Has the mechanism by which the endocrine placenta scavenges the mother whilst sparing the foetus been unmasked?

P J Lowry
School of Animal and Microbial Sciences, University of Reading, Reading RG6 6AJ, UK

Abstract

The endocrine placenta has a dilemma; it shares the foetal circulation and yet it needs to secrete active peptide hormones into the maternal circulation to control her metabolism to meet the demands of the growing foetus. Simultaneously, it needs to allow the endocrine systems of the foetus to develop independently. This article will describe how peptide hormones are processed from inactive intermediates and will propose a hypothesis of how the placenta has revised this process to protect the foetus from the potentially damaging affects of these products.

Introduction

There is no doubt that, throughout pregnancy, the placenta controls much of the metabolism of the mother for the benefit of the foetus, to such an extent that there are many instances in which, in humans, the mother’s health is compromised during pregnancy and parturition. These health problems can persist post partum, often for years. As there are no neural connections between the placenta and the mother, the main means by which the placenta controls the mother’s metabolism must be endocrine in nature. There is, however, a fundamental problem with this situation. The foetal blood that perfuses the placental/uterine boundary cells to carry oxygen and nutrients back to the foetus will also collect the very hormones the placenta is producing in the boundary cells in amounts sufficient to diffuse into the maternal circulation and reach concentrations that can affect the mother’s metabolism. Despite all these same placental endocrine materials circulating in the foetal circulation, the foetus seems to be able to develop on its own with its physiological systems maturing naturally, without any significant interference from these placental endocrine products. Whilst we understand much of the complexity of the peripheral endocrine organs and systems in the non-pregnant state, we know little of the endocrinology of the placenta; in fact, despite the sophisticated level of technology available today most of the workings of the placenta remain an enigma.

Processing of polypeptide hormone precursors

Peptide hormones are by far the most abundant and diverse group of the endocrine signals, so to expand my hypothesis of how the placenta has overcome its endocrine dilemma, I have concluded that the solution lies in the way the placenta biosynthetically processes its peptide hormones. These are normally synthesised in endocrine and neural tissue as large inactive precursors which are

P J Lowry holds the 2003 Society for Endocrinology Medal. This article is based on his presentation at the 194th meeting of the Society for Endocrinology, 3–5 November 2003.
then processed by proteolytic enzymes in the Golgi apparatus and finally converted to active peptide hormones inside mature secretory vesicles. These active hormones are then released from the cell directly into the circulation in response to an external stimulus, be it a hypothalamic releasing factor as in the case of adrenocorticotrophin (ACTH), a neurotransmitter as in the case of oxytocin, or a circulating metabolite such as glucose as is the case for insulin.

This processing of polypeptide hormones appears to have evolved over a billion years ago, as examples of the proteases responsible can be found in single-cell eukaryotes; indeed they were first characterised and cloned in yeast and were called Kex 1 and 2 (Fuller et al. 1988). The first processing step is by the action of an endopeptidase, trypsin-like enzyme, that cleaves at the carboxyl side of the dibasic residue site (and usually at lysine-arginine). The next step is carried out by a carboxypeptidase B-like enzyme that removes the two remaining basic residues from the C-terminal of the resulting intermediate to create the active peptide (see Fig. 1). The mammalian equivalents of these enzymes were cloned by using the conserved motifs around the catalytic pockets of the yeast proteases in a homology cloning strategy and they have been given the name prohormone converting enzymes (Smeekens & Steiner 1990) and carboxypeptidase E (Fricker et al. 1986). If a glycine residue is exposed at the C-terminus after removal of the dibasic residues then it is converted into a C-terminal amide by peptidylglycine α-amidating enzyme (Murthy et al. 1986). There are many examples of processing of polypeptide hormone precursors to create active peptide hormones in endocrine and neural tissues occurring throughout the animal kingdom and there are even homologues of some peptide hormones such as the tachykinins/neurokinins and insulin found in insects. Thus, we can conclude that the mechanism of producing active peptide hormones from biologically inactive precursors was an early event in eukaryotic evolution.

**Evolution of the developing embryo**

A simple way of reproduction in which the developing embryo can have a plentiful supply of food and is able to absorb oxygen and eliminate carbon dioxide is in the form of an egg with a yolk sac. The only problem here is that the egg is vulnerable to predation; eggs may therefore be produced in very large numbers, they may be protected by guarding in a nest or they are held in a pouch. This last method evolved further in the marsupials; in the case of the kangaroo the primitive embryo, after being born, makes a hazardous journey, crawling up through the mother’s fur and eventually reaching the pouch, where it latches onto a teat. This is in stark contrast to wildebeest in which a fully mature calf is born, and within seconds is tottering around finding its feet; half an hour later it is able to run with the herd. It is not therefore surprising that, when the placental mammals arrived in South America, they drove out the indigenous marsupials from most of the ecological niches with only a few exceptions such as the possum remaining.

**Corticotrophin releasing factor and its binding protein**

Some years ago we studied corticotrophin releasing factor (CRF), the hypothalamic factor released by
axons terminating in the median eminence which then passes into the hypophyseal portal system to stimulate the release of ACTH from the anterior pituitary gland. We found that the placenta was capable of secreting this same CRF neuropeptide into the circulation of both the mother and the foetus, reaching concentrations in the third trimester that mimicked those found in the hypothalamic portal blood in stressed animals (Campbell et al. 1987). Paradoxically, neither ACTH nor cortisol was increased in the mother (data from the human foetus are not available). We went on to discover, purify and clone a high affinity plasma binding protein (CRF-BP) that was present in the blood in sufficient concentrations to be capable of neutralising the biological activity of CRF in the peripheral circulation of both mother and foetus (Behan et al. 1989). This provided an explanation for the protection of both maternal and foetal pituitary glands from the effects of placental CRF throughout most of pregnancy (Linton et al. 1990).

An observation relating to the paracrine function of CRF which I find persuasive is one recently proposed by Hillhouse and Grammatopoulos (2002) where they suggested that the CRF receptors in the myometrium are the target for the placental CRF, enhancing the sensitivity of the myometrium oxytocin receptors towards term. We had also found that the increasing concentrations of CRF near term reduced the concentrations of circulating CRF-BP, and concluded that this occurred by specific clearance of the complex (Woods et al. 1994). This results in equimolar concentrations of the two components (CRF and CRF-BP) being reached three weeks before term (MacLean et al. 1995), and hence any further increase in placental CRF circulates in the unbound active form at this time, with an accelerated increase in biological CRF activity in blood towards term allowing it to have a peripheral action in the final moments of parturition, most likely stimulating the release of pituitary ACTH in both mother and foetus and giving rise to the pre-partum increase in cortisol.

This placental CRF plasma CRF-BP phenomenon appears to be unique to the higher apes, both CRF and its binding protein being confined mainly to the brain in other animals studied, such as the rat and sheep. The CRF-BP is the only example of a binding protein for a neuropeptide, and no related genes appear to be present in the human genome. This is in complete contrast to the situation with the neuropeptide receptors: there is often more than one example of a receptor for a particular neuropeptide, and there are also some hundreds of orphan G-protein receptor genes present in the human genome with an as yet unknown partner ligand or function.

In the case of CRF one could argue that this situation arose because the CRF/CRF-BP phenomenon allows CRF to express its dual effects during pregnancy, first in a paracrine fashion influencing oxytocin sensitivity of the myometrium and then later in an endocrine fashion, being available to stimulate both foetal and maternal pituitary glands to release ACTH in both foetus and mother. As this situation of interaction of CRF and its binding protein in the peripheral circulation appears to be confined to the higher apes, this would suggest that it is a very recent evolutionary event. It would be a very costly and illogical situation for the foetus to have to synthesise and secrete a large number of neutralising binding proteins for every possible peptide hormone that the placenta made, because they would also neutralise all its own peptide hormones and interfere with the maturation of the foetal endocrine systems. In this regard, the short period during which hypothalamic CRF is exposed to plasma CRF-BP in the hypothalamic portal system does not appear to be sufficient to block its activity at the pituitary.

The mechanism hypothesis

From recent observations in my laboratory we have hypothesised that the early advantage that the placental mammals had over the marsupials was achieved by the ability of the developing placenta to evolve a novel mechanism by which the processing of precursor polypeptide hormones was arrested at an intermediate stage. Thus, only inactive intermediates processed by endopeptidases (prohormone converting trypsin-like enzymes) are stored and secreted by the placenta, to be activated by carboxypeptidase B-like action as they enter the mother’s circulation. This would result in only inactive intermediates having access to the foetal circulation as they would not be subject to the carboxypeptidase activating step, and would allow

---

www.endocrinology.org
the foetus to develop its own endocrine systems independently, being spared from the potential interfering effects of the biologically active placental peptide hormones.

**Processing of neurokinin B in the placenta**

This simple hypothesis arose from our work on neurokinin B in which we initially found that the placenta secretes neurokinin B (NKB), a tachykinin normally restricted to the brain and spinal cord, into maternal blood. Interestingly, the radioimmunoassay we initially used in this study detected nanomolar concentrations of NKB in the blood of pregnant women suffering from pre-eclampsia which appeared to explain many of the symptoms seen in this condition as it would cause over-stimulation of peripheral neurokinin receptors (Page et al. 2000). More recently, NKB has also been shown to be capable of causing oedema in the lung and liver (two symptoms of pre-eclampsia), possibly by a non-neurokinin receptor-related mechanism (Grant et al. 2002). Our original study was carried out using a classical extracted radioimmunoassay with an antiserum directed towards the NKB specific sequence located at the N-terminal half of the peptide. As the C-terminal of all the tachykinins/neurokinins contains the sequence FXGLM-amide (see Fig. 1), antibodies that recognise this motif would also react with the other members of the family. On reflection, our selection of this antiserum for its non-recognition of C-terminal epitopes was fortuitous as further cross-reaction studies revealed that the same antibody also recognised C-terminal extended NKB peptides (but not N-terminal extended peptides) and was not specific for the C-terminal amide (KJ Brayley & P J Lowry, unpublished observations). The latter property is unusual as antibodies raised against much longer C-terminally amidated peptides (e.g. secretin) can be very specific for the C-terminal amide and often do not recognise C-terminally extended forms of the same peptide (Solomon et al. 1999). This phenomenon of amide-specific antibodies was also very recently observed in those generated towards a viral-derived tachykinin named virokinin (Zimmer et al. 2003).

In an attempt to develop a user-friendly and robust assay that was capable of measuring NKB directly in plasma we raised antibodies to DMHDF (the NKB specific motif) and FVGLM-amide, the motif that is found in both NKB and NKA. The resulting two-site immunoassay was able to measure synthetic NKB added to non-pregnant human plasma and endogenous NKB in rat brain extracts but it detected only trace amounts of the NKB found in plasma samples taken from patients suffering from pre-eclampsia. Even placental extracts were found to contain only minute amounts of NKB when we used this specific NKB two-site assay. The resulting peak of immunoreactivity that was observed after gel filtration of placental extracts on Superdex eluted in a position consistent with a 13-residue peptide rather than the 10-residue synthetic/brain NKB. However, when the original radioimmunoassay (the N-terminal-directed antiserum) was used on the same Superdex fractions, a large peak of NKB immunoreactivity was observed, eluting in the same position consistent with a 13 mer. In contrast, a rat brain extract run on the same Superdex column resulted in a significant peak of NKB immunoreactivity eluting in the same position as the synthetic NKB 10-residue peptide, and the material reacted equally in both the specific two-site assay and the radioimmunoassay. Mass spectrometric analysis confirmed that the placental HPLC-purified placental material had a molecular weight consistent with a 13 mer (KJ Brayley, S Howell & P J Lowry, unpublished observations).

We know that most endocrine and neuroendocrine tissues process and store their peptide hormone products in secretory granules in the final active form and, in the case of NKB, as the amidated 10 mer (see Fig. 1), but the evidence above suggested that the placenta stores NKB in an intermediate processed form, most likely with the C-terminal Gly-Lys-Arg tri-peptide still attached, rendering it biologically inactive. Difficulty in detecting significant amounts of carboxypeptidase E in the syncitium of the placenta would tend to support this observation (Reznik et al. 1998). However, the presence of an abundant carboxypeptidase B-like enzyme that has been reported to be membrane-attached to the maternal side of the microvilli of the placenta (carboxypeptidase M; Skidgel et al. 1989) offers the perfect clue to the next step: not only would it specifically remove the remaining basic residues, this would also result in the glycine-extended form of NKB entering the...
maternal circulation. In neuroendocrine cells, the glycine residue would normally be converted into, and stored as, the C-terminal amide in the secretory vesicle, but the placenta has clearly evolved a different approach, ensuring that only the glycine-extended form enters the maternal circulation. Although amidated neurokinins are the recognised bioactive form at the relevant receptor, synthetic glycine-extended neurokinins have been observed to be equally as potent as the amidated form, e.g. in relaxing aortic strips, appearing to be converted locally to the amide form by the peptidylglycine α-amidating enzyme that is present in endothelial cells (Oldham et al. 1997). Several cell lines not formally associated with neuropeptide amide processing were also recently found to be capable of completely processing a viral fusion protein into a novel tachykinin, virokinin (Zimmer et al. 2003). The placental NKB-Gly-Lys-Arg peptide that enters the foetal circulation would not be subject to the carboxypeptidase M processing step and, being biologically inactive, would be metabolised in the brush border of the foetal kidney and would therefore not be able to interfere with the development of the foetus’s own neurokinin-mediated systems. For overall scheme see Fig. 2.

Other placental peptide hormones

Over the years, I have been puzzled by the fact that, although many peptide hormone precursor mRNAs have been found in significant amounts in the human placenta and have facilitated the cloning of their respective genes, there are very few reports of the expected amounts of resulting immunoreactive or biologically active peptide hormones in placental extracts, and there have been no reports of significant concentrations in either foetal or maternal blood. The explanation may be in the specificities of the antisera used. Had we initially chosen to use in our radioimmunoassay an NKB antibody that required the presence of a C-terminal amide in order to achieve...

Figure 2 In the placenta, neurokinin B is only processed by prohormone convertase action and is stored and released as a biologically inactive intermediate, NKB-GKR. This is then exposed to carboxypeptidase M action on the maternal side of the microvilli removing the basic KR residues. The glycine residue is then converted to the C-terminal amide in peripheral maternal tissues where peptidylglycine alpha-amidating enzyme is found. NKB-GKR is metabolised on the foetal side.
cross-reaction, it would not have detected any C-terminally extended forms; we would have detected only trace amounts of NKB in placental extracts and maternal blood, and would have given the presence of NKB or its potential as a contributing factor in the pathogenesis of pre-eclampsia no further attention. I have alluded above to the serendipity of our choice of antiserum and would suggest that the paucity of papers in the literature reporting significant amounts of immunoreactivity of other peptide hormones in placental extracts is also explained by the inability of the antiserum used to detect the relevant placental form as, in each case, the original immunogen used to generate the respective antiserum would have been the mature active form of the peptide found in peripheral endocrine or neuroendocrine tissue. This situation would be even more exaggerated if a two-site assay technology was used, as one of the antibodies would intentionally have been raised to a C-terminal epitope and would be specific in recognising the normal C-terminal amino acid whether it finished with a free carboxyl group or amide.

The future

If my theory is correct and we are to understand the workings of the placenta and be able to predict and understand, with respect to peptide hormones, pathological pregnancies, it is vital that we re-examine the endocrinology of the placenta with this new hypothesis in mind.

The use of single-site assays should be considered as a first approach and, to minimise false negatives, antisera should be selected that are directed towards N-terminal epitopes, as they will be less susceptible to any variations found at the C-terminal of placental peptides. In my experience, this can easily be achieved by using an N-terminal peptide synthesised with a cysteine-amide substitution as its C-terminal residue. This can then be coupled, using a bi-functional reagent such as maleimido-N-hydrosuccimide, to an antigenic carrier protein that can then be used as the immunogen to raise an antibody. The resulting antisera should be screened with the normal full-length peptide hormone. Using the normal full-length peptide hormone as both label and standard will also ensure that the resulting immunoassay will measure both normal-length and C-terminally extended forms of the relevant peptide hormone. If this assay detects significant amounts of immunoreactivity of the chosen peptide in placental extracts and an immunoassay for the fully processed form only picks up trace amounts, then it can be concluded that this particular peptide hormone is also stored and secreted from the placental cell in an inactive form and will be subjected to carboxypeptidase action on passing into the maternal circulation. If the relevant peptide does not contain an exposed glycine then it will be in its full active state; if it still has a glycine attached then it will be activated in tissues that produce local high concentrations of active peptidyl glycine α-amidating enzyme. It is also possible that the placenta may be adding novel post-translational modifications (PTM) during the processing stage, which may complicate the situation. Amino acids that can carry these should be avoided in the initial immunogen if possible as, again, both the placental form and the form which appears in the maternal circulation may evade detection with antisera in which the recognition of the epitope is destroyed by the PTM.

The fact that the developing placenta is capable of producing significant concentrations of chorionic gonadotrophin in early pregnancy and, later, placental lactogen and that both are easily detected by immunoassay, are testament to the ability of the developing placenta to act as an efficient endocrine organ. The antisera used in the immunoassays to these proteins would, by definition, recognise the placental forms, as these would have been used as the immunogen to generate them. My theory would suggest that the placenta is capable of producing similar amounts of other peptide hormones that are necessary to control the mother’s metabolism for the benefit of the developing foetus. Since the advent of immunoassay in the late 1960s, our understanding of the complexity of endocrinology/neuroendocrinology in the non-pregnant state has been slowly but surely unravelled, whilst the endocrinology of the placenta has largely resisted investigation. There is no doubt in my mind that the placenta constitutes the most complex of endocrine organs, but if we are to be able to predict and treat pathological pregnancies it is vital that we understand its workings. I hope that this article will stimulate interest in the endocrinology of the placenta and
give those working on the various endocrine systems that are known to be affected in the mother during pregnancy some new ideas and ways to study the placenta in their chosen area of physiology.

Acknowledgements

As the Commentary above proposing this hypothesis has been written on the occasion of my being awarded the 2003 Society medal, I would like to thank all my collaborators over the years particularly past and present members of lab 300 in AMS at Reading, Steve Howell at the NIMR, Mill Hill, Sheila Gardiner in Nottingham, Andy Grant and Sue Brain at Kings College, London and Isaac Manyonda at St Georges Hospital, London. I would also like to acknowledge the MRC for funding the work which has led up to this hypothesis.

References


Received 6 August 2003
Accepted 20 October 2003