Mechanism of foetal wastage following immunoneutralization of riboflavin carrier protein in the pregnant rat: disturbances in flavin coenzyme levels

Kavita Krishnamurthy, Namita Surolia and P. Radhakantha Adiga*

ICMR Center for Advanced Study in Reproductive Biology, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Received 29 August 1984; revised version received 4 October 1984

Immunoneutralization of maternal RCP results in a >90% decrease in the content and the incorporation of [2-^{14}C]riboflavin into embryonic FAD as well as a percentage redistribution of both embryonic FMN and riboflavin. This is unaccompanied by any discernible changes in flavin distribution pattern in the maternal liver. Embryonic α-glycerophosphate dehydrogenase and NADPH-cytochrome *c* reductase register significant decreases in activities in the RCP antiserum-treated rats. These alterations readily explain the arrest of foetal growth culminating in pregnancy termination in the antiserum-treated animals.

Immunoneutralization *RCP* *FAD* *Fetal wastage* *Flavin*

1. INTRODUCTION

We have earlier provided evidence for the occurrence and obligatory participation of a specific, high-affinity carrier protein, viz., riboflavin carrier protein (RCP) in transplacental flavin transport in the pregnant rat. The functional importance of the rodent protein during gestation in terms of unremitting flavin supply and hence, uninterrupted foetal growth, was demonstrated by passive immunoneutralization of the endogenous maternal RCP with specific antisera to either the homologous [1] or the heterologous [2] RCP. This precipitated acute foetal wastage and abrupt pregnancy termination. In attempts to delve into the intraembryonic events culminating in foetal death, we have shown that immunoneutralization of the carrier protein leads to drastic curtailment of [2-^{14}C]riboflavin transport to the foetoplacental unit with consequent arrest of growth [3]. Confirmatory evidence for the essentiality of material RCP for foetal survival stems from experiments wherein active immunization of fertile female rats with the heterologous (chicken) RCP terminated their early pregnancies without any detrimental effect on maternal growth, cyclicity, vitamin status and subsequent ability to conceive [4]. In attempts to delineate the molecular processes that follow intraembryonically, we show that marked depletion of embryonic total flavin content, following immunoneutralization of the maternal carrier protein, sets in motion pronounced disturbances in the relative proportions of individual flavins, with changes in FAD being most significant.

* To whom correspondence should be addressed

Abbreviations: RCP, riboflavin carrier protein; a/s, antiserum; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide

2. MATERIALS AND METHODS

The sources of [2-^{14}C]riboflavin and other reagents as well as monitoring of pregnancies of adult female rats have been described [3]. The preparation and characterization of rabbit a/s to purified chicken RCP and its administration to

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00 © 1984 Federation of European Biochemical Societies

rats on day 11 of pregnancy were as in [3]. All operations thereafter were carried out in diffused light. Aqueous tissue homogenates (20% w/v) were heated at 70°C for 3 min prior to quantification of the total radioactivity and total flavin contents in the extracts [3]. For the quantification of [2-^{14}C]riboflavin associated with different flavins and to determine the basal level of endogenous tissue flavins, the heat-denatured extracts were clarified by centrifugation (1000 × *g*, 10 min at 4°C), concentrated to a small volume and the flavin components therein were resolved by descending chromatography on Whatman no.3 filter paper using *n*-butanol/acetic acid/water, 12:3:5, v/v/v, as the solvent system alongside known graded amounts of standard riboflavin, FMN and FAD (Sigma). The flavins separated on the air-dried chromatogram were identified by their fluorescence in UV light and individual flavins were either directly counted for radioactivity by liquid scintillation spectrometry or eluted from the paper with distilled water at 60°C and quantified fluorimetrically [3]. The mitochondrial α-glycerophosphate dehydrogenase (αGPD) activity was determined spectrophotometrically using phenazine methosulphate (PMS) and dichlorophenol indophenol (DCIP) as electron acceptors [5]. The foetal microsomal NADPH-cytochrome *c* reductase activity was also measured spectrophotometrically as in [6].

3. RESULTS

Sharp curtailment of transplacental transport of [2-^{14}C]riboflavin administered to pregnant rats, as a consequence of immunoneutralization of maternal RCP, is revealed by an approx. 80% decrease in the labelled flavin uptake by the foetal tissue when compared to that from control animals treated with nonimmune rabbit serum (NRS); a similar pattern was observed when radioactivity was expressed either per foetus or unit fresh weight of the tissue (table 1). From the relative distribution of radioactivity among individual flavins, it is evident that FAD represented the maximally labelled component which accounted for ~50% of the total ^{14}C-labelled vitamin taken up by the control foetus, the remainder being equally distributed between FMN and riboflavin. In contrast, there was a drastic decrease in radioactivity associated with foetal FAD from the a/s treated animals which represented only 2–3% of the total radioactivity in control foetuses and 13% of total labelled flavins in those from the a/s treated mothers. However, relative decreases in labelled FMN and riboflavin were less marked. Radioactivity associated with FMN in control foetal tissue represented ~7% of the total labelled flavins vis-à-vis 40% in a/s treated animals. Corresponding values for free riboflavin were 8 and 45%, respectively. In other words, labelled FMN plus riboflavin comprised a greater proportion of total radioactive flavins in foetuses from a/s treated animals compared with those from control animals, notwithstanding a steep decrease in [^{14}C]riboflavin transport to the foetuses in immunoneutralized animals. Further, this altered pattern of relative redistribution of labelled vitamin among different flavins in a/s treated animals, was confined to the foetal tissue since, concurrently, there was no discernible change in flavin pattern in the maternal liver (not shown).

Corresponding changes in the relative levels of individual and total flavin contents in the foetuses from the control and a/s treated animals as quantified spectrophotometrically are also depicted in table 1. It was found that a/s administration brought about ~30% reduction in foetal weight which was accompanied by a 50% decrease in total flavin content. A comparison of the levels of individual flavins in foetuses from the control vis-à-vis in those from a/s treated animals revealed that pronounced depletion in both the content and concentration of FAD was the major alteration accompanying RCP immunoneutralization. This was followed by an apparent but statistically non-significant increase in both FMN and riboflavin contents in the affected foetus. The selective nature of such a disturbance in flavin nucleotide levels in the foetal tissue is exemplified by the data of table 2, which shows that in the maternal liver, the flavin distribution pattern, including the contribution of FAD accounting for 80% of total flavins remained unperturbed on RCP a/s treatment.

A drastic decrease in foetal flavin levels in general, and in FAD levels in particular in the RCP a/s treated animals, could be expected to reflect in depleted activities of some of the FAD-dependent foetal flavoenzymes. Two representatives of FAD-

Table 1

Effect of immunoneutralization of maternal RCP in the pregnant rat on incorporation of [2-^{14}C]riboflavin into and contents of different flavins in the foetal tissue

Flavin	Control foetus				
	Radioactivity (cpm × 10^{-3})		Flavin content (ng)		Spec. radioactivity (cpm/ng)
	per mg tissue	per foetus	per mg tissue	per foetus	
Total	0.320 ± 0.012	40.9 ± 2.0	3.4 ± 0.2	705 ± 82	0.058
FAD	0.173 ± 0.006	22.1 ± 0.8	2.4 ± 0.5	470 ± 131	0.047
FMN	0.080 ± 0.010	10.3 ± 1.4	0.2 ± 0.1	44 ± 15	0.234
Riboflavin	0.066 ± 0.010	8.4 ± 1.6	0.3 ± 0.1	59 ± 27	0.142
	RCP a/s-affected foetus				
	Radioactivity (cpm × 10^{-3})		Flavin content (ng)		Spec. radioactivity (cpm/ng)
	per mg tissue	per foetus	per mg tissue	per foetus	
Total	0.073 ± 0.01	6.9 ± 1.0	2.6 ± 0.1	327 ± 52	0.021
FAD	0.009 ± 0.004	0.9 ± 0.4	0.2 ± 0.1	27 ± 17	0.033
FMN	0.029 ± 0.01	2.8 ± 1.1	0.5 ± 0.2	69 ± 15	0.040
Riboflavin	0.033 ± 0.004	3.2 ± 0.4	1.1 ± 0.6	131 ± 67	0.024

Pregnant rats (day 11) were injected either 0.5 ml NRS (control) or 0.5 ml RCP a/s and killed 24 h later. Total and individual flavin contents of clarified embryonic homogenates were estimated fluorimetrically. For the measurement of embryonic uptake and distribution of [2-^{14}C]riboflavin, pregnant rats were injected the labelled vitamin (0.5 μCi equivalent to 6 μg/animal) 1 h after antiserum treatment. Embryos were removed 24 h later and analyzed chromatographically for radioactive flavins. Values repesent mean ± SE (n = 5)

dependent foetal enzymes, viz., the mitochondrial α-glycerophosphate dehydrogenase and the microsomal NADPH-cytochrome c-reductase have been assessed in the control and RCP a/s affected foetal tissues at 24 h after a/s treatment to rats during day 12 of gestation (fig.1). It is clear that both the enzyme activities register a significant decrease in terms of both total and specific activities although the extents of inhibition observed were not commensurate with the corresponding depletion in FAD levels in the affected foetuses.

Table 2

Effect of immunoneutralization of RCP on distribution of flavin coenzymes in maternal liver

Flavin	μg/g Liver	
	Control	RCP a/s
FAD	20.6 ± 2.0	19.5 ± 1.8
FMN	3.6 ± 1.0	5.1 ± 1.2
Riboflavin	3.7 ± 1.3	4.6 ± 1.6

Pregnant rats (day 11) were injected with either NRS or cRCP a/s (intraperitoneally) and killed 24 h later. Livers were analyzed for individual flavins by monitoring flavin fluorescence. Values are mean ± SE (n = 5). Changes in contents of FAD, FMN and riboflavin in livers from the a/s treated group were not statistically significant ($P > 0.25$)

4. DISCUSSION

It is clear that acute riboflavin deprivation culminating in foetal wastage in RCP a/s treated pregnant rats is attended by a selective but drastic disturbance in the labelled vitamin distribution among different foetal flavins with FAD exhibiting the most pronounced decrease. A comparison of specific radioactivities of different flavins in the normal and affected foetuses (table 1) reveals that the major consequence of acute flavin deficiency in the foetus is the pronounced inhibition of FAD

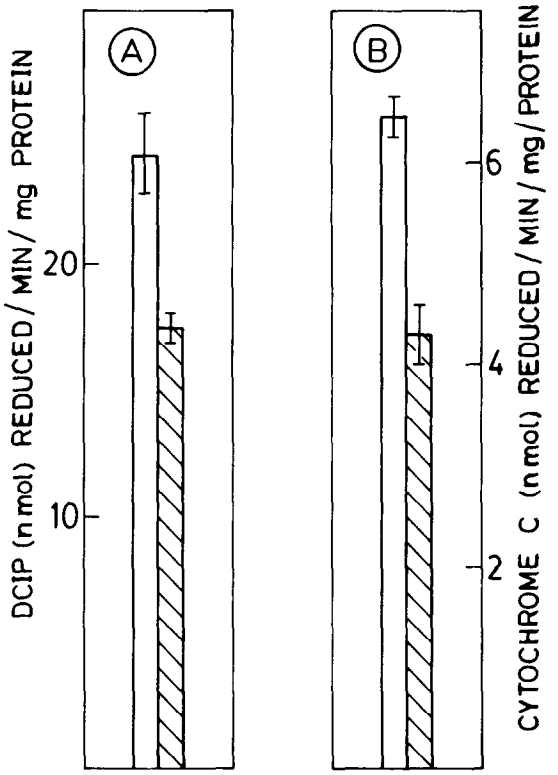

Fig.1. Influence of RCP a/s on the embryonic activities of two FAD dependent flavoenzymes: ▭ control; ▨ RCP a/s treated. (A) Mitochondrial α-glycerophosphate dehydrogenase. The assay mixture (3.0 ml) contained 83.3 mM potassium phosphate buffer (pH 7.4), 33.3 mM KCl, 3 mM KCN, 3.3 mM $MgCl_2$, 1.8 mg BSA, 0.2 mM PMS, 0.26 mmol DCIP, 0.04 M DL-α-glycerophosphate, and 0.5–1.0 mg mitochondrial protein. (B) Microsomal NADPH-cytochrome *c* reductase. The reaction mixture (3 ml) comprised 0.33 M potassium phosphate buffer (pH 7.6), 50 mM cytochrome *c*, 42 mM NADPH and microsomal protein. Vertical bars represent SE (n = 5).

elaboration [7]; that its biosynthesis is presumably affected is supported by the following: in the normal foetus, FMN/riboflavin ratio is 1.6 and that of FMN/FAD is 5, showing that FMN⟶FAD conversion is the rate-limiting step in FAD synthesis in the foetus. On the other hand, in the affected foetus, FMN/riboflavin ratio is comparable to that in the control foetus, while the FMN/FAD value (1.2) shows a 4-fold decrease. This steep decline in foetal FAD under conditions of RCP immunoneutralization points to basic similarities between biochemical events that manifest under conditions of immunoneutralization of the vitamin carrier and those precipitated by chronic flavin deficiency when the pregnant animals are fed either a riboflavin-deficient diet alone or that supplemented with galactoflavin [8]. In these earlier studies on maternal vitamin deficiency, both types of diets brought about comparable extents (30–37%) of total flavin deficiency, but galactoflavin supplementation led to a 60% decline in FAD level [8]. Viewed from this angle, the above novel approach of producing selective foetal vitamin deficiency is far superior and quicker since decreases in total flavin (54%) and FAD (90%) were more severe. Interestingly, unlike under the conditions of hypothyroidism and dietary flavin deficiency in adult animals where hepatic concentrations of FAD are maintained at the expense of the more dispensable FMN and free riboflavin [9], the contents of the latter two flavins are relatively less disturbed in the foetal tissue under conditions of immunoneutralization of maternal RCP. Furthermore, the flavin profile encountered in the affected foetuses is similar to that in hepatocytes during drug-induced carcinogenesis [10], pointing to the basic similarities in flavin metabolism between the two types of rapidly proliferating cell types.

Selective depletion of FAD in the affected foetus reflects itself in decreased activities of the two FAD dependent flavoenzymes (fig.1) and is in conformity with the above findings, although the extents of decline in the two representative flavoenzymes fall short of that expected from the magnitude of FAD depletion in the affected foetus; this may indicate that the enzyme-bound FAD represents a minor fraction of the total foetal FAD and has a slower turnover rate relative to total FAD. Of relevance in this context is that the $t_{1/2}$ of flavin coenzymes and the rate of exchange of free flavins with enzyme bound forms are known to be governed by the amount of free flavin available, the rate constants of association and dissociation and the presence of substrates and inhibitors [11].

From the foregoing it is clear that the acute foetal flavin deficiency setting in rapidly and severely as a consequence of immunological interference with maternal RCP brings about a drastic disturbance in flavin metabolism such that

vital metabolites like FAD fall to critically low levels; faced with this situation the rapidly proliferating foetus is presumably unable to cope with the enhanced demand for more flavin coenzymes for its growth and hence loses its viability.

ACKNOWLEDGEMENTS

Financial assistance from the Indian Council of Medical Research, and the Family Planning Foundation, New Delhi is gratefully acknowledged.

REFERENCES

[1] Ramanamurthy, C.V. and Adiga, P.R. (1982) J. Biol. Sci. 4, 227–237.
[2] Muniyappa, K. and Adiga, P.R. (1980) FEBS Lett. 110, 209–212.
[3] Ramanamurthy, C.V. and Adiga, P.R. (1981) FEBS Lett. 135, 281–284.
[4] Ramanamurthy, C.V. and Adiga, P.R. (1982) Science 216, 191–193.
[5] Arrigoni, B. and Singer, T.P. (1962) Nature 193, 1256–1258.
[6] Rivlin, R.S., Menendez, C. and Langdon, R.G. (1968) Endocrinology 83, 461–469.
[7] Yagi, K., Nagatsu, T., Nagatsu-Ishibashi, I. and Ohashi, A. (1966) J. Biochem. 58, 313–315.
[8] Miller, Z., Poncet, I. and Takacs, E. (1962) J. Biol. Chem. 237, 968–973.
[9] Rivlin, R.S. (1969) Adv. Enzyme Regul. 8, 239–250.
[10] Rivlin, R.S., Hornibrook, R. and Osnos, M. (1973) Cancer Res. 33, 3019–3023.
[11] Okuda, J. and Yagi, K. (1977) in: Biochemical Aspects of Nutrition (Yagi, K. ed.) pp.155–160, Japan Sci. Soc. Press, Tokyo and University Park Press, Baltimore, MD.