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The influence of restraint and infusion on rates of muscle protein synthesis in the rat

Effect of altered respiratory function

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1. Male rats (110–140 g body wt.) were restrained by a standard laboratory technique, by wrapping in a linen towel, and subjected to a constant intravenous infusion of saline (0.15 M-NaCl) for periods of 1 or 6 h. Fractional rates of protein synthesis (k_s, %/day) were estimated at the start and at the end of the infusion period, by injection of a large concentration of [^{3}H]phenylalanine. 2. In fed and overnight-fasted rats, restraint and infusion of saline for 1 and 6 h decreased k_s in skeletal muscle by 15–20% and 30–35% respectively. Plasma glucose, insulin, glucagon and corticosterone concentrations in restrained and infused rats were not characteristic of immobilization stress. 3. Restrained rats responded to nutrient administration; k_s in skeletal muscle increased by 35–40% after infusion of a mixture of amino acids and glucose for 1 or 6 h, as compared with saline-infused rats. 4. Restraint and infusion for 1 or 6 h did not overtly decrease k_s and k_{RNA} (protein synthesis per unit of RNA) in hypoxaemia-sensitive tissues, such as heart and liver. Restraint and infusion in an open cage, or in a cloth of open weave, did not decrease k_s in muscle after 1 h. Blood gas measurements showed that rats restrained in a linen cloth were hypercapnic and acidotic compared with rats in an open cage. 5. It was concluded that respiratory acidosis, rather than hypoxia, resulting from restraint in a linen cloth decreases muscle protein synthesis.

INTRODUCTION

Experimental techniques involving intravenous infusion and subsequent restraint of laboratory animals have been used for a variety of purposes. These include the measurement of rates of protein synthesis in individual tissues and in the whole body by infusion of labelled amino acids into the tail vein of rats for periods of up to 6 h (Garlick *et al.*, 1975; Waterlow *et al.*, 1978). The effects of infusion of hormones and substrates have also been examined by this approach, e.g. insulin and glucose infusions (Garlick *et al.*, 1983). It is an assumption of this approach that the procedures do not themselves alter the functions that are being investigated. To test this hypothesis, we set out to determine if skeletal-muscle protein synthesis was affected by intravenous infusions and consequent restraint of laboratory rats.

Part of this work was carried out at the Clinical Nutrition and Metabolism Unit (London School of Hygiene and Tropical Medicine, London, U.K.) and has previously been presented in abstract form (Preedy & Garlick, 1984).

MATERIALS AND METHODS

Animals

Male rats [Charles River, Margate, Kent, U.K. (Wistar strain) or Rowett Research Institute (Lister strain)] were housed in a humidified temperature-controlled animal house on a 12 h-light/12 h-dark cycle, with light commencing at 08:00 h. They were fed *ad libitum* on a pelleted diet containing 23% (w/w) crude protein (Oxoid, Basingstoke, Hants., U.K.) for at least 5 days, by which time they attained a weight of 110–140 g. Rats designated as 'fasted' had their food removed at 23:00 h the previous night.

Experimental procedures

Rats that were infused were restrained by lightly wrapping in a cloth (linen glass-cloth unless stated otherwise). A 26-gauge needle attached to a length of polypropylene tubing was then inserted into a lateral vein, as described by Garlick *et al.* (1975). The needle was secured in place with adhesive tape, and infusion commenced at a rate of 1.3 ml/h per rat. At 10 min before the end of infusion, rates of protein synthesis were measured by injection into the cannula of a large concentration of L-[4-^{3}H]phenylalanine (Amersham International, Amersham, Bucks., U.K.) at a dose of 150 μmol/100 g body wt., as described by Garlick *et al.* (1980, 1983). The infusion was continued until the animal was killed by decapitation, at which time blood was collected. In uninfused rats, L-[4-^{3}H]phenylalanine was injected intravenously, with the rats lightly restrained in a cloth for a period not exceeding 2 min. In one study (Expt. 3, Table 1) fasted rats were also intravenously infused with a mixture of amino acids (Synthamin 17; Travenol Laboratories, Thetford, Norfolk, U.K.) and glucose at a rate equivalent to 30 mg of N/h per rat and 0.3 g/h per rat respectively. After death, tissues were rapidly removed, frozen in liquid N_2 and stored at −20 °C until analysis.

Abbreviations used: k_s, fractional rate of protein synthesis; k_{RNA}, rate of protein synthesis per unit of RNA per day.

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Table 1. Effect of 6 h immobilization and infusion on fractional rates of muscle protein synthesis *in vivo*

Treated rats were wrapped in a linen towel and intravenously infused with 0.15 M-NaCl for 1 or 6 h. In Expts. 3 and 4, additional fasted rats were infused with a mixture of amino acids and glucose, as described in the Materials and methods section. During the last 10 min of treatment, rates of protein synthesis were estimated by injection of [4-^{3}H]phenylalanine into the cannula and infusion of saline was continued for the remaining 10 min. Controls were neither immobilized (except for a brief 1–2 min period during which they were lightly wrapped in a linen towel to facilitate injection of label to measure protein synthesis) nor infused, but were given label in a single intravenous injection at the beginning or the end of the infusions in corresponding treated rats. Injection of label is indicated by mean time of death. Values are means ± S.E.M. for five or six observations. Significance of differences between controls and immobilized/saline-infused rats in Expts. 1–4: $^{**}P < 0.01$; $^{***}P < 0.001$. Significance of differences between starved saline-infused rats and starved rats infused with amino acids and glucose, in Expts. 3 and 4: †††$P < 0.001$.

Expt. no.	Treatment	Mean time of death (h)	k_s (%/day)	k_{RNA} (g of protein/day per g of RNA)
1	Fed, control	11:00	16.3 ± 0.4	16.4 ± 1.0
	Fed, 6 h infused	17:00	11.1 ± 0.4***	12.1 ± 0.5***
2	Fasted, control	17:00	8.9 ± 0.8	13.2 ± 0.6
	Fasted, 6 h infused	17:00	6.8 ± 0.3**	10.2 ± 0.4**
3	Fasted, control	11:00	11.4 ± 0.4	12.4 ± 0.5
	Fasted, 6 h infused	17:00	7.8 ± 0.7***	8.8 ± 0.7***
	Fasted, 6 h infused + amino acids + glucose	17:00	11.3 ± 0.2†††	12.3 ± 0.6†††
4	Fasted, control	11:00	11.5 ± 0.8	15.4 ± 0.9
	Fasted, 1 h infused	11:00	8.9 ± 0.3**	12.3 ± 0.5***
	Fasted 1 h infused + amino acids + glucose	11:00	11.9 ± 0.3†††	16.2 ± 0.6†††

Assays for RNA, protein, phenylalanine specific radioactivities and glucose were as described by Garlick *et al.* (1980, 1983). Plasma insulin was measured by radioimmunoassay (Herbert *et al.*, 1965). Glucagon, in plasma stored at −20 °C in the presence of Trasylol [Bayer (UK) Ltd., Haywards Heath, Sussex, U.K.; 200000 international units/ml of plasma] was measured by double-antibody radioimmunoassay with a kit obtained from IRE-UK Ltd. (High Wycombe, Bucks., U.K.). Blood gases and derived parameters were measured on samples withdrawn anaerobically from a needle and cannula (as for infusion) inserted in a lateral tail vein a few minutes previously. Analysis was performed with an ABL blood-gas analyser (model 3; Radiometer, Copenhagen, Denmark).

The fractional rate of protein synthesis (i.e. k_s, the percentage of tissue protein synthesized each day, %/day) was calculated from the specific radioactivity of free and protein-bound phenylalanine in tissues taken 10 min after injection of [^{3}H]phenylalanine as described previously (Garlick *et al.*, 1980, 1983). k_{RNA} is the amount of protein synthesized per unit of RNA each day and was calculated by dividing k_s by the ratio RNA/protein.

Statistics

Results are presented as means ± S.E.M. for 5–11 observations. Statistical significance was assessed by two-tailed *t* tests, using the pooled estimate of variance when appropriate.

RESULTS AND DISCUSSION

The results of Expt. 1 (Table 1) demonstrated that the fractional rate of protein synthesis (k_s) in gastrocnemius muscle of rats infused with saline for 6 h, while restrained in a linen glass-cloth, was decreased by 32% compared with the rate before infusion. The amount of protein synthesized per mg of RNA (k_{RNA}) also fell by 26%. However, the rats did not have access to food during infusions, so this decline in k_s and k_{RNA} could have resulted from nutrient deprivation.

Fasting from 23:00 h the previous night caused both k_s and k_{RNA} to decline (Expt. 2, Table 1), but subsequent infusion for a period of 6 h caused k_s to decline by a further 32%, with similar changes in k_{RNA}. In this experiment the control animals were killed at the same time as the infused rats and suffered the same degree of starvation, showing that the procedure adopted inhibited muscle protein synthesis independently from nutritional factors. These effects were also demonstrable after 1 h (Expt. 3, Table 1) suggesting that the effect of restraint/infusion developed rapidly and was independent from nutritional influences. Expts. 3 and 4 (Table 1) showed that, despite the inhibition during the infusion procedure, muscle protein synthesis was still stimulated by the provision of nutrients by intravenous infusion of amino acids plus glucose, by 32–45%.

The effect of infusion/restraint in post-absorptive rats was specific to skeletal muscle, as there was no change in k_s in either heart or liver after 1 h. Values of k_s in heart and liver in unrestrained rats were 16.9 ± 0.5 and 71.4 ± 2.6%/day respectively. After 1 h of restraint/infusion, values were 16.3 ± 0.8 and 71.8 ± 2.6%/day respectively. After 6 h of restraint/infusion, there was a small fall in k_s in both tissues, but this was partly due to a fall in RNA content, and values for k_{RNA} did not differ overtly from control values (results not shown).

The fall in muscle k_s during infusion might have

Table 2. Effects of method of restraint and infusion for 1 h on muscle protein synthesis

Fasted rats were restrained (*a*) by an open cage consisting of parallel metal bars, which caused minimal restriction of movement, or (*b*) by wrapping in a linen towel (glass-cloth), or (*c*) by wrapping in a perforated cloth. Infusions of saline (0.15 M-NaCl) were given into a lateral tail vein for 1 h (when indicated), at the end of which [^{3}H]phenylalanine was intravenously injected for measurement of protein synthesis, as described in the Materials and methods section. Rats that were not otherwise restrained were lightly restrained in a perforated cloth during injection of [^{3}H]phenylalanine and then returned to their cages. Significance of differences from appropriate unrestrained group is shown by $*P < 0.05$, $**P < 0.01$, and from appropriate linen-towel group, by $\dagger P < 0.05$.

Restraint	Infusion	k_s (%/day)
None	−	11.8±0.7
Open cage	−	11.7±0.4
Open cage	+	11.9±0.7
Linen towel	−	9.1±0.6**
Linen towel	+	9.9±0.4*
None	−	11.0±0.4
Linen towel	−	9.3±0.4**
Perforated cloth	−	10.5±0.2†

resulted from one of the following: hormonal differences resulting from stress, limb immobilization or respiratory problems. We were not able to detect any differences in circulating hormones that might have explained the effect. Nakhooda *et al.* (1981) have shown that stress induced by immobilization of the whole rat is associated with hypoglycaemia, hyperglucagonaemia and decreased insulin secretion. However, in overnight-fasted rats the concentrations of insulin and glucagon were not indicative of this. Thus plasma insulin and glucagon concentrations in unrestrained controls were 3.1±1.3 μunits/ml and 356±44 pg/ml respectively, whereas after 1 h of restraint/infusion corresponding values were 7.8±2.2 μunits/ml and 320±46 pg/ml respectively. At 6 h corresponding values were 5.7±2.2 μunits/ml and 395±34 pg/ml respectively. Plasma glucose concentrations at 6 h were indicative of immobilization-stress, i.e. 7.3±0.3 mM in unrestrained rats versus 5.6±0.1 mM in 6 h-restrained/saline-infused ($P < 0.001$). However, as the hormones did not follow the pattern described by Nakhooda *et al.* (1981), we do not think stress induced by restraint was responsible for the decrease in muscle k_s. Furthermore, small changes in insulin and glucagon from normal fasting concentrations have previously been shown to have no effect on the synthesis of mixed proteins of the gastrocnemius muscle (Garlick *et al.*, 1983; Preedy & Garlick, 1985). Corticosterone concentrations were not altered by restraint, either (369±69, 325±71 and 351±58 ng/100 ml in fasted control and 1 h- and 6 h-restrained/infused rats respectively), but these tend to be influenced by the injection of [^{3}H]phenylalanine given 10 min previously (Preedy & Garlick, 1986). It was very unlikely that corticosterone could have been involved in the effect of infusion, as this hormone requires more than 1 h for its effect on muscle protein synthesis to become apparent (Garlick *et al.*, 1987), whereas the effect of restraint/infusion was pronounced at 1 h.

The method of wrapping rats in a linen towel is a recommended technique for restraint of small animals, to facilitate access to venous blood (Weihe, 1987). By this method the animal has one layer of material covering its face, but has some freedom of movement and does not usually struggle or appear distressed. This became our preferred method for constant intravenous infusion (Garlick *et al.*, 1975), as it allowed ready access to the tail vein. There were numerous difficulties in the use of open cages, which necessitated the use of jugular cannulae inserted under general anaesthetic several days previously (Waterlow & Stephen, 1967). However, as the data in Table 2 show, restraint in a linen towel decreased k_s in muscle, whereas restraint in an open cage did not. We also ascertained that the restraint procedure itself was instrumental in decreasing protein synthesis, as infusion of saline had no additional effect on rats either caged or restrained in a linen cloth. Table 2 also shows that restraint in a perforated cloth (J-cloth; Johnson and Johnson, Slough, U.K.) had no effect on muscle protein synthesis, suggesting that restraint in the less open-weave linen towel was interfering with the free passage of respiratory gases.

Table 3. Effect of method of restraint on blood-gas parameters

Fed rats were restrained in open cages, linen towels or perforated cloths as described in Table 2 for 1 h before immersion of the tail in warm water for about 1 min and insertion of a 26-gauge needle connected to a short length of polyethylene tubing into a lateral tail vein as described in the infusion procedure. A few minutes were allowed for the rat to settle, and then a blood sample was anaerobically withdrawn through the needle for measurement of venous blood gases in an automated blood-gas analyser as described in the Materials and methods section. Significance of difference between open cage and linen-towel-wrapped or perforated-cloth-wrapped rats: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

	Open cage	Linen towel	Perforated cloth
pO_2 (mmHg)	58.4±3.0	52.6±2.2	64.5±2.1
% satn. of haemoglobin with O_2	89.4±1.3	81.1±2.0**	91.7±1.0
pCO_2 (mmHg)	39.7±1.3	56.2±1.7***	39.2±1.5
$[HCO_3^-]$ (mmol/l)	24.4±1.1	26.5±0.6	24.7±0.7
Total CO_2 (mmol/l)	25.8±1.2	28.3±0.6*	25.8±0.7
pH	7.401±0.010	7.294±0.015***	7.407±0.011

Severe hypoxia for 6 h, in similar animals to those shown here, inhibits k_s in liver and heart by 20–25%, but has relatively little effect on skeletal muscle (Preedy *et al.*, 1985). Severe hypoxia for 24 h is required to decrease k_s in skeletal muscle (V. R. Preedy & P. H. Sugden, unpublished work). This is a different pattern from that observed in the present study, and suggests that a different aspect of respiratory function might be responsible. This was confirmed by measurement of blood-gas parameters in arterialized-venous blood taken from the tail vein of rats restrained in different ways (Table 3). Jung *et al.* (1966) have demonstrated that arterial pH, pCO_2 and $[HCO_3^-]$ correlate with venous parameters, but pO_2 does not. However, the pO_2 was highest in rats restrained in perforated cloths; the difference from unrestrained rats was small and not statistically significant. There was also no difference in percentage saturation of haemoglobin with O_2 between rats in open cages and in perforated cloths. In general, values for other parameters in unrestrained animals were the same as when restrained in perforated cloths. The pO_2 in rats restrained in linen towels was significantly lower than those in perforated cloths, but the difference from unrestrained animals was not significant. The percentage saturation of haemoglobin with O_2 in towel-restrained animals was significantly lower than in either of the other two groups, and represented only about a 10% decrease in the oxygen content of the blood. The pCO_2 was markedly and significantly elevated in towel-restrained rats ('mild' hypercapnia; Atkinson *et al.*, 1977). This was accompanied by a significantly lower pH and higher total CO_2 content in towel-restrained animals. This pattern and magnitude of change, which is typical of respiratory acidosis, is quite different from that seen in hypoxia, when arterial pO_2 was decreased to lower values than the lowest venous value seen in the present study, yet pCO_2 decreased, pH increased and muscle protein synthesis was unaltered (Van Liere & Stickney, 1963; Preedy *et al.*, 1985). It therefore appears that the changes in pCO_2 and/or pH are more important in altering muscle protein synthesis than are those in pO_2. It is only in more severe forms of hypoxia, when the animal approaches death, that acidosis is produced (Van Liere & Stickney, 1963). Despite this, the muscles of restrained/infused rats were able to respond to stimuli, e.g. parenteral nutrients (Table 1). Similarly, the stimulation of muscle protein synthesis by insulin was the same in rats restrained in linen towels (Garlick *et al.*, 1983) as in later studies when perforated towels were used (Reeds *et al.*, 1985). We therefore have no reason to suppose that the previously reported changes in protein synthesis brought about by diet and hormones in restrained/infused rats (see, e.g., Garlick *et al.*, 1975, 1983; Preedy & Garlick, 1985, 1986) do not remain valid.

In conclusion, restraint in a linen towel decreases k_s in muscle, as a result of hypercapnia and/or acidosis. This can be prevented by use of a perforated cloth. However, the results also have important implications, as hypercapnia and acidosis are also complicating factors in clinical disorders.

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