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ACTION OF THE ANTIEPILEPTIC DRUG, VALPROIC ACID, ON FATTY ACID OXIDATION IN ISOLATED RAT HEPATOCYTES

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Valproate at 0.1 to 5 mM strongly inhibited oxidation of 1-(^{14}C)-palmitate in isolated rat hepatocytes. Valproate at the same concentrations markedly decreased ketogenesis from 1 mM oleate. Valproate in a dose up to 5 mM did not significantly affect cellular concentration of ATP but lowered β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios which paralleled its effect on ketogenesis. Moreover concomitant acetyl-CoA levels were drastically decreased by valproate. From this it may be concluded that inhibition of fatty acid oxidation by valproate results in reduced production of two carbons units and a drop of NADH/NAD+ ratio in rat hepatocyte. This suggests that valproate seriously interfers with β-oxidation of physiological long-chain fatty acids.

Valproate (2(n-propyl)pentanoate), administered either as sodium valproate or as free valproic acid, is an antiepileptic drug which has been successfully used in the treatment of several types of epilepsy, particularly in childhood (1). It has received adverse publicity because of its effects on hepatic function (2). Several fatal hepatic failures associated with valproate administration have been reported (3). Moreover an encephalopathic disorder resembling Reye's syndrome has been also attributed to valproate in a few cases (4, 5). The mechanism by which this hepatic injury occurs is presently unknown. Nevertheless several findings suggest that valproate may seriously interfer with β-oxidation of fatty acids. Indeed valproate is a branched chain fatty acid which undergoes both ω and β-oxidation in liver (6, 7). Main metabolites have been identified in humans and animals (8, 9). They closely resemble to pent-4-enoic acid, a strong inhibitor of fatty acid oxidation (10). Moreover C_6-C_{10} dicarboxylic aciduria has been noted in patients and rats treated with valproate (11, 12). In children dicarboxylic aciduria is usually associated with inherited disorders or acquired illness in which fatty acid oxidation is impaired, i.e. carnitine deficiency (13), defective

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acyl-CoA dehydrogenases (14), hypoglycin toxicity (15). The purpose of this work has been to study the effects of valproate on fatty acid oxidation in isolated rat hepatocytes.

MATERIAL AND METHODS

Albino Wistar male rats (150-200 g),starved for 18h, were used. After anesthetizing the animal with Nembutal (7 mg per 100 g of body weight) the liver was cannulated, isolated and mounted in a recirculation perfusion system as described by WILLIAMSON et al (16). The procedure used for the cell isolation was similar to that of BERRY et al (17) with some modifications (18). The yield was usually 500 mg of dry weight per liver. Cell viability was routinely tested by trypan blue (0.4% w/v) exclusion and phase contrast microscopy. Preparations with more than 10% damaged cells were discarded. The cells were suspended in 2 ml Krebs's bicarbonate medium oxygenated with 95% O_2 + 5% CO_2 , containing 1.3 mM calcium. Incubations were performed for 30 min in 25 ml Erlenmeyer flasks which were shaken at 80 oscillations/min in a water-bath at 37°C. The final concentration of cells was between 3 and 5 mg of dry weight/ml of incubation medium. Assay of total contents of cell metabolites was performed at the end of 30 min incubation by adding 1 ml-aliquot of cell suspension to perchloric acid (4.5%(w/v), final concentration) followed by centrifugation and neutralization to pH 6 with 3N KOH. Long chain fatty acids were complexed to 10% dialyzed fatty acid free bovine serum albumin at a ratio of 20 nmol of fatty acid per mg of albumin. Oxidation of 1-(^{14}C) palmitate was measured by incubating hepatocytes in 2 ml Krebs's bicarbonate medium containing 0.5 mM 1-(^{14}C)-palmitate (2 Ci/nmol). After 30 min at 37°C the reaction was terminated by addition of 0.25 ml of 50% (w/v) trichloracetic acid. The $^{14}(CO_2)$ evolved was trapped on hyamine-coated filter paper during a further 30 min incubation period. The (^{14}C)-labeled trichloracetic acid soluble reaction products were isolated according to the technique of VAN HINSBERGH et al (19). The (^{14}C) activity was measured in a liquid scintillation counter.

The dry weight of the cells was obtained by protein precipitation and washing with trichloracetic acid (20). ATP was measured fluorimetrically by an enzymatic method (21). β-hydroxybutyrate, acetoacetate, lactate and pyruvate were determined by the spectrophotometric method of WILLIAMSON et al (21). Acetyl-CoA was measured using citrate synthase as described by PANDE et al (22). All experiments were repeated 3 to 6 times. Results are presented as means ± SEM. Statistical significance was calculated using the paired Student's t-test. Enzymes, coenzymes and reagents were purchased from Boehringer or Sigma.U (^{14}C)-aspartic acid and 1-(^{14}C)-palmitate were obtained from Amersham.

RESULTS

Fatty acid oxidation ($^{14}CO_2$ + (^{14}C) acid-soluble products) in rat hepatocytes proceeded linearly with time up to 60 min. Valproate at 0.1 to 5 mM strongly inhibited the production rate of acid-soluble radioactive products from 1-(^{14}C)-palmitate which mainly reflects ketone bodies formation (23). However concomitant ($^{14}CO_2$) production was significantly increased, suggesting that acetyl-CoA generated from palmitate was directed away from ketogenesis and into the citric acid cycle (Fig 1). Moreover as

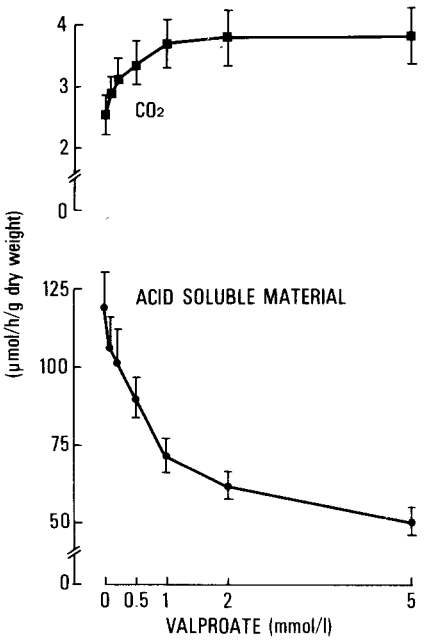


Fig 1 - Effect of valproate on (^{14}C) acid-soluble products and $^{14}CO_2$ formation from 1-(^{14}C)-palmitate in hepatocytes isolated from 18h starved rats.
Rat liver cells (3 to 5 mg dry weight/ml) were incubated for 30 min with 0.1 mM 1-(^{14}C) palmitate. Sodium valproate was added at the indicated concentrations. Values are means ± SEM for 4 experiments.

expected valproate at the same concentrations markedly decreased ketone bodies formation from 1 mM oleate (Fig 2).

Valproate in a dose up to 5 mM did not significantly affect cellular content of ATP (10.8 ± 1.2 umol/g dry weight) but lowered β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios which paralleled its effect on ketogenesis. Concomitant cellular acetyl-CoA levels were also drastically decreased (Fig 3). From this it may be concluded that inhibition of fatty acid oxidation by valproate in rat hepatocytes resulted in reduced production of two-carbon units and in a drop NADH/NAD+ mitochondrial ratio. Furthermore valproate greatly inhibited ketogenesis from 1 mM butyrate in hepatocytes incubated respectively with and without 2 mM valproate (412 ± 127 and 641 ± 187 umol/h/g dry weight).

DISCUSSION

These results demonstrate that the antiepileptic agent, valproic acid, is an exceedingly potent inhibitor of long-chain

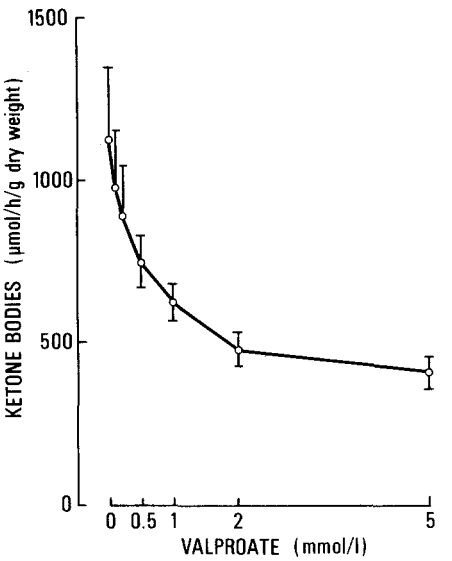


Fig 2 - Effect of valproate on ketogenesis from 1 mM oleate in hepatocytes isolated from 18 starved rats.
Rat liver cells (3 to 5 mg dry weight/ml) were incubated for 30 min with oleate. Sodium valproate was added at the indicated concentrations. Ketone bodies formation was calculated as the sum of B-hydroxybutyrate and acetoacetate. Values are means ± SEM of 3 to 6 experiments.

fatty acid oxidation in rat hepatocytes. The oxidative route open to a long chain fatty acid subsequent to its entry into the liver cell are summarized in schema 1. It is generally agreed (23) that after activation to its acyl-CoA derivative in the cytosol the long chain fatty acid will enter the mitochondrion (under the influence of carnitine acyl-transferase) where upon it is comitted to production of acetyl-CoA via the β-oxidation pathway. Acetyl-CoA can be metabolised through a variety of pathways including the Krebs cycle, lipogenesis or the reactions of the hydroxymethylglutaryl-CoA sequence leading to the synthesis of acetoacetate and β-hydroxybutyrate. Therefore several factors may contribute to an inhibition of long chain fatty acid oxidation. These include : (a) decreased ATP production ; (b) defective carnitine dependent transport ; (c) inhibition of the β-oxidation pathway ; (d) defective hydroxymethylglutaryl-CoA sequence.

A limiting general ATP production may be excluded because no decrease was observed in the ATP content of hepatocytes incubated with valproate. A specific inhibition of the carnitine

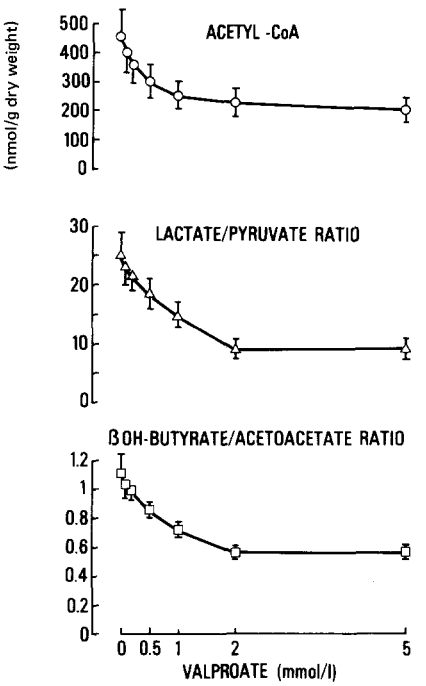
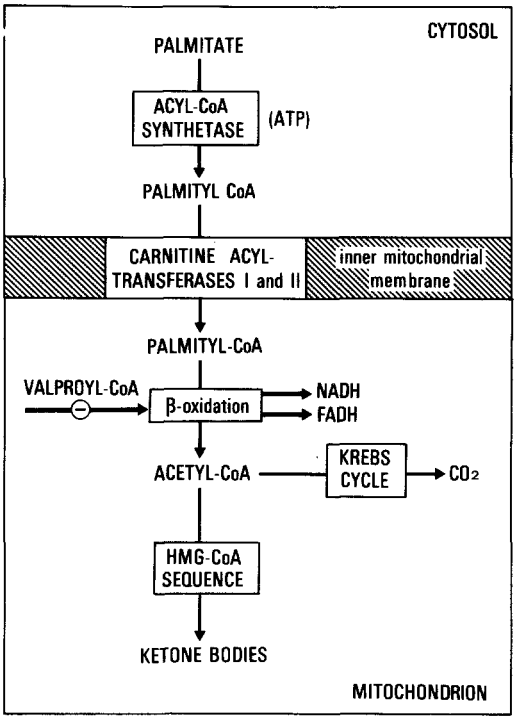


Fig 3 - Effect of valproate on cellular acetyl-CoA levels and mitochondrial and cytosolic NADH/NAD+ ratios in hepatocytes isolated from 18h starved rats. Rat liver cells (5 mg dry weight/ml) were incubated for 30 min with 1 mM oleate. Sodium valproate was added at the indicated concentrations. Assay of total contents of cell metabolites was performed at the end of incubation period on neutrolized cell extracts as described under "Methods".

dependent mitochondrial transport may be excluded because valproate inhibits ketogenesis from butyrate, a short chain fatty acid, which do not requires the carnitine acyl-transferase step for entry into the mitochondria. Therefore it may be concluded that valproate inhibits fatty acid oxidation, subsequently to the initiation of the β-oxidation sequence. Furthermore the concomitant decrease of acetyl-CoA production and NADH/NAD+ ratio formerly excluded a specific inhibition of the hydroxy-methylglutaryl-CoA sequence which would decreased ketogenesis but in contrast would markedly enhanced cellular acetyl-CoA and NADH/NAD+ ratio (see schema 1). Therefore our results strongly suggest that valproate which has been shown to undergo ω and β-oxidation in liver (7, 8) seriously interfers with β-oxidation of physiological fatty acids. They did not preclude of the actual mechanism by which inhibition of fatty acid β-oxidation occurs.



Schema 1 - Long chain fatty acid oxidation in liver
HMG-CoA refers to hydroxymethylglutaryl-CoA

Nevertheless the fact that main metabolites of valproate closely resemble to pent-4-enoic acid, a potent inhibitor of β-oxidation (10) provides a strong support to our hypothesis.

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