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INHIBITION OF UREA SYNTHESIS BY PENT-4-ENOIC ACID : POTENTIATION BY AMMONIA

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Pent-4-enoic acid inhibited ureagenesis approximatively 90 % in rat hepatocytes incubated with pyruvate, ammonia and ornithine. Inhibition of ureagenesis was much less with alanine as substrate (approximatively 10 %). The addition of ammonia led to a drastic dose-dependent inhibition of ureagenesis by pent-4-enoate. Half-maximum effect of ammonia was observed at 0.2 mM concentration. Concomitant cellular concentrations of N-acetylglutamate were also drastically modified by the addition of ammonia as was the accumulation of citrulline. These data suggest that ammonia may seriously interfer with the metabolism of pent-4-enoic acid and leads to a dramatic potentiation of its toxicity.

Pent-4-enoic acid is a chemical analog of hypoglycin, the compound which causes Jamaican vomiting sickness (1, 2). Pent-4-enoate inhibits fatty acid oxidation (3, 4) and gluconeogenesis (5). Moreover it produces in rats many of the essential features of Reye's syndrome (6) including hyperammonemia and the inhibition of urea synthesis by pent-4-enoate has been shown to be mainly due to a decrease in N-acetylglutamate concentration (7) the allosteric activator of carbamoylphosphate synthetase I, a key enzyme of the urea cycle.

We have been studying the effect of pent-4-enoate on ureagenesis in isolated rat hepatocytes. In the course of our studies ammonia was found to play a critical role in the action of pent-4-enoate on urea synthesis. In the present paper we report that ammonia strongly potentiates the inhibition of urea synthesis induced by pent-4-enoate. These data suggest that ammonia may seriously interfer with the metabolism of pent-4-enoate in rat hepatocytes.

MATERIAL AND METHODS

Albino Wistar male rats (150-200g), starved for 18 h, were used. After anesthetizing the animal with Nembutal (7 mg per 100g of body weight) the liver was cannulated, isolated and mounted in a recirculation perfusion system as described by Williamson et al (8). The procedure used for the cell

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isolation was similar to that of Berry and Friend (9) with some minor modifications (10). 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 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 per ml of incubation medium. Experiments were usually terminated after 30 min incubation. For assay of total contents of cell metabolites, 1 ml aliquots were added to perchloric acid (4.5 % (w/v), final concentration) followed by centrifugation and neutralization to pH 6 with 3 N KOH.

The dry weight of the cells was obtained by protein precipitation and washing with trichloracetic acid (11). Urea was determined colorimetrically with an automatic apparatus (12). Citrulline was measured with the same methodology after preincubation of the neutralized perchloric extracts with urease. The assay of N-acetylglutamate was based on measurement of the activation of carbamoylphosphate synthetase I by the neutralized cell extract. The procedure used was that described by Zollner (13). All experiments were repeated 3 to 6 times. Results are expressed as means ± SEM. Statistical significance was calculated using the paired Student's t test.

Enzymes and coenzymes were purchased from Boehringer or Sigma. Na H $^{14}CO_3$ (56 Ci/mol) for the measurement of N-acetylglutamate was purchased from Amersham.

RESULTS AND DISCUSSION

Ureagenesis in isolated hepatocytes was determined in the presence of 10 mM pyruvate, 10 mM NH_4Cl and 3 mM ornithine. Urea synthesis proceeded linearly with time up to 60 min. As expected the addition of 2 mM pent-4-enoate produced a striking inhibition of ureagenesis (140 ± 20 versus 1920 ± 230 µmol/h/g dry weight in control hepatocytes). Surprisingly in the presence of 10 mM alanine and 3 mM ornithine pent-4-enoate inhibited only slightly urea synthesis (Fig. 1). But addition of ammonia which per se stimulated urea synthesis from alanine and ornithine, resulted in a dramatic potentiation of the inhibition induced by pent-4-enoate (Fig. 2). Half-maximum effect of ammonia occurred at 0.2 mM concentration (Fig. 3).

Since it has been demonstrated that variations in the N-acetylglutamate content affect the urea cycle (14, 15) and since it has been shown (7) that the inhibition by pent-4-enoate of ureagenesis in rat hepatocytes was mainly due to decrease in N-acetylglutamate concentration, we have been measuring N-acetylglutamate contents at the end of the urea synthesis assay. As examplified in Table I, N-acetylglutamate and citrulline levels markedly de-

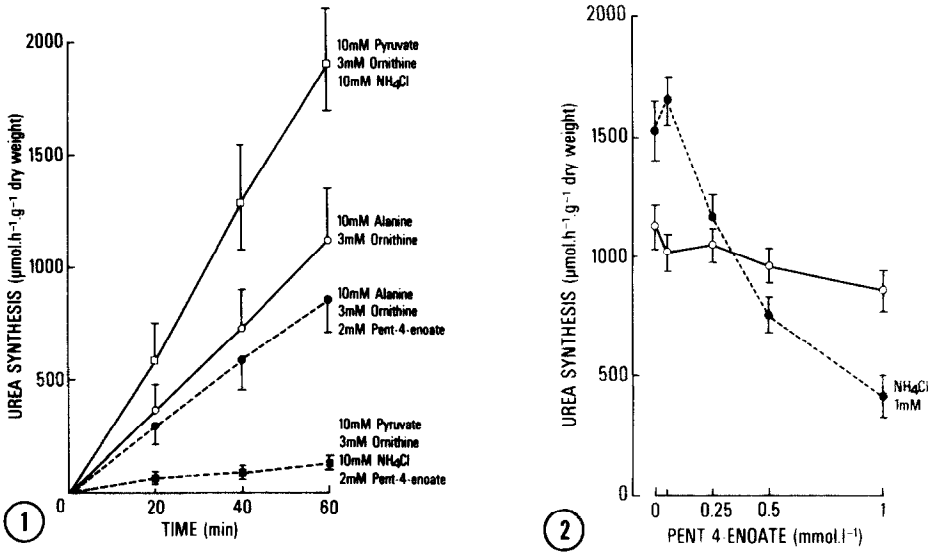

Fig. 1 Effect of pent-4-enoate on urea synthesis in isolated hepatocytes.

Rat liver cells (3 mg dry weight/ml) isolated from 18 h starved rats were incubated for 60 min in the presence of various substrates. Values shown are means ± SEM of three experiments.

Fig. 2 Effect of pent-4-enoate and NH_4Cl on urea synthesis in isolated hepatocytes.

Rat liver cells (3 mg dry weight/ml) isolated from 18 h starved rats were incubated for 30 min in the presence of 10 mM alanine and 3 mM ornithine. Addition of pent-4-enoate and NH_4Cl were as indicated in the figure. Values shown are means ± SEM of four experiments.

creased with addition of pent-4-enoate in the presence of ammonia. In contrast pent-4-enoate alone produced only slight effects. Moreover the rate of urea synthesis was in good parallel with cellular levels of N-acetylglutamate

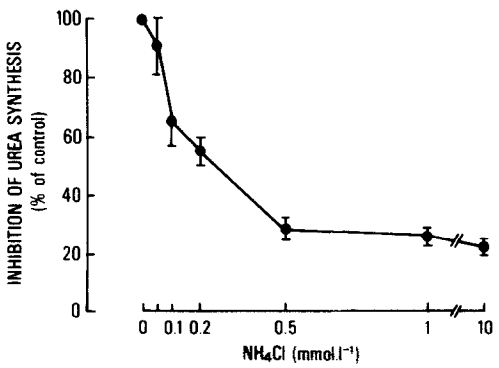

Fig. 3 Dose dependence effect of ammonia on the inhibition of ureagenesis induced by pent-4-enoate in rat hepatocytes.

Rat liver cells (5 mg dry weight/ml) isolated from 18 h starved rats were incubated for 30 min in the presence of 10 mM alanine and 3 mM ornithine. Values shown are means ± SEM of three experiments.

TABLE I : Effect of pent-4-enoate and NH_4Cl on N-acetylglutamate and citrulline levels in isolated rat hepatocytes.

Addition			Urea synthesis μmol/h/g dry weight	N-acetylglutamate nmol/g dry weight	Citrulline μmol/h/g dry weight
None			1 132 ± 220	328 ± 33	36 ± 9
Pent-4-enoate	0.05	mM	1 020 ± 182	326 ± 34	32 ± 8
Pent-4-enoate	1	mM	868 ± 143	274 ± 50+	27 ± 8+
NH_4Cl	1	mM	1 535 ± 294‡	344 ± 27	27 ± 7
Pent-4-enoate + NH_4Cl	0.05 1	mM mM	1 662 ± 323	348 ± 50	31 ± 9
Pent-4-enoate + NH_4Cl	1 1	mM mM	431 ± 52‡	85 ± 12‡	3 ± 1‡

Hepatocytes (14 mg dry weight/ml) isolated from 18 h starved rats were incubated for 30 min in the presence of 10 mM alanine and 3 mM ornithine. Metabolite concentrations were determined on neutralized acidified extracts as described in methods.
+ $P < 0.05$, ‡ $P < 0.01$ relative to control without NH_4Cl or pent-4-enoate.

in control and treated hepatocytes (Fig. 4). These data confirm that the inhibition of ureagenesis by pent-4-enoate was mainly due to decreased levels of N-acetylglutamate and suggest that ammonia potentiates the effect of pent-4-enoate on ureagenesis via a concomitant effect on N-acetylglutamate levels.

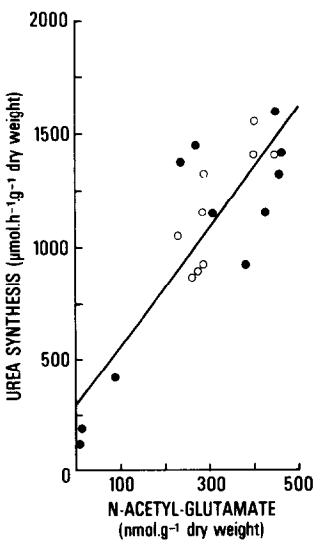

Fig. 4 Relationship between urea synthesis and N-acetylglutamate levels in hepatocytes incubated with different concentrations of pent-4-enoate and NH_4Cl.

Rat liver cells (7 mg dry weight/ml) isolated from 18 h starved rats were incubated for 30 min in the presence of 10 mM alanine and 3 mM ornithine. ($r = 0.8562$, $y = 2.64\ x + 295.82$, $P < 0.01$).

Although the reason for this effect of ammonia is not obvious, it must be stressed that two pathways are open in the mitochondria for pent-4-enoate to be metabolized. The first pathway which is quantitatively minor involves the β-oxidation of pent 2-4 dienoyl-CoA which produces 3 ketopent-4-enoyl-CoA, the metabolite responsible for the inhibition of fatty acid oxidation (16). The second pathway which is quantitatively the most important involves the crucial NADPH-dependent reduction of pent 2-4 dienoyl-CoA (17) producing pent-2-enoyl-CoA which can be degraded via β-oxidation to propionyl-CoA and acetyl-CoA. Thus 2-4 dienoyl-CoA reductase appears to be the rate limiting enzyme in the metabolism of pent-4-enoate as suggested by the observation that the inhibition of fatty acid oxidation by pent-4-enoic acid in rat hepatocytes is relieved by feeding of clofibrate, which causes a several fold increase in the activity of 2-4 dienoyl-CoA reductase (18). Since 2-4-dienoyl-CoA reductase requires NADPH as a cofactor (19, 20) and since, a part from a stimulation of carbamoylphosphate formation, the primary effect of ammonia addition to hepatocytes is a stimulation of reductive amination of α-ketoglutarate via glutamate dehydrogenase, causing a large oxidation of the mitochondrial NADP system (21, 22), ammonia addition would therefore impair the activity of 2-4 dienoyl-CoA reductase with the resulting accumulation of pent 2-4 dienoyl-CoA. Whatever these data demonstrate that ammonia leads to a dramatic potentiation of the toxicity of pent-4-enoate and suggest that ammonia must seriously interfer with the metabolism of pent-4-enoate.

REFERENCES

1. Sherratt, H.S.A. (1969) Br. Med. Bull. 25, 250-255.
2. Bressler, R., Corredor, C., and Brendel, K. (1969) Pharmacol. Rev. 21, 105-130.
3. Corredor, C., Brendel, K., and Bressler, R. (1967) Proc. Natl. Acad. Sci. U.S. 58, 2299-2306.
4. Holland, P.C., and Sherratt, H.S.A. (1973) Biochem. J. 136, 157-171.
5. Toews, C.J., Lowy, C., and Ruderman, N.B. (1970) J. Biol. Chem. 245, 818-824.
6. Glasgow, A.M., and Chase, H.P. (1975) Pediatr. Res. 9, 133-138.
7. Aoyagi, K., Mori, M., and Tatibana, M. (1979) Biochim. Biophys. Acta 587, 515-521.
8. Williamson, J.R., Browning, E.T., and Scholz, R. (1969) J. Biol. Chem. 244, 4607-4616.

9. Berry, M.N., and Friend, D.S. (1969) J. Cell. Biol. 43, 506-520.
10. Meijer, A.J., Gimpel, J.A., Deleeuw, G.A., Tager, J.M., and Williamson, J.R. (1975) J. Biol. Chem. 250, 7728-7738.
11. Berry, M.N., and Kun, E. (1972) Eur. J. Biochem. 27, 395-400.
12. Ceriotti, G., and Spandrio, L. (1965) Clin. Chim. Acta 11, 519-522.
13. Zollner, H. (1981) Biochim. Biophys. Acta 676, 170-176.
14. Shigesada, K., and Tatibana, M. (1978) Eur. J. Biochem. 84, 285-291.
15. Shigesada, K., Aoyagi, K., and Tatibana, M. (1978) Eur. J. Biochem. 85, 385-391.
16. Schulz, H. (1983) Biochemistry 22, 1827-1832.
17. Sherratt, H.S.A., and Osmundsen, H. (1976) Biochem. Pharmacol. 25, 743-750.
18. Borrebaek, B., Osmundsen, H., and Breme, J. (1980) Biochem. Biophys. Res. Commun. 93, 1173-1180.
19. Kunau, W.H., and Dommes, P. (1978) Eur. J. Biochem. 91, 533-544.
20. Cuebas, D., and Schulz, H. (1982) J. Biol. Chem. 257, 14140-14144.
21. Tischler, M.E., Friedrichs, D., Coll, K., and Williamson, J.R. (1977) Arch. Biochem. Biophys. 184, 222-236.
22. Sies, H., Akerboom, T.P.M., and Tager, J. (1977) Eur. J. Biochem. 72, 301-307.