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Inhibition of protein synthesis by glucagon in different rat muscles and protein fractions *in vivo* and in the perfused rat hemicorpus

Victor R. PREEDY*†§ and Peter J. GARLICK*‡
*Clinical Nutrition and Metabolism Unit, Hospital for Tropical Diseases, London NW1 2PE,
†Division of Clinical Cell Biology, Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ,
and ‡Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

The effect of glucagon on the rate of muscle protein synthesis was examined *in vivo* and in the isolated perfused rat hemicorpus. An inhibition of protein synthesis in skeletal muscles from overnight-fasted rats at various plasma concentrations of glucagon was demonstrated *in vivo*. The plantaris muscle (Type II, fibre-rich) was more sensitive than the soleus (Type I, fibre-rich). Myofibrillar and sarcoplasmic proteins were equally sensitive *in vivo*. However, protein synthesis in mixed protein and in sarcoplasmic and myofibrillar fractions of the heart was unresponsive to glucagon *in vivo*. In isolated perfused muscle preparations from fed animals, the addition of glucagon also decreased the synthesis of mixed muscle proteins in gastrocnemius (Type I and II fibres) and plantaris, but not in the soleus. The sarcoplasmic and myofibrillar fractions of the plantaris were also equally affected *in vitro*. Similar results were observed *in vitro* with 1-day-starved rats, but the changes were less marked.

INTRODUCTION

Metabolic stresses, such as starvation and diabetes, are accompanied by loss of muscle protein, mainly as a result of a decrease in the rate of protein synthesis (Waterlow *et al.*, 1978). The role of insulin in such changes has been extensively studied (see, e.g., Jefferson, 1980), but there appear to have been no equivalent studies of the role of the elevated concentrations of glucagon which have been demonstrated in these conditions (Unger & Lefebvre, 1972; Adibi *et al.*, 1976; Stearns & Benzo, 1978). Although there was an early report of a decrease in protein synthesis in incubated muscle treated with glucagon (Beatty *et al.*, 1963), there have been few subsequent measurements of its effect on muscle protein synthesis. Reviews have tended to ignore (Millward, 1979) or even to deny (Alford & Chrisholm, 1979) its possible role in muscle. This may be due to the original studies on glucagon failing to induce changes in muscle cyclic AMP (Rall & Sutherland, 1958). However, more recently it has been shown that in liver glucagon may also mediate its actions via a non-cyclic-AMP-inducible mechanism, i.e. inositol trisphosphate (Petersen & Bear, 1986; Wakelam *et al.*, 1986). Furthermore, a role for glucagon in protein metabolism is illustrated by studies in the perfused rat hindquarter (Rennie *et al.*, 1986) and the whole body (Nair *et al.*, 1987), which have shown effects on muscle glutamine efflux and whole-body protein breakdown respectively. Our own studies previously demonstrated an effect of glucagon in decreasing muscle protein synthesis *in vivo* (Preedy & Garlick, 1985). The three outstanding questions which we have now attempted to answer are (*a*) whether glucagon can exert its actions at concentrations within the physiological range, (*b*) whether different muscles and protein fraction show different sensitivities to the hormone, and (*c*) whether glucagon acts on muscle directly.

We examined the effects of hyperglucagonaemia, at various concentrations *in vivo*, on the synthesis of mixed tissue protein in Type I (aerobic, oxidative, slow-twitch, i.e. soleus and heart) and Type II (anaerobic, glycolytic, fast-twitch, i.e. plantaris) fibre-rich muscles. Synthesis of myofibrillar and sarcoplasmic proteins was also measured. Similar muscles and protein fractions were also studied in the perfused isolated rat hemicorpus.

Some of the data have been published previously in abstract form (Preedy *et al.*, 1980*a*,*b*,*c*).

MATERIALS AND METHODS

Materials

L-[4-^{3}H]Phenylalanine and L-[U-^{14}C]tyrosine were obtained from Amersham International (Amersham, Bucks., U.K.). Monocomponent glucagon was from Novo Laboratories (Basingstoke, Hants., U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.).

Animals

Male Wistar rats, from Charles River, Margate, Kent, U.K., were housed singly in a temperature-controlled environment with a 12 h-light/12 h-dark cycle. They were fed *ad lib.* on a commercially available cubed diet containing 23% (w/w) crude protein (studies *in vivo*) or a synthetic powdered diet containing 18% (w/w) casein (studies *in vitro*) for a minimum of 5 days before measurement of synthesis. Food was removed from 'fasted' rats either 10 h before infusion *in vivo* or 25 h before perfusion *in vitro*. The average weight of rats for the studies *in vivo* and *in vitro* was 120 and 160 g respectively.

Measurements *in vivo*

Experiments were performed between 09:00 and

§ To whom correspondence and reprint requests should be sent, at: MRC Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

17:00 h. Rats were immobilized by wrapping in a linen towel, and glucagon was infused, via a tail vein, at various concentrations, i.e. 10-fold increments from 0.0002 to 2.0 mg/ml, at a rate of 0.3 ml/h per rat. The mean concentrations of glucagon in the plasma of treated rats at the end of the infusions are displayed in Table 1. Controls were infused with diluent (Preedy & Garlick, 1985). At the end of 6 h, [4-^{3}H]phenylalanine (150 mM, 25 μCi/ml; 1 ml/100 g body wt.) was injected into the cannula and the infusion continued for a further 10 min. At the end of this period, rats were killed and muscles rapidly removed and frozen in liquid N_2, as described by Garlick *et al.* (1980). Fractional rates of protein synthesis (K_s, defined as the percentage of tissue protein renewed each day by synthesis, %/day) were calculated from the specific radioactivities of free phenylalanine in tissue homogenates (S_I) and protein-bound phenylalanine in tissue hydrolysates (S_B) from the formula:

$$K_s = (S_B \times 100)/(S_I \times t)$$

where t is the period of incorporation in days (McNurlan *et al.*, 1979; Garlick *et al.*, 1980).

Rates of protein synthesis were measured in mixed tissue proteins of soleus, heart and plantaris muscles and different protein fractions of heart and gastrocnemius. In the absence of performing biochemical analysis on individual fibres, a variety of muscles were selected to represent the different fibre types. For the purposes of this paper we have defined the terminology 'Type I' as aerobic, oxidative or slow-twitch fibres. 'Type II' fibres are those also referred to as anaerobic, glycolytic or fast-twitch. Although histochemical analysis was not performed on muscles reported here, this has been described in the literature. The proportions of Type I fibres in soleus, plantaris and gastrocnemius are 84, 6 and 5% respectively. However, in the plantaris, the proportion of Type II fibres (i.e. 53%) is greater than in gastrocnemius (i.e. 38%). Remaining fibres are intermediate (Ariano *et al.*, 1973). Thus the soleus and plantaris were considered to be Type I and II fibre-rich respectively, and the gastrocnemius as an intermediate muscle. Myofibrillar and sarcoplasmic fractions were separated on the basis of their relative solubilities in buffers of different ionic strength (Helander, 1961) as described previously (Preedy, 1981).

At the end of *in vivo* measurements, plasma was also collected for immunoassay of glucagon, using a kit supplied by IRE-UK Ltd. (High Wycombe, Bucks., U.K.). Plasma was stored at -20 °C in the presence of Trasylol (200 K.I. units/ml of plasma) prior to assay.

Measurements *in vitro*

Preparation and perfusion of the rat hemicorpus were performed basically as described by Ruderman *et al.* (1971) and Jefferson (1975). After pentobarbitone anaesthesia (50 mg/kg body wt.), heparinization (5000 units/kg body wt.) and oxygenation (10 litres/min per kg body wt.), rats were subjected to operative procedures. This included evisceration, aortic cannulation and hemisection above the diaphragm. Perfusate flow was 10 ml/min per preparation and was gassed with O_2/CO_2 (19:1) at a rate of 300 ml/min. The perfusate (37.5 °C) consisted of (per 100 ml): aged washed human erythrocytes, 45 ml; bovine serum albumin, 5.25 g; pyruvate, 0.043 mM; glucose, 11 mM; heparin, 1 i.u./ml; plasma amino acids, at normal plasma concentrations (Preedy, 1981) in bicarbonate buffer (Krebs & Henseleit, 1932) at pH 7.45. Rates of protein synthesis *in vitro* were measured as described by Preedy *et al.* (1979, 1984) and Preedy & Garlick (1981, 1983). Briefly, 15 min after the start of perfusion, the concentration of tyrosine in the perfusate was raised by replacing 38 ml of recirculating medium with an equal volume of fresh perfusate containing 28 μmol and 4 μCi of L-[U-^{14}C]tyrosine. This procedure raises the concentration of free tyrosine in the perfusate to 248 nmol/ml, compared with 60 nmol/ml in preparations perfused without additional tyrosine (Preedy & Garlick, 1983). We have also shown that, with this technique, intracellular and plasma free tyrosine specific radioactivities are comparable at the end of the experiment, and incorporation of label into tissue protein is linear (Preedy & Garlick, 1983). After a further 35 min, the experiments were terminated and muscles dissected out and stored in liquid N_2. After acid precipitation of powdered muscle, values for S_I and S_B were obtained (Preedy *et al.*, 1984; Preedy & Garlick, 1981, 1983). Fractional rates of protein synthesis were calculated by the same formula as used for studies *in vivo*. Plantaris muscles were also separated into sarcoplasmic and myofibrillar proteins as for studies *in vivo*. Where appropriate, glucagon (Novo Laboratories) was added at an initial concentration of 1 μg/ml.

The radioimmunoassay of Alford *et al.* (1977) was used to measure concentrations of glucagon in perfusate plasma (previously stored at -20 °C with trasylol, as for studies *in vivo*).

Statistics

Results are presented as means ± S.E.M. for 4–12 observations. Differences were assessed by analysis of variance and Student's t tests. Significance was determined when $P < 0.05$.

RESULTS

The results in Table 1 shows that glucagon infusions decreased the synthesis rate of mixed muscle protein in soleus and plantaris *in vivo* by 15–25%. The lowest concentration at which glucagon significantly inhibited protein synthesis in the soleus was 1.3 μg/ml, well above the patho-physiological range. In contrast, significant effects on the plantaris were observed at lower concentrations of glucagon, i.e. 11.5 ng/ml. There was also a significant increase ($P < 0.01$) in the ratio of synthesis rates in these two muscles as plasma glucagon increased. This confirms that *in vivo* Type II fibre-rich muscles are more sensitive to hyperglucagonaemia.

Table 2 shows that both myofibrillar and sarcoplasmic protein synthesis were responsive to glucagon treatment *in vivo*. Significant decreases in synthesis (i.e. 18% fall) were observed in the myofibrillar protein fraction when glucagon was raised by as little as 0.8 ng/ml to a plasma value of 1.6 ng/ml. The effects on the sarcoplasmic fraction were similar, though they only reached statistical significance when glucagon was increased to 11.5 ng/ml. There was no significant effect on the ratio of synthesis rates in the two fractions, suggesting both fractions responded equally.

Table 3 shows the effect of glucagon infusion on fractional rates of protein synthesis in the heart *in vivo*. In these studies a large amount of glucagon was infused,

Table 1. Effect of glucagon on the synthesis of mixed protein in soleus and plantaris *in vivo*

Overnight-fasted rats were infused with various concentrations of glucagon at a rate of 0.3 ml/h per rat for 6 h. After 6 h, rates of protein synthesis in soleus and plantaris muscles was measured as described in the Materials and methods section. Amounts of immunoreactive glucagon at the end of synthesis measurements were also measured in samples of blood collected at death. Values are means ± S.E.M., for 4 to 12 observations. Differences from control groups: $*P < 0.05$; $**P < 0.01$.

Glucagon (ng/ml)	Fractional synthesis rates (%/day)		$\frac{\text{Soleus}}{\text{plantaris}}$ ratio
	Soleus	Plantaris	
0.8	14.32±0.53	9.19±0.47	1.528±0.033
1.6	12.96±0.60	8.13±0.58	1.625±0.117
11.5	12.87±0.39	7.60±0.37*	1.705±0.061
138	12.85±0.54	7.29±0.24**	1.763±0.048**
1315	11.55±0.87**	6.86±0.77**	1.786±0.098**

Table 2. Effect of glucagon on the synthesis of sarcoplasmic and myofibrillar proteins *in vivo*

Data relate to rats described in Table 1. Gastrocnemius muscles were separated into sarcoplasmic and myofibrillar protein fractions on the basis of their solubilities in buffers of various ionic strength; for details see the text. Values are means ± S.E.M., for 4–12 observations. Difference from control groups: $*P < 0.05$; $**P < 0.01$.

Glucagon (ng/ml)	Fractional synthesis rates (%/day)		$\frac{\text{Sarcoplasmic}}{\text{myofibrillar}}$ ratio
	Sarcoplasmic	Myofibrillar	
0.8	7.82±0.35	7.77±0.33	1.008±0.022
1.6	6.54±0.33	6.35±0.29**	1.029±0.015
11.5	6.17±0.44*	5.96±0.35**	1.034±0.030
1315	6.47±0.86	6.39±0.73*	1.005±0.020

Table 3. Effect of glucagon infusion on synthesis of mixed, sarcoplasmic and myofibrillar proteins of the heart *in vivo*

Rats were fasted overnight and infused with either carrier or carrier plus glucagon for 6 h as described in the text. Amounts of plasma glucagon in the treated group was the same as the highest dose in Tables 1 and 2 (i.e. approx. 1 μg/ml). Values are means ± S.E.M. for 5–6 observations. There was no significant difference between results from glucagon and carrier-infused rats.

Treatment	Fractional synthesis rates (%/day)			$\frac{\text{Sarcoplasmic}}{\text{myofibrillar}}$ ratio
	Mixed	Sarcoplasmic	Myofibrillar	
Carrier	15.64±0.72	13.37±1.68	17.47±0.60	0.760±0.080
Glucagon	16.60±0.89	14.35±0.82	16.39±1.35	0.889±0.039

i.e. 2 mg/ml solution infused at 0.3 ml/h/rat, whereupon plasma concentrations of 1 μg/ml were attained. Glucagon infusion had no effect on the synthesis of mixed protein nor any of the fractions.

The effects of glucagon on rates of protein synthesis measured *in vitro* in hemicorpuses from fed and fasted rats are displayed in Tables 4 and 5. The rate of synthesis of mixed protein in plantaris muscle of fed rats was significantly decreased by the addition of glucagon to the perfusate. A significant, but smaller, decrease was also seen in gastrocnemius muscle, but the soleus was not affected (Table 4). In preparations from rats that had been fasted for 1 day, rates of synthesis in plantaris and gastrocnemius were lower than those from fed animals, and in both muscles a further decrease in synthesis occurred when glucagon was added. The decrease,

Table 4. Effect of glucagon on protein synthesis *in vitro*, in three muscles of perfused preparations from fed and fasted rats

Rates of muscle protein synthesis *in vitro* were measured in isolated hemicorpus preparations as described in the text. The concentration of glucagon at the start of synthesis measurement was 11 ng/ml, which was decreased to 2 ng/ml at the termination of the experiment, as measured by immunoassay. Similar concentrations were achieved in preparations from fed and fasted rats. Values are means ± S.E.M. for 4–6 observations. Differences between control and treated preparations: $*P < 0.05$; $**P < 0.01$.

	Fractional synthesis rates (%/day)			
	Fed		Fasted	
Muscle	Control	Glucagon	Control	Glucagon
Plantaris	15.01 ± 0.68	11.94 ± 0.66**	10.29 ± 0.99	7.29 ± 0.53*
Gastrocnemius	10.75 ± 0.44	9.07 ± 0.38*	7.35 ± 0.78	6.91 ± 0.36
Soleus	24.18 ± 1.39	23.82 ± 1.29	24.32 ± 1.21	27.76 ± 3.99

Table 5. Effect of glucagon on synthesis of sarcoplasmic and myofibrillar proteins in muscle of perfused preparations from fed and fasted rats

Data relate to experiments described in Table 4. Plantaris muscles were separated into protein fractions on the basis of their solubilities in buffers of different ionic strengths. For other details see the text. Values are means ± S.E.M. for 4–6 observations. Differences between control and treated perfusions: $*P < 0.05$; $**P < 0.01$.

	Fractional synthesis rates (%/day)			
	Fed		Fasted	
Protein fraction	Control	Glucagon	Control	Glucagon
Sarcoplasmic	13.75 ± 0.91	11.03 ± 0.45*	10.13 ± 1.27	8.39 ± 0.60
Myofibrillar	14.30 ± 1.11	11.96 ± 0.78**	9.41 ± 1.17	8.01 ± 0.56
$\frac{\text{Sarcoplasmic}}{\text{myofibrillar}}$ ratio	0.969 ± 0.032	1.018 ± 0.039	1.075 ± 0.016	1.047 ± 0.077

however, was only significant for the plantaris. The soleus muscle was not affected either by starvation or by the addition of glucagon (Table 4).

Table 5 shows the rates of synthesis of sarcoplasmic and myofibrillar proteins from perfusion experiments described in Table 4. Synthesis of both fractions from fed rats was significantly decreased by addition of glucagon. Although fasting decreased the synthesis of both protein fractions, the addition of glucagon had only a small additional effect (15% decline), and the changes were not statistically significant. With both fed and fasted rats there was an equal effect of glucagon on sarcoplasmic and myofibrillar proteins, since the ratio of sarcoplasmic to myofibrillar protein synthesis was unaltered. This ratio was, however, lower in fed than in fasted rats.

DISCUSSION

These results show that under certain circumstances glucagon can inhibit protein synthesis in skeletal muscle *in vivo* and *in vitro*. Previous studies have not consistently demonstrated this effect. Peterson *et al.* (1963) and Beatty *et al.* (1963) showed that glucagon decreased the net release of amino acids and inhibited protein synthesis in incubated muscle fibre preparations. However, Garber *et al.* (1976) failed to show any effect of the hormone on alanine, glutamate or glutamine release from incubated epitrochlearis muscles. Similarly, in the perfused rat hindquarters glucagon did not significantly affect the release of glutamine and alanine (Haas *et al.*, 1975; Ruderman & Berger, 1974). However, more recently Rennie *et al.* (1986) have proposed that protein synthesis in skeletal muscle may be decreased by glucagon via a decrease in the concentration of intra-muscular glutamine *in vitro*.

The results presented here may give some explanation for the inconsistency in the literature. Different muscles and protein fractions have different sensitivities to glucagon. *In vivo* the smallest increment in glucagon able to produce an effect was 0.8 ng/ml, but these observations were only significant for myofibrillar proteins. By comparison with mixed protein, synthesis in plantaris muscles was significantly decreased when glucagon was increased by 11 ng/ml. However, it is notable that, whenever a supression by the hormone was significant at high concentrations, there was a similar though smaller effect at the lowest increment. The plantaris displayed greater sensitivity *in vivo* than did soleus or heart. Indeed, using high (1 μg/ml) concentrations of glucagon *in vivo*, we were unable to determine any effect on mixed

protein synthesis in heart, nor on either of the protein fractions. A similar relationship of differential sensitivity was also observed in the isolated hemicorpus, i.e. Type II fibre-rich muscles were more responsive to glucagon treatment than Type I fibre-rich muscles, whereas both principal protein fractions were equally responsive.

The different response of fed and fasted rats to glucagon *in vitro* may also account for some of the discrepancies in the literature. Synthesis in fasted rats is already low, and it is possible that any additional effect of glucagon was not sufficiently adverse to decrease synthesis further. It is interesting that the relative sensitivity of the individual muscles to glucagon *in vivo* and *in vitro* is the same as their relative sensitivity to fasting. A similar ranking was also observed for the stimulatory effect of insulin in perfused hemicorpuses from starved rats (Preedy & Garlick, 1983). The insensitivity of Type I fibre-rich muscles relative to other muscles has also been noted in diabetic rats (Flaim *et al.*, 1980) and chronically treated ethanol-fed rats (V. R. Preedy & T. J. Peters, unpublished work). These observations suggest that the insensitivity of Type I fibre-rich muscles to starvation (Li & Goldberg, 1976) might result from their lack of response to circulating hormones, such as glucagon.

A possible factor that might account for the variability in response to glucagon in different laboratories is the disappearance of the added glucagon. This was observed in both sets of experiments, with the intact rat *in vivo* and the isolated hemicorpus *in vitro*. For studies *in vivo*, glucagon at an initial concentration of 10 mg/ml was diluted with the protein-free commercial carrier to concentrations of between 0.0002 and 2 mg/ml, and infused at a rate of 0.3 ml/h per rat. At the end of treatment, plasma concentrations of immunoreactive glucagon in carrier-infused rats (controls 0.8 ± 0.1 ng/ml, $n = 6$) was the same (0.8 ± 0.1 ng/ml, $n = 6$) as at the lowest dose (50 ng/h per 100 g body wt.). This probably reflects adsorption of the hormone on to the plastic components of the laboratory apparatus used to dilute the glucagon, and on to the plastic syringe and infusion cannula during the experiment, as has been reported for insulin (Garlick *et al.*, 1983). For this reason, data from the groups with glucagon infusions of zero and 50 ng/h per 100 g body wt. were combined to form a single control group with $n = 12$. For studies *in vitro*, the calculated amount of glucagon added was 1 μg/ml. At 5 min after addition of the label to the hemicorpus the measured concentration was 11–12 ng/ml, and after 35 min it had fallen to 2.3 ng/ml. This was probably the result of adsorption on to plastic and glass components of the perfusion apparatus. Similar observations were obtained by Parrilla *et al.* (1974) in perfused liver. To necessitate efficient oxygenation of the erythrocytes, the perfusion medium has to flow over a large surface area of glass. The rapid disappearance of the hormone illustrates the importance of adding quite large quantities of glucagon initially to counteract the losses during the experiment. In our studies we paid particular attention to the cleaning of the apparatus in detergent, acid and distilled water to remove any bound hormones between each perfusion (Preedy, 1981).

Literature values for glucagon concentrations in rat plasma are very variable. The highest pathological plasma concentrations that we have observed are those reported by Muller *et al.* (1973) and Katsilambros *et al.* (1970) for streptozotocin-induced diabetes (4.7 ng/ml) and for diabetic ketoacidosis (1 ng/ml). These values are similar to the lowest values capable of decreasing protein synthesis *in vivo* and *in vitro*. However, in both diabetic and starved animals plasma insulin concentrations are low in the presence of hyperglucagonaemia. The infusion of a large concentration of glucagon for 6 h increases circulating insulin concentrations by 20–30 μ-i.u./ml above fasting plasma values (Preedy & Garlick, 1985). This might have attenuated the effect of glucagon. The ability of small amounts of insulin, by itself or as a contaminant, to mask the effects of glucagon are well documented (Randle, 1958; Peterson *et al.*, 1963; Parrilla *et al.*, 1974). For example, when we used a different batch of glucagon *in vitro*, which raised circulating concentrations of insulin, we were unable to observe a decrease in synthesis in skeletal muscle. This was the result of adding a large initial amount of glucagon (1 μg/ml), which has a small proportion of insulin as a contaminant (results not shown).

The mechanism whereby glucagon might exert its effects on muscle protein synthesis is not known. Studies by Rall & Sutherland (1958) indicated that skeletal muscle is unresponsive to changes in cyclic AMP brought about by glucagon. In contrast, there is evidence that heart contains a glucagon-mediated adenylate cyclase (Sutherland & Robinson, 1969). However, protein synthesis in heart is unresponsive to supraphysiological concentrations of glucagon (Table 3), whereas skeletal muscle is responsive to physiological concentrations of the hormone *in vivo* (see above). This suggests that alterations in concentrations of cyclic AMP may not be an essential component of the catabolic effects of glucagon on protein metabolism. Evidence for this hypothesis is supported by studies by Martin-Requero *et al.* (1981), which demonstrated that glucagon-mediated decreases in hepatic protein synthesis were also unrelated to alterations in cyclic AMP. Furthermore, it has been shown in liver that a mechanism exists whereby glucagon may induce alterations in intracellular concentrations of inositol triphosphate as a secondary messenger, rather than cyclic AMP (Petersen & Bear, 1986; Wakelam *et al.*, 1986), although we have no evidence to suggest that a similar mechanism occurs in our studies on muscle. *In vivo* it has been suggested that the actions of glucagon on muscle may be indirect, via changes in the concentrations of other metabolites (i.e. amino acids; Rennie *et al.*, 1986; Daniel *et al.*, 1977). However, the results in the perfused rat hemicorpus demonstrate that glucagon may indeed influence the rate of protein synthesis in muscle by a direct mechanism.

Our studies were designed to answer a number of outstanding questions about the actions of glucagon on muscle. The experiments showed that: (*a*) glucagon exerted its effects on muscle protein synthesis at concentrations in the pathophysiological range; (*b*) glucagon had different effects on different muscle types and protein fractions; (*c*) glucagon exerted its effects directly on skeletal muscle. Further work will be needed to determine the importance of these responses in the regulation of protein metabolism in normal and pathological states in the context of the whole animal.

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