Nutritional regulation of IGF-II, but not IGF-I, is age dependent in sheep

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Abstract

In post-natal animals, plasma concentrations of IGF-I are tightly regulated by nutritional status. The current study reports that plasma levels of IGF-II in sheep are also regulated by nutrition, but whether plasma IGF-II is increased, decreased or remains the same, depends on the age of the animal. Ewe lambs, ranging in age from 2 days to 2 years, were fed or fasted for lengths of time between 24 and 72 h. Blood samples were taken at intervals of 24 h throughout the treatment period and immediately before slaughter.

Plasma concentrations of IGF-I increased with advancing age in fed animals ($P < 0.001$) and were reduced by fasting in all age groups ($P < 0.001$). Plasma concentrations of IGF-II also increased as animals matured ($P < 0.001$), but did not show an overall effect of the fasting treatment. An interaction between age and nutrition ($P < 0.001$) resulted from a decrease in plasma IGF-II in response to fasting in neonatal animals ($P < 0.01$) and, conversely, increased levels of plasma IGF-II in fasted mature animals ($P < 0.01$).

or $P < 0.001$). Fasted sheep of peripubertal age showed no change in plasma levels of IGF-II.

The nutritional sensitivity of serum IGF-binding proteins (BPs) also changed with age. The 29 kDa BP, which we presume to be BP1, was elevated by fasting in young animals and reduced slightly in older animals. BP2 was increased to a similar magnitude by fasting at all ages. BP3 was depressed by fasting in young animals and showed little change in adults. In contrast, a 24 kDa BP, which is probably BP4, showed little change in young animals and was reduced substantially in older sheep.

In conclusion, the response of plasma IGF-II to fasting suggests that this peptide has functions in mediating nutritional stress which depend on the age of the animal, and also that the role of IGF-II may differ from that of IGF-I in adults.

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Introduction

A major role of insulin-like growth factors (IGFs) is considered to be the regulation of tissue growth and differentiation. IGF-II is considered to be predominantly a foetal growth factor while IGF-I is of primary importance during post-natal growth. In many species it is now recognised that both IGFs are present in plasma and tissues of post-natal animals, and IGF-II is often at higher concentrations in plasma than IGF-I (Sohlstrom *et al.* 1998).

While it is well documented that the nutritional status of an animal markedly affects plasma levels of IGF-I (Bass *et al.* 1991, Monaco & Donovan 1997), the response of IGF-II to poor nutrition has not been studied as extensively. In young rats, IGF-II in serum is decreased by nutritional restriction but is less sensitive in the short term than IGF-I (Donovan *et al.* 1991). Malnourished children have reduced serum concentrations of IGF-II (Soliman

et al. 1986), although studies done in adults report no change in IGF-II levels following 5 days of fasting (Davenport *et al.* 1988). In ruminants, a number of reports suggest that fasting alters plasma levels of IGF-II. McGuire *et al.* (1995a) reported a reduction following 2 days of food deprivation in dairy cattle while Bass *et al.* (1984) also observed lower plasma IGF-II in 10 month sheep that were fasted for 5 days. A further study has also provided evidence that nutritional regulation of plasma IGF-II is age dependent in sheep, as fasting increased plasma IGF-II in mature ewes but not 4 month lambs (Hua *et al.* 1995).

These reports suggest that both IGF-I and IGF-II are regulated by nutrition, but they may be differentially controlled. We have systematically studied the effects of post-natal age and food deprivation on plasma levels of IGF-I, IGF-II and IGF-binding proteins (BPs) to investigate whether nutrition is a controlling factor of plasma IGFs at all ages in sheep, and whether the manner of regulation differs for each IGF.

Table 1 Random allocation of sheep

Treatment	Age and developmental stage of sheep					
	Neonatal		Peripubertal		Adult	
	2 days	14 days	4 months	6 months	12 months	24 months
Fed (n)	4	5	4	5	5	5
Fasted (n)	4	6	4	4	5	5
Fasting period (h)	24	24	48	72	72	72

Materials and Methods

Animals

Female sheep ranging in age from 2 days to 2 years were obtained from the farm at Ruakura Research Centre and randomly allocated to experimental treatments as in Table 1. The ages for study were selected to encompass significant phases of post-natal development from birth to maturity, i.e. the initial growth response after birth, rapid growth around puberty and the slowing of growth at maturity. Details of care and feeding are published elsewhere (Oldham *et al.* 1996). Blood samples were taken from all animals at intervals of 24 h throughout the fasting period until slaughter. Blood (5 or 10 ml) was collected by jugular venepuncture into tubes containing EDTA for assay of IGFs, IGFBNs and glucose, or sodium oxalate for assay of non-esterified fatty acids (NEFA). The samples were chilled on ice, centrifuged (2000 g, 4 °C) and the plasma stored at -20 °C until assay. This experiment received approval from the Animal Ethics Committee at Ruakura Research Centre.

IGF RIA

Ovine IGF-I and IGF-II were radiolabelled using Iodogen as previously reported (Hodgkinson *et al.* 1989). The ¹²⁵I-IGF peptides were purified using reversed-phase Sep-Pak (Waters Associates, Milford, MA, USA) and stored at -20 °C until use. IGF-I was dissociated from IGFBNs in plasma using acid/ethanol cryoprecipitation (Hodgkinson *et al.* 1991), which routinely has a recovery efficiency of ~91% in adult sheep plasma (Hodgkinson 1991). IGF-II was extracted from plasma using C18 Sep-Pak (Waters Associates) (Jackson *et al.* 1991), showing a recovery of 82%. RIA for IGF-I and IGF-II was performed as reported by Hua *et al.* (1995). The IGF-I antibody was generated against a recombinant human amino-terminal methionyl IGF-I (IMC/Pitman Moore, Terre Haute, IN, USA) and the IGF-II antibody was a mouse monoclonal antibody against rat IGF-II (Amano Pharmaceutical Company Limited, Nagoya, Japan). Both assays cross-react <1%

with the heterologous IGF and <0.1% with insulin. Within and between assay variations were <4 and <10% respectively for IGF-I and <6 and <11% respectively for IGF-II.

Assay of plasma metabolites

Glucose in plasma was measured in duplicate using the Beckman Glucose Analyser 2 (Beckman, Palo Alto, CA, USA). Glucose standard (150 mmol/l) was measured after every 12 unknown samples and had a coefficient of variation of 1.8%. An enzymatic colorimetric method of micro-assay was used for the measurement of NEFA (Demacker *et al.* 1982). Samples were assayed in duplicate, with a coefficient of variation of 1.52%.

Ligand blotting for IGFBNs

Aliquots of plasma were prepared for SDS-PAGE using a modified method of Laemmli (1970). Pooled plasma (n=3 per group) samples from blood collected at slaughter, and controls of normal sheep plasma and foetal lamb plasma, were diluted in sample buffer and dissociated by boiling, then electrophoresed on an acrylamide gel and transferred onto nitrocellulose membrane. The method for ligand blotting was modified from Hossenlopp *et al.* (1986). Membranes were washed and incubated with ¹²⁵I-IGF-II (800 000 c.p.m.) for 24 h at room temperature. Subsequently, they were washed, dried and exposed against XAR-5 film (Eastman Kodak, Rochester, NY, USA) for 5 days with intensifying screens at -70 °C. Optical density values from the radiographic bands in each sample were determined by densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

Statistics

Data from plasma samples at slaughter were adjusted before analysis by using pre-treatment concentrations as a covariate. Other data were log-transformed when necessary. Main effects analysis by ANOVA was used and data are presented as age and treatment means ± S.E.D. or S.E.M.

Figure 1 (A) Plasma concentrations of IGF-I at slaughter from fed and fasted sheep of different ages. (B) Plasma concentrations of IGF-I in fasted sheep of different ages at 24 h intervals during the treatment period. Time 0=pre-treatment (mean $\pm$ pooled S.E.M., $**P<0.01$, $***P<0.001$). D=days, M=months.

Results

Plasma IGFs

At slaughter, plasma concentrations of IGF-I (Fig. 1A) increased with age ($P<0.001$) and were reduced by fasting ($P<0.001$). Changes within individual groups showed progressively reduced concentrations of IGF-I at all ages in fasted lambs (Fig. 1B, $P<0.01$ or $P<0.001$). In fed lambs, the levels of IGF-I were relatively high in the neonatal period, before dropping and subsequently rising steadily to peak at 12 months. Developmental changes followed a similar pattern in fasted animals, with values 40–80 ng/ml lower than fed counterparts at the same age.

Plasma IGF-II increased with age ($P<0.001$) and there was a significant interaction between age and nutrition ($P<0.001$), but no main effect of the fasting treatment

Figure 2 (A) Plasma concentrations of IGF-II at slaughter from fed and fasted sheep of different ages. (B) Plasma concentrations of IGF-II in fasted sheep of different ages at 24 h intervals during the treatment period. Time 0=pre-treatment (mean $\pm$ pooled S.E.M., $*P<0.05$, $**P<0.01$, $***P<0.001$). D=days, M=months.

(Fig. 2A). This was due to age-related differences in the directional change of IGF-II as a result of fasting. The fasting treatment (Fig. 2B) reduced concentrations of IGF-II in younger animals ($P<0.05$) and increased concentrations in older animals ($P<0.01$ or $P<0.001$). Developmental changes in the fed animals followed a similar pattern to that for IGF-I, with the exception that levels peaked at 6 months instead of 12 months.

Plasma IGFBPs

IGFBPs in circulation, as assessed qualitatively by a ligand blot of pooled plasmas at each age (Fig. 3A and B), were of samples taken from fasted animals at the onset of fasting and then at slaughter. This was 24 h later for 2 day and 14 day lambs, 48 h later for 4 month lambs and 72 h later for 6, 12 and 24 month sheep. The IGFBPs showed

Figure 3 (A) Ligand blot of serum IGFFBPs in pooled pre-treatment (P) and final (F) samples from fasted sheep of different ages. (B) Fasting-induced changes in serum IGFFBPs have been estimated by differences in optical density between pre-treatment samples and final samples at slaughter. Final samples were taken at 24 h for 2 day and 14 day lambs, 48 h for 4 month lambs and 72 h for 6, 12 and 24 month sheep. Arrows indicate overall trends for younger and older sheep. D=days, M=months.

Figure 4 Plasma concentrations of (A) glucose and (B) NEFA at slaughter from fed and fasted sheep of different ages (mean $\pm$ pooled S.E.M.). D=days, M=months.

different patterns of sensitivity to fasting, some of which were influenced by age of the lambs. The 29 kDa protein, which is likely to be BP1, was increased by fasting in young animals and depressed by fasting in older animals. BP2 was elevated in response to fasting at each age. BP3 was depressed by fasting in younger animals and the 24 kDa protein, possibly BP4 (Carr *et al.* 1994), was also depressed by fasting.

Plasma metabolites

Changes in glucose concentrations with age and nutrition were both significant (Fig. 4A, $P < 0.001$). Glucose levels in fed lambs declined steadily with increasing age but were always higher than in fasted counterparts. NEFA concentrations (Fig. 4B) declined with age in fed animals

($P < 0.05$) and concentrations were increased overall by fasting ($P < 0.001$). There was an interaction between these variables ($P < 0.001$) which is likely to have arisen from the greater response of NEFA to fasting in older animals.

Discussion

The data we present document responses of the endocrine IGF system to fasting. The primary finding is that plasma levels of IGF-II are nutritionally sensitive throughout much of post-natal life, but that the directional change as a result of fasting differs between young and older sheep. IGFBPs also show age-related responses to nutritional stress. If the expendable IGF function in the face of nutritional deficit is cellular growth, as suggested by McCusker (1998), then IGF levels in fasted animals may reflect an absolute requirement for cellular maintenance that is greatest in mature animals.

Generally, the pattern of increasing concentrations of IGF with advancing age is the same for IGF-I and IGF-II, so it may be speculated that both growth factors are regulated by growth hormone (GH) and the appearance of the hepatic GH receptor (Gluckman *et al.* 1983). While there is no doubt that plasma IGF-I is positively regulated by GH (Bass *et al.* 1991), the relationship of GH with IGF-II has not been well defined. GH treatment reduces plasma IGF-II in sheep, regardless of age or nutritional status (Hua *et al.* 1995) so it is possible that a decline in plasma GH with advancing age (data not presented) is permissive in increasing post-natal levels of IGF-II in plasma.

The resistance of plasma IGFs to change in the face of nutritional stress is also developmentally regulated, tolerance in very young animals being less than in older animals. The more immediate reduction of plasma IGF-I in young fasted animals may be a response reflecting a greater dependence on glucose synthesis and metabolism than mobilisation of fatty acids. This is supported by metabolite data showing greater reductions in glucose and smaller increases in NEFA in fasted young lambs when compared with mature sheep. The response of NEFA to treatment with GH or IGF-I may also be age dependent. In sheep, Bass *et al.* (1992) reported a dose-dependent increase in NEFA as a result of daily treatment with GH, but neonatal calves do not alter NEFA in response to treatment with GH or IGF-I (Hammon & Blum 1997).

Results from other studies suggest that IGF-I clearance from plasma in older sheep does not alter with fasting (Hodgkinson *et al.* 1987). Further, hepatic IGF-I mRNA is reduced in poorly fed lambs (Pell *et al.* 1993). These studies indicate that nutritionally driven changes in plasma IGF-I in older animals are probably the result of altered hepatic production of IGF-I. IGF clearance rates in sheep of different ages have not been investigated, but a comparison of two independent reports suggests that the

metabolic clearance of IGF-I is greater in 6- than 9-month-old wethers (Hodgkinson *et al.* 1987, Davis *et al.* 1989). Also, the clearance rate is greater for IGF-II than IGF-I (Hodgkinson *et al.* 1989). Regardless of changes in IGF production and secretion, a change in clearance rate may also contribute significantly to the developmental increases in circulating concentrations of IGFs that are reported in the current study.

Ratios of IGFBPs are useful correlates of clearance rate. A comparison of BP3:BP1+BP2 at different ages in the fed animals (data not shown) shows an increase in the ratio with advancing age, which supports the concept of a relative shift from smaller to larger IGFBP complexes and a possible decrease in IGF clearance associated with this. Plasma levels of BP3 and acid-labile subunit increase in concert with IGF-I during post-natal development of rats (Frystyk *et al.* 1998), and together may provide the binding capacity to account for some of the increase in plasma IGF observed with advancing age.

Our data on changes in plasma IGF-II as a result of fasting demonstrate the complexity of this response, and provide a framework for the evidence that IGF-II is nutritionally regulated at some ages but not others. Serial IGF-II levels throughout each fasting period corroborates the comparison made between fed and fasted lambs at slaughter. Overall, the response of IGF-II to fasting cannot be considered less sensitive than that of IGF-I, as two of the four groups in which changes in IGF-II were apparent differed significantly from pre-treatment by 24 h.

The directional change in plasma IGF-II in response to fasting occurs as plasma levels of IGF-II peak developmentally at 6 months. This age coincides with both puberty and a significant decline in growth rate (Fourie 1965) and we have previously speculated that it may also be associated with a transition for the IGF axis from growth promotion to metabolic homeostasis (Oldham *et al.* 1996, Martyn *et al.* 1997). In the adult animal, elevated levels of IGF-II as a consequence of fasting may be associated with a down-regulation of metabolic IGF actions in mature muscle. If secretion of IGF-II is increased in the adult as a result of fasting, this suggests that IGF-II may play a positive role in the adaptive response of the animal. Evidence for such a role has been reported previously, with IGF-II infusion blocking the anabolic effects of IGF-I on animals in a catabolic state (Koea *et al.* 1992a,b). Alternatively, elevated IGF-II together with reduced IGF-I may allow metabolic homeostasis to continue at the expense of cellular growth. Such actions may be controlled by insulin. Under conditions of hyperinsulinaemia and euglycaemia, IGF-I concentrations in adult cattle are elevated while IGF-II levels are depressed (McGuire *et al.* 1995b), so it may be a reduction of insulin by fasting which is directly regulating the changes in plasma IGFs.

Species and developmental differences exist in the response of IGF-II to nutritional stress. IGF-II is reduced with fasting in adult cattle (McGuire *et al.* 1995a) and

humans (Merimee *et al.* 1982) and by malnutrition in children (Soliman *et al.* 1986, Pucilowska *et al.* 1993). In contrast, nutritional restriction but not fasting causes a reduction of plasma IGF-II in guinea-pigs (Sohlstrom *et al.* 1998). Pregnancy is a further state in which the response of IGF-II may differ from that which we report, as fasting of pregnant ewes reduces IGF-II in plasma while restricted nutrition does not (Bauer *et al.* 1995, Lee *et al.* 1997).

The preliminary data that we report for patterns of fasting-induced changes in serum IGFBPs, differed for each of the four main species of IGFBP. The 29 kDa IGFBP, likely to be BP1, changed in a manner inversely mirroring the response to nutrition by IGF-II. This relationship has not been previously demonstrated, although the relationship of BP1 with insulin and glucagon (Nygren *et al.* 1997) and the role of BP1 in glucose regulation (Mortenson *et al.* 1997) are well documented. The nutritional change in BP1 from an increase in young animals to a small decrease in older animals may also be indicative of a change in emphasis from glucose to fatty acid metabolism when animals are nutritionally stressed. As in other studies (McGuire *et al.* 1995a, Monaco & Donovan 1997), levels of BP2 were increased by nutritional stress and Murphy (1998) suggests that this assists in the maintenance of nutritional homeostasis, in contrast to glucose regulation by BP1, by retarding IGF clearance from plasma.

BP3, like BP1, showed a developmental change in response to fasting. Adult animals did not alter plasma levels of BP3, as is also the situation in fasted dairy cattle (McGuire *et al.* 1995a), despite marked differences in both IGF-I and IGF-II. The ratio of tertiary and binary IGF and IGFBP complexes is a regulatory control of IGF clearance from plasma (McCusker 1998). In nutritional deficit, the ratio of BP3:BP1+BP2 reduces, the consequence of this being the increased clearance of IGFs to peripheral tissues. Certainly, this appears to be the case in young animals but the relationship breaks down in older animals as neither BP3 nor BP1 levels alter markedly. The 23 kDa IGFBP, which we presume to be BP4, was depressed at most ages by fasting. BP4 impedes IGF action in culture systems (McCusker 1998) and is reduced by poor nutrition (Holt *et al.* 1996, Takenaka *et al.* 1996). Reduction of BP4, in conjunction with changes in clearance and/or secretion of IGF, seems to be an appropriate response if basal metabolic functions of IGFs are to be maintained in the face of nutritional stress.

In conclusion, we believe that the differential response of plasma IGF-II to fasting in young and mature sheep is a consequence of a change in the post-natal role of IGF-II from promoting growth to controlling cell metabolism, possibly by regulation of IGF-I actions in target tissues.

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