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Effect of Insulin on Protein Synthesis in Skeletal Muscle of an Isolated Perfused Preparation of Rat Hemicorpus

(psoas muscle/gastrocnemius muscle/ribosomal subunits/fatty acid/fasting)

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ABSTRACT **A method for perfusion *in vitro* of a preparation of rat hemicorpus was developed for study of the metabolism of skeletal muscle. The preparation was stable during perfusion, as indicated by maintenance of ATP concentration, perfusion pressure, and oxygen consumption for up to 90 min. The perfused hemicorpus provided the following advantages for study of protein synthesis in skeletal muscle: (*a*) hormones and substrates reached the muscle cells through an intact capillary bed, and (*b*) the preparation included the psoas muscle, which was sufficiently large to allow measurements of intermediates in the pathway of protein synthesis and was readily homogenized for preparation of ribosomes and ribosomal subunits. Perfusion of psoas muscle from fasted rats with buffer containing glucose and insulin reduced the concentration of ribosomal subunits and increased phenylalanine incorporation as compared to perfusion with buffer containing glucose alone. In addition, the hormone increased glucose uptake from the perfusate and inhibited release of free fatty acids from the preparation. When the muscle was perfused with buffer that contained glucose and palmitate, the concentration of ribosomal subunits and phenylalanine incorporation were unchanged. Since fatty acid is known to stimulate protein synthesis in heart muscle, these results indicated that rates of protein synthesis in heart, but not in skeletal, muscle would be maintained during fasting or in diabetic animals by increased plasma concentration of fatty acid.**

Regulation of protein synthesis in skeletal muscle is poorly understood. Lack of an *in vitro* preparation of skeletal muscle suitable for the study of control mechanisms of protein metabolism has been partially responsible for this situation. The isolated rat diaphragm muscle has been used in numerous studies on the incorporation of labeled amino acids into protein (1–8). However, this preparation does not provide optimal amounts of tissue for analysis of intracellular concentrations of intermediates within the pathway of protein synthesis, such as amino acids, aminoacyl tRNAs, polysomes, and ribosomal subunits. In recent studies, the rat extensor digitorum longus muscle has been used for investigation of the amino-acid pool that functions as a precursor for protein synthesis (9, 10), but this preparation, like the diaphragm, requires that oxygen and other substrates diffuse through the tissue to reach the innermost cells.

The use of isolated perfused rat hindlimbs has been reported by several investigators (11–15), suggesting that this preparation is suitable for a detailed study of protein metabolism in skeletal muscle. Studies of control mechanisms in isolated perfused organs have several advantages, including delivery of substrates and hormones to the cells by the normal capillary bed. In addition, adequate amounts of tissue and perfusate can be obtained for estimation of concentrations of substrates and intermediates.

Initial attempts to prepare homogenates of rat hindlimb skeletal muscle for the isolation of polysomes and ribosomal subunits met with difficulty. On the other hand, psoas muscle was found to be readily homogenized by the gentle techniques used for heart muscle (16). In the present experiments, we have developed an isolated perfused rat hemicorpus preparation to study protein synthesis in psoas muscle. The preparation remains in a good physiological state for as long as 90 min and responds to insulin. A site of insulin action on protein synthesis is identified.

EXPERIMENTAL PROCEDURES

Perfusion of the eviscerated hemicorpus

Male rats of the Sprague-Dawley strain, weighing 250–350 g, were used for hemicorpus perfusion. The animals were heparinized (10.0 mg/kg) and anesthetized with Nembutal (50 mg/kg). The abdomen was opened through a midline incision, and the following structures were ligated with surgical silk: coeliac artery, superior mesenteric artery, inferior mesenteric artery, renal and suprarenal arteries and veins, spermatic cord, and rectum (Fig. 1). The chest was opened through vertical incisions along either side of the anterior thorax. A loose ligature was placed around the descending aorta immediately above the diaphragm, and a cannula was inserted and tied into place. Perfusion was begun immediately with Krebs-Henseleit bicarbonate buffer gassed with humidified O_2–CO_2 (95:5%) and containing 25% bovine erythrocytes, 3% bovine serum albumin, and the substrates that are listed in the Tables. The buffer was passed through a Millipore filter (3 μm) before the addition of erythrocytes. After perfusion was begun, the animal was transected above the level of the aortic cannula; the stomach, large and small bowels, liver, and testes were removed; and the preparation was covered with polyvinyl film.

The hemicorpus was supported on a stainless steel screen that rested on a stainless steel trough. Perfusate dripped from the vena cava into the trough from which it was pumped through a glass wool filter into oxygenating chambers of an apparatus that had originally been designed for liver perfusion (17). The hemicorpus preparation and oxygenating chambers were enclosed within a Plexiglas box that was maintained at 37°. Perfusate was recirculated at a flow rate of 7.0 ml/min by a peristaltic pump (Harvard Apparatus Co.). The first 50 ml of perfusate that dripped from the vena cava were discarded, and 100 ml of perfusate were recirculated for 90 min.

Analytical methods

Glucose was determined with glucose oxidase (18), free fatty acids by titration as described by Noble (19), oxygen tension

TABLE 1. *Properties of the perfused hemicorpus preparation*

Parameter	Unperfused	Perfused
Water content (ml/100 g)		
Psoas	74.0 ± 0.6* (7)	73.6 ± 0.4 (12)
Gastrocnemius	73.4 ± 0.6 (6)	73.6 ± 0.4 (12)
Sorbitol space (ml/100 g)		
Psoas		21.0 ± 0.6 (6)
Gastrocnemius		18.1 ± 0.5 (6)
ATP content (μmol/g)		
Psoas	6.45 ± 0.45 (6)	6.00 ± 0.24 (6)
Gastrocnemius	6.63 ± 0.18 (6)	6.27 ± 0.30 (6)
Perfusion pressure (mm Hg)		
Initial		137 ± 4 (7)
Final		136 ± 10 (4)
Oxygen tension of perfusate (mm Hg)		
Arterial		293 ± 25 (6)
Venous		55 ± 2 (12)

Preparations were perfused for 90 min with buffer containing 15 mM glucose and normal plasma amounts of all amino acids (22). The figures in parentheses denote the number of observations. Water content, sorbitol space, and ATP content are expressed per weight of wet muscle.

* Standard error of mean.

with an oxygen electrode, ATP by enzymatic analysis of perchloric acid extracts (20), and RNA by alkaline hydrolysis (21). Water content and sorbitol and phenylalanine spaces were determined as described (22). Perfusion pressure was measured with Statham transducers (model P23AC) that were attached to a Beckman Dynograph. Perfusate radioactivity was estimated with a liquid scintillation spectrometer. Radioactivity of muscle protein was determined with a gas-flow planchet counter after plating of samples that had been freed of nucleic acids, lipids, and soluble radioactivity (23).

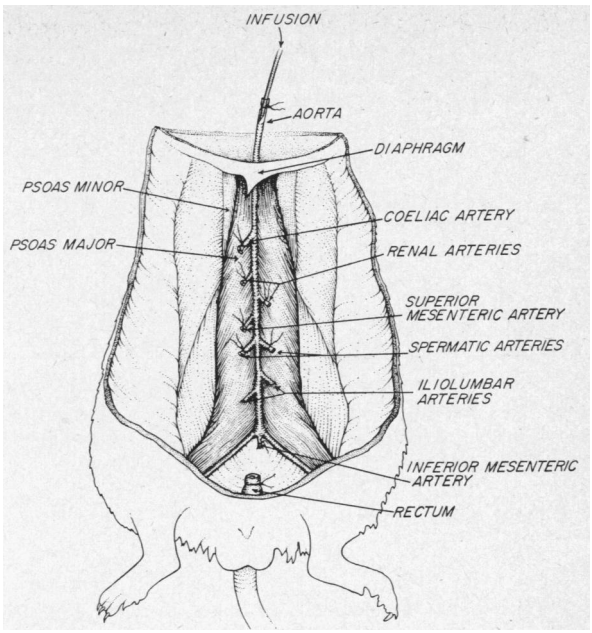

FIG. 1. The perfused eviscerated hemicorpus.

Sucrose density gradient analysis of muscle ribosomes was done as described (16).

RESULTS

We performed preliminary studies to determine the suitability of the perfused hemicorpus preparation for investigation of skeletal muscle metabolism. Several parameters were measured and indicated that the preparation remained in a good physiological state during a 90-min perfusion (Table 1). Water content of psoas and gastrocnemius muscle did not change during perfusion, and the value obtained for the sorbitol space of the psoas and gastrocnemius muscles agreed with similar determinations in diaphragm (24, 25) and with

TABLE 2. *Metabolism of glucose and fatty acids by the perfused eviscerated hemicorpus*

Substrate	Insulin	Perfusate concentration (mM)		Uptake (−) or release (+) of substrate (μmol/100 g per 90 min)
		Initial	Final	
		Glucose		Glucose
Glucose	0	18.0 ± 1.3	13.6 ± 0.8* (12)	−380 ± 19*
Glucose	+	18.0 ± 1.3	2.5 ± 0.3 (16)	−940 ± 44†
Glucose + palmitate (1.45 mM)	0	16.7 ± 1.4	11.1 ± 0.6 (7)	−400 ± 22
Glucose + palmitate (1.45 mM)	+	16.7 ± 1.4	2.1 ± 0.3 (5)	−870 ± 26†
		Fatty acid		Fatty acid
Glucose (18 mM)	0	0.30 ± 0.09	1.39 ± 0.24 (10)	+69 ± 7
Glucose (18 mM)	+	0.30 ± 0.09	0.57 ± 0.13 (11)	+25 ± 5†
Glucose (16.7 mM) + palmitate	0	1.45 ± 0.10	1.68 ± 0.09 (6)	+17 ± 5
Glucose (16.7 mM) + palmitate	+	1.45 ± 0.10	1.07 ± 0.14 (5)	−21 ± 7‡

Preparations were perfused for 90 min with buffer containing the glucose and palmitate concentrations that are listed. Insulin, 25 mU/ml, was added as indicated. Metabolism of substrates was calculated as described (28). Uptake and release data are presented per 100 g of wet weight of the preparation. The figures in parentheses indicate the number of preparations that were perfused.

* Standard error of mean.

† $P < 0.001$ against corresponding control.

‡ $P < 0.005$ against corresponding control.

TABLE 3. *Effect of fasting on amount of ribosomal subunits in psoas muscle*

Condition of animal	RNA (mg/g)	Sucrose gradient peaks (mg RNA in peaks/mg RNA in psoas homogenate) 60 S	40 S
Fed	1.94 ± 0.18* (18)	0.067 ± 0.004	0.045 ± 0.002
Fasted			
48 hr	1.90 ± 0.14 (5)	0.103 ± 0.004†	0.067 ± 0.003†
72 hr	1.93 ± 0.18 (4)	0.107 ± 0.014†	0.070 ± 0.006†

Psoas muscle was removed from fed and fasted rats and analyzed for RNA content and distribution of RNA in sucrose gradient fractions of the homogenate (16). Peaks were collected from gradients that were centrifuged for 15 hr (see Fig. 2).

* SE.

† $P < 0.01$ against fed rats.

estimations *in vivo* of extracellular space in skeletal muscle (26). These findings and the observation that the perfusion pressure remained constant indicated that swelling had not occurred during perfusion. When the flow rate was 7.0 ml/min, a mean perfusion pressure of 136 mm Hg was maintained. This pressure is similar to the systolic pressure of the rat (27). Oxygen tension of the arterial and venous perfusate indicated that the perfusion conditions provided an adequate oxygen supply to the tissues of the hemicorpus preparation. In support of this suggestion, the ATP content of psoas and gastrocnemius muscles was well maintained at *in vivo* concentrations during perfusion.

Measurements of substrate metabolism provided another index of the viability of the perfused hemicorpus preparation (Table 2). When the hemicorpus was perfused with buffer containing glucose (18 mM), uptake of this substrate averaged 380 ± 19 μmol/100 g per 90 min. Addition of insulin to the perfusate increased glucose uptake by about 2.5-fold. Provision of palmitate (1.45 mM) as an alternative substrate had no effect on the basal rate of glucose metabolism and did not alter the stimulatory effect of insulin on glucose uptake. When the hemicorpus was perfused with buffer that contained glucose and albumin, fatty acid release was 69 ± 7 μmol/100 g per 90 min. The albumin preparation used in these studies initially contained 0.3 mM fatty acid. When insulin was added to the perfusate, a net accumulation of fatty acid still occurred, but the rate of release was significantly restrained. When palmitate was added to the perfusate at an initial concentration of 1.45 mM, no significant change in the perfusate concentration of fatty acid was observed. Under these conditions, however, addition of insulin resulted in net uptake of fatty acid from the perfusate. Rates of fatty acid metabolism are thought to reflect primarily changes in the metabolism of adipocytes contained within the preparation and agree with the known effects of insulin on these cells (29).

We undertook additional studies with the perfused hemicorpus preparation to investigate the regulation of protein synthesis in skeletal muscle. These studies were prompted by observations that suggested that protein synthesis was regulated differently in skeletal and heart muscle (30). In these earlier studies, skeletal muscle of diabetic animals contained increased numbers of ribosomal subunits and decreased numbers of polysomes, whereas the amount of subunits and polysomes in heart muscle of diabetic rats was the same as that of the controls. The number of polysomes and ribosomal subunits is influenced by rates of initiation and elongation of peptide chains. Increased numbers of subunits result when initiation is retarded relative to elongation (16). Fasting had an effect similar to that of diabetes on the aggregation of ribosomes in psoas muscle (Fig. 2). Compared to fed controls, psoas muscle of rats that were fasted for 48 hr contained increased amounts of subunits and decreased amounts of polysomes. Changes in amounts of subunits were seen more clearly in sucrose gradients that were centrifuged for 15 hr (*right panel*, Fig. 2). We quantitated the amount of subunits by collecting and analyzing the RNA content of fractions from these gradients. When compared to fed animals, rats

TABLE 4. *Effects of insulin and palmitate on phenylalanine incorporation and ribosomal aggregation in perfused skeletal muscle*

Additions to perfusate	[^{14}C] Phenylalanine incorporation (cpm/mg) Psoas	Gastrocnemius	Psoas muscle RNA (mg/g)	Sucrose gradient peaks mg RNA in peaks/mg RNA in homogenate 60 S	40 S
Glucose, 18 mM (6)	13 ± 2*	34 ± 3	2.24 ± 0.26	0.098 ± 0.011	0.073 ± 0.009
Glucose, 18 mM + insulin (11)	28 ± 5†	54 ± 5†	2.67 ± 0.22	0.045 ± 0.006†	0.030 ± 0.004†
Glucose + palmitate, 1.45 mM (5)	16 ± 3	40 ± 5	2.65 ± 0.16	0.105 ± 0.019	0.057 ± 0.009
Glucose, 18 mM + palmitate, 1.4 mM + insulin (5)	27 ± 3†	64 ± 5†	2.29 ± 0.24	0.040 ± 0.004†	0.028 ± 0.006†

Hemicorpus preparations were perfused for 90 min with buffer containing normal plasma levels of amino acids, [^{14}C]phenylalanine (0.04 μCi/ml), and the substrates that are indicated. Insulin, 25 mU/ml, was added as indicated. After 90 min of perfusion, gastrocnemius muscles were removed and rapidly frozen in Wollenberger tongs at the temperature of liquid nitrogen. Psoas muscles were homogenized and the homogenates were used for estimation of [^{14}C]phenylalanine incorporation and RNA content and for sucrose density gradient analysis of ribosomes. Peaks were collected from gradients that were centrifuged for 15 hr. The figures in parentheses represent the number of preparations that were analyzed.

* SE.

† $P < 0.05$ against glucose or palmitate + glucose, no insulin.

that were fasted for 48 hr showed a significant increase in the RNA content of the fractions representing the 60S and 40S subunits. The effect of fasting on the amount of ribosomal subunits was not amplified by increasing the duration of the fast to 72 hr (Table 3). Fasting for either 48 or 72 hr had no effect on the total RNA content of psoas muscle.

Since incorporation of amino acid into protein was inhibited in muscle of diabetic (31, 32) and fasting (33) animals, increased amounts of ribosomal subunits and decreased amounts of polysomes would indicate that protein synthesis was inhibited due to a block in peptide-chain initiation. In other studies, the decline in the rate of protein synthesis that occurred during perfusion of rat heart *in vitro* was also related to the development of a block in peptide-chain initiation (16). Since these changes could be prevented or reversed in the perfused heart by addition of insulin or palmitate, effects of these agents on protein synthesis in perfused skeletal muscle were investigated. In these experiments, rats were fasted for 48 hr before perfusion to increase the amounts of ribosomal subunits. As seen in Table 4, perfusion for 90 min with buffer containing glucose did not increase the amounts of ribosomal subunits over those seen in psoas muscle of fasted unperfused animals (Table 3). When insulin was added to perfusate that contained glucose, the amount of ribosomal subunits was reduced more than 50% and the rate of incorporation of [^{14}C]-phenylalanine into protein of psoas and gastrocnemius muscles was increased about 2-fold. Insulin did not alter the [^{14}C]-phenylalanine space (0.82 ± 0.05 ml/g) of the gastrocnemius muscle when compared to controls perfused without the hormone. Perfusion with buffer containing palmitate (1.45 mM) had no effect on either the rate of incorporation of [^{14}C]-phenylalanine into protein or on the amount of ribosomal subunits. Insulin was as effective in the presence of the fatty acid as in its absence. These observations are in contrast to similar studies in heart muscle in which both insulin and palmitate were observed to stimulate the rate of protein synthesis and to decrease the amount of ribosomal subunits to the same degree (34, 35). Since insulin increased incorporation of [^{14}C]phenylalanine into protein and decreased the amount of ribosomal subunits in perfused skeletal muscle, the hormone appeared to have facilitated peptide-chain initiation in this tissue as it had appeared to do in perfused heart muscle (16).

Since insulin was found to inhibit protein degradation in perfused liver (36) and heart (16) preparations, it was possible that the increase in [^{14}C]phenylalanine incorporation after addition of the hormone may have resulted, in part, from a

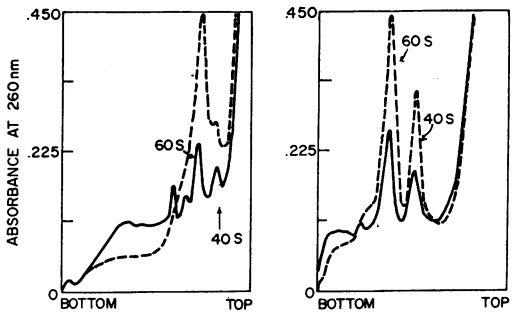

FIG. 2. Effect of fasting on ribosomal aggregation in psoas muscle. Psoas muscle from fed rats and from rats not fed for 48 hr was homogenized (16) in four volumes of a solution containing 0.25 M KCl-2 mM $MgCl_2$-10 mM Tris·HCl (pH 7.4). Sucrose gradient analysis of ribosomal aggregation was performed with the supernatant of muscle homogenate that had been centrifuged at 10,000 × *g* (16). Exponential sucrose gradients were formed in tubes of an SW-40 rotor with 15% and 2 M sucrose dissolved in homogenization buffer (16). After centrifugation at 40,000 rpm for 3.25 (*left*) or 15 (*right*) hr, gradients were monitored by pumping contents of the tube through the flow cell of a Gilford recording spectrophotometer. Ribosomal profiles from psoas muscle of fed and fasted rats are indicated by the *solid* and *dashed lines*, respectively. Large and small ribosomal subunit peaks are labeled as 60 S and 40 S, respectively. The profiles presented are representative of at least six observations.

diminution in the rate of dilution of the specific activity of the intracellular phenylalanine pool that supplied protein synthesis. In other studies it was found that the specific activity of intracellular phenylalanine could be kept constant by perfusing psoas muscle with buffer containing 15 times the normal plasma amount of this amino acid. Under these conditions, insulin still increased the rate of incorporation of [^{14}C]-phenylalanine into protein of psoas and gastrocnemius muscles by about 2-fold over the control rate (Table 5). The [^{14}C]-phenylalanine space of these tissues was not affected by the hormone. These observations indicated that the stimulation of phenylalanine incorporation by insulin involved an increase in the rate of protein synthesis rather than an effect of the hormone on the specific activity of the intracellular phenylalanine pool.

DISCUSSION

The isolated perfused rat hemicorpus has several advantages for studies of protein synthesis in skeletal muscle. The prepa-

TABLE 5. *Effect of insulin on incorporation of* [^{14}C] *phenylalanine into protein of skeletal muscle*

Additions to perfusate	[^{14}C]Phenylalanine space (ml/g)		[^{14}C]Phenylalanine incorporation (cpm/mg)	
	Psoas	Gastrocnemius	Psoas	Gastrocnemius
Glucose, 15 mM (6)	0.79 ± 0.04*	0.91 ± 0.01	12 ± 2	28 ± 2
Glucose, 15 mM + insulin (6)	0.77 ± 0.03	0.89 ± 0.01	25 ± 4†	46 ± 1†

Hemicorpus preparations were perfused for 90 min with buffer containing 15 times the normal plasma amount of phenylalanine (1.2 mM), [^{14}C]phenylalanine (0.15 μCi/ml), normal plasma amounts of the other 19 amino acids, and the additions that are indicated. Insulin, 25 mU/ml, was added as indicated.

* SE.

† $P < 0.001$ against control, no insulin.

ration is stable during perfusion, as indicated by maintenance of ATP concentration, perfusion pressure, and oxygen consumption during periods up to 90 min. The preparation includes the psoas muscle, which is sufficiently large to allow measurements of intermediates in the pathway of protein synthesis and is readily homogenized. Hormones and substrates are able to reach the muscle cells through an intact capillary bed. The major disadvantage of the preparation is the inclusion of adipose tissue, connective tissue, skin, and bone. Hormonal effects on these tissues will affect the composition of the perfusate (Table 2). Changes in composition of the perfusate could modify the rate of protein synthesis and could be confused with a direct effect of hormones on muscle tissue.

Protein synthesis may be regulated at several steps, including amino-acid transport and activation, DNA transcription, and peptide-chain initiation and elongation. Relative rates of chain initiation and elongation can be assessed by measurements of the rate of phenylalanine incorporation and of the concentrations of ribosomal subunits and polysomes. The amount of ribosomal subunits can be quantitated, but a portion of the polysomes is lost in a pellet of myofibrils and mitochondria (16). Only qualitative comparisons of polysome profiles are possible. An increase in ribosomal subunits together with a decrease in the concentration of polysomes and the rate of amino-acid incorporation indicates that initiation of peptide chains is restrained to a greater extent than elongation. Changes of this type were observed in skeletal muscle of fasted rats (Tables 3 and 4). This restraint was overcome by perfusion of the muscle with buffer containing insulin, indicating that the hormone had stimulated peptide-chain initiation. These studies do not define the mechanism of the effect of insulin on initiation or rule out the possibility of effects on other steps in the pathway of protein synthesis.

Control of protein synthesis in skeletal muscle appears to differ from control of the process in cardiac muscle. Fatty acid stimulates protein synthesis in the heart but not in psoas muscle. Oxidation of fatty acid by cardiac muscle accounts for a larger fraction of oxygen consumption than in skeletal muscle (37). Addition of fatty acid inhibited glucose metabolism by heart muscle (38), but had no effect in the perfused hemicorpus (Table 2), rat hindlimbs (39), rat diaphragm (40), or skeletal muscle fibers (41). Ketone bodies were readily metabolized when added to the perfusate of isolated rat hindlimbs (15). In other experiments, addition of acetoacetate (5 mM) had no effect on incorporation of phenylalanine into protein of psoas or gastrocnemius muscles. Stimulation of protein synthesis in cardiac muscle by the increased plasma concentrations of fatty acids and ketone bodies, normally associated with fasting, may serve to maintain heart size and function during periods of food deprivation. During these periods, skeletal muscle protein is degraded and the amino acids are used as substrates for glucose production by the liver. If cardiac muscle were subjected to a similar fate, survival of the animal would be severely compromised.

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