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PROTECTIVE EFFECT OF D,L-CARNITINE ON VALPROATE-INDUCED HYPERAMMONEMIA AND HYPOKETONEMIA IN PRIMARY CULTURED RAT HEPATOCYTES

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Abstract—The effect of D,L-carnitine on sodium valproate (VPA)-induced hyperammonemia and hypoketonemia was investigated in primary cultures of rat hepatocytes. Administration of VPA (0.1 to 1.0 mM) resulted in an increase of ammonia and a decrease of ketone bodies in culture medium. When D,L-carnitine was added with VPA to the medium, the level of ammonia decreased significantly and that of ketone bodies increased. A significant negative relationship was found between the concentrations of ammonia and the ketone bodies in the medium following administration of D,L-carnitine. Our results suggested that VPA suppressed the urea cycle metabolism and that a protective effect of D,L-carnitine on ketone metabolism was probably due to the reversal of the inhibition of beta-oxidation.

The antiepileptic property of sodium valproate (VPA), a branched short chain fatty acid, was first described by Meunier *et al.* [1]. Although VPA has been in world-wide clinical use, it has been incriminated in a number of instances as a cause of severe hepatic injury [2–4], producing hyperammonemia, usually without liver dysfunction [5, 6]. The mechanism of VPA-induced hyperammonemia has not been clearly established. Coude *et al.* [7] reported that VPA-induced hyperammonemia is secondary to an inhibition of *N*-acetylglutamate (NAGA) synthesis. It is also known that VPA inhibits ketogenesis [8]. Recently, it was reported that the administration of carnitine has a protective effect on VPA-induced hyperammonemia [9, 10].

The present study investigated the effect of D,L-carnitine on VPA-induced hyperammonemia and hypoketonemia in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Male Wistar strain rats, weighing 150–200 g were used. They were fed a standard commercial rat chow (not containing L-carnitine) and water *ad lib.* Rats were anesthetized intraperitoneally with sodium pentobarbital.

Williams' medium E was purchased from Flow Laboratory (Rockville, MD). D,L-Carnitine was obtained from the Sigma Chemical Co. (St Louis, MO). VPA was a gift from the Kanebo Yakuhin Co. (Tokyo). Ammonia was determined by the glutamate dehydrogenase method (Determiner NH_3, Kyowa Medex Co., Tokyo). Ketone bodies were examined by the diazo method (Keton Test "Sanwa", Sanwa Kagaku Kenkyusho, Nagoya).

Isolation of hepatocytes was performed by a modification of the method of Seglen [11]. All components of the perfusion apparatus were autoclaved, and collagenase solution was filter-sterilized. Hanks' solution with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer was used as the perfusion fluid. After perfusion with 0.05% collagenase solution at 37° for 15–20 min, the liver was decapsulated. The decapsulated cell mass was placed for 5 min (without any addition of collagenase) in Krebs–Ringer–bicarbonate buffer containing 0.2% albumin in a gently rotating flask with constant oxygenation. The cells were washed three times at 50 *g*, for 3 min. Cell viability was determined with trypan blue (0.4%, w/v) exclusion and phase contrast microscopy. Preparations with more than 10% damaged cells were discarded. For the cell culture, culture medium in a humidified incubator at 37° under 5% CO_2 in air was inoculated with 5–10 × 10^6 cells/ml. The time-concentration relation was studied by using 0.1 to 1 mM VPA concentrations. The medium was changed after the first 24 hr and then was incubated with VPA, VPA plus D,L-carnitine, and normal medium (control) respectively.

Results were analyzed statistically with Student's *t*-test.

RESULTS

Ammonia concentrations in the medium proportionally increased as the incubation time was prolonged, and the presence of VPA (1 mM) significantly enhanced its production 0.5 to 24 hr after the start of the culture (Fig. 1). Figure 2 shows the relationship between the VPA added to the medium and the production of ammonia, and ketone bodies.

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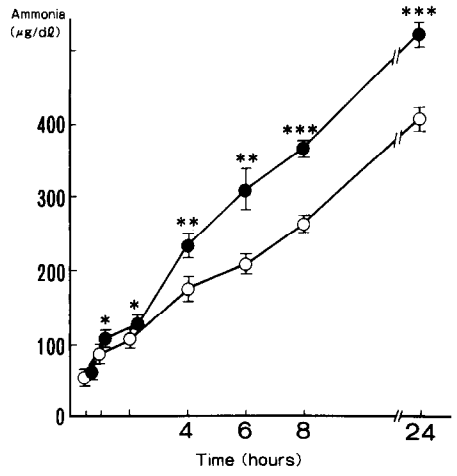

Fig. 1. Relation between ammonia concentration and duration of culture with and without VPA. Key: (○) control, and (●) VPA, 1 mM. Results are means ± SD of five experiments. Asterisks indicate significant difference from control: (*) $P < 0.02$, (**) $P < 0.01$, and (***) $P < 0.001$.

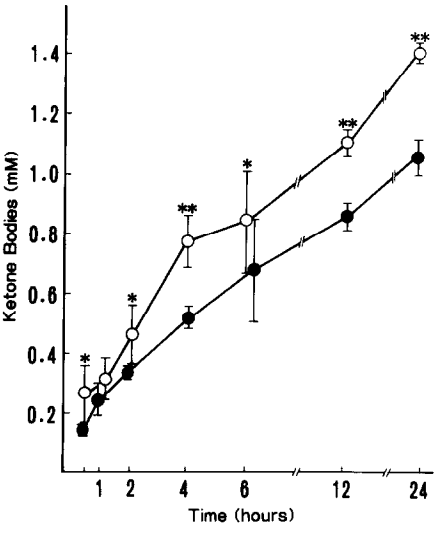

Fig. 3. Relation between production of ketone bodies and time course of culture with (●) and without (○) VPA (0.5 mM). Results are means ± SD of six experiments. Asterisks indicate a significant difference from control: (*) $P < 0.05$, and (**) $P < 0.001$.

Figure 3 shows the relationship between the incubation time and the production of ketone bodies. Unlike ammonia, ketone bodies in the cultures containing VPA concentrations were significantly lower than in the control. Ketone bodies in the medium decreased linearly as the VPA concentration added to the medium was increased (Fig. 2). VPA shows a concentration-related inhibition of the liberation of ketone bodies in the medium.

The effect of the administration of D,L-carnitine on the production of ammonia and ketone bodies in the VPA-added medium is shown in Fig. 4. In the medium containing VPA, the concentration of ammonia decreased and the concentration of ketone bodies increased following the administration of D,L-carnitine.

The relation between ammonia and ketone body levels when 1 mM D,L-carnitine and 0.5 mM VPA were added to the medium is shown in Fig. 5. A significant negative relation was found between the concentration of ketone bodies and ammonia in the medium.

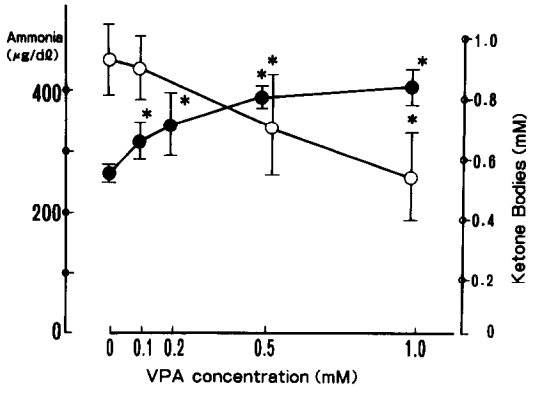

Fig. 2. Relation between VPA added to the medium and production of ammonia (●) and ketone bodies (○). Culture duration: 6 hr. Results are means ± SD of five experiments. Asterisks indicate a significant difference ($P < 0.01$) from control (without VPA added).

DISCUSSION

The results in this paper show that VPA affected the production of ammonia and ketone bodies in primary cultured rat hepatocytes. The level of

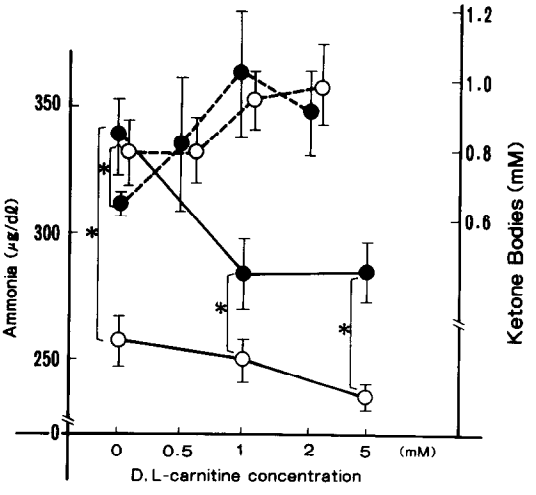

Fig. 4. Relation between ammonia concentration and D,L-carnitine concentration (———) in D,L-carnitine without VPA (○) and with VPA (1 mM) (●). Relation between D,L-carnitine concentration and production of ketone bodies (– – –) VPA with (0.5 mM) (●), or without VPA (○). Culture duration: 6 hr. Results are means ± SD of five experiments. Asterisks indicate a significant difference ($P < 0.001$).

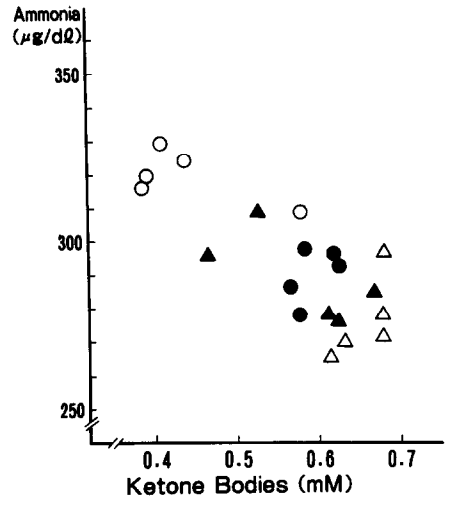

Fig. 5. Relation between ammonia and ketone body concentrations. Culture duration: 6 hr without D,L-carnitine (○), and in the presence of D,L-carnitine at concentrations of 0.5 mM (●), 0.8 mM (▲) and 1 mM (△), respectively, and VPA (0.5 mM) added to the medium. $y = -160x + 384$, $N = 20$, $r = -0.80$.

ammonia was elevated and that of ketone bodies inhibited by VPA at concentrations of 0.1 to 1.0 mM. In this series, the VPA concentration in the medium was similar to the levels of VPA blood concentration in clinical use.

Hyperammonemic encephalopathy in a patient on VPA medication was first reported by Coulter and Allen [5]. The principal mechanism, as suggested by Coude *et al.* [7], was that VPA or VPA metabolites reduced the activity of NAGA synthetase, which decreased the activity of carbamyl phosphate synthetase I (CPS I), inducing hyperammonemia. Recently, Aramaki [12] reported that the concentration of NAGA was reduced, but the activity of NAGA synthese was not inhibited in isolated rat mitochondria by the administration of VPA. VPA-induced hyperammonemia, including reduced urea synthesis, probably occurred with a reduced level of NAGA, which resulted in a high ratio of acyl-CoA/CoASH and reduced levels of acetyl CoA [13]. In this paper, the high ratio of acyl-CoA/CoASH and the reduced availability of acetyl-CoA were shown indirectly by the inhibition of ketone body production.

Thurston *et al.* [8] reported reduced levels of ketone bodies in the blood of epileptic patients on VPA medication. Becker and Harris [13] and Turnbull *et al.* [14] proposed that either valpronyl CoA itself or the sequestration of CoA caused inhibition of beta-oxidation of fatty acid and hyperammonemia *in vitro*. Hyperammonemia could be due to the proposed indirect inhibition of ureagenesis which resulted in the inhibition of beta-oxidation in liver mitochondria by valpronyl CoA.

Recently, Ohtani *et al.* reported secondary carnitine deficiency associated with VPA therapy [9], and Böhles *et al.* showed hypocarnitinemia in a case with Reyes-like syndrome due to VPA medication [15]. We also reported hypocarnitinemia due to VPA medication in clinical and experimental studies in rats [16, 17]. The results in this series of experiments indirectly indicate a depressed carnitine level in the medium containing VPA, because the abnormality in ammonia and ketone body metabolism due to the presence of VPA was reversed by the administration of D,L-carnitine. Although there have been a few papers on the protective effect of carnitine on ammonia metabolism [9, 10], there has been no study of the influence of different concentrations of carnitine on ammonia and ketone body metabolism. Our study has shown that the most effective concentration of D,L-carnitine was 1 mM, which may be attainable in clinical applications of D,L-carnitine treatment of the abnormal status due to VPA therapy.

In this paper, a significant negative relationship was shown between the concentrations of ketone bodies and ammonia in medium containing VPA (0.5 mM) after the administration of D,L-carnitine (0–1 mM). The production of ketone bodies indicates the function of beta-oxidation of fatty acids, and the ammonia concentration shows the function of ureagenesis. Carnitine plays an essential role in the mechanism of fatty acid oxidation, as a carrier for the entry of acyl groups into the mitochondria [18]. The mechanism of the protective effect may be indicated from our study and the following considerations: (1) after the administration of carnitine, valpronyl CoA is metabolized to valpronylcarnitine, which is excreted in urine; (2) subsequently, the ratio of acyl CoA/CoASH is normalized in mitochondria, and the inhibition of beta-oxidation of fatty acids is reversed; and (3) increased acetyl-CoA may lead to increased synthesis of NAGA and to physiological activation of CPS I and, therefore, to a higher rate of urea production.

This study indicates that the inhibition of beta-oxidation may occur in patients on VPA medication and that the administration of D,L-carnitine may have a protective effect on the abnormal metabolic state that is evidenced by hyperammonemia and the inhibition of ketogenesis *in vitro*.

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