Co-localisation of prostaglandin endoperoxide synthase and immunoreactive adrenocorticotrophic hormone in ovine foetal pituitary

S K Reimsnider and C E Wood

Department of Physiology and Functional Genomics, PO Box 100274, University of Florida College of Medicine, Gainesville, Florida 32610-0274, USA
(Requests for offprints should be addressed to S K Reimsnider; Email: sharonr@phys.med.ufl.edu)

Abstract

Previous studies have shown that both an intact hypothalamic–pituitary–adrenal axis and prostaglandin E_2 (PGE_2) are involved in the timing of parturition in sheep. PGE_2 is known to be synthesised by the placenta but has also been found in the foetal brain and pituitary. We propose that the enzymes necessary for production of PGE_2 are found in the ovine foetal pituitary and may be able to exert an autocrine and/or paracrine influence on corticotropes, resulting in an increased secretion of immunoreactive adrenocorticotrophin (irACTH). Pituitary tissues from foetal sheep, of gestational ages 119–126 days, were examined by immunohistochemistry. Primary antibodies for prostaglandin H synthase-1 and -2 (PGHS-1 and PGHS-2), microsomal prostaglandin

endoperoxide synthase (mPGES) and irACTH were used to probe expressed proteins and Alexa-Fluor red- and green-fluorescent secondary antibodies were used to visualise the bound primary antibody. Staining for PGHS-1, PGHS-2 and mPGES was found throughout the foetal anterior pituitary. PGHS-1 and mPGES were widely distributed, including but not restricted to corticotropes. PGHS-2 was less widely distributed but occasionally was found in cells adjacent to corticotropes. The results indicate that locally produced prostaglandins may have an influence on the secretion of ACTH, independent of placental PGE_2 .

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Introduction

Foetal plasma cortisol concentration rises spontaneously during late gestation in the foetal sheep (Challis & Brooks 1989). The increase in cortisol results in the activity of 17 α -hydroxylase (cytochrome P450_{C17}) in the placenta, allowing an increase in the concentration of oestrogen relative to the concentration of progesterone. The increased oestrogen-to-progesterone ratio increases myometrial tone and augments the contractility of the uterus (Liggins *et al.* 1973), and ultimately triggers labour and delivery. While the basic sequence of events which trigger labour is now known, the mechanism underlying the increase in activity of the foetal hypothalamus–pituitary–adrenal (HPA) axis is not. Exogenous prostaglandin E_2 (PGE_2) stimulates HPA axis activity (Louis *et al.* 1976, Young & Thorburn 1994). Inhibition of endogenous prostaglandin biosynthesis reduces the magnitude of HPA responses to hypotension in the foetus (Tong *et al.* 1998, Tong & Wood 1999). Thorburn and others have proposed that PGE_2 might play a critical role in the stimulation of HPA axis activity at the end of gestation. While PGE_2 might play an important role in modulating foetal

adrenocorticotrophin (ACTH) secretion, the source of prostaglandin affecting pituitary function in the foetus is controversial. We and others have demonstrated the presence of prostaglandin biosynthetic enzymes in the brain and pituitary (Breder *et al.* 1992, 1995, Tong *et al.* 1999, 2000, 2002, Deauseault *et al.* 2000). These enzymes include prostaglandin H synthase-1 and -2 (PGHS-1 and PGHS-2), which are involved in the conversion of PGG_2 to PGH_2 , and microsomal prostaglandin endoperoxide synthase (mPGES), which is one of two known enzymes in the final enzymatic process in the production of PGE_2 . On the basis of these experiments, we have proposed that local biosynthesis of prostaglandins in the brain and/or pituitary modulates the activity of the HPA axis (Cudd & Wood 1991, 1992).

Prostaglandins are known modulators of corticotrope function (Brooks & Gibson 1992). Brooks and co-workers demonstrated that PGE_2 increases the ACTH response to arginine vasopressin (AVP), but that it reduced the ACTH response to corticotrophin-releasing hormone (CRH). In the sheep, both of these releasing factors act as secretagogues (Rose *et al.* 1985, Brooks & Gibson 1992, Currie & Brooks 1992). Local generation of PGE_2 within or in

close proximity of the corticotrope would therefore be expected to modulate corticotrope responsiveness to the hypothalamic releasing factors. The purpose of the present study was to test the hypothesis that either PGHS-1 or PGHS-2 is expressed within corticotropes in the foetal anterior pituitary. The secondary aim of these experiments was to test the hypothesis that PGE₂ synthase is expressed in or adjacent to corticotropes which express either isoform of PGHS.

Methods

We studied pituitaries obtained from foetal sheep of known gestational age (123–140 days gestation). All of the pituitary tissue was obtained as a part of another study that was designed to investigate neuronal and pituitary expression of Fos (the protein product of the *c-fos* gene) in response to oestradiol administration and cerebral hypoperfusion (Purinton & Wood 2002). Pituitaries used in the present study were from foetuses that had not been subjected to cerebral hypoperfusion. Each foetus was chronically instrumented with vascular catheters and an extravascular balloon occluder around the brachiocephalic artery. Nevertheless, the circulating ACTH and cortisol concentrations in these foetuses was appropriate for gestational age and the foetuses were not, therefore, chronically stressed.

Immediately after killing, the foetal brain and pituitary were perfused with 2 litres of heparinised saline, followed by 2 litres of 4% paraformaldehyde. Tissue was processed for embedding by dehydration with progressively increasing concentrations of ethanol followed by xylene. Tissue was embedded in paraffin and cut into 5–7 µm sections using a Zeiss HM-325 microtome. Sections were then mounted on poly-L-lysine-coated slides. Sections were deparaffinised with xylene. The sections were then rehydrated using successive 2-min rinses in 100, 90, 70, 50 and 25% solutions of ethanol in distilled and deionised water (ddH₂O), followed by two washes in ddH₂O. Immunohistochemistry was performed using commercially available primary and secondary antisera. Primary antibodies included anti-ACTH (Zymed Co., San Francisco, CA, USA), anti-PGHS-1 and anti-PGHS-2 (Oxford Biomedical, Oxford, MI, USA) and anti-mPGES (Oxford Biomedical).

Tissue sections were blocked with 10% goat serum (Sigma) in PBS with 0.2% Tween 20 for at least 10 min. Sections were incubated with primary antisera for 30 min at room temperature, washed again with PBS, then incubated with secondary antibodies for 30 min. After a final wash, the sections were coverslipped using Fluoromount (Zymed Co.). Secondary antisera were goat anti-rabbit IgGs conjugated with a green (Alexa Fluor 488; Molecular Probes, Eugene, OR, USA) or red (Alexa Fluor 594; Molecular Probes) fluorophore. Immunostaining was

visualised using a Zeiss Axioplan 2 fluorescence microscope and/or a Bio-Rad 1024 ES confocal microscope in the McKnight Brain Institute Optical Microscopy Center (University of Florida, Gainesville, FL, USA). Each set of slides examined included negative controls for each primary antibody (i.e. slides were stained with one of the primary antibodies and without the other). These slides were visualised and photographed for both red and green secondary antibodies. Specific immunofluorescence was eliminated in the negative control slides.

Results

Staining and co-localisation of ACTH with PGHS-1 and PGHS-2

The distribution of corticotropes within the anterior pituitary was typical for foetuses of this gestational age (~120 days). At low magnification, cells containing immunoreactive ACTH (irACTH) were found in clusters (Fig. 1A). Although no attempt was made to map the distribution of these clusters, the irACTH-positive cells were readily identifiable and were interpreted to be corticotropes. Islands and cords of immunoreactive cells were scattered throughout the anterior pituitary and appeared to be a mixture of adult-type (strongly reactive and cuboidal) and foetal-type (weaker reactivity and more elongated) corticotropes (Fig. 1).

PGHS-1 was found in a majority of cells within the anterior pituitary, including those positive for ACTH and those not positive for ACTH (Fig. 1B). As shown in Fig. 1B, most of the cells of the anterior pituitary were positive for PGHS-1 (red fluorescence, Fig. 1B). Cells co-localising immunoreactive PGHS-1 and irACTH were identified by the yellow-orange colour of the superimposed green (ACTH) and red (PGHS-1) fluorescence. Random samples were analysed using the confocal microscope, confirming co-localised staining within individual cells (data not shown).

PGHS-2 was found in fewer cells, generally in groups of cells scattered throughout the anterior pituitary (Fig. 1C). The intensity of the immunostaining for PGHS-2 was also not as high as the intensity of the PGHS-1 immunostaining. PGHS-2 was often found in cells in very close proximity to ACTH-producing cells, and occasionally these two were found within the same cell (Fig. 1C).

Cells within the neurointermediate lobe (NIL) are known to express pro-opiomelanocortin (POMC) and were readily identifiable using the anti-ACTH antiserum (Fig. 2A and B, green fluorescence). At high magnification, staining for PGHS-2 (Fig. 2A and B, red fluorescence) was seen within the cells of the NIL. Throughout the NIL, there was extensive immunostaining of immunoreactive ACTH and PGHS-2, although the immunostaining did not appear to directly overlap.

The staining for irACTH within this lobe was probably POMC, α -melanocyte-stimulating hormone (α -MSH) and corticotrophin-like intermediate lobe peptide (CLIP).

The posterior lobe (PL) was not specifically investigated in this study. However, in PL tissue adjacent to the anterior pituitary, we observed what appeared to be isolated islands of corticotropes (Fig. 2C). The endothelial cells lining the blood vessels within the PL stained very strongly for both PGHS-1 and PGHS-2 (Fig. 2D and E respectively).

Staining and co-localisation of mPGES with PGHS-1 and PGHS-2

mPGES immunostaining was widespread within the anterior pituitary. The degree of staining within the cells varied, some were very strong positives while others appeared only weakly reactive to this antibody (Fig. 3A, red fluorescence). As shown by the yellow colour of overlapping red (mPGES) and green (PGHS-1) fluorescence, mPGES was found co-localised with PGHS-1 in many cells (Fig. 3B). PGHS-2-positive cells (green fluorescence) and mPGES-positive cells (red fluorescence) were found in close proximity to each other, but rarely did the same cells stain for both (Fig. 3C). Nevertheless, there were occasional islands of cells in which mPGES and PGHS-2 co-localised, as illustrated by the yellow colour in Fig. 3C. mPGES did not seem to be abundant within the NIL (note the relative lack of red fluorescence in the NIL shown in Fig. 3F). Those cells that were positive for mPGES were very weak, and did not appear to be co-localised with either of the other enzymes.

Co-localisation of ACTH and mPGES

In some sections, ACTH-positive cells were clustered near mPGES-positive cells (Fig. 3D), while in other sections ACTH and mPGES were co-localised (Fig. 3E). The staining of mPGES within the NIL was very weak, but appeared to be present in most cells. As shown in Fig. 3F, co-localisation of irACTH (green fluorescence) and immunoreactive mPGES (red fluorescence) was more common in the anterior pituitary than in the NIL. Staining of mPGES within the posterior pituitary was not apparent (not shown).

Discussion

The results of the present study strongly suggest that PGE₂ is synthesised locally in the foetal anterior pituitary; the

Figure 1 Co-staining of immunoreactive ACTH and PGHS-1 or PGHS-2. (A) Immunoreactive ACTH (green fluorescence) in a group of corticotropes ($\times 40$ magnification). (B) Immunoreactive PGHS-1 (red fluorescence), immunoreactive ACTH (green fluorescence) and co-staining of immunoreactive PGHS-1 and ACTH (yellow fluorescence, $\times 80$ magnification). (C) Immunoreactive PGHS-2 (red fluorescence), and immunoreactive ACTH (green fluorescence, $\times 80$ magnification).

Figure 2 Co-staining of immunoreactive ACTH and PGHS-2 in neurointermediate lobe. (A) Immunoreactive ACTH (green fluorescence) and PGHS-2 (red fluorescence) in the NIL (× 20 magnification). (B) Immunoreactive ACTH (green fluorescence) and PGHS-2 (red fluorescence) in the NIL (× 40 magnification). (C) Immunoreactive ACTH (green fluorescence) in an apparent island of corticotropes within the posterior pituitary (× 20 magnification). (D) Immunoreactive PGHS-1 (red fluorescence) in microvasculature (× 20 magnification). (E) Immunoreactive PGHS-2 (red fluorescence) in microvasculature (× 20 magnification).

Figure 3 (A) Immunoreactive microsomal PGE synthase (mPGES, red fluorescence) in the anterior pituitary ($\times 40$ magnification). (B) Immunoreactive mPGES (red fluorescence) and immunoreactive PGHS-1 (green fluorescence). Nearly all of the immunostaining for these two proteins is co-localised within the same cell, indicated by the yellow fluorescence ($\times 40$ magnification). (C) Immunoreactive mPGES (red fluorescence) and immunoreactive PGHS-2 (green fluorescence). The Figure shows one island of cells which contained both proteins (yellow fluorescence, $\times 20$ magnification). (D) Immunoreactive mPGES (red fluorescence) and immunoreactive PGHS-2 (green fluorescence). Compare with (A), which is the same section showing only mPGES immunoreactivity ($\times 40$ magnification). (E) Immunoreactive mPGES (red fluorescence) and immunoreactive ACTH (green fluorescence, $\times 20$ magnification). (F) Immunoreactive mPGES (red fluorescence) and immunoreactive ACTH (green fluorescence, $\times 20$ magnification); cells expressing both proteins are indicated by the yellow fluorescence; in this section, the NIL and the immunoreactive ACTH-containing cells of the NIL are clearly visible (left).

late-gestation ovine foetal anterior pituitary expresses PGHS-1, PGHS-2 and mPGES. The distribution of the enzymes suggests that PGE₂ can be synthesised in foetal corticotropes, probably as a result of the action of PGHS-1 and mPGES. The effect of prostanoids and prostanoid inhibitors on the ability to modulate the release of ACTH from the pituitary has been examined at both hypothalamic and pituitary levels. There is convincing evidence that metabolites of arachadonic acid can modulate the secretory response to CRH (Cowell *et al.* 1991) and AVP (Brooks 1992, Brooks & Gibson 1992). Studies performed *in vitro* (Vlaskovska & Knepel 1984, Abou-Samra *et al.* 1986, Wilson & Guild 2002) and *in vivo* (Hedge 1977) have shown that inhibition of PGE₂ and/or injection of PGE₂ has a direct modulatory effect on corticotropes.

Ovine parturition is initiated by an increase in the activity of the HPA axis. Prior to parturition, there is an increase in the circulating concentrations of ACTH (Rose *et al.* 1978, Wintour *et al.* 1975) and cortisol (Bassett & Thorburn 1969), as well as an increase in the mass and responsiveness of the foetal adrenal to ACTH (Durand *et al.* 1981, 1982, 1984, Boshier & Holloway 1991). Disruption of the axis at any level defeats or delays the initiation of parturition. Cutting the infundibular stalk (Antolovich *et al.* 1990), lesion of the paraventricular nuclei (McDonald & Nathanielsz 1991), and destruction of the pituitary itself (Liggins *et al.* 1967) can all delay parturition in sheep. Thorburn and colleagues have demonstrated that PGE₂ circulates in foetal plasma, and that the concentrations of PGE₂ increase prior to parturition (Challis *et al.* 1976, Fowden *et al.* 1987, Deayton *et al.* 1993). The primary source of the circulating PGE₂ is the placenta, and the activity of PGHS in the placenta increases proportionately as a function of foetal gestational age (Rice *et al.* 1990, 1992). Thorburn and colleagues have also demonstrated that exogenous PGE₂ stimulated ACTH secretion (Hollingworth *et al.* 1995) and that continuous intravenous infusion of PGE₂ into the foetus can stimulate premature parturition. These observations support Thorburn's proposal that PGE₂, released by the placenta in increasing concentrations prior to parturition, is a stimulus to the foetal HPA axis and that it is the 'drive' from the PGE₂ that ultimately triggers parturition (Young & Thorburn 1994).

Experiments performed in this and other laboratories, however, have suggested that local production of prostaglandins might be more important than circulating sources. We have demonstrated that infusion of PGE₂ into the carotid arteries did stimulate foetal ACTH secretion, but only at concentrations which exceeded those produced by placental secretion (Cudd & Wood 1992). In a separate study, Brooks found that PGE₂, delivered either intravenously or intracerebroventricularly, increased ACTH secretion in foetal sheep (Brooks 1992). It is possible that PGE₂ delivered intravenously acts at the pituitary gland

to augment the corticotrope response to hypothalamic releasing factor. Intracerebroventricular injections of PGE₂ probably mimic neuronal or glial synthesis of PGE₂. In previous experiments, we have used the technique of microdialysis to find that brainstem and hypothalamic interstitial fluid concentrations of PGE₂ are increased during hypotension. We and others have found that the foetal brain contains significant amounts of both isoforms of PGHS (Tong *et al.* 1999, Deauseault *et al.* 2000). Blockade of the synthesis of PGE₂ with indomethacin greatly attenuates the HPA response to hypotension (Tong *et al.* 1998).

The foetal pituitary secretes ACTH in response to stimulation by either AVP or CRF. The corticotrope response to these two hormones changes as a function of gestational age/maturity of the foetus (Norman & Challis 1987, Brooks & Gibson 1992). In the foetal pituitary, PGE₂ acts synergistically with AVP to produce a greater increase in the production of irACTH from the anterior pituitary than does AVP alone (Brooks & Gibson 1992). Interruption of the hypothalamic-pituitary stalk ablates the response of the pituitary (secretion of ACTH) to the infusion of PGE₂, suggesting that PGE₂ may act at the hypothalamic level (Young *et al.* 1996), or that it acts by augmenting the ACTH response to endogenous releasing factors. We propose that prostanoids, including but not restricted to PGE₂, are synthesised within the foetal pituitary and act in a paracrine or autocrine manner to modify the corticotrope response to CRH and AVP. Although we have focused our present efforts on the biosynthetic enzymes required for PGE₂ synthesis, it is possible that other prostanoids or PGHS products are important, as well. For example, we have found that U46619, the thromboxane A₂ mimetic, stimulated foetal ACTH secretion. This drug was more effective when infused into the carotid arteries as compared with infusion into the vena cava (Wood *et al.* 1993). This suggests that thromboxane acted at some site within the region perfused by the carotid vasculature (potentially the anterior pituitary) to stimulate ACTH secretion. The anterior pituitary contains immunoreactive thromboxane synthase (Wood *et al.* 1997, Tong *et al.* 1999). On the other hand, there are no data available concerning the potential influence of thromboxane A₂ on corticotrope function.

The development of corticotropes within the anterior pituitary shows a progressive maturation from a 'foetal' type, which predominates at 115 days gestational age, to an 'adult' type which predominates at 135 days (Perry *et al.* 1985). The 'foetal' type shows less intense staining for ACTH immunoreactive cells, which are large and columnar in shape, and arranged in clumps or palisades; in contrast, the adult-type has more intense staining, they occur individually or in small clusters, are usually angular and occasionally have cellular processes enveloping adjacent cells (Perry *et al.* 1985, Antolovich *et al.* 1989). Maturation of the foetal pituitary is also reflected in

ontogenetic changes in responsiveness to AVP. Durand and co-workers reported that corticotrope responsiveness to AVP was reduced *in vitro* at 133 and 144 days gestation compared with younger foetuses. In that study, responsiveness to CRH was not changed. *In vivo*, on the other hand, responsiveness to both AVP and CRH were decreased in the 135–140 day gestation foetus, but this was possibly due to the elevated foetal plasma cortisol concentration at that time (Durand *et al.* 1986, Norman & Challis 1987). The morphology of the tissue examined in the present study is consistent with the previously published results. The pituitary tissue used in this study was from foetuses between 123 and 140 days gestation, and the majority of the corticotropes appeared to be consistent in morphology and staining intensity with the 'adult'-type corticotropes, although the overall abundance of the corticotropes in these foetal pituitaries was far greater than in adult ovine pituitaries prepared and analysed similarly (Reimnsnider and Wood, unpublished observations). Because of the specific range of foetal gestational ages used in this study, it is important to recognise that the patterns of co-expression of ACTH, PGHS-1, PGHS-2 and mPGES might have been different in younger foetuses. Nevertheless, we chose this age for study because it is the foetal gestational age at which the activity of the HPA axis is rapidly increasing, approximately 2 weeks prior to the initiation of labour (Rose *et al.* 1978, 1981, Challis & Brooks 1989).

In summary, we conclude that the prostaglandin biosynthetic enzymes are expressed in the foetal pituitary which would allow local biosynthesis of, and autocrine or paracrine action of, PGE₂ or other prostanoids. Additional work will be required to assess the importance of this biosynthesis *in vivo*, exclusive of an effect of changes in circulating PGE₂ concentrations. Nevertheless, the results of this study suggest that the local biosynthesis of prostanoids might play a physiologically relevant role in the control of foetal ACTH secretion.

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