The circulating molecular weight forms of infused recombinant insulin-like growth factor-I and effects on glucose and fat metabolism in lambs

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Summary. We have investigated the relationship between the plasma distribution of infused recombinant insulin-like growth factor-I across the insulin-like growth factor binding proteins and the resultant effects on glucose and fat metabolism. The studies were performed in 24-h fasted ram lambs which received primed constant infusions of ³H labelled glucose tracer. When isotopic equilibrium had been reached, the animals received 90-min infusions of human insulin-like growth factor-I at various doses (2.5, 20, 40 and 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, $n=3$ for each dose). Total plasma insulin-like growth factor-I was significantly elevated by infusion at a rate of 40 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (from $185 \pm 14 \mu\text{g/l}$ to $442 \pm 41 \mu\text{g/l}$, $p < 0.05$) and 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (from $181 \pm 2 \mu\text{g/l}$ to $953 \pm 39 \mu\text{g/l}$, $p < 0.005$). The plasma concentrations of insulin-like growth factor-I not associated with binding proteins remained undetectable ($< 15 \mu\text{g/l}$) at the end of the 2.5 and 20 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ doses, but were significantly elevated at the end of the 40 and 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusions (to $71 \pm 14 \mu\text{g/l}$, $p < 0.05$ and $176 \pm 55 \mu\text{g/l}$, $p < 0.01$ respectively). The infused insulin-like growth factor-I associated primarily with 35–60 kilodalton binding proteins. Glucose kinetics were significantly altered only by the highest dose infusion,

during which there was a fall in plasma glucose concentration from $3.5 \pm 0.2 \text{ mmol/l}$ to $1.9 \pm 0.2 \text{ mmol/l}$ ($p < 0.05$). This was due to a 51 % increase in the rate of glucose clearance. There was no significant change in the rate of glucose production. The plasma concentrations of glycerol and non-esterified fatty acid were not changed by any of the doses infused. We conclude that the hypoglycaemic action of infused recombinant insulin-like growth factor-I relates to a marked elevation of free insulin-like growth factor-I in the plasma, but that a threshold concentration of free insulin-like growth factor-I must be exceeded before this action is observed. The hypoglycaemic action of recombinant insulin-like growth factor-I results primarily from an increase in glucose clearance while glucose metabolism was more sensitive than fat metabolism to infused recombinant insulin-like growth factor-I. Both these actions contrast with those of insulin, and suggest that the acute metabolic effects of recombinant insulin-like growth factor-I are not mediated simply by cross-reaction with insulin receptors.

Key words: Insulin-like growth factor-I, insulin-like growth factor binding proteins, glucose metabolism, isotopes.

As the major constituent of the non-suppressible insulin-like activity of plasma, circulating insulin-like growth factor-I (IGF-I) has been attributed with considerable hypoglycaemic potential [1]. The normal animal is protected from IGF-I induced hypoglycaemia because of its association with specific plasma binding proteins which prevent IGF-I from exerting insulin-like actions [2]. Whether there are physiologically significant amounts of free IGF-I present in the plasma under physiological conditions has been debated [3, 4]. However, if the plasma binding capacity is saturated by the administration of a sufficiently high dose of IGF-I, the resultant free IGF-I can lower the blood glucose concentration [2].

We examined the effects of infusion of several different doses of IGF-I on glucose metabolism in order to clarify how the distribution of IGF-I across the IGF-I binding protein influences its action on glucose kinetics. Although the hypoglycaemic action of IGF-I administration has been reported in both animal [2] and human studies [5], this has been ascribed to its affinity for the insulin receptor as a result of the considerable homology between the peptides and their receptors [6]. Few *in vivo* studies have addressed the mechanism of IGF-I induced hypoglycaemia. We have used isotopic tracer methodology to determine the effects of IGF-I infusion on glucose production and peripheral glucose clearance, and have measured the ef-

fects of IGF-I infusion on glycerol and non-esterified fatty acid concentrations.

Materials and methods

The animals involved in these studies were cryptorchid crossbred lambs of an age range between 3 and 5 months which were fasted for 24 h prior to being studied. On the morning of the study they were weighed and placed in Pavlov slings to which they had become accustomed. Diazepam (0.3 mg/kg i.m.) was then administered and SURFLO teflon catheters (Terumo, Tokyo, Japan) inserted into the external jugular veins. A 20 gauge catheter was inserted rostrally into one external jugular vein to enable infusion of isotopes and hormones and a 16 gauge catheter was inserted caudally into the contralateral vein for the taking of blood specimens.

The priming dose for the isotopic infusions was administered at 09.00 hours, and during the first 3 h 154 mmol/l NaCl and glucose tracer were infused simultaneously while isotopic equilibrium was being reached. Blood specimens were collected at 10-min intervals over the last 20 min of this period to enable baseline measurements to be made. Following this, the test infusions were started and continued over a 90-min period during which time plasma specimens were taken at 70, 80 and 90 min. All the blood specimens were collected in chilled, heparinized tubes and were immediately centrifuged. The separated plasma was frozen prior to being analysed.

Hormone infusions

The animals were infused during the test period with recombinant human IGF-I (rhIGF-I) at doses of 2.5, 20, 40 and 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($n=3$ for each dose). The rhIGF-I was generously donated by Dr. A. Skottner of Kabi Vitrum Laboratories (Stockholm, Sweden). Immediately prior to infusion, all of the hormones were diluted in 35 ml 154 mmol/l NaCl and 0.5 ml of the plasma from the animal to be infused was added to reduce hormone binding to the surface of the syringe and plastic tubing. The infusates were infused at a rate of 20 ml per h.

Isotopic infusions

The rate of glucose appearance was determined by a primed constant infusion of 6- ^3H glucose of 5.0 nCi $\cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (prime ratio 80:1). The glucose tracer was obtained from Amersham Laboratories (Amersham, Buckinghamshire, UK).

Sample analysis

The plasma specific activity of 6- ^3H glucose was determined by a previously described method [7]. Non-esterified fatty acid concentrations were quantified using a gas chromatography technique [8], and glycerol concentrations by the spectrophotometric method described by Galletti [9]. The plasma concentrations of glucose, urea and potassium were determined using a Hitachi 704 Autoanalyser (Hitachi, Tokyo, Japan).

Plasma hormone concentrations

a) *IGF-I.* Plasma IGF-I was measured by radioimmunoassay [10] using a rabbit antiserum to rh-metIGF-I (878/4) at a final titre of 1:150,000. The antiserum has a cross-reaction with IGF-II of <0.05%, a minimal detectable dose of 0.06 ng/tube and a half displacement dose of 0.40 ng/tube.

Recombinant y-rhIGF-I was iodinated by a modified chloramine-T method [11]. The ^{125}I labelled y-rhIGF-I was purified by exclusion chromatography on a pre-albuminated Sephadex G-50 (Pharmacia,

Uppsala, Sweden) column (10 $\times$ 600 mm). Fractions of 40 drops were collected using 0.01 mol phosphate buffered saline (pH 6.2) containing 0.1% gelatin (weight/volume) as eluent. The specific activity of ^{125}I labelled y-rhIGF-I was 120–160 $\mu\text{Ci}/\mu\text{g}$. Only the eluates corresponding to the IGF-I monomer were pooled and stored in aliquots at 4°C. This material was used within 2 weeks of iodination.

Before immunoassay plasma samples were subject to acid-ethanol extraction with an additional cryo-precipitation step (AEC extraction [10]). The correlation between values for sheep plasma obtained using this extraction technique and those of Sephadex G75 chromatography in 1 mol/l formic acid was $r=0.98$ ($p<0.001$, $n=10$) and the slope of the linear regression was 1.02. Using this extraction system, the interassay coefficient of variation was 9.8% and the within assay coefficient of variation was 5.0%.

The incubation mixture consisted of 100 μl diluted neutralized acid-ethanol solution containing the standard or test plasma and 200 μl of assay buffer containing IGF-I antiserum. After preincubation for 60 min at room temperature, ^{125}I labelled y-rhIGF-I (20,000 cpm) was added in 200 μl of assay buffer and the incubation was continued for 18–24 h at 4°C. Bound and free IGF-I were separated by the addition of 1 ml of a pre-precipitated second antibody containing 0.01 mol/l phosphate buffered saline, 1% (volume/volume) sheep antirabbit gammaglobulin, 0.1% (volume/volume) normal rabbit serum and 5% polyethylene glycol (PEG 6000). The test tubes were then incubated for 1 h at room temperature and centrifuged at 3000 g for 30 min at 4°C. The supernatant was decanted and the radioactivity in the remaining pellet was determined. Plasma concentrations of IGF-I are expressed in terms of World Health Organisation reference standard IRRIGF-I batch 87/518 (National Institute for Biological Standards and Control, Potters Bar, Herts, UK).

b) *Measurement of circulating molecular forms of IGF-I.* The circulating molecular forms of IGF-I in plasma were measured using a modification of the method of Butler and Gluckman [12]. In brief, aliquots of 500 μl were subjected to high-performance gel chromatography on a Superose 12 column (10 $\times$ 300 mm, Pharmacia) fitted to a Pharmacia FPLC System calibrated as previously described [13]. The column was equilibrated with 0.1 mol/l phosphate buffer containing 0.15 mol/l NaCl and 0.02% NaN_3 (pH 7.2) and the samples were eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected after an 8-min delay into silicone-coated tubes containing 20 μl of 1% bovine serum albumin (weight/volume) in water. The fractions containing free IGF-I eluted at fraction 49–60. Fractions in the range of 35–60 kilodalton (kDa) (fraction 39–48) containing predominantly insulin-like growth factor binding protein (IGFBP)-1 and IGFBP-2 and fractions in the range of 150 kDa (fraction 29–38) containing IGFBP-3 were pooled for the analysis of circulating molecular weight forms of IGF-I. Aliquots of the pooled fractions were subjected to AEC extraction before RIA. Recoveries of unlabelled y-rhIGF-I added to the plasma before high-performance gel chromatography, extraction and RIA were in the order of 95–110%. Complete linearity was found in the range of 0–500 ng rhIGF-I added per ml plasma.

The superose 12 column was calibrated before use with the following markers: dextran blue ($M_r = 2000000$), thyroglobulin ($M_r = 330000$), alcohol dehydrogenase ($M_r = 150000$), bovine serum albumin ($M_r = 68000$), carbonic anhydrase ($M_r = 29000$), ^{125}I labelled rhIGF-I ($M_r = 7650$), aprotonin ($M_r = 6500$), glucagon ($M_r = 3000$) and ^{125}I ($M_r = 125$).

c) *Measurement of plasma insulin concentrations.* Plasma insulin was measured by radioimmunoassay using a guinea pig antiserum to ovine insulin at a final titre of 1:400,000. The assay has a minimal detectable dose of 0.15 ng/tube and a half displacement dose of 0.40 ng/tube. The incubation mixture consisted of 100 μl of standard or test plasma and 200 μl of assay buffer containing ovine insulin antiserum. After preincubation at room temperature for 24 h, ^{125}I labelled insulin (15,000 cpm) was added in 200 μl of assay buffer and the incubation was continued for 18–24 h at 4°C. Bound and free insulin were separated by addition of 1 ml of a second antibody complex containing 4% (weight/volume) polyethylene glycol 6000 made

Fig. 1. Total plasma insulin-like growth factor-I (IGF-I) concentrations over the duration of rhIGF-I infusion at doses: $2.5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\square$), $20 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\blacklozenge$), $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\blacksquare$) and $120 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\diamond$). * $p < 0.05$, ** $p < 0.005$

up in 0.01 mol/l phosphate buffered saline. The test tubes were then incubated for 1 h at room temperature and centrifuged at 3000 g for 30 min at 4°C. The supernatant was decanted and the radioactivity in the remaining pellet determined. The interassay coefficient of variation was 11.5% and the within assay coefficient of variation was 6.7%.

Calculations

The rate of appearance (Ra) of glucose was calculated using the Steele equation [14]. The rate of peripheral clearance was determined by dividing the Ra glucose by the prevailing plasma glucose concentration [8].

Statistical analysis

Dunnett's test was applied to repeated measures taken over the duration of the infusions, and Dunnett's variation of one way factorial analysis of variance was used to compare the concentrations of IGF-

I binding forms at the end of the 90-min infusions of variable doses. All data are expressed as the mean $\pm$ SEM.

Results

Plasma IGF-I concentrations

The total plasma IGF-I concentration was not altered by the infusion of rhIGF-I at $2.5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (Fig. 1). The mean plasma IGF-I level increased from $237 \pm 44 \mu\text{g/l}$ to $293 \pm 22 \mu\text{g/l}$ by the end of the $20 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion, but this change did not reach statistical significance. Infusion of rhIGF-I at $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ increased the plasma IGF-I concentration from $185 \pm 14 \mu\text{g/l}$ to $442 \pm 41 \mu\text{g/l}$ ($p < 0.05$), and the $120 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ dose increased the plasma IGF-I concentration from $181 \pm 2 \mu\text{g/l}$ to $953 \pm 39 \mu\text{g/l}$ ($p < 0.005$). The plasma concentrations of the circulating molecular weight forms at the end of the baseline 180-min infusion of 154 mmol/l NaCl were: 7 kDa, below the detectable limits of measurement ($< 15 \mu\text{g/l}$); 35–60 kDa, $62 \pm 7 \mu\text{g/l}$; and 150 kDa, $44 \pm 15 \mu\text{g/l}$. The plasma concentration of free (7 kDa form) IGF-I remained below the limit of the sensitivity by the end of the two lower dose infusions (Fig. 2 A, B). There were detectable amounts of free IGF-I present by the end of the 40 and $120 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusions ($71 \pm 14 \mu\text{g/l}$ and $176 \pm 52 \mu\text{g/l}$ respectively) (Fig. 2 C, D). The mean plasma concentration of 35–60 kDa binding forms of IGF-I was almost two times higher by the end of the $20 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. The 35–60 kDa form concentration was significantly higher at the end of the $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion ($276 \pm 33 \mu\text{g/l}$, $p < 0.05$) and of the $120 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($646 \pm 60 \mu\text{g/l}$, $p < 0.01$), when compared to plasma concentration of this binding form at the end of the $2.5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion. None of the higher doses of rhIGF-I infusion increased the 150 kDa form significantly above the concentration measured at the end of the $2.5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion.

Fig. 2A–D. Insulin-like growth factor-I (IGF-I) binding forms after 90 min of recombinant IGF-I (rhIGF-I) infusion at a rate of $2.5 \mu\text{g/kg} \cdot \text{h}$ (A), $20 \mu\text{g/kg} \cdot \text{h}$ (B), $40 \mu\text{g/kg} \cdot \text{h}$ (C), and $120 \mu\text{g/kg} \cdot \text{h}$ (D). The concentration is expressed in terms of $\mu\text{g/l}$ of IGF-I found in each pool. The 7 kilodalton (kDa) fraction represents free IGF-I and the 35–60 and 150 kDa forms are IGF associated with low and high molecular weight binding proteins respectively. * $p < 0.05$, ** $p < 0.01$ compared to 154 mmol/l NaCl infused controls

Fig. 3A–C. Effects of recombinant human insulin-like growth factor-I (rhIGF-I) infusion on glucose concentration (A), glucose rate of appearance (Ra) (B) and glucose clearance (C). Rates of infusion; 2.5 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\square$), 20 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\blacklozenge$), 40 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\blacksquare$) and 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($\diamond$). * $p < 0.05$

Plasma insulin concentrations

The plasma insulin concentrations were below the limit of sensitivity of the radioimmunoassay employed (0.15 $\mu\text{g/l}$) both before and during 154 mmol/l NaCl and IGF-I infusion.

Plasma glucose kinetics

The 2.5, 20 and 40 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusions of rhIGF-I did not alter the plasma glucose concentration, whereas the 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion reduced the plasma glucose concentration from 3.5 ± 0.2 mmol/l to 1.9 ± 0.2 ($p < 0.05$) (Fig. 3A). None of the infusions significantly changed the rate of appearance of glucose into the plasma compartment, although there was a tendency for the Ra glucose to be lower by the end of the 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion (Fig. 3B). The rate of glucose clearance, unchanged by the lower dose infusions of rhIGF-I, was significantly increased by the 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion from 3.8 ± 0.4 ml $\cdot$ kg $^{-1} \cdot$ min $^{-1}$ to 5.6 ± 0.4 ml $\cdot$ kg $^{-1} \cdot$ min $^{-1}$ ($p < 0.05$) (Fig. 3C).

Plasma non-esterified fatty acid and glycerol concentrations

The plasma concentrations of non-esterified fatty acid and glycerol were not significantly changed by any dose of rhIGF-I infusion.

Plasma electrolytes

Plasma potassium concentration fell over the course of the 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ rhIGF-I infusion from 3.7 ± 0.1 mmol/l to 2.6 ± 0.5 mmol/l ($p < 0.05$), but was not altered by infusions of a lower dose. Plasma urea concentrations were not changed by any of the doses infused.

Discussion

Our data support the assertion that the acute hypoglycaemic action of circulating IGF-I is attributable to the free form. The highest dose of rhIGF-I infusion (120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was associated with a high plasma concentration of free IGF-I and significantly lowered the plasma glucose concentration, but when the infused dose was insufficient to saturate the plasma IGF-I binding capacity, plasma glucose levels remained unchanged. However, an intermediate dose of 40 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ saturated the plasma binding capacity but did not lower the plasma glucose concentration. This may reflect the relatively weaker hypoglycaemic potency of free IGF-I compared to insulin [6], and subsequent requirement for a threshold concentration to be passed before hypoglycaemic effects are seen.

The role of the plasma IGF binding proteins has been a source of considerable discussion [15]. They were initially viewed as having primarily a reservoir function, as there is no tissue or organ which specifically stores IGF [16]. However, in vitro studies have suggested that the mitogenic potential of rhIGF-I may be either potentiated or inhibited by association with binding proteins [17–19]. Combined with other reports of the ability of IGF-I associated to IGFBP to pass freely from the plasma to the tissues [20], the possibilities are raised of IGF-IBP complexes being

the biologically active forms or that they may target IGF delivery. In our study, the majority of infused IGF-I was found in the 35–60 kDa fraction. The relatively lower concentration of free IGF-I reflects in part the more rapid rate of clearance of free IGF-I relative to the 35–60 kDa and 150 kDa forms [15] as well as the presence of unsaturated high affinity binding proteins in the circulation. We and others observed a similar pattern of distribution of infused IGF-I in a previous study in which the action of IGF-I on protein metabolism was determined [21, 22]. It was found that acute IGF-I infusions in two doses (15 and 50 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$), the higher of which lowered the plasma glucose concentration and the lower of which did not, despite doubling the plasma IGF-I concentration, both reduced the rate of protein loss in fasted lambs. It is tempting to speculate that the anabolic action of rhIGF-I observed in these lambs was attributable to the marked increase of the 35–60 kD molecular weight form, which consists of IGF-I associated with IGFBP-1, IGFBP-2 and/or the β subunit of IGFBP-3 [23]. However, the *in vivo* physiological roles of the IGFBPs will probably not be definitively elucidated until sufficient quantities are synthesized to enable infusion into animal models.

The 120 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion lowered plasma concentration of glucose primarily by increasing the rate of glucose clearance by 51 % ($p < 0.05$). This effect was associated with a fall in the mean rate of glucose production by 16 %, which did not reach statistical significance. The predominance of the glucose clearing effect of IGF-I has been previously reported in fasted rats [24]. The kinetics of the hypoglycaemic action of IGF-I contrast with those of insulin, to which glucose production is more sensitive than glucose uptake [25]. We speculate that this disparity may be a result of the hypoglycaemic actions of IGF-I being mediated by IGF type 1 receptors which are numerous in many tissues including muscle [26] but sparse in hepatic tissue [27]. However, attributing an observed response to a particular hormone-receptor interaction is difficult in the case of insulin and IGF-I, because of the structural homology shared by both the hormones and their receptors and subsequent overlapping of receptor affinities. The concept of insulin acting primarily on glucose and amino acid transport and of IGF-I having a predominantly mitogenic role now appears to be an oversimplification, as it becomes increasingly clear that both hormones can exhibit metabolic and mitogenic actions [28–30]. The physiological function performed by these hormones probably relates to the number of receptors and their relative affinities in various tissues.

Although insulin is anti-lipolytic when infused at doses below that required to alter glucose kinetics [31], we have observed that glucose metabolism is more sensitive than fat metabolism to infused IGF-I. This observation is consistent with those of others who have observed relatively much smaller decreases in plasma non-esterified fatty acid levels than plasma glucose levels following bolus injections of IGF-I in both normal rats [2] and human subjects [5]. The absence of an anti-lipolytic effect of IGF-I in our study provides further evidence for the supposition that the metabolic actions of IGF-I are not simply mediated by cross-reaction with insulin receptors.

In summary, we have observed that IGF-I infusion is hypoglycaemic if the dose infused is sufficient to saturate the plasma binding protein capacity, although it appears that a threshold plasma concentration of free IGF-I concentration must be exceeded. The predominant effect of IGF-I on glucose clearance and lack of anti-lipolytic action contrasts with the previously reported properties of insulin, and suggests the IGF-I may be acting via IGF rather than insulin receptors to modulate carbohydrate metabolism.

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