The GH/IGF-I axis in the brushtail possum (*Trichosurus vulpecula*) pouch young

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Abstract

Plasma and pituitary GH concentrations and liver GH receptor (GHR), IGF-I and IGF-binding protein-3 (IGFBP-3) mRNA expression were determined in brushtail possum (*Trichosurus vulpecula*) pouch young aged 12–150 days post-partum and in adults. Mean plasma GH concentrations were highest, measuring around 150 ng/ml, from 12 to 100 days post-partum, and thereafter declined so that by 150 days post-partum levels were not significantly different from those in adults (10.8 ± 1.8 ng/ml (S.E.M.)). In contrast to plasma levels, pituitary GH content increased markedly throughout pouch life, with an 87-fold increase between 12 and 150 days post-partum. However, when expressed per gram body weight, pituitary content was relatively constant between 25 and 150 days post-partum, indicating that the decline in plasma GH after 100 days post-partum

was not due to decreased synthesis and/or storage of GH in the pituitary gland. Expression of GHR, IGF-I and IGFBP-3 mRNAs was determined by semi-quantitative RT-PCR. Liver GHR and IGF-I mRNA expression were low at 12 and 25 days post-partum and did not show sustained and significant increases ($P < 0.05$) until 125 and 150 days post-partum. IGFBP-3 expression was also low at 12 days post-partum but then increased rapidly to a maximum at 50 days post-partum and thereafter declined. For all three mRNAs, liver expression at day 150 was not significantly different from that in adults. These patterns of gene expression for GHR and IGF-I suggest that the possum liver is resistant to the high plasma GH concentrations during early pouch life and in this way is similar to the fetal liver of some eutherian mammals.

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Introduction

In contrast to eutherian mammals, marsupials have a very short period of growth and development *in utero* and give birth to a relatively immature pouch young that then undergoes a lengthy period of further growth and development in the mother's pouch. We have recently shown that for two species of marsupial, the bandicoot (Saunders *et al.* 2000) and brushtail possum (Saunders *et al.* 2002), plasma growth hormone (GH) concentrations are very high for about the first half of pouch life before gradually declining to adult levels. Growth rate is slower during the first half of pouch life (Gemmell & Hendrikz 1993) when plasma GH concentrations are at their highest (Saunders *et al.* 2002), which suggests that GH does not play a major role in stimulating growth at this time. One possible explanation for this apparent paradox would be if GH-stimulated insulin-like growth factor-I (IGF-I) production from the liver were low during early pouch life and then subsequently increased around the time of increasing growth rate after about 96 days post-partum. In this study of the brushtail possum (*Trichosurus vulpecula*), we therefore set out to quantify gene expression for two elements of this

pathway in the liver, GH receptor (GHR) and IGF-I. In several eutherian species, expression of both genes is stimulated by GH as well as other hormones (Mathews *et al.* 1986, 1989, Sauerwein *et al.* 1991, Ambler *et al.* 1992, Lemmey *et al.* 1997). We also chose to quantify liver gene expression of IGF-binding protein-3 (IGFBP-3) because this is also stimulated by GH (Lemmey *et al.* 1997) and so could provide another indicator of tissue responsiveness to GH. In order to measure expression of these genes, it was first necessary to obtain the possum sequence for each gene so that PCR primers could be designed.

Materials and Methods

Animals and collection of blood and tissue

A breeding colony of brushtail possums was maintained as previously described (Saunders *et al.* 2002) with food and water freely provided. Blood, pituitary gland and liver samples were obtained from brushtail possum adults and pouch young aged 12, 25, 50, 75, 100, 125 and 150 days post-partum ($n \geq 3$ at each age). All pouch young were bred in captivity and their ages precisely known from

Figure 1 Schematic diagram of cDNAs encoding GHR, IGF-I and IGFBP-3. The approximate positions of primer combinations and PCR products used for cloning (dotted line) and semi-quantitative RT-PCR (dashed line) are shown; ** indicates the transmembrane region of GHR. The 5'- and 3'- untranslated regions are not shown.

regular examination of pouches to identify the day of birth. The ages of 12-day-old pouch young were accurate to within 1 day whereas older age groups were ± 3 days. All samples were collected during the normal breeding season (Gemmell 1990) between 0900 and 1300 h. A permit for the collection and maintenance of possums was obtained from the Queensland National Parks and Wildlife Service and experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Queensland.

Blood samples were obtained by cardiac puncture from pouch young and adults that were lightly anaesthetised with a 3% halothane (Rhone Merieux, West Footscray, Australia) in oxygen mixture (0.5 l/min). All blood samples were centrifuged (2000 g, 10 min, 4 °C) and the plasma collected and stored at -20 °C prior to assay for GH. After blood was collected, animals were killed by intracardiac injection of Nembutal (pentobarbitone sodium 60 mg/ml; Boehringer Ingelheim, Artarmon, Australia; 0.25 ml/100 g). The pituitary gland (only from pouch young) and a piece of liver (pouch young and adults) were removed, frozen on dry ice and then stored at -80 °C until pituitary homogenates were prepared or RNA was extracted from liver tissue.

Pituitary gland homogenates

Pituitary glands were homogenised on ice, in 500 μ l ammonium bicarbonate (0.2 M, pH 8.3) with a glass-Teflon homogeniser. Homogenates were transferred to 1.5 ml tubes and centrifuged at 10 000 g for 5 min in a

microcentrifuge and then the supernatant was stored at 20 °C until GH assays were performed.

GH RIA

Plasma and pituitary gland GH concentrations were determined in a heterologous RIA (Saunders *et al.* 2002). This assay uses an antiserum against wallaby GH (wGH) (G5/3; Curlewis & McNeilly (1992)), brushtail possum GH purified from pituitary glands as standard and wGH (cWB-9) as the radioiodinated ligand. Intra- and inter-assay coefficients of variation were 3.5 and 18.0% respectively and the assay sensitivity was 0.8 ng/ml.

PCR cloning of possum GHR, IGF-I and IGFBP-3

cDNA sequences for GHR, IGF-I and IGFBP-3 had not been reported for any marsupial species when these experiments began so we first obtained cDNA partial clones of each gene by RT-PCR/cloning and then, in most cases, used these sequences to design new primers for semi-quantitative RT-PCR. The initial sets of primers were designed on regions of nucleotide sequence that were well conserved between a range of mammalian and non-mammalian species and to cover as much of the protein coding region as possible (Fig. 1).

Total RNA was extracted from the liver of a single adult possum with TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) and single-strand cDNA (ss cDNA) was synthesised using the Superscript Preamplification System (Gibco BRL) as previously described (Saunders *et al.*

2001). GHR cDNA was isolated using a touchdown PCR method that involved denaturation at 94 °C (40 s), annealing from 55 °C to 41 °C (2 °C decrease every three cycles; 2 min), and extension at 72 °C (3 min). This was followed by 15 cycles at an annealing temperature of 55 °C. Forward and reverse primers were (i) 5' GGTCACCTGA RCTGGAGAC 3' and (ii) 5' GGCTAAGGCATGAT TTTGTTCAG 3' and their approximate location is shown in Fig. 1. IGF-I and IGFBP-3 clones were isolated by PCR carried out for 35 cycles (94 °C, 60 s; 55 °C, 60 s; 72 °C, 60 s) with primers (iii) 5' AATCAGCAGTC TTCCAAC 3' and (iv) 5' GATC ACAGCTCCGG AAGC 3' for IGF-I and primers (v) 5' TGCGARCCGT GCGACGCGCG 3' and (vi) 5' GCTTYCTGCCTTT GGAAGGGCG 3' for IGFBP-3. Reactions for all genes contained 0.5 µl ss cDNA, 0.5 µM each primer, 0.2 mM each dNTP, 2 mM MgCl₂ and 1.3 U Taq DNA polymerase (Biotech International, Perth, Australia) in 50 µl 1 × Taq reaction buffer (Biotech International). PCR products were cloned and sequenced as previously described (Saunders *et al.* 2001). For each cDNA, two clones were sequenced in both directions. Nucleotide sequences were aligned using the AssemblyLIGN software program (Oxford Molecular Ltd, Oxford, UK) and compared with sequences in the GenBank database. Final sequences were submitted to Genbank.

Semi-quantitative RT-PCR for GHR, IGF-I and IGFBP-3

This method has been previously described by us for quantification of IGF-II mRNA expression in possum liver (Saunders *et al.* 2001). Extraction of total RNA from liver tissue and preparation of ss cDNA were performed as described above except that 3 µg total RNA were used in the RT reaction. Control reactions were also routinely performed. These included negative controls where water was substituted for RNA and reactions in which reverse transcriptase was omitted. Initially, non-quantitative PCR was performed with ss cDNA and primers to either GHR ((vii) 5' GAGCTGGACATCG ATGAC 3' and (viii) 5' GACAGGTAGA CCAGTG GAC 3'), IGF-I ((ix) 5' TCRCATCTCTTCTAYCT GGC 3' and (iv)), IGFBP-3 ((x) GTGCGGCGTCTAC ACGAGCG 3' and (xi) 5' GTCTTCCATTCTCT CCG 3') or possum β-actin ((xii) 5' CCTGAAGAGC ACCCTGTGC 3' and (xiii) 5' GTCAGGATCTTCAT GAG GTAG 3') using the conditions specified above for IGF-I and IGFBP-3, except that an initial denaturation step (94 °C, 1 min) and final extension step (72 °C, 10 min) were added. PCR with these primers gave single products of the expected sizes which were 375, 216, 415 and 294 bp for GHR, IGF-I, IGFBP-3 and β-actin respectively. The relative positions of these primers are shown in Fig. 1. As no PCR products were generated when reverse transcriptase was omitted from the RT reaction, these products were presumed to be of cDNA

rather than genomic DNA origin. The amount of product generated in PCR was quantified after electrophoresis through 1.2% agarose gels containing ethidium bromide. PCR products were visualised by UV illumination and quantified using the Multi-Analyst program on the Gel Doc 2000 Gel Documentation System (Bio-Rad, Hercules, CA, USA). Each of the PCR products was quantified in terms of the area covered by the product and the pixel density.

Using adult and pouch young liver ss cDNA as template, PCR conditions with GHR, IGF-I, IGFBP-3 and β-actin primers were optimised so that the amount of product generated was directly proportional to the amount of mRNA in the original sample. Linear assay conditions were determined from a series of validation experiments in which the number of PCR cycles or the amount of starting template (total RNA and ss cDNA) used in PCR was altered. Based on results from validation experiments (data supplied for review), the following PCR conditions and reagent concentrations were used for all further semi-quantitative analysis. For GHR and IGF-I individual reactions contained 0.25 µl ss cDNA as template, 0.5 µM each primer, 0.2 mM each dNTP, 2 mM MgCl₂ and 1.3 U Taq DNA polymerase in a 50 µl volume of 1 × Taq reaction buffer. Identical reactions were prepared when amplifying the IGFBP-3 and β-actin genes except that 0.4 µl ss cDNA was used. PCR conditions were the same for all genes examined (31 cycles; 94 °C, 40 s; 56 °C, 40 s; 72 °C, 40 s), along with an initial denaturation step (94 °C, 40 s) and final extension step (72 °C, 10 min).

GHR, IGF-I and IGFBP-3 mRNA expression in liver from pouch young (12–150 days post-partum) and adults ($n=4$ /group) was then determined and expressed relative to β-actin, which did not show changes between age groups (Saunders *et al.* 2001). The precision of the semi-quantitative PCR was assessed on five replicates of an adult liver cDNA sample. The intra-assay coefficients of variation for GHR, IGF-I and IGFBP-3 were 24.3, 20.5 and 20.0%.

Statistics

All data were log transformed before analysis. Plasma GH concentrations, pituitary GH contents and level of gene expression from the differing age groups were compared using one-way ANOVA. Where significant effects were obtained ($P<0.05$), Duncan's new multiple range test was then used for pairwise comparisons.

Results

Plasma and pituitary gland GH concentrations

Mean plasma GH concentrations were highest between 12 and 100 days post-partum, measuring between about

Figure 2 (A) Plasma GH concentrations, (B) GH content per pituitary gland and (C) pituitary GH content expressed per gram body weight in brushtail possum pouch young. Values are means $\pm$ S.E.M. The number of animals per group is shown within or above the bars. Groups with the same letters are not significantly different ($P > 0.05$).

100 and 180 ng/ml (Fig. 2A). Thereafter they declined so that by 150 days post-partum levels approached those in adults. Overall, there was a highly significant ($P < 0.001$, ANOVA) effect of age with GH concentrations in pouch young between 12 and 100 days post-partum significantly ($P < 0.05$) above those at 125 and 150 days and in adults. GH levels were not influenced by the sex of the pouch young (data not shown).

In contrast to plasma levels, GH content in the pituitary gland increased markedly through pouch life (Fig. 2B), with an 87-fold increase from 12 to 150 days post-partum ($P < 0.001$, ANOVA). Pituitary GH content was lowest at 12 days and increased significantly ($P < 0.05$) by 50 days and older. A further large increase in GH content was seen between 125 and 150 days post-partum ($P < 0.05$). There was a significant ($P < 0.05$) negative correlation ($r = -0.77$) between plasma GH concentration and pituitary GH content. Because it was not possible to weigh the anterior pituitary gland to enable us to calculate GH content per gram pituitary gland, pituitary GH content was also expressed per gram body weight of the individual animal to give an estimate of GH-synthesising ability of the pouch young at different post-natal ages (Fig. 2C). GH content (per gram body weight) was highest at 12 days post-partum and thereafter declined to be significantly lower ($P < 0.05$) in all age groups other than at 50 days post-partum.

cDNA sequences of possum GHR, IGF-I and IGFBP-3 genes

The partial clones for possum GHR, IGF-I and IGFBP-3 showed a high degree of similarity with other mammalian sequences at both the nucleotide and amino acid level. The possum GHR cDNA (Genbank Accession No. AF467545) was 1705 bp and comprised 91% of the protein coding region. The nucleotide sequence identity (amino acid identity) over this region was 79.4% (77.6%) with pig (X54429), 78.6% (77.4%) with rabbit (AF015252), 78.2% (72.7%) with human (X06562), 77.7% (72.5%) with sheep (M82912), 74.1% (67.9%) with rat (J04811) and 71.2% (64.9%) with chicken (M74057).

The IGF-I partial clone (AF467543) was 260 bp, which corresponds to 118 bp of the signal peptide and 142 bp of the coding region. The nucleotide sequence identity (amino acid identity) over the coding region was 81.0% (95.7%) with chicken (M32791), 81.0% (89.4%) with human (X00173), 81.0% (89.4%) with pig (M31175), 79.6% (89.4%) with sheep (M31736), 77.5% (87.2%) with rat (X06107), and 77.5% (87.2%) with *Xenopus* (M29857). Amino acid sequence for IGF-I is also available for one marsupial species, the Western grey kangaroo (*Macropus fuliginosus*) (Yandell *et al.* 1998). Of the 47 amino acids, deduced from possum cDNA, there was 100% identity with the kangaroo sequence.

The possum IGFBP-3 cDNA (AF467544) was 613 bp, which corresponds to 77% of the total protein coding region. The nucleotide sequence identity (amino acid identity) over this region was 72.8% (77.3%) with pig (J05228), 74.5% (74.6%) with human (X64875) and 72.2% (71.5%) with rat (M31837).

Figure 3 Pattern of liver gene expression throughout pouch life and in adult brushtail possums: (A) GHR, (B) IGF-I and (C) IGFBP-3. Values are means $\pm$ S.E.M. ($n=4$ /group). Where no S.E.M. is shown the value is less than the symbol depicting the mean. Groups with the same letters are not significantly different ($P>0.05$).

Semi-quantitative analysis of GHR, IGF-I and IGFBP-3 gene expression

The ontogeny of liver GHR, IGF-I and IGFBP-3 mRNA expression is shown in Fig. 3. For each mRNA, differences between age groups were highly significant

($P<0.001$; ANOVA). Liver GHR expression was low early in pouch life and then increased more than 4-fold to reach a maximum at 150 days post-partum, which was similar to that in adults (Fig. 3A). This increase was gradual during early pouch life and did not reach statistical significance ($P<0.05$) until day 125 (vs day 12 and day 25). The developmental profile for IGF-I gene expression (Fig. 3B) was similar to that for GHR. IGF-I mRNA levels increased 5-fold between 12 and 150 days post-partum with only minor differences between age groups through to day 100. One such increase was at day 75 ($P<0.05$ compared with day 25) but it was followed by a return to lower levels of expression at day 100. Thereafter, liver IGF-I mRNA increased more rapidly to be similar to that in adults by day 150. There was a highly significant ($P<0.01$) correlation between GHR and IGF-I mRNA levels ($r=0.93$) and both also showed significant negative correlations ($r=-0.83$ and -0.89 respectively) with plasma GH ($P<0.05$ and 0.01 respectively).

In contrast to GHR and IGF-I, liver IGFBP-3 mRNA (Fig. 3C) increased significantly ($P<0.05$) after day 12 to peak at 50 days post-partum. Levels then gradually declined to be significantly lower by 100 days post-partum. IGFBP-3 expression in adult liver was not significantly different from that in pouch young at days 12, 125 and 150 days post-partum. There were no significant correlations between IGFBP-3 and plasma GH, pituitary GH content, GHR mRNA or IGF-I mRNA.

Discussion

This study of the brushtail possum provides the first detailed account of the ontogeny of the GH/IGF-I axis during pouch life of a marsupial. Here we show that liver GHR and IGF-I mRNA expression are low early in pouch life, do not show large and sustained increases until 125 days post-partum and are similar to those in adult liver by 150 days post-partum. In contrast, we have previously shown that IGF-II gene expression is high from 12 through to 150 days post-partum and significantly above that of the adult for this entire period (Saunders *et al.* 2001). These results suggest that IGF-II, which is regarded as a fetal growth factor in eutherian mammals, could play a similar role in post-natal growth of marsupials during the first half of pouch life when GHR and IGF-I expression is low.

In this study we chose to examine hepatic mRNA expression of GHR, IGF-I and IGFBP-3 because GH has been shown to regulate the expression of these proteins in other mammalian species (Mathews *et al.* 1986, 1989, Isgaard *et al.* 1988, Zapf *et al.* 1989, Sauerwein *et al.* 1991, Ambler *et al.* 1992, Bichell *et al.* 1992, Baumbach & Bingham 1995, Lemmey *et al.* 1997). Since the liver is a primary target for GH, expression of GHR, IGF-I and IGFBP-3 in this tissue is likely to reflect the effects of circulating GH. Our results for GHR and IGF-I, which

are negatively correlated with plasma GH, suggest that the possum liver is resistant to GH early in pouch life, possibly because GHR expression is low. In support of this explanation, plasma GH-binding protein concentration, which correlates with liver GHR levels in eutherian mammals (Mulumba *et al.* 1991, Ambler *et al.* 1992), is also low early in pouch life (days 45–87) but then increases more than 3-fold by about 120 days post-partum (Saunders *et al.* 2002). In eutherian mammals, the fetal liver is relatively insensitive to GH and only post-natally does GH become an important regulator of hepatic IGF-I production. This insensitivity to GH is thought to result from a relative absence of GHR in the liver (Gluckman 1984, Badinga *et al.* 1991), and as GHR expression increases post-natally (Mathews *et al.* 1989, Peng *et al.* 1996) IGF-I expression and growth become GH-dependent. In sheep, the increase in hepatic GHR and IGF-I expression that occurs in the last 10 days of gestation is due to the pre-partum rise in cortisol and thyroid hormone (Li *et al.* 1996, Forhead *et al.* 2000). In the brushtail possum pouch young, plasma cortisol concentrations have not been reported but Buaboocha & Gemmell (1995) have shown that plasma triiodothyronine and thyroxine concentrations are low up until about 60 days post-partum then gradually increase to peak at about days 100–130 post-partum. Although this increase in thyroid hormones slightly precedes the increase in hepatic GHR and IGF-I expression, further experiments would be justified to establish whether this represents a causal relationship.

In contrast to the pattern seen for GHR and IGF-I, IGFBP-3 mRNA expression peaked at 50 days post-partum then declined over the subsequent 50 days. In eutherian mammals, GH and IGF-I are thought to regulate IGFBP-3 expression (Villafuerte *et al.* 1996, Lemmey *et al.* 1997). However, if, as discussed above, the liver is resistant to GH early in pouch life, factors other than GH itself and IGF-I must be involved in stimulating IGFBP-3 expression between birth and 50 days post-partum. Based on research on eutherian mammals, candidates for this role would include IGF-II, acting at the type-1 IGF receptor (Villafuerte *et al.* 1996). In the possum, we have observed high hepatic IGF-II mRNA expression through pouch life. If IGF-II protein is also increased, either locally in the liver or in plasma, this hormone could be a key regulator of IGFBP-3 in pouch young. However, IGFBP-3 expression declines after day 75, at a time when there is no decrease in IGF-II expression and presumably increased GH signalling through GHR as evidenced by increased GHR and IGF-I mRNA expression. Clearly further studies that include estimates of protein expression should be undertaken to resolve this apparent paradox.

We have previously reported that plasma GH concentrations in the possum pouch young are very high early in life but then decline to reach adult levels by about 96–120 days post-partum (Saunders *et al.* 2002). In the present study on precisely aged pouch young, plasma GH

concentrations remained elevated above those in the adult until 125 days post-partum, even though they had declined from those seen in younger animals. This difference between studies is most probably due to the errors involved in estimating age from published growth curves (Lyne & Verhagen 1957) which we used in our earlier study (Saunders *et al.* 2002). GH is not present in possum milk (Saunders *et al.* 2002), so the source of high GH concentrations in pouch young is most probably the anterior pituitary gland. In the present study we measured pituitary GH content, and in contrast to plasma concentrations there was a substantial increase through pouch life, which presumably reflects the increased total body weight, and therefore weight of pituitary tissue. In an attempt to compensate for this effect, we calculated pituitary GH content per gram body weight and although there was a significant decline after 12 days post-partum, GH content was relatively constant between 25 and 150 days post-partum. Therefore, the decline in plasma GH after 100 days post-partum is not due to decreased synthesis and/or increased storage in the pituitary gland.

Very high plasma GH concentrations also occur during development of eutherian mammals but these peak *in utero* or around the time of birth. For example, human and sheep fetuses have elevated GH concentrations that are well in excess of those in the adult. For humans, a major decline in GH concentrations occurs mid-gestation (Gluckman *et al.* 1981) while for sheep it occurs around the time of parturition (Bassett *et al.* 1970, Gluckman *et al.* 1981). In contrast in the rat, GH concentrations increase rapidly during late gestation, peak at birth and then decline over the next 10 days (Rieutort 1974). This decline in plasma GH levels in fetal and neonatal eutherians appears to be associated with key developmental or maturational changes such as the onset of inhibitory control by somatostatin, decreased secretion of GH-releasing hormone (Gluckman *et al.* 1979) and the development of the negative feedback mechanism, where increasing IGF-I concentrations act directly on the pituitary gland to decrease GH secretion (Berelowitz *et al.* 1981, Tannenbaum *et al.* 1983). Our observation in the present study of the possum, that the first significant increase in hepatic IGF-I expression coincided with the decline in plasma GH at 125 days post-partum, lends support to the possibility that the decline in plasma GH is also due to an increase in the negative feedback from IGF-I in this species.

In summary, this study revealed that GHR and IGF-I gene expression in the liver show an inverse relationship with plasma GH concentration. This pattern is similar to that observed in some eutherian species, except that in the possum these major changes in plasma GH and GHR and IGF-I gene expression occur much later in relation to the time of birth. Given the immaturity of the marsupial at birth, it would appear that this transition occurs at a similar developmental stage in both eutherian mammals and the possum. In contrast to GHR and IGF-I, IGFBP-3

expression increased much earlier in pouch life than would be expected from studies of eutherian mammals.

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