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Significance of Riboflavin Carrier Protein in Animal Reproductive Physiology

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Estrogen-inducible riboflavin-carrier (or-binding) protein (RCP) obligatorily involved in yolk deposition of the water-soluble vitamin in the developing oocyte in egg laying hen is evolutionarily conserved in mammals including primates in terms of physicochemical, immunological and functional characteristics and serves as the primary mediator of transplacental vitamin transport to support uninterrupted embryonic vitamin nutrition and hence its development. Passive immunoneutralisation of the vitamin carrier in rodents drastically and selectively curtails flavin transport to the fetoplacental unit resulting in rapid tissue wastage culminating in abrupt pregnancy termination. The active immunisation with the heterologous RCP elicits bioneutralizing antibodies which interfere with pregnancy establishment without any discernible adverse effect on the maternal vitamin status, reproductive hormonal profiles and general well-being in rodents and bonnet monkeys. Recent data reveal that RCP is also estrogen-inducible in the mammary gland to become a constituent of the mammalian milk and hence serves to sequester the vitamin from the maternal system into milk for neonatal vitamin nutrition. Additionally, the vitamin carrier is produced by Leydig and Sertoli cells of the mammalian testis to become a constituent of the germ cells including mature spermatozoa, presumably to function in facilitating vitamin transport through the blood-testis barrier offered by the Sertoli cell tight junctions. These findings emphasize the importance of RCP in different facets of reproductive biology of the animals through evolution.

Key Words: Riboflavin-carrier protein, Egg-yolk, Estrogen, Evolutionary conservation, Mammals, Placental transport, Mammary gland, Milk, Testis, Germ cells

Introduction

I am thankful to the President and the Council of the Indian National Science Academy for bestowing on me the honour of receiving the first endowment lecture award instituted in memory of the late Prof. M R N Prasad. In this context, it is appropriate that I pay tribute to the late Prof. Prasad who not only made landmark contributions to Reproductive Physiology during his scientific career in India but also trained and inspired several generations of young scientists in the country. Prof. Prasad spent the last couple of years before his untimely demise, at the Indian Institute of Science and I had the privilege of coming into close contact with him during this period and was inspired to pursue some of the lines of investigation on the roles of vitamin carrier proteins in mammalian reproduction which form the subject matter of this lecture.

The Background

As you are all aware, the embryonic development represents the most intensive growth phase in the life of an individual. It has also been realised that under normal circumstances, the mammalian embryo *in utero* enjoys a remarkable degree of autonomy in its developmental regulation such that even under marginal nutrient supply to the maternal system the embryo continues to accumulate several vital nutrients against concentration gradients for its uninterrupted growth unperturbed by the competing demands of the vastly bigger maternal system. While details underlying this enigmatic phenomenon remain to be elucidated, it is generally agreed that endocrine principles secreted into the maternal circulation by the fetoplacental unit influence appropriate biochemical and physiological changes. One such hormone steadily elevated in maternal circulation during gestation is estrogen. However the biochemical significance of this enhanced estrogen production is incompletely understood despite the observation that in pathologic pregnancies there is a positive correlation between depressed maternal estrogen levels and intrauterine fetal growth retardation (Beling 1978).

If however, one examines the evolutionary aspects of estrogen action in reproductive processes, the dominant role of this steroid hormone in oviparous vertebrates in preparing the maternal system to provide adequate nutritional support for embryonic growth becomes conspicuous. Thus in these egg-laying species during the reproductive phase, estrogen induces massive amounts of the egg yolk and white proteins in the liver and the oviduct respectively for oocyte deposition for nutrition of the prospective embryos (Chan et al. 1978, Tata & Smith 1979). Then the question arises as to whether this important function of estrogen in maternal nutrient mobilization is conserved atleast in part during evolutionary transition to viviparity in view of the fact that viviparity has evolved from oviparity. Of relevance in this context is the fact that the evolutionary transition has been continuous, innovations compatible and that fundamental similarities exist between the two types of reproductive phenomena. Based on the above premise, I would like to place before you today evidence accumulated in our laboratory that an estrogen-inducible riboflavin-carrier (or -binding) protein (RCP) obligatorily involved in yolk deposition of the vitamin in the developing chicken oocyte is scrupulously conserved through evolution all the way to primates in terms of physicochemical and immunological characteristics, estrogen inducibility at its sites of synthesis and finally its function as the primary vehicle for transport of the vitamin from the maternal organism to the developing embryo.

RCP in the Chicken Egg

The major functions of the vitamin carrier proteins which non-covalently interact reversibly with high-affinity and selectivity in 1:1 molar ratio with fat- and water-soluble vitamins are dietary absorption, circulatory transport and the conservation of these micro-nutrients. Among these, retinol-binding proteins, transcobalamines and folate binding proteins are ubiquitous in the animal kingdom. Neither the natural occurence nor the possible physiological functions of carrier proteins for other major vitamins (eg. riboflavin) was known until the discovery of an unique non-enzymatic flavoprotein in the chicken egg white and yolk which interacts with riboflavin in preference to FMN and FAD (Clagett 1971). That egg-laying birds elaborate this unique flavoprotein solely as a reproductive stratagem to ensure adequate vitamin deposition in the developing oocyte is indicated by the following: (a) such a protein is undetectable in the adult male or rapidly growing immature pullets of either sex; (b) unhampered growth rate, sexual maturity and

egg laying capacity of a mutant strain of the chickens lacking functional gene coding for this protein; and (c) the fertilized eggs from these mutant birds fail to hatch since flavin deficiency precipitated by the maternal failure to deposit the vitamin in the egg leads to early embryonic mortality (Adiga & Murty 1983).

The chicken egg RCP is a well-characterized single chain phosphoglycoprotein (Mr 37 K, 219 amino acids) with a highly folded structure reinforced by 9 disulphide bridges and containing 2 N-linked oligosaccharides and 8 phosphoserine residues clustered in a highly anionic region near the C-terminus. The amino acid architectures of the RCPs from the egg white produced in the oviduct and the serum synthesized in the liver are identical implying a single structural gene encoding the protein. As expected from its precursor product relationship with the serum RCP, the yolk RCP has an identical sequence except that it lacks 11-13 amino acids at the C-terminus presumably due to limited proteolytic cleavage during yolk deposition. The vitamin carriers from all the three sources otherwise exhibit identical features including post-translational modifications (White & Merrill 1988).

Estrogen specificity of hepatic induction has been unequivocally demonstrated in the chicken (Adiga et al. 1983, 1988a). Recent studies using labelled cDNA probe have confirmed that RCP production under estrogenic influence reflect enhanced transcription of RCP mRNA in addition to its cytoplasmic stabilization and increased recruitment for polysomal function (Murti 1990).

Mammalian RCP and Roles in Reproduction

As indicated earlier, in higher mammals including humans, the rapidly growing fetoplacental unit with its parasitic existence *in utero* obtains continuous and unremitting nourishment from the maternal supply line and consequently accumulates several essential nutrients including riboflavin which exhibits high fetal: maternal ratio of 4:1 despite the barrier offered by the placental membranes (Dancis & Schneider 1975). The molecular basis of this enigmatic reproductive phenomenon was unknown until recently. As a working hypothesis, it was attractive to visualize that the placental barrier to the vitamin may be similar to that offered by the plasma membrane of the avian oocyte and hence an RCP-mediated delivery mechanism may be operational to account for preferential vitamin accumulation by the fetal side of the conceptus. If this premise is correct, it is to be anticipated that the RCP gene is conserved in mammals to be expressed under instruction from estrogen; consequently the major structural features relevant to its function are retained during evolution and hence would enable its detection and quantitation by immunochemical analysis.

The above premise is amply borne out by immunological and biochemical evidence that in pregnant (but not adult male or immature) rats, measurable amounts of an RCP-like entity could be identified and quantified by the specific radioimmunoassay standardized for chicken RCP (Adiga et al. 1988b). The rodent RCP purified by bioaffinity chromatography could bind free riboflavin with high-affinity and specificity and its presence in rodent circulation during gestation and after estrogen treatment clearly favour its involvement in transplacental vitamin transport to subserve embryonic growth.

If RCP is evolutionarily conserved form the egg laying lower vertebrates to viviparous rodents despite the different strategies employed by the latter to nourish their embryos, such an essential mechanism is expected to be retained during further evolution of the species. Recent purification and characterisation of RCPs from the pregnant bonnet monkey (*M. radiata*) and human (maternal and the umbilical cord) sera are in line with the above premise. The primate

RCPs display marked similarities to their chicken egg counterpart in terms of molecular size, pI, preferential binding to free riboflavin and gross immunological characteristics. Sequence similarities amongst the RCPs is supported by the overall patterns of distribution of their ^{125}I-labelled tryptic peptides on finger printing (Adiga et al. 1988b). Recent availability of a battery of monoclonal antibodies (MAbs) recognising different non-overlapping epitopic conformations on the surface of the avian RCP afforded an opportunity to compare the immunotopological characteristics of the various RCPs. The remarkable finding that all the MAbs recognise the various mammalian RCPs albeit with different affinities, leads to the inevitable conclusion that the overall surface contour of RCP is scrupulously conserved as expected from the homologous proteins during evolution (Adiga et al. 1988a, Karande et al. 1991).

Estrogen Modulation of Biosynthesis

There is unequivocal evidence to show that the similarities with regard to molecular characteristics between the avian and mammalian RCPs extends to the estrogenic modulation of its gene expression at its biosynthetic loci (eg. the maternal liver). Thus in the female rat, the circulatory concentrations of RCP change in concert with the steroid hormone levels during the estrous cycle. During pregnancy, elevated levels of the vitamin carrier are encountered coincident with intense embryonic growth. Unequivocal proof for estrogen being the primary signal for hepatic induction stems from experiments wherein the male rats were administered pharmacological doses of the hormone. The kinetic analysis of the inductive response exhibited overall qualitative similarities between the avian and rodent systems which include ineffectiveness of progesterone and inhibition by antiestrogen of RCP gene expression. The plasma RCP concentrations in female primates are also physiologically modulated by the steroid hormone. Furthermore, administration of pharmacological doses of estradiol-17β to immature female or male monkeys enhances the plasma RCP concentrations in concert with the pharmacokinetics of the hormone, the males exhibiting a relatively sluggish response as a consequence of the faster rate of hormonal clearance from circulation (Adiga et al. 1988a,b).

Functional Significance in Early Embryonic Development

Since protein evolution is guided by its indispensibility for the survival of the organisms, it is reasonable to assume that RCP is an integral part of the evolutionarily conserved primary mechanism mediating transplacental vitamin transport in mammals. This is supported by experiments wherein endogenous RCP in pregnant rats was immunoneutralised with potent antibodies to either the homologous or the heterologous vitamin carrier which led to embryonic loss due to severe curtailment of vitamin supply to the fetal side of the conceptus (Adiga & Murty 1983, Adiga et al. 1988a). Similarly, active immunisation of proven fertile female rats with chicken RCP prevented pregnancy progression without impairing the maternal vitamin status and general well-being (Murty & Adiga 1982).

Evidence for the operation of a similar phenomenon in the primates is provided by experiments wherein active immunisation of proven fertile female monkeys with chicken RCP elicited high titre antibodies without any adverse effect on their vitamin status, menstrual cyclicity and gonadal hormonal profiles. The latter findings support the contention that RCP is dispensable for the maternal well-being and that its role is confined to the reproductive process, a view further strengthened by the plasma appearance of RCP in plasma in high

concentration only during the luteal phase of the menstrual cycle and pregnancy (Adiga et al. 1991). An examination of the reproductive performance of the actively immunised monkeys revealed that more than 50% of the recognised early pregnancies were terminated provided antibody titres were high. This clearly testifies that the primate RCP plays a vital role as an obligatory vitamin carrier through the placental barrier (Adiga et al. 1988b). Later experiments using SDS-denatured, unfolded (by reduction-carboxymethylation of disulphide bonds) chicken RCP as the immunogen suggested that immunogenic efficacy of chicken RCP in terms of eliciting bioneutralizing antibodies in monkeys and rats can be substantially improved and that early pregnancy suppression due to active immunisation can approach 100%. These findings show that many of the native epitopic conformations at the surface of chicken RCP may be redundant in terms of bioneutralisation which can be accomplished by a restricted population of antibodies recognizing segmental epitopes (Adiga et al. 1991).

Mechanism of Embryonic Wastage in the Rodents

In attempts to understand mechanism(s) responsible for embryonic demise following passive immunoneutralisation of RCP in pregnant rats, it was remarkable to find that severe fetal flavin deficiency was precipitated due to drastic curtailment of [^{14}C]-riboflavin influx from the maternal supply line to the fetoplacental unit. This seemed to set in motion a series of intraembryonic events characterized by major disturbances in relative as well as absolute concentrations of flavin co-enzymes with depletion in FAD being most dramatic. Thus in the absence of sustained carrier-mediated flavin delivery, the very survival of the growing embryo gets jeopardized. Histological examination of the affected fetoplacental unit revealed: (1) detachment of the placental membrane from the decidua; (2) severe degeneration of trophoblast (Adiga et al. 1988b).

RCP in Other Reproductive Processes

Lactation

Since riboflavin concentration in mammary gland secretions such as colostrum and milk is several-fold higher than in maternal circulation during lactation, it is conceivable that RCP occurs and functions as the protein carrier for vitamin secretion into milk for neonatal nutrition. This premise is further strengthened by the fact that the mammary gland is yet another estrogen dependent female reproductive system where an established physiological barrier due to circumferential light junctions between epithelial cells prevents passage of blood constituents (Linzell & Peaker 1971). This working hypothesis could be clearly confirmed by purification and molecular characterization of RCP from the milks of variety of mammals including monkeys and humans. The bovine milk RCP could be isolated in bulk and shown to share immunological and physicochemical proper- ties with its avian egg counterpart. The ability of the rat mammary gland to synthesize RCP during pregnancy and lactation and upon estrogen administration to virgin animals could be shown by (1) the detection and quantification of RCP-mRNA by dot blot and Northern blot analysis using ^{32}P-labelled chicken RCP-cDNA; (2) ^{35}S-methionine incorporation into immunoprecipitable RCP during *in vitro* culture of mammary explants (Prasad 1992). The proposed function of the mammary RCP appears to be to sequester the available vitamin and secrete the same in a concentrated form into milk for neonatal nutrition (Prasad 1992).

Spermatogenesis

From the foregoing, one would get the impression that the elaboration of RCP dictated by a female sex steroid (viz., estrogen) as a stratagem to ensure adequate vitamin nutrition of the growing organisms is confined to female reproductive physiology. However, recent investigations in our laboratory show this not to be the case, following the discovery that RCP is a germ-cell component in the adult mammalian testis. These investigations were prompted by the following known facts: (i) in the adult mammals, spermatogenesis in the testis involves very active and continuous germ cell proliferation and differentiation; (ii) an effective blood-testis barrier in the form of tight junctions between adjacent Sertoli cells in the seminiferous tubules isolate the germ cells from the extra tubular environment thus necessitating the passage of all nutrients and growth factors across the Sertoli cells (Bardin et al. 1988); (ii) estrogen is produced by the Leydig and Sertoli cells and is involved in the regulation of testicular function (Dufau 1985). It was therefore attractive to visualize that RCP may be produced by the appropriate cell types in the testis to target the vitamin through the physiological barrier to support profound germ cell proliferation and differentiation. This hypothesis is amply borne out by our finding that a measurable amount of RCP-mRNA in total testicular polysomal RNA is present as assessed by hybridization with ^{32}P-labelled chicken RCP-cDNA. That this mRNA is translationally active could be shown by synthesis of radiolabelled RCP immunoprecipitable with specific RCP-antibodies during *in vitro* culture of rat testis in presence of ^{3}H-labelled amino acids. Additionally, RCP could be immunocytochemically localized in both the Leydig and Sertoli cells as well as in the pachytene spermatocytes, round spermatids and in the acrosomal region of mature spermatozoa from the rat, cattle, monkeys and humans (Bhat et al. unpublished observations). It is thus tempting to speculate that RCP mediates transport and sequestration of the vitamin by the germinal cells on lines suggested for testicular transferrin (Griswald 1988).

Conclusion

It is thus clear that RCP mediating the yolk deposition of the vitamin in egg-laying lower vertebrates for use by the prospective embryo is remarkably conserved through evolution of the species all the way upto humans. The fact that the mammals unlike the oviparous species, have built-in mechanisms to provide nutrients continuously to their developing embryos *in utero* and yet have not eliminated the carrier-mediated vitamin delivery mechanism to support fetal growth emphases the indispensibility of RCP in the survival of the species. It is remarkable that not only basic structural features, immunochemical and molecular characteristics but also such details as biosynthetic loci and hormonal specificity of induction are retained throughout. The estrogen specificity of RCP gene modulation endows on this hormone an important molecular function during reproduction viz., the continued production during gestation of the vitamin carrier to deliver riboflavin in adequate amounts to ensure optimal embryonic growth uninfluenced by the competing demands of the mother. It is likely that progressive increase in estrogen levels in maternal circulation during pregnancy represents one of the endocrine communications originating from the fetoplacental unit to command the mother to mobilize her resources to provide adequate micronutrients for sustained growth. Since immunointerference with the carrier function terminates pregnancy, the possibility exists that genetic/endocrine defects in production/functionality/transport of RCP may explain some of the ill-defined causes of habitual/spontaneous abortions/ and intra-uterine growth retardation. Finally, the finding that

immunoneutralization of RCP produces acute yet selective fetal vitamin deficiency opens new vistas of research on fetal vitamin metabolism in relation to its development.

Recent unequivocal evidence for the occurrence and functionality of RCP in tissues and processes other than those directly involved in embryonic development has broadened the functions of RCP to other reproductive processes such as lactation and spermatogenesis. The only common denominator shared by these systems are: (i) they all have well-defined physiological barriers; and (ii) are estrogen-dependent target tissues undergoing cellular proliferation and differentiation. It is conceivable therefore that there may be other physiological systems satisfying the above criteria in the body where RCP is produced and functionally required. Further research on these lines should be fruitful.

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