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INHIBITION OF MITOCHONDRIAL PYRUVATE TRANSPORT BY PHENYLPYRUVATE AND α-KETOISOCAPROATE

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SUMMARY

1. Pyruvate oxidation by coupled rat heart and brain mitochondria is inhibited by phenylpyruvate and α-ketoisocaproate but not by α-ketoisovalerate or α-keto-β-methyl valerate; none of these compounds inhibit pyruvate dehydrogenase.

2. Transport of pyruvate but not acetate into rat liver and brain mitochondria is inhibited by both phenylpyruvate and α-ketoisocaproate.

3. Phenylpyruvate inhibits the transport of pyruvate but not acetate into human red blood cells.

5. It is suggested that inhibition of pyruvate transport by phenylpyruvate and α-ketoisocaproate may be involved in the pathology of phenylketonuria and maple syrup urine disease.

INTRODUCTION

The recent discovery that pyruvate transport across mitochondrial and erythrocyte membranes is specifically inhibited by α-cyano-4-hydroxy-cinnamate implies that a specific carrier is involved in the transport of pyruvate across both types of membrane [1]. This raises the possibility that other inhibitors of either physiological or pathological importance may exist. In phenylketonuria, plasma phenylpyruvate levels of 0.5 mM may occur [2, 3] and in maple syrup urine disease plasma concentrations of α-ketoisocaproate and other branched-chain ketoacids may reach a total of 2–4 mM [4, 5]. It has been reported that both phenylpyruvate and α-ketoisocaproate inhibit pyruvate oxidation by brain and liver homogenates and mitochondria [5–9] although no effect of either keto acid on pyruvate dehydrogenase has been observed (refs 10 and 11 and Denton, R. M., unpublished). Phenylpyruvate has also been shown to inhibit pyruvate carboxylation by rat brain mitochondria [3] and gluconeogenesis from pyruvate by the perfused rat liver [6] and lactate by kidney cortex slices [12]. These findings suggest that both phenylpyruvate and α-ketoisocaproate may inhibit pyruvate transport into the mitochondrion and in this paper we present results indicating that such inhibition does occur.

METHODS

Rat liver mitochondria and human red blood cells were prepared and incubated as described previously [1]. Rat brain mitochondria were prepared by the method of Clark and Nicklas [13] and rat heart mitochondria were kindly prepared by Professor P. J. Randle and Mr A. L. Kerbey of this laboratory without the use of Nagarse. Measurements of pyruvate and acetate uptake were performed by both radioactive and enzymic assay as described previously [1] with the exception of Expt 3 in Table II. In this experiment standard incubation medium (0.8 ml) containing mitochondria (approx. 6 mg protein) was layered onto 0.25 ml of oil (silicone oil, specific gravity 1.06–dinonyl phthalate, 1 : 1) covering 0.2 ml of 3 % (w/v) $HClO_4$ containing 25 % (w/v) glycerol in a plastic centrifuge tube. 2 min after addition of substrate to the incubation medium the tubes were centrifuged for 1.5 min at 15 000 × *g* in an Eppendorf 3200 centrifuge. The supernatant was removed with a syringe and brought to 2 % $HClO_4$; after removal of the oil the mitochondrial pellet was sonicated for 15 s at 0 °C with the remaining aqueous layer containing $HClO_4$ and glycerol. The analysis of the $HClO_4$ extracts for substrate was performed as described previously [1].

O_2 uptake by mitochondria was measured with a Clark-type oxygen electrode.

The sodium salts of phenylpyruvate, α-ketoisocaproate, α-keto-isovalerate and α-keto-β-methylvalerate were obtained from Sigma Chemical Co. Ltd. London, S.W. 6., U.K., and were used as 100 mM solutions in water. Silicone fluid (specific

TABLE I

EFFECT OF PHENYLPYRUVATE AND BRANCHED-CHAIN KETO ACIDS ON PYRUVATE OXIDATION BY RAT BRAIN AND HEART MITOCHONDRIA

Brain mitochondria (2 mg protein) were incubated at 25 °C in 1.2 ml of medium containing 225 mM mannitol, 75 mM sucrose, 50 μM EDTA, 5 mM KCl, 10 mM Tris–HCl, 5 mM potassium phosphate, 5 mM ADP, 0.5 mM potassium malate, pH 7.4. Heart mitochondria (2 mg protein) were incubated at 30 °C in 4 ml of medium containing 125 mM KCl, 10 mM Tris–HCl, 5 mM potassium phosphate, 5 mM ADP, 0.5 mM potassium malate, pH 7.4. Pyruvate was present at the concentrations shown. After a steady rate of oxygen uptake was achieved inhibitor was added and the new rate measured. Results are means of at least two determinations.

Source of mitochondria	Inhibitor	Concentration of inhibitor (mM)	Concentration of pyruvate (mM)	Inhibition of rate of O_2 uptake (%)
Brain	α-cyano-4-hydroxycinnamate	0.2	2.5	97
Brain	β-phenylpyruvate	5.0	1.25	57
Brain	β-phenylpyruvate	5.0	2.5	39
Brain	α-ketoisocaproate	2.5	1.0	15
Brain	α-ketoisocaproate	5.0	1.0	41
Brain	α-keto-β-methyl-valerate	5.0	1.0	0
Brain	α-ketoisovalerate	5.0	1.0	0
Heart	β-phenylpyruvate	2.5	2.8	46
Heart	β-phenylpyruvate	5.0	1.4	75
Heart	α-ketoisocaproate	2.5	0.5	14
Heart	α-ketoisocaproate	5.0	0.5	45

gravity 1.06) M.S. 550 was obtained from Hopkin and Williams, Ltd., Chadwell Heath, Essex, U.K.

RESULTS

In Table I we report the results of experiments on the effect of phenylpyruvate and branched chain ketoacids on pyruvate oxidation in the presence of malate by coupled rat brain and heart mitochondria. It can be seen that both phenylpyruvate and α-ketoisocaproate inhibit pyruvate oxidation significantly although the inhibition by α-ketoisocaproate was less pronounced than that of phenylpyruvate, especially at high pyruvate concentrations. Neither 5 mM α-ketoisovalerate nor 5 mM α-keto-β-methylvalerate showed inhibition even at low pyruvate concentrations. No uncoupling

TABLE II

THE EFFECT OF PHENYLPYRUVATE ON THE TRANSPORT OF PYRUVATE INTO RAT LIVER AND BRAIN MITOCHONDRIA

Mitochondria were preincubated at room temperature (22–23 °C) for 2 min in 1 ml medium (125 mM KCl, 20 mM Tris–HCl (pH 7.4)) containing 2.5 mM succinate, rotenone (5 μg/ml), [6,6′(n)-$^3H_2$]-sucrose (2 μCi/ml) as an extramitochondrial marker and other additions as indicated. Substrate (1.4 mM and 0.2 μCi/ml) was added and after 2 min further incubation 0.5 mM α-cyano-4-hydroxycinnamate was added. The mitochondria were centrifuged and the mitochondrial substrate content determined as described previously [1]. In Expt 3 mitochondria were centrifuged through oil as described in Methods. In Expts 1–4 rat liver mitochondria (approximately 8 mg protein per ml) were used; in Expt 5 rat brain mitochondria (approximately 2 mg protein per ml) were used. The intramitochondrial water volume was calculated from the distribution of ^3H_2O and [U-^{14}C]sucrose to be 0.62±0.03 (Expt 1), 0.86±0.04 (Expt 2), 0.63±0.02 (Expt 3) and 1.00±0.05 (Expt 4) (values expressed as μl/mg mitochondrial protein and mean ±S.E. of 6 observations). All values in the table are given as the mean ±S.E. for the number of observations shown in parentheses. Abbreviation: FCCP, p-trifluoromethoxycarbonyl cyanide phenylhydrazone.

Expt No.	Substrate	Other additions	Concentration of phenylpyruvate (mM)	Uptake of substrate (nmoles/μl intramitochondrial space)		
				Without phenylpyruvate	With phenylpyruvate	
1	[U-^{14}C]pyruvate	–	5	4.85±0.20	2.69±0.05**	(4)
	[U-^{14}C]pyruvate	0.5 mM α-cyano-4-hydroxycinnamate	5	0.72±0.18	0.64±0.10	(4)
2	[U-^{14}C]pyruvate	–	1	1.95±0.09	1.39±0.04**	(4)
	[U-^{14}C]pyruvate	–	5	1.95±0.09	1.25±0.12**	(4)
	[U-^{14}C]acetate	–	5	2.52±0.28	2.58±0.24	(4)
3	[U-^{14}C]pyruvate	–	5	3.33±0.21	1.82±0.19**	(4)
	pyruvate	–	5	3.42±0.28	1.58±0.10**	(6)
4	[U-^{14}C]pyruvate	0.1 μM FCCP	5	0.80±0.14	0.25±0.06*	(3)
	[U-^{14}C]acetate	0.1 μM FCCP	5	1.29±0.09	1.11±0.19	(4)
5	[U-^{14}C]pyruvate	–	5	1.33±0.18†	0.43±0.08†,**	(4)

* $P < 0.02$ versus control incubated in the absence of phenylpyruvate.

** $P < 0.01$.

† Values expressed as nmoles pyruvate/mg mitochondrial protein.

or inhibition of O_2 uptake was observed by either 5 mM phenylpyruvate or 5 mM α-ketoisocaproate when 2 mM 2-oxoglutarate or 2 mM glutamate (both in the presence of 0.5 mM malate) or 2 mM succinate were substrates. No significant effect of 5 mM phenylpyruvate or 5 mM α-ketoisocaproate on purified pig heart pyruvate dehydrogenase has been detected even at pyruvate concentrations of 0.1 mM (Denton, R. M., unpublished). Similar results have also been reported for brain pyruvate dehydrogenase [10, 11]. These results suggest that the inhibition of pyruvate oxidation by intact mitochondria may be due to inhibition of pyruvate transport into the mitochondria.

This possibility was investigated more directly by measuring pyruvate accumulation by rat liver and brain mitochondria incubated with succinate and rotenone to allow accumulation of pyruvate without its metabolism. The accumulation of pyruvate was stopped when desired by the addition of 0.5 mM α-cyano-4-hydroxycinnamate and the mitochondria separated by centrifugation. Results shown in Table II indicate that phenylpyruvate (5 mM) inhibits pyruvate accumulation assayed both by enzymic and radioactive assay, but not that of acetate. It appears unlikely that the effect is due to inhibition of pyruvate binding by phenylpyruvate since when transport is inhbited by α-cyano-4-hydroxycinnamate, the pyruvate content of the mitochondria is not decreased by phenylpyruvate (Expt 1). An effect of 1 mM phenylpyruvate was also seen with pyruvate at 1.4 mM (Expt 2). The lack of inhibition of acetate accumulation by phenylpyruvate suggests the inhibition was specific and not, for example, due to an uncoupling effect. In any case phenylpyruvate inhibition of pyruvate transport into

TABLE III

THE EFFECT OF α-KETOISOCAPROATE ON THE TRANSPORT OF PYRUVATE INTO RAT LIVER AND BRAIN MITOCHONDRIA

Experimental conditions were the same as those in Table II except that pyruvate where present was added at 0.2 mM and acetate at 2.00 mM. Pyruvate uptake was stopped by addition of 0.5 mM α-cyano-4-hydroxycinnamate after 45 s for brain mitochondria and 2.0 min for liver mitochondria. Liver mitochondria (Expt 1) were used at approximately 4 mg/ml and brain mitochondria (Expt 2) at approximately 2.5 mg/ml. The uptake of substrate expressed as nmoles substrate per mg mitochondrial protein is given as the mean ±S.E. of four separate observations

Expt No	Mitochondria	Substrate	Other additions	Uptake of substrate (nmoles/mg mitochondrial protein)	
				Without α-keto-isocaproate	With 5 mM α-ketois-ocaproate
1	Liver	[U-^{14}C]pyruvate	–	0.82 ±0.05	0.49 ±0.01**
		[U-^{14}C]pyruvate	0.5 mM α-cyano-4-hydroxycinnamate	0.10 ±0.01	0.18 ±0.03
		[U-^{14}C]acetate	–	5.30 ±0.20	5.10 ±0.20
2	Brain	[U-^{14}C]pyruvate	–	0.306±0.020	0.232±0.010*
		[U-^{14}C]pyruvate	0.5 mM-α-cyano-4-hydroxycinnamate	0.048±0.030	0.028±0.024
		[U-^{14}C]acetate	–	3.76 ±0.38	4.28 ±0.36

* $P < 0.02$ versus control without added α-ketoisocaproate.
** $P < 0.01$ versus control without added α-ketoisocaproate.

uncoupled mitochondria could be demonstrated (Expt 4). The transport of pyruvate into rat brain mitochondria was also markedly inhibited by phenylpyruvate (Expt 5). In Table III we show experiments with α-ketoisocaproate which indicate that this ketoacid also inhibits the transport of pyruvate but not acetate into both rat liver and brain mitochondria. However this inhibition was less potent than that seen with phenylpyruvate and was not observed unless low pyruvate concentrations (0.2 mM) were used at an inhibitor concentration of 5 mM.

We have previously shown that α-cyano-4-hydroxycinnamate inhibits the efflux of pyruvate from preloaded mitochondria [1]. We have attempted similar experiments using phenylpyruvate where mitochondria incubated with succinate and rotenone were loaded in pyruvate (2.8 mM) and resuspended in wash medium for 1 min in the presence or absence of inhibitor. The pyruvate retained (expressed as nmoles/μl intramitochondrial space and the mean ±S.E. of four separate experiments) decreased from 0.46±0.03 in control experiments to 0.195±0.04 when phenylpyruvate (5 mM) was present in the wash, but increased to 1.25±0.04 when α-cyano-4-hydroxycinnamate (0.5 mM) was used. This appears to indicate that phenylpyruvate may exchange for pyruvate as suggested by Paradies and Papa [14].

TABLE IV

THE EFFECT OF PHENYLPYRUVATE ON THE TRANSPORT OF PYRUVATE AND ACETATE INTO HUMAN ERYTHROCYTES

Erythrocytes were incubated for 5 min at 22–23 °C in 1 ml medium (84 mM sodium citrate, 10 mM Tris, brought to pH 7.4 with 1 M NaH_2PO_4), containing [6,6′(n)-$^3H_2$]sucrose (2.0 μCi/ml) as extracellular marker and where indicated 5 mM phenylpyruvate or 0.5 mM α-cyano-4-hydroxycinnamate. Substrate (1.5 mM and 0.2 μCi/ml) was then added and after a further 2-min incubation 0.5 mM α-cyano-4-hydroxycinnamate. The erythrocytes were immediately separated by centrifugation and the extracellular and intracellular substrate concentrations determined by either enzymic or radioactive assay as described previously [1]. The intracellular volume was calculated from the distribution of ^3H_2O and [U-^{14}C]sucrose to be 0.12 ml of a total pellet volume of 0.20 ml. Results are given as the mean ±S.E. of four separate observations.

Substrate	Inhibitor	Extracellular substrate concentration (mM)	Intracellular substrate concentration (mM)	Concentration ratio intracellular/extracellular
Pyruvate	none	1.34±0.05	5.88±0.13	4.42±0.24
Pyruvate	phenylpyruvate	1.86±0.04*	3.33±0.08	1.79±0.02*
Pyruvate	α-cyano-4-hydroxycinnamate	2.10±0.03*	2.37±0.10	1.11±0.07*
[U-^{14}C]pyruvate	none	1.43±0.03	6.03±0.11	4.23±0.17
[U-^{14}C]pyruvate	phenylpyruvate	1.88±0.01*	2.91±0.06*	1.55±0.03*
[U-^{14}C]pyruvate	α-cyano-4-hydroxycinnamate	1.96±0.03*	2.77±0.06*	1.42±0.05*
[U-^{14}C]acetate	none	1.72±0.05	8.26±0.19	4.83±0.20
[U-^{14}C]acetate	phenylpyruvate	1.88±0.05	7.08±0.07**	3.78±0.14**
[U-^{14}C]acetate	α-cyano-4-hydroxycinnamate	1.74±0.02	7.90±0.20	4.52±0.12

* $P < 0.001$.
** $P < 0.01$.

Phenylpyruvate also inhibits pyruvate accumulation by human erythrocytes whilst having very little effect on acetate accumulation (Table IV). Thus, like α-cyano-4-hydroxycinnamate, phenylpyruvate inhibits both the mitochondrial and erythrocyte pyruvate carrier.

DISCUSSION

The existence of a specific pyruvate transport system in mitochondria was first suggested by the work of Papa et al. [15]. Paradies and Papa [14] also provided some evidence for pyruvate/phenylpyruvate exchange on this carrier. However this work is open to criticism [16], and it is only with the discovery that α-cyano-4-hydroxycinnamate specifically inhibits pyruvate transport into mitochondria [1] that it has been possible to study this transport process with confidence. In this paper we have provided evidence that both phenylpyruvate and α-ketoisocaproate also inhibit the mitochondrial transport of pyruvate.

The powerful inhibition of mitochondrial pyruvate transport by phenylpyruvate found in these studies may provide an explanation for the wide range of inhibitory effects of this compound on pyruvate metabolism. For example the inhibition of gluconeogenesis from pyruvate and lactate [6, 12], the inhibition of pyruvate carboxylation by brain mitochondria [3] and the inhibition of pyruvate oxidation by homogenates and mitochondrial preparations of brain and liver [5–9, 17] may all be explained by an inhibition of pyruvate transport into the mitochondria. In phenylketonuria it is thought that accumulation of phenylpyruvate in the blood to concentrations as high as 0.1–0.5 mM may be responsible for the far reaching consequences of the disease [3, 18]. Most apparent of these is the mental retardation and impaired myelination which is characteristic of phenylketonuria [18]. The mechanism by which phenylpyruvate exerts its pathological effect is not known although it has been suggested that inhibition of brain fatty acid synthesis by inhibition of pyruvate carboxylase [3] or fatty acid synthetase [19] may be responsible. In the brain, mitochondrial pyruvate metabolism is important for the formation of acetyl-CoA which is used in both fatty acid synthesis and the production of ATP through the citrate cycle. Thus it might be expected that inhibition of pyruvate entry into the mitochondria could inhibit both these processes and have detrimental effects on the development and metabolism of the brain. That a general impairment of mitochondrial pyruvate metabolism may occur in phenylketonuria is suggested by the elevated blood lactate concentration seen in such patients [20].

α-Ketoisocaproate also appears to inhibit mitochondrial pyruvate transport (Table III) but less powerfully than phenylpyruvate. This ketoacid may accumulate to concentrations of 2–4 mM in the blood of patients suffering from maple syrup urine disease and is believed to be the cause of the metabolic disturbances associated with the disease [4, 5]. There are many clinical similarities between maple syrup urine disease and phenylketonuria such as mental retardation and impaired myelination in the central nervous system [21]. It is possible that these similarities reflect a common pathological mechanism which we would suggest may be the inhibition of mitochondrial pyruvate transport by phenylpyruvate and α-ketoisocaproate. The concentrations of phenylpyruvate and α-ketoisocaproate required to demonstrate inhibition of pyruvate transport in our experiments show that phenylpyruvate is the more potent

inhibitor. However the plasma concentration of α ketoisocaproate may reach 2–4 mM in maple syrup urine disease [4, 5] whereas phenylpyruvate only reaches concentrations of 0.1–0.5 mM in phenylketonuria [3, 18]. Since the concentration of pyruvate in the brain is of the order of 0.1 mM [22], it would seem that the ratios of inhibitor concentration to pyruvate concentration used in our experiments may be similar to those occurring in the respective diseases. As far as we are aware the only other inborn error of metabolism whose pathological effects may be exerted through inhibition of a mitochondrial anion transport process is methylmalonic aciduria where malate transport into the mitochondria may be inhibited by methylmalonate [23].

Since the completion of the work reported here Clark and Land [24] have published results of experiments which show inhibition of pyruvate oxidation by intact mitochondria, but not of pyruvate dehydrogenase itself, by α-ketoisocaproate and phenylpyruvate. These results led Clark and Land to anticipate some of the findings we report here.

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