Effects of Pentachlorophenol, Pentylenetetrazol and Carnitine on Mitochondria

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Abstract : Pentachlorophenol (PCP) increased oxygen consumption and lowered the respiratory control ratio (RCR) in mitochondria from rat liver. These effects of PCP were lessened by 1 mM L-carnitine but not by D-carnitine. In contrast, up to 150 mM of pentylenetetrazol (PTZ) added at state 4 of respiration did not accelerate oxygen consumption. When mitochondria were incubated with 3.3 mM of PTZ, oxygen consumption, RCR and ADP/O ratio were all decreased. Moreover, these could not be suppressed even by high concentrations (— 20 mM) of L-carnitine. Thus, while L-carnitine could suppress effects of PCP, it could not counteract PTZ in mitochondria. It appears that anticonvulsive effects of carnitine in PTZ-induced seizures may not be due to mitochondrial protection.

Key words pentachlorophenol, pentylenetetrazol, carnitine, seizure, anticonvulsive.

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Introduction

Carnitine (β -hydroxy- γ -N-trimethylbutyrate) is widely distributed among tissues including the brain. In extraneural tissues, carnitine is an essential cofactor for the transport of fatty acids through the inner mitochondrial membrane [1]. In the brain, however, fatty acids are not utilized as an energy source, and physiological functions of carnitine are unknown. Previous studies have indicated that carnitine can protect the brain from various insults such as hyperammonemia [2, 3] and severe ischemia [4]. We have observed that carnitine can also suppress convulsions induced by pentylenetetrazol (PTZ) [5], which is one of the most commonly used epileptogenic agents. However, its mechanism is not clear. We therefore examined effects of PTZ and carnitine on mitochondria comparing them with those of pentachlorophenol (PCP) [6—8] which is a typical chemical that impairs biological membranes including mitochondria.

Materials and Methods

Chemicals: L-Carnitine (inner salt), D-carnitine (inner salt), pentachlorophenol (PCP) and pentylenetetrazol (PTZ) were purchased from Sigma (St. Louis, Mo). All other chemicals were reagent grade.

Mitochondria: Livers from male Wistar rats were minced quickly in a solution containing 250 mM sucrose, 0.5 mM EDTA and 10 mM Tris-HCl (pH 7.4), and homogenized in a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged at $800 \times g$ for 10 min to obtain supernatant, which was then centrifuged at $10000 \times g$ for 8 min. After the sediment was resuspended in the solution and centrifuged at $8000 \times g$ for 8 min, the supernatant was discarded. These procedures were all done at 4°C . Protein of the mitochondrial suspension was determined by the method of Lowry *et al* [9].

Oxygen consumption: The solution used to measure oxygen consumption (3 ml) contained 225 mM sucrose, 10 mM KCl, 5 mM MgCl_2 , 5 mM potassium phosphate buffer (pH 7.4), 0.5 mM EDTA, 20 mM Tris-HCl (pH 7.4) and mitochondrial suspension (4.5 mg of protein). A YSI Model 5300 Biological Oxygen Monitor (Yellow Spring Instrument, Yellow Spring, Ohio) equipped with a Clark-type oxygen electrode was used and the changes were recorded with a Hitachi 056 recorder. The temperature of the reaction mixture (30°C) was controlled with a Lauda RM3 high precision circulating water bath (Lauda-Köningshofen). Unless otherwise stated, mitochondrial suspension was first incubated with or without carnitine (L or D-form) for 10 min at 30°C , and then with or without a chemical (PCP or PTZ) for another 10 min. The reaction was monitored after adding $5 \mu\text{l}$ of 80 mM ADP and $10 \mu\text{l}$ of 200 mM α -ketoglutarate. When necessary, chemicals were added through the port of the reaction chamber. PCP was first dissolved in ethanol and then added to the reaction mixture. The final concentration of ethanol was less than 0.3% which did not affect the reaction.

The respiratory control ratio (RCR) was calculated as (oxygen consumption at state 3 of respiration) / (oxygen consumption at state 4), and ADP/O ratio as (ADP added) / (oxygen consumed).

Results

PCP dose-dependently increased oxygen consumption and lowered RCR (Fig. 1). These changes were less marked when 1 mM L-carnitine was present. In addition, L-carnitine dose-dependently suppressed effects of PCP while D-carnitine did not (Fig. 2).

Fig. 1. Effects of pentachlorophenol (PCP) and L-carnitine on oxygen consumption and respiratory control ratio (RCR) in mitochondria from rat liver (Mean $\pm$ SE, $n = 6$). Mitochondria were incubated with PCP and L-carnitine ($1 \mu\text{M}$ or 1mM) or without it for 10 min at 30°C , and changes in the amount of oxygen in the solution were monitored. Oxygen consumption is expressed as nmol oxygen consumed/mg protein/min. Asterisks indicate differences from the control (no carnitine) (** $P < 0.01$, by Student t -test).

—○— No Carnitine, —△— $1 \mu\text{M}$ L-Carnitine, —●— 1mM L-Carnitine

On adding PTZ to the reaction mixture successively over a wide range of concentrations ($3, 6, 9, 12$ and $15 \mu\text{M}$; $30, 60, 90, 120$ and $150 \mu\text{M}$; $3, 6, 9, 12$ and 15mM ; $30, 60, 90, 120$ and 150mM) at state 4 of respiration, no increase of oxygen consumption was observed. When mitochondria were incubated with 3.3mM of PTZ for 10 min, oxygen consumption, RCR and ADP/O ratios were all decreased (Fig. 3). L-Carnitine even at 20mM did not suppress these effects of PTZ (Fig. 4).

Fig. 2. Effects of L- and D-carnitine on pentachlorophenol (PCP)-induced alterations of oxygen consumption and respiratory control ratio (RCR) in mitochondria from rat liver (Mean $\pm$ SE, $n = 6$). Mitochondria were incubated with carnitine (L- and D-form) for 10 min at 30°C and then with PCP (3 μ M) or without it for 10 min and changes in oxygen consumption in the solution were monitored. Asterisks indicate difference from the effect of D-carnitine (** $P < 0.01$, *** $P < 0.001$, by Student *t*-test).

—○— No PCP + L-Carnitine, —●— 3 μ M PCP + L-Carnitine
 —△— No PCP + D-Carnitine, —▲— 3 μ M PCP + D-Carnitine

Fig. 3. Effects of pentylenetetrazol (PTZ) on oxygen consumption, respiratory control ratio (RCR) and ADP/O ratio in mitochondria from rat liver (Mean $\pm$ SE, $n = 6$). After mitochondria were incubated with PTZ or without it for 10 min at 30°C, ADP and α -ketoglutarate were added and the amount of oxygen in the solution were measured. Asterisks indicate difference from the control (without PTZ) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by Student t -test).

—○— O₂consumption, —●— RCR, —△— ADP/O

Fig. 4. Effects of pentylenetetrazol (PTZ) and L-carnitine on oxygen consumption, respiratory control ratio (RCR) and ADP/O ratio in mitochondria from rat liver (Mean $\pm$ SE, $n = 6$). After mitochondria were incubated with PTZ and L-carnitine for 10 min at 30°C, the reaction was started by adding ADP and α -ketoglutarate. Similar results were obtained when mitochondria were first incubated with L-carnitine (2 and 10 mM) at 30°C for 10 min and then for another 10 min with PTZ (3.3 mM). Asterisks indicate differences from the control (without PTZ and carnitine) (*** $P < 0.001$, by Student t -test).

■ control, ■ 10mM L-Carnitine, □ 20mM L-Carnitine,
▨ 3.3mM PTP + 10mM L-Carnitine, ▩ 3.3mM PTP + 20mM L-Carnitine,

Discussion

Although PCP has been used widely, it can be toxic to humans and even fatal cases of intoxication with PCP have been reported [7]. The mechanisms of the toxicity have not been fully clarified but PCP has been known to disrupt biological membranes including mitochondria and erythrocyte membrane; PCP is a potent uncoupler of oxidative phosphorylation in mitochondria [6], and it can hemolyze erythrocytes [7]. The increase of oxygen consumption and decrease of RCR in mitochondria seen in the present experiments are consistent with the uncoupling of oxydative phosphorylation by PCP. And these could be suppressed by L-carnitine. This indicates that L-carnitine can protect mitochondria not only in hyperammonemia [10] or damage induced by octanoic acid [11] but also from impairment caused by PCP. Hence, it is of interest that mitochondrial dysfunction caused by different kinds of chemicals can be ameliorated by L-carnitine which plays an important physiological role in the inner mitochondrial membrane. Furthermore, it is also of interest that, in the case of PCP as seen in the present experiments, only L-carnitine exerted protective effects, while D-carnitine did not because D-carnitine is believed not to occur naturally and has been shown to interfere with some effects of L-carnitine [12]. However, much more work is necessary before any conclusion can be drawn concerning whether carnitine is effective in the prevention or treatment of intoxication with PCP.

PTZ is one of the most commonly used epiletogenic agents to evaluate possible anticonvulsive drugs. Although the mechanism of epileptogenesis by PTZ has not been well understood, drugs effective in suppressing PTZ-induced seizures often affect GABAergic systems [13]. On the other hand, it has been reported that GABA caused swelling of mitochondria *in vitro* [14]. Furthermore, ATP content in the brain decreased prior to convulsions induced by chemicals including PTZ [15]. We previously observed that L-carnitine could also suppress seizures induced by PTZ as well as alterations of high energy phosphate compounds in the brain in mice [5]. Hence, it would be of interest to examine effects of PTZ and carnitine on mitochondria *in vitro*, particularly because the mechanism of the anticonvulsive effects of carnitine is unclear, and because the protective effects of carnitine on mitochondria have been suggested in ammonium intoxication and in octanoic acid-induced brain damage [10, 11]. Nevertheless, we observed no changes indicating that PTZ uncoupled oxidative phosphorylation in mitochondria i.e. we saw no increase of oxygen consumption by PTZ. But we did observe that PTZ could impair mitochondrial functions; oxygen consumption, RCR and ADP/O ratio were lowered by PTZ. However, these effects of PTZ are most likely nonspecific because only high concentrations of

PTZ could induce them and because all indices were lowered. Furthermore, while toxicities of PCP could be suppressed by L-carnitine, no protection against PTZ toxicities were provided by L-carnitine, even at high concentrations. Thus, at least *in vitro*, while L-carnitine could suppress effects of PCP, it did not show any protective effects on mitochondria from PTZ. Hence, it is likely that the anticonvulsive effects of carnitine in PTZ-induced seizures may not be due to mitochondrial protection.

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ペンタクロロフェノール，ペンチレンテトラゾール，カルニチンの ミトコンドリアへの影響

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要 旨： ペンタクロロフェノール (PCP) は，ラット肝臓より得たミトコンドリアで酸素消費増加と呼吸調節比 (RCR) 低下をもたらした。これらの PCP の効果は，1 mM の L-カルニチンによって抑制されたが，D-カルニチンは無効であった。これと対照的に，ペンチレンテトラゾール (PTZ) は，状態 4 呼吸時に 150 mM まで加えても，酸素消費を亢進させなかった。ミトコンドリアを 3.3 mM の PTZ とインキュベートすると，酸素消費，RCR，ADP/O 比いずれも低下した。しかも，これらは高濃度 (20 mM 以下) の L-カルニチンによって抑制され得なかった。すなわち，L-カルニチンは PCP の影響に対しては明確な保護効果を示したが，PTZ の作用からミトコンドリアを保護することはなかった。PTZ 誘発けいれんにおけるカルニチンの抗けいれん作用は，ミトコンドリア保護によるのではない可能性が考えられる。

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