Regulation of insulin-like growth factor-binding protein-3 ternary complex in feline diabetes mellitus

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Abstract

The 140 kDa ternary complex of insulin-like growth factor-binding protein-3 (IGFBP-3), IGFs and an acid-labile subunit (ALS) has previously been shown to be decreased in diabetes mellitus in humans and rats. We have studied IGF-I levels and ternary complex formation in normal and diabetic cats. Total IGF-I concentrations, measured by RIA using des(1–3)-IGF-I as tracer were ($\pm$ s.e.m.) 54 ± 13 nmol/l in eight normal and 227 ± 57 nmol/l in eight diabetic cats ($P < 0.01$). The size-distribution of IGFBPs in the cat circulation was determined by incubation with ¹²⁵I-IGF-II and Superose 12 chromatography. In normal animals $26 \pm 2\%$ of the ¹²⁵I-IGF-II were in a 140 kDa form compared with $48 \pm 5\%$ in diabetic cats ($P < 0.01$). When samples from normal and diabetic animals were co-incubated $52 \pm 3\%$ were at 140 kDa. A similar shift was seen when normal cat and normal human serum were co-incubated. A 2-fold increase in the 140 kDa form in diabetic cats was con-

firmed first by size-fractionating samples and then performing a ligand-binding assay with ¹²⁵I-IGF-I or -II and charcoal separation. SDS-PAGE and Western ligand blotting demonstrated a 45 kDa doublet (presumably IGFBP-3) and 30–35 kDa forms. There were no apparent differences between normal and diabetic profiles on SDS-PAGE, suggesting that a proportion of IGFBP-3 which circulates 'free' in normal cats forms a ternary complex in the diabetic circulation. We conclude that (i) in contrast to humans and rats, ALS is the limiting factor for ternary complex formation in normal cats, (ii) ALS concentrations increase in feline diabetes mellitus and, by promoting ternary complex formation, this leads to an increase in total IGF-I concentrations, and (iii) total IGF-I concentrations may not be reliable in the diagnosis of acromegaly in diabetic cats.

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Introduction

Previous studies have shown that insulin-like growth factor-I (IGF-I) in the human circulation is predominantly bound to IGF-binding protein-3 (IGFBP-3) and an acid-labile subunit (ALS), forming a 140 kDa complex. In this form IGF-I has a half-life of many hours (Guler *et al.* 1989), whereas when it is in the 'free' form or in binary complexes with IGFBPs, the half-life is a few minutes (Guler *et al.* 1989, Lewitt *et al.* 1993b). Human IGFBP-5 is also able to form this complex with IGF-I and ALS (Twigg & Baxter 1998), but represents a minor proportion of IGF-I in the circulation. One group has suggested that IGFBP-3 can form a binary complex with ALS *in vitro* (Lee & Rechler 1995) and in the rat circulation *in vivo* (Lee *et al.* 1997). In adult humans approximately 50% of ALS are not in the ternary complex (Baxter 1990, Khosravi *et al.* 1997) and there is indirect evidence that

this is true also in the rat (Lewitt *et al.* 1993a, Baxter & Dai 1994, Lee *et al.* 1997). In rat liver cells, growth hormone (GH) stimulates transcription of ALS (Ooi *et al.* 1997) while cAMP (Delhanty & Baxter 1998), dexamethasone (Dai *et al.* 1994) and epidermal growth factor (Dai *et al.* 1994) inhibit ALS synthesis and secretion.

IGF-I concentrations are decreased in diabetes mellitus in humans (Bach & Rechler 1992) and rats (Lewitt *et al.* 1993a). IGFBP-3 and ALS concentrations also decrease in the diabetic state in human (Baxter & Martin 1986) and rat (Dai & Baxter 1994, Frystyk & Baxter 1998) studies. Diabetes mellitus is a common clinical problem in cats. It is characterised by insulin resistance and impaired insulin secretion and, similar to human diabetes, can be insulin-dependent or non-insulin-dependent in type (Lutz & Rand 1995). In contrast to human diabetes, we have observed that circulating IGF-I levels are often elevated in cats in the

absence of any clinical features of acromegaly (D B Church, unpublished observation). The aim of this study was to determine the IGF-I concentrations and IGFBP profile in the serum of normal and diabetic cats.

Materials and Methods

Samples

Serum from eight diabetic cats (age range 6–18 years) was obtained from the University Veterinary Centre, Sydney. None of the study group had clinical features of acromegaly. Three had had computerised tomography scanning, which showed no pituitary tumour. Three animals had a past history of thyrotoxicosis but were not thyrotoxic at the time of study. For comparison serum was collected from eight normal cats (age range 5–14 years).

IGF assays

After acid-ethanol extraction, total IGF-I concentrations were measured in cat serum by RIA using human IGF-I standard, anti-human IGF-I antibody and human IGF-I tracer, as previously described (Church *et al.* 1994). It is well documented that IGFbps potentially interfere with IGF in radioligand assays and therefore recombinant human des(1–3)-IGF-I (GroPep, Adelaide, Australia) was used to prepare tracer for duplicate assays. All serum samples were analysed in both assays. In addition, samples from four normal and four diabetic cats were applied at pH 2.8 to an HPLC column (Protein-Pak 125, Waters/Millipore, Lane Cove, Australia) in order to separate IGFs from IGFbps before assay.

SDS-PAGE and electroblotting

Electrophoresis was carried out on 12% SDS-polyacrylamide gels (5 cm × 8 cm × 1.5 mm). Samples were prepared by the addition of sample buffer to final concentrations of 15.5 mmol/l Tris-HCl, 3% SDS, 10% glycerol and 0.02% bromophenol blue, pH 6.8, and boiled for 5 min. After electroblotting onto Hybond-ECL (Amersham Pharmacia, Biotech, Uppsala, Sweden), IGFbps were detected by incubating with biotinylated IGF-II and then by enhanced chemiluminescence after the method described by Op De Beeck *et al.* (1997). In the case of the samples first separated on Superose chromatography (see below), the fractions were dialysed to remove salt and lyophilised before being taken up in sample buffer and subjected to SDS-PAGE.

Size-separation chromatography

Sera were size-fractionated under neutral conditions using methodology similar to that previously described (Gargosky *et al.* 1991). Serum samples (25 µl) were

Figure 1 IGF-I concentrations in normal and diabetic cats. IGF-I was measured on acid-ethanol extracted serum samples by RIA using des(1–3)-IGF-I as tracer, as outlined in the Materials and Methods. The results for eight normal and eight diabetic cats are represented as a box plot showing the median, the 10th and 90th percentiles and the standard error of the mean. Outliers are shown as ○. The values for diabetic cats were significantly higher than those for normal animals ($P < 0.01$).

incubated for 16 h at 4 °C with 50 000 c.p.m. 125 I-IGF-II in a final volume of 100 µl in 50 mmol/l sodium phosphate, 0.15 mol/l sodium chloride, and 0.02% sodium azide, pH 6.5. Samples were then size-fractionated on a Superose 12 fast protein liquid chromatography (FPLC) column (Pharmacia, Uppsala, Sweden), calibrated as described previously (Baxter 1988), and run at 1 ml/min. Fractions of 0.5 ml were collected and their radioactivity counted using a γ -counter. Fractions 22–24 represent approximately 140 kDa, fractions 26–28, 30–50 kDa and fractions 32–34, approximately 7 kDa (free 125 I-IGF-II).

In some experiments serum was first separated in the absence of tracer. For these studies 50 µl serum were applied to the Superose 12 column in a final volume of 100 µl with FPLC buffer. Samples were separated at physiological pH and collected as described in the preceding paragraph, and then subjected to ligand-binding assay as described below or SDS-PAGE as described above.

Ligand-binding assays

Serum samples were size-fractionated as described above and then 250 µl were incubated with 20 000 c.p.m.

Figure 2 SDS-PAGE of IGFBPs in normal and diabetic cats. Serum samples, 0.5 μ l, were subjected to gel electrophoresis as outlined in the Materials and Methods. Lanes 1, 3, 5, 7 and 9 are samples from diabetic cats and lanes 2, 4, 6 and 8 are normal cat samples. Molecular mass markers, run in an adjacent lane, are indicated in kDa by the arrows.

125 I-IGF-I or -II, added in 50 μ l 0.05 mol/l sodium phosphate, 1.5% BSA, pH 6.5 for 16 h at 22 °C. The free tracer was then separated by precipitation with charcoal. In brief 1 ml ice cold 1% charcoal, 1.5% BSA was added and, after a 10 min incubation at 4 °C, centrifuged for 15 min at 1500 g. Radioiodine in half of the supernatant was counted in a γ -counter and the results multiplied by 2 and expressed as a percentage of total counts after correction for non-specific binding (<10% of total for both IGF-I and -II).

Statistics

Comparisons between normal and diabetic groups were made by *t*-test or by Mann-Whitney rank sum test as appropriate, and correlations were by Spearman rank order correlation (SigmaStat; Jandel Scientific Software, San Rafael, CA, USA).

Results

The IGF-I concentrations were measured by RIA using a human standard. Values were similar when des(1-3)-IGF-I was used as the tracer and the displacement curves for cat serum and the human standard were parallel (data not shown). The concentrations of IGF-I in normal and diabetic cats are shown in Fig. 1, here determined using truncated IGF-I as tracer. Concentrations were 4-fold higher in the diabetic animals ($P<0.01$). Similar results were obtained using intact IGF-I as tracer. The lack of interference of IGFBPs in the assay was confirmed by HPLC separation of normal and diabetic cat serum ($n=4$; data not shown).

We determined the molecular size distribution of IGFBPs in the serum of normal and diabetic cats by SDS-PAGE and by Superose 12 chromatography. Figure 2 shows a Western blot using biotinylated IGF-II as ligand. After SDS-PAGE, cat serum contains a doublet of approximately 45 kDa, consistent with IGFBP-3, and a number of 25–35 kDa forms. There were no qualitative or quantitative differences apparent between the normal and diabetic samples under these, denaturing, conditions and co-incubation of samples from normal and diabetic cats showed an additive effect (data not shown), suggesting the absence of circulating protease activity.

There were clear differences, however, between control and diabetic serum when samples were size-fractionated at physiological pH on a Superose 12 column. After overnight incubation with radiolabelled IGF-II, $26 \pm 2\%$ (S.E.M) of 125 I-IGF-II were in a 140 kDa form in serum from normal animals compared with $48 \pm 5\%$ in diabetic animals ($P<0.001$; Fig. 3). The percentage at 30–50 kDa was $38 \pm 3\%$ in normal animals and $12 \pm 2\%$ in diabetic animals ($P<0.001$). The percentage at 140 kDa correlated with IGF-I concentrations ($r=0.571$; $P<0.05$).

In order to confirm the observed increase in the 140 kDa form, we first separated the serum under neutral conditions on Superose 12 chromatography and then performed ligand-binding assays with 125 I-IGF-II, or lyophilised the samples and subjected them to Western ligand blotting with IGF-II tracer. Figure 4A shows that there was more 125 I-IGF-II binding at 140 kDa in diabetic samples than in normal cat serum. Under these conditions there was no difference in the binding at 30–50 kDa. When samples were acidified for 1 h at 37 °C and then neutralised, before size-separation, all high molecular mass

Figure 3 Size-distribution of IGFBPs in the cat circulation. Serum samples from eight normal (●) and eight diabetic (○) cats were incubated for 16 h at 4 °C with ^{125}I -IGF-II and then fractionated on a Superose 12 size-exclusion column under neutral conditions, as outlined in the Materials and Methods. The approximate molecular masses of the major peaks are indicated. The values are the means $\pm$ S.E.M.

binding (140 kDa and greater) was abolished (data not shown). Figure 4B shows that, when Superose fractions are separated on SDS-PAGE, the 140 kDa peak fractions appear as a 45 kDa doublet. When the ligand-binding assay was performed with ^{125}I -IGF-I (Fig. 5) an increase in the 140 kDa peak and a decrease in the 30–50 kDa peak were also observed in diabetic animals. Binding at a molecular mass greater than 140 kDa, which was observed for ^{125}I -IGF-II, was not observed when ^{125}I -IGF-I was used as the ligand.

We speculated that, in contrast to the situation in humans (Baxter 1990, Khosravi *et al.* 1997) and rats (Lewitt *et al.* 1993a, Baxter & Dai 1994, Lee *et al.* 1997), in which ALS circulates in excess, low ALS concentrations in the circulation of normal cats might limit formation of the IGFBP-3 ternary complex. We therefore determined the IGFBP size-distribution in the presence of added 'free' ALS. We did this by co-incubation of normal cat serum with normal human serum, which contains 'excess' ALS. Human serum and normal cat serum (12.5 μl of each) were incubated overnight in the presence of ^{125}I -IGF-II. Figure 6A demonstrates a shift from $17 \pm 5\%$ in the 140 kDa form to $48 \pm 1\%$ ($P < 0.05$), similar to that present in pooled human serum alone (48%). In order to determine whether diabetic cat serum contained excess ALS, 12.5 μl diabetic serum were incubated with 12.5 μl normal cat serum in the presence of ^{125}I -IGF-II. As shown in Fig. 6B, in three experiments conducted under these conditions, there was a shift from $20 \pm 1\%$ in the 140 kDa form to $52 \pm 2\%$ ($P < 0.05$), similar to that present in diabetic serum alone ($48 \pm 7\%$).

Figure 4 (A) IGF-II ligand-binding assay of IGFBPs in the cat circulation. Serum samples from eight normal (●) and eight diabetic (○) cats were fractionated on a Superose 12 size-exclusion column under neutral conditions. Each fraction was subjected to ^{125}I -IGF-II ligand binding with charcoal precipitation, as outlined in the Materials and Methods. The approximate molecular masses of the major peaks are indicated. The values are the means $\pm$ S.E.M. (B) SDS-PAGE of IGFBPs in the cat circulation after size-separation chromatography. A representative gel from a diabetic cat sample is shown. The lanes represent fraction numbers after Superose 12 chromatography of serum. The samples were dialysed and lyophilised prior to electrophoresis. Serum, 0.5 μl , was run in the lane marked S. Molecular mass markers, run in an adjacent lane, are indicated in kDa by the arrows.

Discussion

In this paper we have shown that, in contrast to human serum, normal cat serum appears to have limited IGFBP-3 ternary complex formation. In feline diabetes, the amount of ternary complex increases substantially, and is associated with 4-fold higher serum IGF-I concentrations compared with normal cats. The values were derived in a human RIA and so the absolute values could not be determined in

Figure 5 IGF-I ligand-binding assay of IGFBPs in the cat circulation. Serum samples from three normal (●) and three diabetic (○) cats were fractionated on a Superose 12 size-exclusion column under neutral conditions. Each fraction was determined by ^{125}I -IGF-I ligand binding with charcoal precipitation, as outlined in the Materials and Methods. The approximate molecular masses of the major peaks are indicated. The values are the means $\pm$ S.E.M.

the absence of a cat IGF-I standard. However, the possibility that normal cat serum is low in IGF-I relative to other species could explain why the adipogenic potential of cat serum has previously been observed to be low (Kuri-Harcuch & Green 1978, Guller *et al.* 1989).

We determined the IGFBP species in cat serum in several ways. Methods in which the distribution of radioactivity is determined after an overnight incubation *in vitro* may be difficult to interpret because of the presence of several 20–35 kDa IGFBPs, which may each have different IGF affinities and may be differentially influenced by diabetes. For example, a decrease of a 30 kDa IGFBP with a high very high affinity for IGF-II might account for a shift to IGFBP-3 in the ternary complex in diabetes. Therefore we also looked at the IGFBP profile after size-separation of serum and confirmed an increase in the 140 kDa form and the presence of a 45 kDa doublet in those chromatography fractions, consistent with IGFBP-3. In the presence of labelled IGF-II, very high molecular mass binding (>140 kDa) was also demonstrated. This was not present when IGF-I was used as the ligand, raising the possibility that this represents a soluble form of the IGF-II/mannose 6-phosphate receptor, which has been detected in human serum and urine (Kiess *et al.* 1987, Causin *et al.* 1988, Gelato *et al.* 1988, 1989, Valenzano *et al.* 1995). The serum IGF-II/mannose 6-phosphate receptor increases in pregnant diabetic women (Gelato *et al.* 1993) and is stimulated by insulin injection in rats (Clairmont & Czech 1990). Whether there is an increase in feline diabetes will require further study.

Figure 6 (A) Size-distribution of IGFBPs after co-incubation of normal cat serum and pooled normal human serum. Serum samples, 12.5 μl , from three normal cats (○) and a pool from several healthy human volunteers (●), were incubated overnight in the presence of ^{125}I -IGF-II, alone, and in combination (▼). (B) Size-distribution of IGFBPs after co-incubation of normal and diabetic cat serum. Serum samples, 12.5 μl , from three normal (○) and three diabetic (●) cats were incubated overnight alone, and in combination (▼), in the presence of ^{125}I -IGF-II. The samples in both panels were fractionated after incubation on a Superose 12 size-exclusion column under neutral conditions, as outlined in the Materials and Methods. The approximate molecular masses of the major peaks are indicated. The values are the means $\pm$ S.E.M.

There were no apparent differences between normal and diabetic profiles on SDS-PAGE and in particular there was no increase in the intensity of the 45 kDa doublet. This was surprising in view of the clear increase in the levels of IGF-I and ternary complex in diabetic cats. A likely explanation for this phenomenon is that a proportion of IGFBP-3 circulates 'free' in normal cats and is available

to participate in ternary complex formation both *in vitro* and *in vivo*, in diabetes.

These studies suggest that ALS does not circulate in excess of the ternary complex in normal cats. When incubated *in vitro* with normal human serum, which contains substantial concentrations of 'free' ALS, there was a shift of the labelled IGF-II to a pattern in which 140 kDa binding predominated. An alternative explanation for this would be that there is substantially greater affinity of the human IGF-II label for human ALS even in the presence of cat IGFBP-3. However, the pattern was identical when normal and diabetic cat serum were co-incubated. An increase in ALS in feline diabetes mellitus would contrast with the situation in humans and in rats and the mechanism of such an increase is the subject of ongoing investigation.

Our study sample was recruited from veterinary patients. Animals were not fasting and were not controlled for the time since the last meal. Three of the diabetic cats had a history of treated hyperthyroidism, which is known to be associated with impaired glucose tolerance (Hoenig *et al.* 1992). While we cannot exclude an effect of previous thyroid disease on the IGF axis, none was currently thyrotoxic and there was no difference between this group and the rest of the study series.

The observations we have made in this study are important for at least three reasons. First, they indicate that the use of IGF-I concentrations to screen for GH excess may be unreliable in diabetic cats. Secondly, they add to the current knowledge of ALS regulation and indicate that an important stimulator might exist in feline diabetes mellitus or that the nature of ALS regulation differs between species. Finally, they raise the possibility that the IGF system might contribute to the pathophysiology of feline diabetes mellitus. Further carefully controlled studies are required to address these issues.

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