Horse serum contains a novel insulin-like growth factor binding protein

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ABSTRACT

Ligand blotting analysis of serum from the horse using radiolabelled IGF-I revealed a protein at 96 kDa which was not present in serum from goat, cow, sheep, deer or donkey. These latter species all displayed five labelled bands in the range 24 to 41 kDa. Conversely these were only weakly labelled in serum from the horse. Size exclusion chromatography of horse serum pre-incubated with radiolabelled IGF-I revealed reduced binding in the 130 kDa peak compared with goat plasma and ligand blotting analysis indicated the 96 kDa protein was present in this peak. The 96 kDa protein from horse serum binds IGF-I and IGF-II specifically and appears to be unique to this species. The nature of this protein is at present unknown.

Keywords: IGF, binding proteins, horse

INTRODUCTION

As their name implies insulin-like growth factor I and II (IGF-I and -II) are peptides closely related to insulin, possessing both mitogenic and metabolic properties (see Sara and Hall, 1990). IGFs circulate in plasma bound to specific high affinity binding proteins (BPs; Baxter and Martin, 1989a; Clemmons, 1991; Drop *et al.*, 1991). The biological significance of the BPs lies with their ability to alter the circulating half-life, transfer across capillary endothelium and interaction of IGFs with specific cellular receptors. The majority (80-90%) of IGF-I and -II circulates as a complex of approximate molecular weight of 130 kDa. This complex consists of three subunits; IGF, an acid-stable IGF binding unit and an acid-labile unit which helps form the 130 kDa complex, but does not itself bind IGF (Baxter and Martin, 1989b). The remainder of the circulating IGF is bound to BPs of approximately 40 kDa.

The complexity of the IGF/BP system is highlighted by the fact that at least 4 distinct circulating forms of BP have been identified after separation by SDS polyacrylamide gel electrophoresis which discriminates these on the basis of their molecular weight (Drop *et al.*, 1991). These are BP3, which exists as a doublet of 38 and 41 kDa, BP2 (32 kDa), BP1 (28 kDa) and BP4 (24 kDa). In addition to these, several other distinct BPs have been reported in tissues or extracellular fluid (Drop *et al.*, 1991).

The plasma profile of the BPs seem to be relatively conserved within the species surveyed to date, with BP-3 being predominant. This protein has been identified as the IGF-binding unit of the 130 kDa complex and therefore binds most circulating IGF. In this paper we report the existence of a novel high molecular weight BP in equine serum, which appears to be the major binding protein present.

MATERIALS AND METHODS

Samples

Sera from horses were obtained from the Animal Health Laboratory at Ruakura Agricultural Centre. These were collected by different veterinary practices and submitted for routine blood analyses. The horses ranged in age, sex and pedigree. Sera and plasma, collected in EDTA, were obtained from donkey, deer, sheep, goat and cow by venipuncture. All samples were stored frozen at -20°C until analysis.

Ligand blotting

A modification of the Western blotting technique described by Hossenlopp *et al* (1986) was employed to detect IGF-binding activity in samples. Samples (2 ml) were separated on 1.5 x 80 x 55 mm SDS-polyacrylamide gels under non-reducing conditions and protein bands were electrophoretically transferred to 45 mm nitrocellulose using a Novablot Electrophoretic Transfer Unit (LKB, Bromma, Sweden). The nitrocellulose membrane sheets were then treated as described by Hossenlopp *et al* (1986). Radiolabelled human recombinant IGF-I or -II were used to detect BP activity.

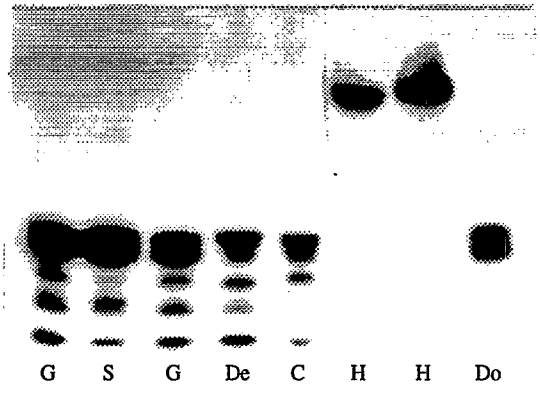
Size exclusion chromatography

Serum (500 ml) was incubated at 4°C overnight with radiolabelled IGF-I with or without 500 ng unlabelled IGF-I. Samples of 200 ml were applied to a Superose 12 FPLC column (1 x 30 cm) previously equilibrated in 0.05 mol/l phosphate buffer (pH 7.4) containing 0.02% (w/v) sodium azide. The column was eluted with the same buffer at 0.5 ml/min, and fractions of 0.25 ml were collected. Radioactivity in each fraction was detected by gamma counter. The column was calibrated with bovine IgG (M_r 160 kDa), ovalbumin (M_r 43 kDa), β-lactoglobulin (M_r 35 kDa), α-lactalbumin (M_r 14.3 kDa) and IGF-I (M_r 7.5 kDa).

RESULTS AND DISCUSSION

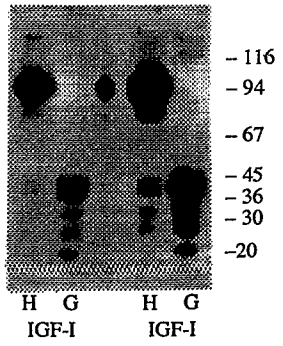
Analysis of serum or plasma from different species, including human (Hossenlopp *et al.*, 1986: Hardouin *et al.*, 1987), sheep (Lord *et al.*, 1991; Davis *et al.*, 1992), goat (Prosser, Baucells and Fleet, 1992), cow (Skaar *et al.*, 1991; Cohick *et al.*, 1992), pig (McCusker *et al.*, 1989), mouse (Fielder *et al.*, 1990) and rat (Donovan *et al.*, 1989; Donovan *et al.*, 1991), by ligand blotting have identified three distinct bands possessing IGF binding activity at 24, 28 and 32 kDa and a doublet at 38 and 41 kDa. The present study shows the only exception to this pattern is horse sera, where labelling of these BPs is either absent or greatly reduced, whereas an additional protein migrating at 96 kDa is the predominant BP present. The 96 kDa protein was not found in serum from the donkey (Fig 1) or zebra (data not shown), also members of the equus order.

FIGURE 1: Ligand blot of serum from goat (G), sheep (S), deer (De), cow (C), horse (H) and donkey (Do) using ^{125}I-labelled IGF-I as the ligand.



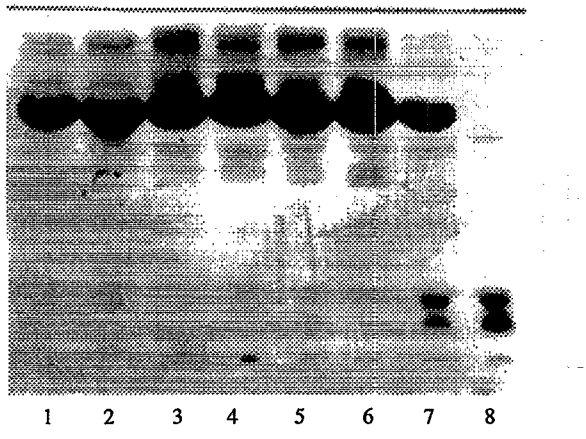
Essentially the same pattern of binding was observed whether IGF-II was used as the ligand or IGF-I, although the intensity of labelling was slightly greater with radiolabelled IGF-II (Fig 2). However, the common BPs were also more apparent after binding to IGF-II, suggesting the greater intensity may be due more to a higher specific activity of the labelled IGF-II than greater affinity of the BPs for this peptide. Although not shown here, the binding of radiolabelled IGF was competed out by inclusion of unlabelled IGF in the binding media. Radiolabelled insulin did not bind to any of the BPs present in horse serum (data not shown).

FIGURE 2: Ligand blot of serum from horse (H) or goat (G) using ^{125}I-labelled IGF-I or IGF-II as ligand. Samples of 2 µl were separated on 8.5% SDS-PAGE.



While the 96 kDa BP was found in sera from many different horses of either sex and varying ages, not all horses exhibited this same unique BP profile (Fig 3). In one animal tested, no 96 kDa BP was identified. Its absence from this animal cannot be readily explained as although it was a pony, a further pony of similar age and sex did exhibit binding at 96 kDa. Interestingly in this animal the intensities of the other smaller BPs appeared similar to those of other species.

FIGURE 3: Ligand blot of sera from a 5 y/o female thoroughbred (lane 1), 16 y/o male Arab (lane 2), 6 y/o male thoroughbred (lane 3), 3 y/o female thoroughbred (lane 4), 7 y/o female pony (lane 5), 3 y/o male standardbred (lane 6), 3 y/o female thoroughbred (lane 7) and 9 y/o female pony (lane 8). Samples (2 µl) were separated on 10% SDS-PAGE prior to electrotransfer to nitrocellulose.

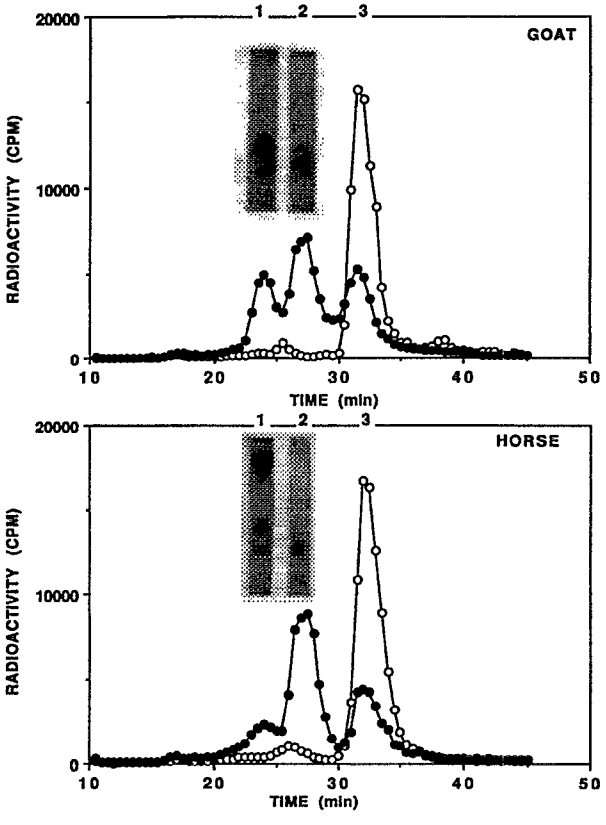


Size exclusion chromatography of goat or horse sera pre-incubated with labelled IGF-I revealed three peaks of radioactivity corresponding to 130, 35 and 7.5 kDa goat (Fig 4). The first two represented specific binding of tracer to BPs as they were significantly reduced in size by inclusion of unlabelled IGF-I in the incubation medium, whereas the latter peak represented the elution position of free IGF-I. In the goat the radioactivity was distributed fairly equally between the first two peaks, similar to previous data for the sheep (Hodgkinson *et al.*, 1989). In contrast, the major BP peak in horse serum occurred at 35 kDa with only a minor component eluting at approximately 130 kDa.

Ligand blotting analysis of the two BP peaks generated by size exclusion chromatography revealed the presence of the 96 kDa BP in the 130 kDa peak, but not the 35 kDa peak (Fig 4), suggesting its size on SDS-PAGE is not due to artifactual aggregation of smaller BPs occurring during processing of the samples. The 130 kDa peak also contained the doublet at 38 and 41 kDa and a further band at 28 kDa after electrophoresis. The doublet most likely is the horse equivalent of BP3, while the band at 28 kDa may be degraded BP3 as suggested by the studies of Zapf *et al.*, (1989) and Binoux *et al.*, (1991). Unfortunately, we were not able to determine whether the 96 kDa BP in horse serum is a distinct and new species of BP or one that is composed of a combination of other classic BPs, a dimer of BP3 for instance.

The possibility that the 96 kDa BP represents components of the 130 kDa complex which are only partially dissociated must not be discounted at this stage. It is notable

FIGURE 4: Size exclusion chromatography of serum from the goat or horse pre-incubated with ^{125}I-labelled IGF-I with (○) or without (●) unlabelled IGF-I. Approximate molecular weight of the three peaks of radioactivity were 130 (1), 35 (2) and 7.5 kDa (3). Following chromatography, fractions from peaks 1 and 2 were ligand blotted using ^{125}I-labelled IGF-I. These are shown aligned to the appropiate fraction.



that Bicsak *et al.*, (1990) found BP-3 in rat serum remaining as a high molecular weight complex with IGF-I after SDS-PAGE. However, they were only able to detect this with antibodies to BP-3 or IGF-I and not by ligand blotting, as the 96 kDa BP was identified in the present study. Moreover, boiling or acid treatment prior to electrophoresis reduced the BP-3/IGF-I complex. In the present study samples were routinely boiled prior to electrophoresis, and treatment with acid had no effect on the pattern of labelling in horse serum (data not shown).

This study raises several questions concerning the IGF/BP system in the horse. Perhaps the most important of these is the relationship between the 96 kDa and other BPs in horse serum. By ligand blotting analysis there appears to be an inverse relationship between the intensity of labelling of the 96 kDa band and the smaller BPs. A further question relates to the presence of the BP in other physiological fluids as well as its influence on IGF action in the horse *in vivo*. Since the BP is not present in serum from all horses we must assume normal IGF function is attained. This also leads one to ask what regulates this BP? From the limited information currently available the BP does not appear to be related to sex or age of the horse, although serial sampling of the same animal over time is required to adequately address this latter issue. The last question is perhaps more fundamental and asks why should the plasma BP profile of the horse be so different from other species? We are unaware of any sequence analysis of IGFs from the horse. In the species analysed to date, IGFs are very highly conserved with the major sequence difference (3-4 amino acids for IGF-I and 4-6 for IGF-II) occurring in rodent IGFs (Sara and Hall, 1990), yet their BP profile is the same as other species. The physiological significance of such a different BP profile for the horse awaits clarification.

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