adult male Sprague–Dawley rats (200–250 g) were obtained from Simonsen Laboratories Inc., housed, and given food and water *ad libitum* for 7 days prior to use. Rats were anesthetized with pentobarbital and killed by exsanguination, and plasma was separated from the collected whole blood. The liver was homogenized in homogenate buffer (250 mM sucrose, 0.5 mM EGTA, 0.1% BSA, 5 mM HEPES, pH 7.4), and the homogenates were centrifuged at 600 *g* for 5 min at 4°. The supernatant was then centrifuged at 10,300 *g* for 10 min at 4°, and the pellet was resuspended in the homogenate buffer (5 mL). Mitochondria were then purified with Percoll medium. Briefly, 2.5 mL of mitochondrial suspension was loaded on top of 20 mL of 30% (v/v) Percoll in 225 mM sucrose, 1 mM EGTA, 25 mM HEPES, 0.1% (w/v) BSA (pH 7.4), and centrifuged at 95,000 *g* for 30 min at 4°. Mitochondria were collected from the lower part of the dense layer; a brownish yellow mitochondrial band was washed twice with homogenate buffer in the absence of EGTA.

2.2. Esterase activity

Porcine liver carboxylesterase (15 mg protein/mL, specific activity: 250 U/mg protein) was obtained from the Sigma Chemical Co. Carboxylesterase activity was determined at 23° by the measurement of the formation of PNP from PNPA. The reaction medium (1 mL in a final volume) contained 20 mM Tris–HCl buffer (pH 8.0), 50 mU esterase, 10 μL mitochondrial suspension (10 mg protein/mL) or 10 μL plasma (50 mg protein/mL). The reaction was

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Abbreviations: TTFA, thenoyltrifluoroacetone; BNPP, bis-(*p*-nitrophenyl) phosphate; PNP, *p*-nitrophenol; PNPA, *p*-nitrophenyl acetate.

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initiated after PNPA (1 mM in final concentration) was added. The increase of absorbance at 405 nm was recorded every 10 s for 10 times. Carboxylesterase activity was calculated based on the formation of PNP and expressed as nmol/min/mU (purified enzyme) or nmol/min/mg protein (mitochondria and plasma). For all inhibition experiments, the enzyme, mitochondria, or plasma was incubated with TTFA or BNPP at 23° for 20 min since TTFA provided poor inhibition without preincubation.

2.3. Mitochondrial complex II activity

Mitochondrial complex II (succinate dehydrogenase) activity was determined according to the method of Kruidering *et al.* [9]. The assay was performed at 23° with 100 μg of mitochondrial protein in a final volume of 1 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 100 μM EDTA, 1 mM KCN, and 0.1% (w/v) BSA. After the addition of 95 μM ubiquinone-0 and stabilization of the signal, the reaction was started by the addition of 100 μL of 0.1 M sodium succinate with or without TTFA. The activity was calculated from the rate of the decrease in ubiquinone and expressed as nmol/min/mg protein. For the inhibition study, preincubation of samples with TTFA was not required.

3. Results and discussion

3.1. Inhibition of purified liver esterase

A 20-min preincubation of commercial purified porcine liver carboxylesterase with TTFA (0.5–10 μM) resulted in a concentration-dependent inhibition of esterase activity (Fig. 1A). The IC_{50} was estimated from the plot of the inhibition of enzyme activity as a function of log TTFA concentration (see inset in Fig. 1A). Under the experimental conditions (50 mU purified enzyme and a 20-min preincubation), the IC_{50} for TTFA was about 0.54 μM. The influence of incubation time on the inhibition of the enzyme activity by TTFA is shown in Fig. 1B. Preincubation of the carboxylesterase with TTFA (0.5 μM) up to 30 min caused a time-dependent inhibition; within 10 min about 30% of the enzyme activity was inhibited and by 20 min of incubation the activity was decreased to about 50% of the control. In addition, we compared the inhibitory effect of TTFA with BNPP, a well-established esterase inhibitor. TTFA at 1 μM resulted in about 70% inhibition of esterase, whereas BNPP at the same concentration caused 85% inhibition (Fig. 2). Taken together, the results demonstrate that TTFA is an inhibitor of carboxylesterase with potency similar to that of BNPP.

The mechanism by which TTFA inhibits carboxylesterase activity is not known, as this is a new discovery. The structure of TTFA is similar to that of the trifluoromethyl ketones, as it contains trifluoro and ketone moieties, and these ketones have been shown to reversibly inhibit esterases such as acetylcholinesterase and pseudocholinesterase [10–12]. Both keto and trifluoromethyl groups are important for esterase inhibition [11–13], and Rosell *et al.* [11] suggested that the 3-keto group may be the actual reactive species to interact with a serine residue of the carboxylesterase enzyme. The active site serine is likely to add to the 3-keto group to form a hemiketal, and the trifluoromethyl group may serve as a powerful electron withdrawing group which would facilitate this addition reaction. Another possible mechanism for TTFA inhibition may be related to the integrity of the esterase thiol groups, which are essential for the catalytic efficiency of the enzyme. In support of this possibility, esterases from rat liver microsomes were inactivated by a number of classical thiol reacting reagents [14], and we have shown that TTFA

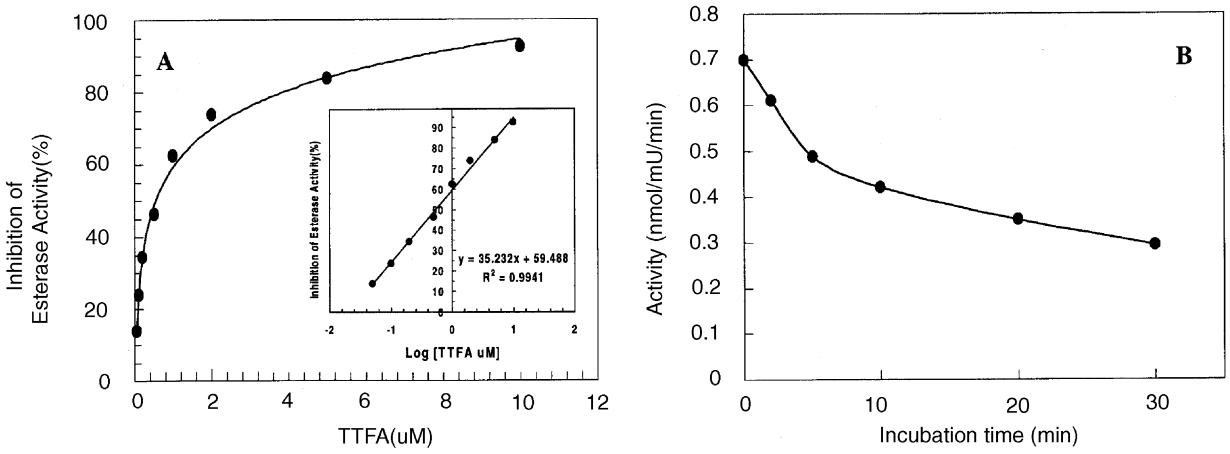

Fig. 1. Concentration- (A) and time- (B) related effects of TTFA on purified liver carboxylesterase activity. The activity was determined by the formation of PNP from PNPA by 50 mU carboxylesterase at 23° after preincubation of the enzyme with TTFA (0.05–10 μM) for 20 min or 0.5 μM TTFA for different incubation times up to 30 min. Inset: plot of inhibition of enzyme activity vs. logarithmic concentrations of TTFA. The graphs represent the means of three experiments.

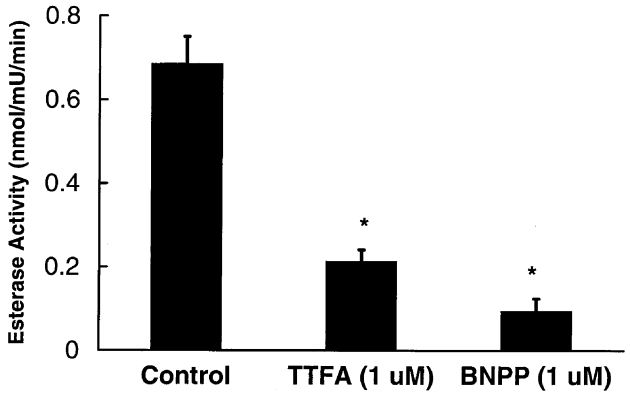

Fig. 2. Comparison of the inhibitory effect of purified liver carboxylesterase activity by TTFA and BNPP. The esterase (50 mU) was incubated with TTFA or BNPP at 23° for 20 min. Activity was determined by the formation of PNP from PNPA (1 mM in final concentration) by the carboxylesterase. Data are the means ± SD of three separate experiments. (*) $P < 0.01$, significantly different from the control, using Student's *t*-test.

dramatically depletes glutathione in isolated hepatocytes [15].

3.2. Inhibition kinetics

To investigate the type of enzyme inhibition exhibited by TTFA, we assayed the esterase activity in the presence of different concentrations of the substrate PNPA (0.05–2.0 mM) and TTFA (0.5 and 2.0 μM). Data from the double-reciprocal plots (Lineweaver–Burk) for the purified porcine liver carboxylesterase (Fig. 3) indicate that the inhibition was non-competitive with a calculated K_i value of about 0.61 ± 0.03 μM ($R^2 = 0.9821$–0.9906) and a K_i' value of about 0.72 ± 0.02 ($R^2 = 0.9721$–0.9910). The results suggest that TTFA does not interfere with the substrate binding site of esterase but instead may bind to a second site (sulfhydryl or serine residues) on the enzyme surface (not the active site) to inhibit the catalytic step.

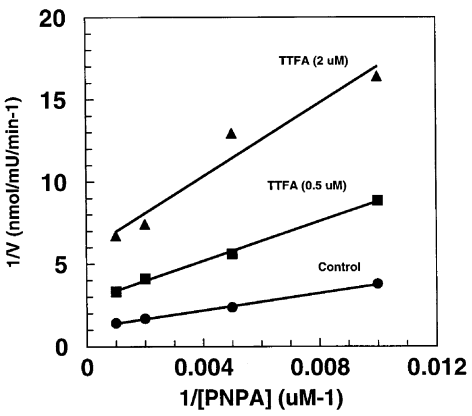

Fig. 3. Double-reciprocal plots of purified liver carboxylesterase inhibition by TTFA. Activity was determined by the formation of PNP from PNPA by 50 mU esterase at several substrate concentrations in the absence (control) and presence of TTFA (0.5 and 2.0 μM) at 23° after a 20-min preincubation of the enzyme with TTFA. The graph represents the mean of three experiments.

3.3. Effect of TTFA on rat plasma, liver mitochondrial esterase, and mitochondrial complex II activity

We further examined the effect of TTFA on rat plasma and liver mitochondrial carboxylesterase activity. Plasma esterase activity was 0.26 ± 0.02 μmol/min/mg protein and liver mitochondrial carboxylesterase activity was 1.36 ± 0.22 μmol/min/mg protein. These results are consistent with the findings of Chanda *et al.* [16] in which carboxylesterase activity in the plasma was much lower than in the tissue. As shown in Fig. 4, addition of TTFA (1–25 μM) dramatically inhibited mitochondrial complex II activity (46.4 ± 3.2 nmol/min/mg protein for control activity) in a concentration-dependent fashion, confirming that TTFA is a potent inhibitor of mitochondrial complex II. The mechanism by which TTFA inhibits mitochondrial complex II activity is unknown. Early studies suggest that TTFA binds to iron–sulfur clusters of succinate-ubiquinone reductase (complex II) [3]. Therefore, the mechanisms of TTFA-induced inhibition of mitochondrial complex II and carboxylesterase activity seem different. This notion may be further supported by the fact that inhibition of the esterase activity by TTFA requires preincubation, whereas inhibition of mitochondrial complex II activity does not. Using the same concentrations, TTFA significantly inhibited both plasma and mitochondrial car-

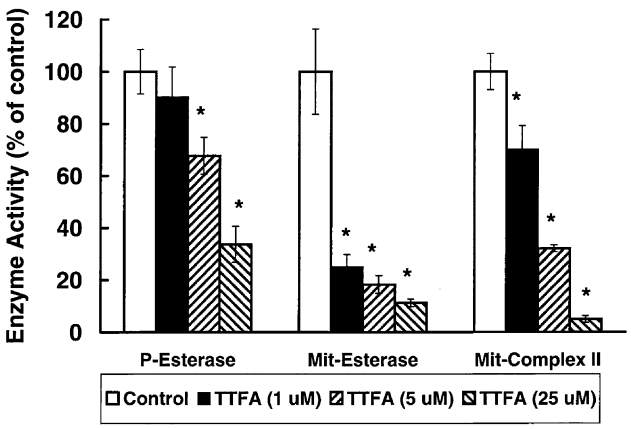

Fig. 4. Effect of TTFA on rat plasma, mitochondrial esterase, and mitochondrial complex II activity. Plasma and mitochondria were isolated from rats, as described in Section 2. Plasma (500 μg of protein) and mitochondria (10 μg of protein) were incubated with TTFA at 23° for 20 min. The esterase activity was determined by the formation of PNP from PNPA (1 mM in final concentration) by carboxylesterase. Mitochondrial complex II activity was determined by the reduction of ubiquinone by mitochondria (100 μg). Data are the means ± SD of three separate experiments. (*) $P < 0.01$, significantly different from the control, using Student's *t*-test. Plasma and mitochondrial carboxylesterase activities and mitochondrial complex II activity in the control were 0.26 ± 0.02 μmol/min/mg protein, 1.36 ± 0.22 μmol/min/mg protein, and 46.4 ± 3.2 nmol/min/mg protein, respectively.

boxylesterase activity after 20 min of incubation. For example, in the presence of 25 μM TTFA, plasma esterase activity was reduced to about 35% of control, while mitochondrial esterase activity was reduced to approximately 10% of control. Therefore, liver mitochondrial carboxylesterase appears to be more sensitive than plasma carboxylesterase to inhibition by TTFA.

The assay used in this study measures predominantly carboxylesterase activity [16], and thus it is possible that TTFA may also inhibit other esterases such as acetylcholinesterase and lipases. Since TTFA is a widely used inhibitor of mitochondrial electron transport, caution should be used when combining it with potential esterase substrates. For example, fluorescence probes containing an ester bond, such as 2′,7′-dichlorohydrofluorescein diacetate and the acetoxymethyl ester of fura, are routinely used for investigating the production of reactive oxygen species from mitochondria or calcium mobilization in mitochondria [4,5,7]. Use of TTFA in these studies may prevent the hydrolysis of these probes and provide confounding results. A final example is the use of TTFA in combination with D-α-tocopheryl succinate, a vitamin E derivative that requires hydrolysis by esterase to release the antioxidant, D-α-tocopherol, and provide mitochondrial protection [17,18]. We have found that 25 μM TTFA inhibits the release of tocopherol from tocopheryl succinate by 75% in rat hepatocytes after a 60-min incubation of the hepatocytes with tocopheryl succinate in the presence of TTFA (unpublished data), as well as preventing tocopheryl succinate-mediated cytoprotection [15].

In conclusion, we have discovered that TTFA is a potent inhibitor of carboxylesterase activity, in addition to its inhibitory effect on mitochondrial complex II activity.

Acknowledgments

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