INHIBITION OF PYRUVATE AND β-HYDROXY BUTY RATE OXIDATION IN RAT BRAIN MITOCHONDRIA BY PHENYLPYRUVATE AND α-KETOISOCAPROATE

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1. Introduction

In studies [1–3] concerning the biochemical basis of the inborn errors of metabolism, phenylketonuria (PKU), and maple syrup urine disease (MSUD), we have reported [3] that both phenylpyruvate and α-ketoisocaproate, which accumulate in tissues and plasma in PKU and MSUD respectively, markedly inhibit the oxidation of pyruvate by rat brain mitochondria. Furthermore, we suggested [3] that this may be due to an impairment of the access of pyruvate across the inner mitochondrial membrane to the pyruvate dehydrogenase complex. Recently it has been reported [4] that a specific transport reaction exists for pyruvate across the rat liver mitochondrial membrane based on the discovery of a specific inhibitor for this process (α-cyano(4 hydroxy) cinnamate). We have therefore extended our studies on the effects of phenylpyruvate and α-ketoisocaproate on pyruvate oxidation by rat brain mitochondria to include α-cysnocinnamate and have also studied the effects of each of these inhibitors on β-hydroxybutyrate oxidation by brain mitochondria. These latter experiments are particularly pertinent for PKU and MSUD since in the young brain, ketone bodies (mainly β-hydroxybutyrate) rather than pyruvate [5] are utilised as the main respiratory fuel, whereas in the adult brain pyruvate is the main energy source [5].

The data indicate that not only pyruvate oxidation but also that of β-hydroxybutyrate is markedly inhibited by phenylpyruvate and α-ketoisocaproate and even more so by α-cyanocinnamate. It is suggested that one of the primary lesions in both PKU and MSUD may be the impaired metabolism of pyruvate and β-hydroxybutyrate by inhibition of the transport of these metabolites across the brain mitochondrial membrane.

2. Materials and methods

Brain mitochondria were prepared from male adult rats, (150–160 g) for pyruvate studies or 22-day-old rats (50–60 g) for β-hydroxybutyrate studies, of the Wistar strain by the method of Clark and Nicklas [6]. Mitochondrial respiration was measured polarographically at 28°C in an incubation medium consisting of (final concentrations) : 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM phosphate–Tris, 10 mM Tris–HCl, 0.05 mM EDTA : final pH 7.4. To the medium, rat brain mitochondria were added (~ 1–2 mg protein/ml) followed by 2.5 mM malate and 5 mM pyruvate or 10 mM DL β-hydroxybutyrate (final concentrations). State 3 [7] was induced by the addition of 250 μM ADP. The inhibitor under investigation was added during the state 4 [7] after one ADP cycle had been completed. Protein was measured by the method of Lowry et al. [8]. Phenylpyruvate and α-ketoisocaproate were obtained from the Sigma Chemical Co., St. Louis, Mo., USA and were used as 200 mM aqueous solutions. α-Cyanocinnamate was obtained from R. Emmanuel, Wembley, Middx., UK and was added as 5 mM solution in ethanol: ethanol alone was added as control.

3. Results

Figs. 1 and 2 represent the effects of increasing concentrations of phenylpyruvate and α-ketoisoca-

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proate on the oxidation of pyruvate and malate or β-hydroxybutyrate and malate by rat brain mitochondria in state 3 (+ ADP) [7]. The results are expressed as a per cent of the control oxidation rate in the absence of inhibitor. With both inhibitors pyruvate oxidation was inhibited to a greater extent than β-hydroxybutyrate oxidation – pyruvate oxidation was inhibited by 54% and 40% of the control by 2 mM phenylpyruvate or α-ketoisocaproate respectively, whereas β-hydroxybutyrate oxidation was inhibited by only 36% and 30% under similar conditions.

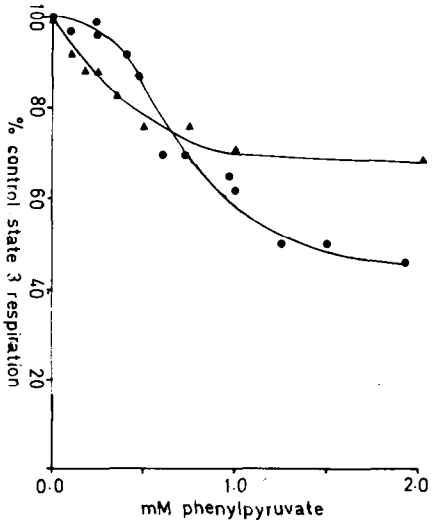

Fig. 1. Effect of phenylpyruvate on the oxidation of pyruvate (●) and β-hydroxybutyrate (▲) by rat brain mitochondria. The mitochondria were prepared by the method of Clark and Nicklas [6] and were incubated in a medium containing 100 mM K^+ (see Materials and methods) together with 5 mM pyruvate and 2.5 mM malate or 10 mM DL β-hydroxybutyrate and 2.5 mM malate. State 3 [7] was induced by the addition of 250 μM ADP and the inhibitor added during the ensuing state 4. The respiration rates (state 3 and 4) in the presence of inhibitor was then estimated by the addition of 2 or 3 aliquots of ADP. The results are the averages of at least 2 estimations of the state 3 rate expressed as a per cent of the average of the comparable state 3 rates in the absence of inhibitor. The control of respiration rates for 5 mM pyruvate and 2.5 mM malate were state 3 = 183 ± 13 (SD – n=15)natoms 0 min^{-1} mg $protein^{-1}$ state 4 = 23 ± 5 (SD – n=15) natoms 0 min^{-1} mg $protein^{-1}$ (adult rats); for 10 mM DL β-hydroxybutyrate and 2.5 mM malate, state 3 = 106 ± 7 (SD – n=5) natoms 0 $min.^{-1}$ mg. $protein^{-1}$, state 4 = 26 ± 4 (SD – n=15) natoms 0 min^{-1} mg $protein^{-1}$ (22-day-old rats).

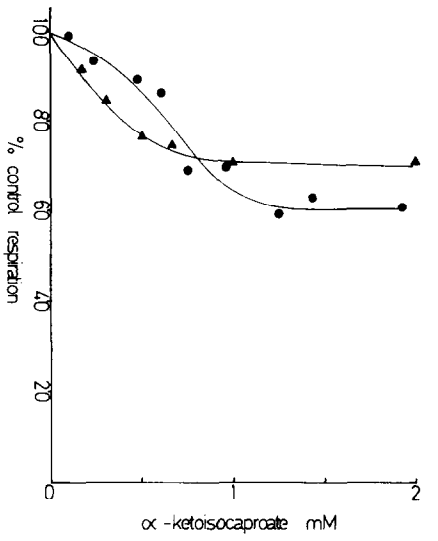

Fig. 2. Effect of α-ketoisocaproate on the oxidation of pyruvate (●) and β hydroxybutyrate (▲) by rat brain mitochondria. The experimental conditions were as in fig. 1 except that α-ketoisocaproate was used as the inhibitor.

Furthermore, for both substrates phenylpyruvate appears to be more inhibitory than α-ketoisocaproate. Figs. 3 and 4 show similar experiments using α-cyanocinnamate as an inhibitor. This compound inhibited

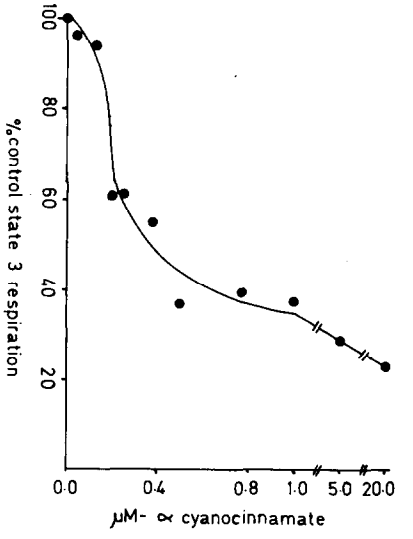

Fig. 3. Effect of α-cyanocinnamate on the oxidation of pyruvate by rat brain mitochondria. Experimental conditions were as in fig. 1 except that α-cyanocinnamate was used as the inhibitor.

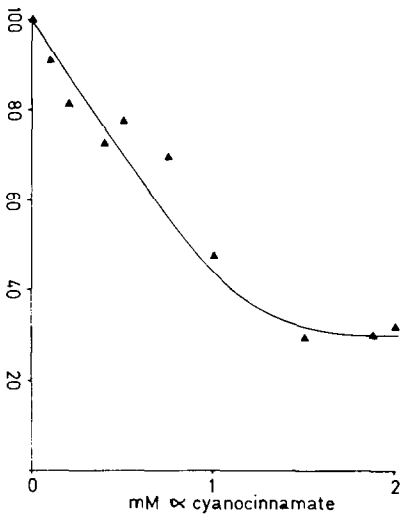

Fig. 4. Effect of α-cyanocinnamate on oxidation of β-hydroxybutyrate by rat brain mitochondria. Experimental conditions as in fig. 3.

pyruvate oxidation by 75% at a final concentration of 20 μM (fig. 3) whereas β-hydroxybutyrate oxidation was inhibited by 70% at a final concentration of 2 mM α-cyanocinnamate (fig. 4). There were no inhibitory effects of phenylpyruvate, α-ketoisocaproate or α-cyanocinnamate on the state 4 (-ADP) respiration rate of brain mitochondria under similar conditions to those in fig. 1–4. In addition no significant inhibitory effects have been observed on the state 3 oxidation rate of glutamate and malate or succinate by up to 2 mM phenylpyruvate or α-ketoisocaproate. The oxygen uptakes, in natoms 0 min^{-1}. mg $protein^{-1}$ in state 3 were: for glutamate and malate (control), 159 ± 3.5; for control + 2 mM phenylpyruvate, 139 ± 4; for succinate oxidation were (control) 165 ± 4; for control + 2 mM phenylpyruvate, 163 ± 5; for control + 2 mM χ-ketoisocaproate, 164 ± 2. See also [4] for α-cyanocinnamate. Studies with semi-purified preparations of pyruvate dehydrogenase and β-hydroxybutyrate dehydrogenase indicate that phenylpyruvate, α-cyano (4-hydroxy) cinnamate have little or no inhibitory effects on the isolated enzyme system at the concentrations used in these experiments ([1,3,4] Clark and Land, unpublished observations).

The absence of any effect of these compounds on the isolated enzymes (pyruvate dehydrogenase and β-hydroxybutyrate dehydrogenase) and their marked and specific effects on the oxidation of pyruvate and β-hydroxybutyrate, is consistent with the interpretation that phenylpyruvate, α-ketoisocaproate and α-cyanocinnamate are inhibiting the transport of pyruvate and β-hydroxybutyrate across the inner mitochondrial membrane, which in itself may be taken as evidence for the existence for both a pyruvate and β-hydroxybutyrate carrier system. Such an interpretation is supported by the recent report of Halestrap and Denton [4] who have suggested (on the basis of a specific inhibition by α-cyano(4 hydroxy) cinnamate) that in liver mitochondria and human erythrocytes a pyruvate carrier exists. What is of particular interest, however, especially in the brain, is the existence of a mitochondrial carrier for β-hydroxybutyrate, which if not part of the same system as the pyruvate carrier, is clearly closely related to it since all three inhibitors affect both pyruvate and β-hydroxybutyrate oxidation. Further evidence for the existence of both a pyruvate and β-hydroxybutyrate carrier and their close interrelationship has been derived from experiments with heart and liver mitochondria which have been preloaded with [^{14}C] pyruvate or β-hydroxybutyrate [9]. Studies have indicated that pyruvate will exchange for β-hydroxybutyrate and vice versa and that both phenylpyruvate and α-ketoisocaproate will inhibit these exchanges. Similar exchange reactions between β-hyfroxybutyrate and pyruvate in whole brain mitochondria have also been found to be inhibited by phenylpyruvate and α-ketoisocaproate (J. M. Land, J. B. Clark and T. Mowbray, unpublished observations).

Although at the lower inhibitor concentrations β-hydroxybutyrate oxidation appears more inhibited than pyruvate oxidation, the ultimate extent to which oxidation is inhibited appears always greater with pyruvate as substrate (figs. 1–4). A possible explanation of this is that at the higher inhibitor concentrations the decrease in intramitochondrial pyruvate content [9] results additionally in an inactivation of the pyruvate decarboxylase unit of the pyruvate dehydrogenase complex [10]. Halestrap and Denton [4] suggested that pyruvate is transported as the enol form and in that case presumably it is the enol forms of phenylpyruvate and α-ketoisocaproate which are the inhibitory species. Precise data concerning the enolisation of phenylpyruvate and α-ketoisocaproate are not available [1,11], although at the pH of the experiments the concentration of the enol forms of these

two α-keto acids would be several orders of magnitude less than that of the keto form. This may account for the apparent effectiveness of α-cyanocinnamate as an inhibitor of pyruvate oxidation (33% inhibition at 0.75 – 1 mM).

These inhibitions of both pyruvate and β-hydroxybutyrate oxidation in brain mitochondria by phenylpyruvate and α-ketoisocaproate may well have important consequences for the understanding of PKU and MSUD. In both these inborn errors of metabolism, if untreated, a defective brain myelination and mental retardation occurs [12,13]. Myelination occurs fairly early on during brain development [14] and during this period the brain of the young animal is mainly dependent on ketone bodies for its respiratory fuel [5], pyruvate mainly being involved in an anaplerotic function [15] and Land and Clark, in preparation). Hence any inhibition of ketone body (β-hydroxybutyrate) utilisation in the brains of young animals by phenylpyruvate or α-ketoisocaproate would have deleterious effects not only for the provision of carbon skeleton for fatty acid and cholesterol synthesis, for myelin deposition, but also for general energy provision. Even when the brain has developed and utilises mainly glucose for its respiratory fuel [5] the presence of phenylpyruvate and α-ketoisocaproate would still effectively prevent pyruvate utilisation. There have been a number of reports concerning the concentrations to which phenylpyruvate and α-ketoisocaproate rise in PKU and MSUD [1–3, 12,13], the consensus of which seems to be that levels 0.5–1 mM in the plasma and tissues are most likely. Thus from our data it may be seen that quite a marked inhibition of pyruvate and β-hydroxybutyrate oxidation will be achieved under these conditions. It may be proposed, therefore, that a primary lesion in phenylketonuria and maple syrup urine disease is the inhibition of pyruvate and β-hydroxybutyrate utilisation by the inhibition of the transport of these substrates across the mitochondrial membrane.

Acknowledgements

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