The relationship between placental protein synthesis and transfer of amino acids

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The relationship between placental protein synthesis and transfer of amino acids from mother to foetus was studied in the guinea pig, by using [U-¹⁴C]-lysine, -leucine, -glycine, -aspartate and - α -aminoisobutyrate. The uptake of label by protein was 12–16% of total label transferred. Cycloheximide inhibited incorporation of all naturally occurring amino acids into protein by 81–96% and transfer by 62–75%; the concentration of label in the free pool was increased for each. These findings were confirmed when specific-radioactivity measurements were made with L-[U-¹⁴C]lysine. The transfer of the non-protein amino acid α -aminoisobutyrate was not significantly decreased by cycloheximide. A model, linking protein synthesis to the generation of a transfer pool of amino acids, is proposed whereby inhibition of protein synthesis decreases the amount of amino acid available for transfer.

Transport studies are most frequently concerned with the events at the plasma membrane, in isolation from the long chain of biochemical processes within the cell which must ultimately determine metabolite flow. However, investigators of protein-turnover rate have been most concerned about the origin of the amino acids providing the precursor amino acyl-tRNA for protein synthesis (Waterlow *et al.*, 1978). Evidence for two sources has been presented from experiments on isolated mammalian tissues. Some observers consider that the free acid-soluble pool provides the precursors, the incoming molecules being transported 'uphill', against considerable concentration gradients, to pass into the pool, where they eventually become equilibrated before linear kinetics of incorporation into protein are observed (Robinson, 1977). Others find no delay before linear incorporation is attained and consider that uptake from the external medium is directed by the protein-synthetic machinery and by-passes the acid soluble pool (van Venrooij *et al.*, 1974). Wheatley & Robertson (1981) have pointed out that the size of the acid-soluble pool was unrelated to the relative proportion of an amino acid in protein, and that there was no correlation between the capacity of labelled essential and non-essential amino acids to equilibrate with either acid-soluble or protein pool

for each type of amino acid. Moreover, they found that linear kinetics of incorporation into protein began before equilibration of the acid-soluble pool occurred, and so favour the hypothesis that this is by-passed.

No relationship between the uptake of the model amino acid α -aminoisobutyrate and protein synthesis was found in fresh human placental tissue slices by Gusseck *et al.* (1975), but they confirmed the findings of Smith & Depper (1974), in isolated human placental villous tissue, that the enhanced uptake after preincubation could be inhibited by the protein-synthesis inhibitor cycloheximide. Since there is much disturbance of the normal cellular relationships in isolated tissues, observations in the more intact model, the guinea-pig placenta perfused *in situ*, were carried out; the placenta has the unique feature of a dual circulation, one on each side of the trophoblast membrane, so that transfer from mother to foetus and accumulation of a radioisotope by the placental protein may be measured at the same time to investigate the relationship between the two. Naturally occurring amino acids, labelled with ¹⁴C and belonging to the main transport groups described by Christensen (1973), were used, as it has been shown that they are transferred across the placenta according to the same groupings (Young & McFadyen, 1973). The transfer of the non-protein amino acid α -aminoisobutyrate was also investigated to determine if this was decreased by inhibition of protein synthesis. The amount of label

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transferred was compared with the amount taken up by the placental protein. Cycloheximide inhibited both protein uptake and transfer and, additionally, increased the labelling of the free acid-soluble pool for the four naturally occurring amino acids (leucine, lysine, aspartate, glycine). The transfer of α -aminoisobutyrate was not significantly inhibited.

Materials and methods

Reagents and radiochemicals

L-[U- 14 C]-Leucine, -lysine, -glycine, -aspartate, α -aminoisobutyrate and $^3\text{H}_2\text{O}$ were obtained from Amersham International, Amersham, Bucks., U.K. Cyclohexamine, lysine decarboxylase and dinitro-fluorobenzene were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Instagel and Soluene 50 were obtained from Packard Chemicals, Caversham, Berks., U.K. Lactate and pyruvate assay kits were obtained from Boehringer Corp., Lewes, East Sussex, U.K.

Animals

Forty-five pregnant albino guinea pigs (Duncan-Hartley strain), mean body wt. 1.24 ± 0.05 kg (s.e.m.) and kept on a 12h-light (07:00–19:00h)/12h-dark cycle were used between days 58 and 65 of gestation. Before the experiments, selection was made from measurements of the crown-rump length of foetuses from X-ray photographs and gestational age was determined from the graphs of Draper (1920). At the end of experiments, the gestational age was determined from foetal weight (Draper, 1920). In the latter stages of the work, initial selection was made from foetal skull length measured on X-ray photographs and related to weight and gestational age by the method of Thomas & Lowey (1982).

Experimental technique

The placentas were perfused *in situ* by cannulating the umbilical vessels, by the method of Reynolds & Young (1971), a modification of the technique as described by Money & Dancis (1960). Perfusion was carried out at a rate of $2\text{ ml} \cdot \text{min}^{-1}$ and a pressure of 35–45 mmHg (4.7–6.0 kPa) approximate to those found *in vivo* by using Krebs solution (Hill, 1975) containing foetal blood concentrations of amino acids (Hill & Young, 1973) and equilibrated with a mixture containing 4% O_2 , 5% CO_2 and 91% N_2 to give partial pressures equivalent to those found in foetal arterial blood.

Maternal arterial blood pressure was monitored from the carotid artery throughout the experiments and arterial samples were taken for pH/blood-gas analysis at the beginning and end of perfusion experiments; samples were also taken during the course of the placental perfusions for lactate and

pyruvate determinations to assess maternal metabolic status and for radiolabel measurements.

Protocol

After an initial perfusion lasting 20 min, $10\text{ }\mu\text{Ci}$ of the ^{14}C -labelled amino acid being studied was injected into the maternal jugular vein as a 1.0 ml bolus in 0.9% NaCl together with 5 mCi of $^3\text{H}_2\text{O}$. A constant infusion of $0.09\text{ }\mu\text{Ci}$ of amino acid/min and $8.3\text{ }\mu\text{Ci}$ of $^3\text{H}_2\text{O}$ /min was then infused over 40 min. All the perfusate was collected in 2- and 4-min samples.

Cycloheximide at a concentration of 5 mM in the perfusate was used to inhibit placental protein synthesis in some experiments; otherwise, the procedure was identical with that above. Cycloheximide is a small (mol.wt. 180) polar molecule and traverses plasma membranes readily (Grollman & Huang, 1973). A set of experiments were undertaken in which $50\text{ }\mu\text{Ci}$ of L-[U- ^{14}C]lysine was injected into the maternal circulation so that sufficient radioactivity (c.p.m.) was available for specific-radioactivity measurements to be made on this amino acid in the maternal arterial plasma, perfusate and placental free and protein pools.

Tissue preparation, radioisotope and metabolite determinations

At the end of the experimental period, the perfused placenta, together with two or three non-perfused placentas, were weighed and freeze-clamped between aluminium plates to the temperature of liquid N_2 and stored in this until analysed.

Placentas were pulverized under liquid N_2 to a fine powder, and 1–2 g was dispersed in 3 vol. of 0.6 M- HClO_4 . The samples were homogenized with a Silverson emulsifier on ice and centrifuged at 1500 g for 20 min. The supernatant fluids and protein pellets were retained for assay.

The ^{14}C and ^3H radioactivities in $100\text{ }\mu\text{l}$ of the placental tissue supernatants, perfusates and blood HClO_4 -extract supernatants were counted in 10 ml of Instagel with an Intertechnique SL30 liquid-scintillation spectrometer. The protein pellets were dispersed and washed three times in 0.6 M- HClO_4 and three times in acetone. The powder was solubilized in 1.5 ml of Soluene and 0.5 ml of H_2O_2 at 60°C over a 2 h period. Duplicate 1 ml samples were counted for ^{14}C radioactivity in 10 ml of Instagel. Label in protein was expressed per g wet weight and label in the free pool was corrected for foetal and maternal blood spaces from the measurements of Bloxam *et al.* (1981).

For specific-radioactivity measurements of L-lysine in maternal blood and placental free pool, the HClO_4 -soluble extracts were neutralized with 1.0 M-KOH. Perfusate samples were used without extrac-

tion. Lysine was decarboxylated to cadaverine and the $1\text{-}^{14}\text{C}$ of lysine liberated as $^{14}\text{CO}_2$ was trapped on filter-paper wicks, which were counted for radioactivity in 10 ml of Instagel. The non-radioactive lysine was measured by preparing a dinitrofluorobenzene derivative of cadaverine, and determining the product at 400 nm spectrophotometrically (Hutzler *et al.*, 1967). The protein pellets were washed as above and 20 mg of the powder was hydrolysed in HCl (Aristar) under nitrogen in sealed vials for 24 h at 120°C . The hydrolysate was evaporated to dryness, then taken up in 2 ml of 0.09 M-HCl, neutralized with 1.0 M-KOH, and the specific radioactivity was determined by the decarboxylation method described above.

In some experiments, placental extracts after immediate freeze-clamping and maternal arterial blood samples were analysed for lactate and pyruvate to determine if cycloheximide affected maternal and placental cytosol redox state. The supernatants from HClO_4 extracts were assayed by enzymic methods using the $\text{NAD}^+ = \text{NADH} + \text{H}^+$ principle. Lactate was determined by the method of Gutmann & Wahlefeld (1974) and pyruvate by the method of Czok & Lamprecht (1974).

Statistical analysis

Statistical comparisons were made by Student's *t* test. All the mean values are given $\pm$ S.E.M.

Results

Homeostasis of preparation

In the 24 control perfusions, the initial mean arterial pH was 7.41 ± 0.02 , pO_2 73 ± 0.10 mmHg and pCO_2 36.20 ± 0.61 mmHg. A rise in pH to 7.43 ± 0.04 and pO_2 to 86 ± 0.09 mmHg and a decrease in pCO_2 to 32.75 ± 0.58 mmHg occurred after the 40 min perfusion period. In 21 cycloheximide-treated guinea pigs, similar changes occurred; pH rose from 7.41 ± 0.03 to 7.43 ± 0.02 , pO_2 rose from 74 ± 0.08 mmHg to 82 ± 0.13 mmHg, and pCO_2 decreased from 35.83 ± 1.42 mmHg to 32.05 ± 0.72 mmHg.

In 15 control perfusions, the foetal:maternal ^3H ratio was measured, and decreased from 0.87 ± 0.05 to 0.63 ± 0.08 over the course of the experiments. Similarly, in cycloheximide-treated guinea pigs, the ratio decreased from an initial value of 0.72 ± 0.12 to a final value of 0.61 ± 0.05 , indicating that a decrease in the maternal circulation had occurred.

In four control perfusions, the initial maternal arterial blood lactate and pyruvate concentrations were 1.32 ± 0.11 and 0.11 ± 0.004 mM respectively (ratio = 12.0) and final concentrations were 1.18 ± 0.05 and 0.11 ± 0.003 mM respectively (ratio = 10.7).

In four cycloheximide-treated guinea pigs, similar changes were observed; the initial lactate and pyruvate concentrations were 1.25 ± 0.09 and 0.12 ± 0.002 mM respectively (ratio = 10.4) and the final values were 1.13 ± 0.03 and 0.17 ± 0.006 mM respectively (ratio = 10.3). The placental concentrations of lactate and pyruvate were 3.71 ± 0.34 and 0.13 ± 0.006 mM respectively (ratio = 28.5, $n = 5$) in controls and 3.15 ± 0.36 and 0.13 ± 0.006 mM respectively (ratio = 23.9, $n = 7$) in cycloheximide experiments.

Maternal plasma ^{14}C contents

A steady state was not achieved in maternal plasma with the constant infusion after a bolus injection of ^{14}C -labelled amino acid. The amount of ^{14}C originating from leucine and lysine decreased by 50% over the first 15 min of perfusion and remained constant for the remaining 25 min of the experiment, although the content of leucine was higher. The amount of ^{14}C originating from glycine and aspartate decreased by 50% in the first 20 min of perfusion and remained constant over the remainder of the experimental period. These values were similar to those of leucine. The amount of α -amino[^{14}C]-isobutyrate decreased by 50% after 5 min of perfusion and remained constant for the remainder of the experiment.

Transfer of ^{14}C -labelled amino acids

Leucine, glycine, lysine and aspartate were chosen to represent the four major transport groups; branched-chain neutral, straight-chain neutral, basic and acidic respectively. Perfusate samples were taken initially every 2 min and then at 4 min intervals. After 10 min perfusion, 73% of the ^{14}C originating from leucine was transferred. Similarly, over the same period the total transfer of ^{14}C was for lysine 78%, aspartate 73% and glycine 48%. The concentration of unlabelled lysine was constant in the effluent perfusate, but the specific radioactivity of lysine decreased by 93% in the first 10 min of perfusion. Lysine and aspartate profiles have been chosen as typical of the results obtained (Figs. 1a and 1b). Transfer of α -amino[^{14}C]-isobutyrate was similar in profile to that of aspartate (Fig. 1c). Transfer of ^{14}C originating from leucine was greater than that from the other amino acids and, taking this as 100%, transfer of label from the other amino acids was, for lysine, 35%, aspartate, 29%, glycine, 28%, and α -aminoisobutyrate, 13% (Fig. 2) over 40 min of perfusion.

The profile of specific radioactivity of lysine in the perfusate effluent (Fig. 1d) was similar to the profile of ^{14}C originating from lysine (Fig. 1a). Approx. 90% of the label was in lysine in the specific-radioactivity experiments.

Fig. 1. Profile of appearance of ^{14}C label in foetal placental perfusate after administering ^{14}C -labelled amino acids into the maternal circulation

For details see the text. ●, Control; ○, cycloheximide. Values shown are the means of three or four determinations. Vertical bars indicate s.e.m.

Placental pools of ^{14}C -labelled amino acids

For each amino acid, the incorporation into placental protein represented 12–16% of the total transferred. Because of the large protein pool, the total amount of ^{14}C originating from each amino acid incorporated into protein was greater than in the corresponding free pool (Fig. 3). Label originating from leucine showed the maximal incorporation into protein, followed by lysine, aspartate and glycine.

There was no statistically significant difference between the amount of ^{14}C label present in the placental free pools of the four naturally occurring amino acids used. The amount of labelled α -amino-

isobutyrate in the free pool was significantly higher ($P < 0.01$). The amount of label in the free pools represented 1–2% of the total transferred of the four natural amino acids, and 75% for α -aminoisobutyrate (Fig. 3). Specific-radioactivity measurements were only made when lysine transfer was studied, and approx. 60% of the label in the free pool and 95% in the protein pool was found to be L- ^{14}C lysine. The specific radioactivity of lysine in the free pool was $2.24 (\pm 0.53) \times 10^4 \text{ d.p.m./}\mu\text{mol}$, 11 times higher than the value of $2.00 (\pm 0.32) \times 10^3 \text{ d.p.m./}\mu\text{mol}$ found in the protein pool.

The amount of label in the protein and free pools was found to be similar in non-perfused placentas

Fig. 2. Effect of inhibition of protein synthesis on transfer of ¹⁴C label originating from amino acids

For details see the text. □, Control; ■, cycloheximide. Values shown are the means of three or four determinations. Vertical bars indicate S.E.M. Statistical comparison was made by Student's *t* test, control versus cycloheximide in each case (NS: not significant). The total label transferred in 40 min is expressed relative to that of leucine, which was taken as 100%.

sampled from the same animal in which perfusion *in situ* was carried out.

Influence of cycloheximide on transfer and placental pools of ¹⁴C-labelled amino acids

Cycloheximide altered the profile of ¹⁴C appearance in the essential amino acids. Taking lysine as representative (Fig. 1a), the large initial transfer of label was absent. The profiles of the non-essential amino acids and α-aminoisobutyrate were unaffected by cycloheximide (Fig. 1b). The transfer of ¹⁴C, originating from each of the four naturally occurring amino acids, was significantly decreased; leucine by 75% (*P* < 0.05), lysine 72% (*P* < 0.05), aspartate 72% (*P* < 0.01) and glycine 62% (*P* < 0.05). α-Aminoisobutyrate transfer was decreased by 50%, though not significantly (Fig. 2).

The incorporation of ¹⁴C label originating from each naturally occurring amino acid into protein was decreased by 96% for leucine and lysine, by 81% for aspartate and by 87% for glycine. The amount of label present in the free pool was increased for each amino acid, though only significantly with glycine (*P* < 0.05) (Fig. 3). Inhibition of protein synthesis decreased the proportion of label incorporated as a percentage of total transfer from 12–16% to 2–5%. Protein-synthesis inhibition increased the specific radioactivity of free-pool lysine 10-fold to 2.5 (± 0.38) $\times 10^5$ d.p.m./ μ mol and decreased that in placental protein to 0.82 (± 0.04) $\times 10^3$ d.p.m./ μ mol (*P* < 0.05). These changes were solely an alteration of labelled lysine.

The effect of cycloheximide on free and protein pools was similar in non-perfused placentas sampled from the same animal in which perfusion *in situ* was performed.

Discussion

Maternal acid–base balance measurements gave results similar to previously published data (see Young, 1981). These parameters, together with maternal arterial blood and placental redox states, were unaffected by cycloheximide, indicating that it had a specific effect on protein synthesis only. Maternal arterial blood lactate concentrations were similar to those found by Sparks *et al.* (1981). The ³H ratio between the maternal circulation and the perfusate decreased during the experiments, which may have been due to the small fall in maternal blood pressure, as the metaraminol used previously (Reynolds & Young, 1971) was not employed to maintain it.

The branched-chain amino acid leucine, as expected, showed the fastest rate of transfer of label to the foetal circulation over the first 15 min of perfusion (Christensen, 1973). Over this period, leucine and lysine transfer was faster than that of glycine and

Fig. 3. Influence of cycloheximide on the total ¹⁴C label in bound and free amino pools in the placenta

For details see the text. Values shown are the means from three or four perfused placentas. Vertical bars indicate S.E.M. ■, Free pool, control; ■, free pool, + cycloheximide; ▨, protein pool, control; ▩, protein pool, + cycloheximide.

aspartate. Interestingly, α -aminoisobutyrate, although belonging to the same transport group as glycine, was transferred at the slowest rate, which may have been due to the absence of unlabelled α -aminoisobutyrate. There was a consistent relationship between the amount of ^{14}C incorporated into placental protein and the amount transferred. The decrease in transfer, owing to protein-synthesis inhibition by cycloheximide, may be related to the decreased incorporation of label into placental protein. The transfer of the non-metabolizable amino acid α -aminoisobutyrate was also decreased by cycloheximide, though not significantly. The prevention of protein production *de novo* may affect enzyme and carrier protein synthesis as well as general protein turnover.

The results also show that the label in the placental free pool is increased when protein synthesis is inhibited; this was only significant for glycine. Specific-radioactivity measurements for lysine demonstrated a 10-fold increase in the free-pool specific radioactivity after inhibition of protein synthesis. If cycloheximide affected membrane transport only, it would be reasonable to expect a significant fall in the uptake of label into the free pool. As cycloheximide had no significant effect on α -aminoisobutyrate transfer, it appears that there is no direct effect on membrane transport proteins; however, it may be that the turnover time for these proteins is not exceeded in these experiments.

It is appreciated that the ^{14}C label, measured in the various pools, may not all belong to the amino acid studied. Only lysine specific radioactivities were measured, and were found to represent 90% of the label transferred. Hill (1975), using labelled leucine, found that 90% of the radioactivity transferred across the guinea-pig placenta was specifically in leucine. No similar measurements have been made for glycine. Stegink *et al.* (1979) found little transfer of labelled aspartate across the placenta of the monkey. Most of the label appeared as glucose and lactate. These observations may explain why the label incorporated into protein, from the non-essential amino acids, was lower. In addition, aspartate and glycine may be incorporated into nucleic acids after metabolism to nucleosides.

The present findings are in contrast with those of Gussek *et al.* (1975), who suggested that there is no link between placental trophoblast protein synthesis and membrane transport. They explained a pre-incubation effect, first observed by Smith & Depper (1974), whereby human placental slices, incubated in an amino acid-free medium, showed a stimulated amino acid uptake in terms of increased synthesis of transport protein. Only the increased transport capacity could be removed by inhibitors of protein synthesis such as cycloheximide.

Hume & Lamb (1976) have presented evidence

for nuclear control of amino acid uptake in cultured cells, suggesting that mRNA synthesis is required for maintenance of normal uptake. Since cycloheximide inhibits mRNA translation, this may provide a molecular explanation of the drug's effect on amino acid transfer.

It has been proposed by several authors (Kipnis *et al.*, 1961; Mortimore *et al.*, 1972) that the free amino acid intracellular pool is heterogeneous. Ward & Mortimore (1978) suggested that it must contain at least two components, an uptake pool and a catabolic component. Wheatley & Inglis (1980) have proposed that protein-synthetic requirements determine the size and nature of these distinct intracellular pools. A rapid turnover of placental protein (Young *et al.*, 1981) places a large requirement on the availability of amino acids. Wheatley & Inglis (1980) suggest that a portion of free intracellular amino acid forms a pool of discarded protein precursors not selected for synthesis. In addition, this pool contains amino acids from protein catabolism. It is suggested that this pool of discarded and catabolic amino acids may provide the source of amino acids which are transferred to the foetal circulation (Fig. 4). By inhibiting protein synthesis, the generation of this pool is decreased, and thus overall transfer may be diminished. α -Aminoisobutyrate transfer is not significantly decreased when protein synthesis is inhibited and, although α -aminoisobutyrate is not incorporated into protein, cycloheximide may alter the rate at which amino acids enter the post-selection pool. The data presented show that the decrease in transfer of label and amino acids is immediate and considerable for the four naturally occurring amino acids used. This would preclude cycloheximide affecting only the

Fig. 4. Suggested interrelationship between free and bound amino acid pools within the placenta effective during transport

(a) Pre-selection, catabolic free pool; (b) post-selection free pool.

generation of catabolic amino acids. Wheatley (1979), using cultured HeLa cells, specifically decreased the generation of catabolic amino acids with cycloheximide; the entry of amino acids directly to the post-selection pool was unaffected. This would therefore be at variance with the present suggestion, but the concentration of cycloheximide was only 0.03 mM and no pretreatment was used before administration of label.

Placental protein synthesis, and therefore turnover, may play important roles in the transfer of amino acid across the placenta. Cycloheximide appears to have no direct effect on membrane carrier protein. The increased concentration of label in the placental free pool suggests that protein turnover is necessary for optimal transfer and that its cellular need also dictates how much amino acid traverses the syncytiotrophoblast.

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