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THE ROLE OF MALIC ENZYME IN THE MALATE DEPENDENT BIOSYNTHESIS OF PROGESTERONE IN THE MITOCHONDRIAL FRACTION OF HUMAN TERM PLACENTA

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Summary—Mitochondria isolated from human term placenta were able to form citrate from malate as the only added substrate. While mitochondria were incubated in the presence of Mn^{2+} the citrate formation was stimulated significantly both by NAD^+ and $NADP^+$ and was inhibited by hydroxymalonate, arsenite, butylmalonate and rotenone. It is concluded that NAD(P)-linked malic enzyme is involved in the conversion of malate to citrate in these mitochondria. It has also been shown that the conversion of cholesterol to progesterone by human term placental mitochondria incubated in the presence of malate was stimulated by NAD^+ and $NADP^+$ and inhibited by arsenite and fluorocitrate. This suggests that the stimulation by malate of progesterone biosynthesis depends not only on the generation of NADPH by NAD(P)-linked malic enzyme, but also on NADPH formed during further metabolism of pyruvate to isocitrate which is in turn efficiently oxidized by $NADP^+$-linked isocitrate dehydrogenase.

INTRODUCTION

Mitochondrial fraction isolated from human term placenta carries out the conversion of cholesterol to progesterone [1–3]. This process is strongly stimulated by tricarboxylic intermediates of the Krebs cycle [4, 5]. It is due to the $NADP^+$-linked isocitrate dehydrogenase activity which results in the generation of NADPH, a highly specific electron donor essential for cytochrome P-450 dependent mixed functions oxidases. We have shown previously that the dicarboxylic intermediates of the Krebs cycle also stimulate the conversion of cholesterol to progesterone by human term placental mitochondria [6–8]. It is believed that this is caused by the $NADP^+$-linked malic enzyme activity, catalysing the production of NADPH. However, we have shown recently in the mitochondria isolated from human term placenta, the activity of NAD(P)-linked malic enzyme, catalysing the oxidative decarboxylation of malate in the presence of either NAD^+ or $NADP^+$, the former being much more effective [9]. Therefore it is reasonable to think that the malate dependent progesterone biosynthesis in the mitochondrial fraction of human term placenta is due to both, the production of NADPH by $NADP^+$-dependent malic enzyme activity as has been suggested previously [6–8] and to the conversion of pyruvate (formed in the reaction catalysed by NAD(P)-linked malic enzyme) to the tricarboxylic intermediates of the Krebs cycle and a subsequent NADPH production by $NADP^+$-linked isocitrate dehydrogenase. To check these predictions the experiments presented in this paper have been carried out.

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EXPERIMENTAL

Chemicals

Malic acid, NAD^+, $NADP^+$, NADH, tartronic acid (hydroxymalonic acid), rotenone, lactic dehydrogenase, malic dehydrogenase and citrate lyase (from *Enterobacter aerogenes*) were from Sigma Chemical Co., St Louis, U.S.A. The $NaAsO_2$ was from E. Merck A. G., Darmstadt, West Germany. The carbonyl cyanide, *m*-chlorophenyl hydrazone (CCCP) and DL-fluorocitrate (barium salt) were from Calbiochem, U.S.A. [4-^{14}C]cholesterol (sp. act.: 58 mCi/mmol) and [^{3}H]progesterone (sp. act.: 12 Ci/mmol) were products of Radiochemical Centre, Amersham, U.K. All other chemicals were analytical grade products purchased from POCH Gliwice, Poland.

Mitochondria isolation

Human term placental mitochondria were prepared as described previously [10].

Citrate measurements

Citrate formation was measured in the reaction mixture (final vol 1 ml) containing: 25 mM KCl, 50 mM Tris–HCl (pH 7.2), 1 mM potassium phosphate (pH 7.2), 50 μM DL-fluorocitrate (sodium salt), 1 μM CCCP, 10 mM L-malate (except in Fig. 1 where different concentrations were used) and 1 mM

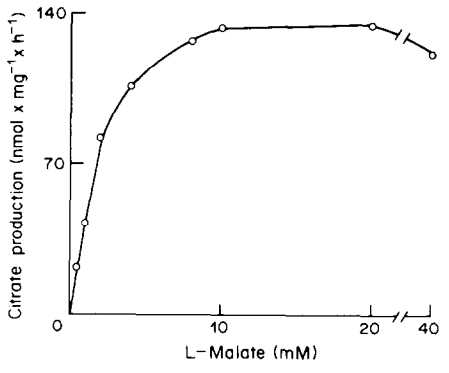


Fig. 1. The effect of malate concentration on citrate formation by human placental mitochondria. For experimental conditions see Experimental.

$MnCl_2$ unless otherwise indicated. Other additions were as indicated in the corresponding Tables. The reaction was started by adding 0.1 ml (approx 3.5 mg protein) mitochondrial suspension, incubation was carried out at 30°C with shaking for 15 min. The reaction was terminated by the addition of 0.1 ml $HClO_4$ (60%). After neutralization with K_2CO_3 citrate was assayed enzymatically by a standard spectrophotometric procedure [11].

Progesterone biosynthesis estimation

The incubation was carried out at 37°C for 60 min with 4 mg of mitochondrial protein in the reaction mixture (final volume 2.5 ml) containing: 0.3 μCi of [4-^{14}C]cholesterol, 25 mM KCl, 50 mM Tris–HCl (pH 7.2) and 1 mM $MnCl_2$. Isolation of the steroid, radioactivity measurements and calculations were as described by Klimek *et al.* [8].

Protein concentration was determined by the biuret method [12].

RESULTS AND DISCUSSION

The exact role of the recently discovered mitochondrial NAD(P)-linked malic enzyme in the metabolism of human placenta is not yet known. One could expect that the enzyme is converting the excess of malate (and its precursors) to pyruvate. The NADPH and NADH formed in this reaction could be either used for steroid hydroxylation or oxidized by the respiratory chain. The pyruvate could be subsequently oxidized by pyruvate dehydrogenase to CO_2 and acetyl-CoA. The acetyl-CoA produced would permit the removal of oxaloacetate (formed from malate in the reaction catalysed by malate dehydrogenase) by condensation to citrate.

To check this prediction the experiments presented in Fig. 1 have been carried out. As can be seen human term placental mitochondria incubated in the presence of varying concentrations of malate (as the only exogenous substrate) and constant amount of fluorocitrate which inhibits the conversion of citrate to isocitrate synthesized citrate with V_{max} being reached at 10 mM L-malate concentration. In this respect human term placental mitochondria resemble mitochondria from the adrenal glands [13] and from the heart [14] which are also able to synthesize citrate from malate as the only exogenous substrate. It seems likely that the NAD(P)-linked malic enzyme plays an essential role in this process. However it should be stated that two other pathways leading from malate to pyruvate (and then to citrate) are possible. One involving the reactions catalyzed by phosphoenolpyruvate carboxykinase and pyruvate kinase (the cytosolic enzyme absorbed to mitochondrial membrane), the other one catalyzed by oxaloacetate decarboxylase. The experiments presented in Fig. 1 were conducted without added ADP, one of the substrates for pyruvate kinase. This suggests that the phosphoenolpyruvate carboxykinase and pyruvate kinase are not involved in the conversion of malate to pyruvate. Extremely low activity of oxaloacetate decarboxylase in the human placental mitochondria (J. Świerczyński, unpublished results) suggests that this enzyme could not account for the pyruvate formation. Therefore it seems reasonable to think that the pathway involving the NAD(P)-linked malic enzyme activity is responsible for the conversion of malate to citrate in human placental mitochondria.

To clarify this point, studies presented in Table 1 and 2 were performed. The stimulation of malate dependent citrate formation in human term placental

Table 1. The effect of NAD^+ and $NADP^+$ on the malate dependent citrate formation by human term placental mitochondria

Additions	Citrate production (nmol × h^{-1} × mg^{-1} mitochondrial protein)
None	146.5 ± 13.6 (6)
+ NAD^+, 1 mM	147.6 ± 17.7 (6)
+ $NADP^+$, 1 mM	179.2 ± 34.9 (6)
+ Mn^{2+}, 1 mM	156.2 ± 8.8 (3)
+ Mg^{2+}, 1 mM	184.1 ± 23.1 (3)
+ NAD^+, 1 mM + Mn^{2+}, 1 mM	438.5 ± 20.8 (3)
+ NAD^+, 1 mM + Mg^{2+}, 1 mM	220.1 ± 19.6 (3)
+ $NADP^+$, 1 mM + Mn^{2+}, 1 mM	417.8 ± 24.2 (3)
+ $NADP^+$, 1 mM + Mg^{2+}, 1 mM	224.9 ± 5.7 (3)

Experimental conditions as described under Experimental except that Mn^{2+} was omitted from incubation medium. The values shown are means from the number of experiments given in parentheses ± SD.

Table 2. The effect of some inhibitors on the malate dependent citrate production by human term placental mitochondria

Additions	Citrate production (nmol × h^{-1} × mg^{-1} mitochondrial protein)
None	137.2 ± 13.3 (7)
+ Hydroxymalonate, 4 mM	58.2 ± 13.0 (4)
+ $NaAsO_2$, 2 mM	50.6 ± 4.9 (4)
+ Butylmalonate, 10 mM	51.3 ± 8.5 (4)
+ Rotenone, 5 μg/ml	10.0 ± 7.0 (3)

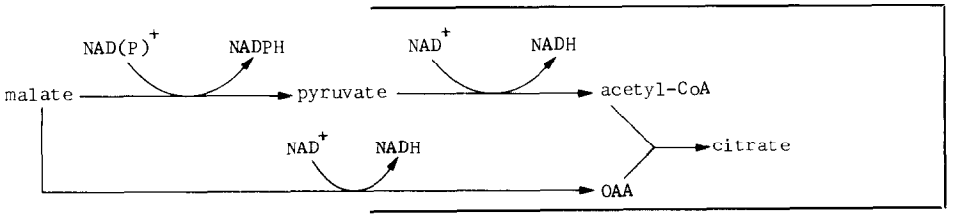
For experimental conditions see Experimental. The results are presented as means from the number experiments indicated in parentheses ± SD.

Table 3. Inhibition of the malate dependent progesterone biosynthesis in human term placental mitochondria by fluorocitrate and arsenite

Additions	Progesterone formation (dpm ^{14}C per sample)	(% of conversion)
L-Malate, 10 mM	1400 ± 60	0.21
+ Fluorocitrate, 50 μM	770 ± 160	0.12
+ $NaAsO_2$, 2 mM	680 ± 120	0.10
L-Malate, 10 mM + $NADP^+$, 1 mM	11500 ± 650	1.7
+ Fluorocitrate, 50 μM	6800 ± 620	1.0
+ $NaAsO_2$, 2 mM	6700 ± 650	1.0
L-Malate, 10 mM + NAD^+, 1 mM	4800 ± 360	0.73
+ Fluorocitrate, 50 μM	1800 ± 310	0.27
+ $NaAsO_2$, 2 mM	1100 ± 150	0.17

Experimental conditions as described under Experimental. Values shown are means ± SD from three experiments.

mitochondria by NAD^+ and $NADP^+$ added together with Mn^{2+}, an activator of malic enzyme, provides convincing evidence that the synthesis of citrate from malate is involving NAD(P)-linked malic enzyme activity. It has been shown previously that Mn^{2+} was by far more effective activator of the NAD(P)-linked malic enzyme than Mg^{2+} [9]. In accordance with this, Mg^{2+} added together with NAD^+ or $NADP^+$ stimulated citrate formation only slightly. To test further the possible contribution of NAD(P)-linked malic enzyme in the conversion of malate to citrate, studies were performed using hydroxymalonate, an inhibitor of both cytosolic [15] and mitochondrial [16] malic enzyme from other sources than human placenta. We have shown that hydroxymalonate inhibited also partially purified NAD(P)-linked malic enzyme from human placental mitochondria (not shown). Data presented in Table 2 indicates that hydroxymalonate also inhibited the conversion of malate to citrate, providing further evidence that the malic enzyme is playing an essential role in this pathway. Assuming that the conversion of malate to citrate in human term placental mitochondria undergoes according to scheme:



one could anticipate that this process should be inhibited by: (a) arsenite an inhibitor of pyruvate dehydrogenase; (b) respiratory chain inhibitor which would prevent the oxidation of the NADH formed; (c) butylmalonate an inhibitor of malate entry into mitochondria. Data presented in Table 2 indicate that the malate dependent citrate formation was in fact inhibited by these inhibitors. The data of experiments presented so far clearly indicate that the mitochondria from human term placenta are able to catalyse the conversion of malate as the only exogenous substrate to citrate and that NAD(P)-linked malic enzyme is involved in this process.

To elucidate the role of NAD(P)-linked malic enzyme in the conversion of cholesterol to progesterone in human term placenta, the experiments presented in Table 3 were performed. As can be seen, both $NADP^+$ and NAD^+ which stimulated citrate formation caused also an increase of the progesterone biosynthesis. On the other hand arsenite which inhibited citrate formation, produced a decrease of progesterone biosynthesis. The inhibition of citrate metabolism by fluorocitrate also caused a significant decrease of progesterone biosynthesis. These results strongly support the idea that the reduction of $NADP^+$ by isocitrate dehydrogenase (in the presence of isocitrate formed from malate) plays an essential role in the malate dependent progesterone biosynthesis.

The data presented above indicate that malate can

increase progesterone biosynthesis in human term placental mitochondria by NADPH generation in the following reaction sequence: Malate is reducing $NADP^+$ in the reaction catalyzed by NAD(P)-linked malic enzyme. Next the pyruvate formed from malate is converted to citrate, which is subsequently oxidized by $NADP^+$-linked isocitrate dehydrogenase. Under conditions described in Table 3 approx. 50% of NADPH (while mitochondria were incubated in the presence of $NADP^+$) required for progesterone biosynthesis was generated by $NADP^+$-linked activity of malic enzyme (synthesis insensitive to fluorocitrate and arsenite). However when mitochondria were incubated with NAD^+, fluorocitrate almost completely abolished the stimulatory effect of this nucleotide on progesterone biosynthesis. This means that under these conditions the effect of malate on progesterone biosynthesis is completely dependent on the conversion of pyruvate (formed by NAD^+-linked activity of malic enzyme) to isocitrate.

Previous studies have shown that energy dependent transhydrogenase could drive the formation of NADPH required for the conversion of cholesterol to progesterone [8]. Therefore it is likely that NADH formed during conversion of malate to citrate (in the reactions catalyzed by NAD(P)-linked malic enzyme, malate dehydrogenase and pyruvate dehydrogenase) could be oxidized by the respiratory chain (in the uncoupled conditions) or by the energy linked transhydrogenase (in the coupled state) providing additional NADPH for progesterone biosynthesis.

Thus the malic enzyme may promote progesterone synthesis in human placental mitochondria not only by providing directly the reducing equivalents from malate, but also by taking part in the conversion of pyruvate to isocitrate, the oxidation of which may be another essential source of reducing equivalents required for progesterone biosynthesis.

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