PROTEIN SYNTHESIS IN A POSTMITOCHONDRIAL SUPERNATANT SYSTEM FROM RAT LIVER. AN EFFECT OF DIABETES AT THE LEVEL OF PEPTIDE CHAIN INITIATION

Virginia M. PAIN

Department of Human Nutrition, London School of Hygiene and Tropical Medicine, Clinical Nutrition and Metabolism Unit, Hospital for Tropical Diseases, 4 St. Pancras Way, London NW1 2PE, UK

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1. Introduction

Hepatic ribosome preparations from diabetic rats have a lower polysome content than those from normal animals, and show reduced protein synthetic activity in cell-free systems [1–4]. More detailed analysis of the effects of diabetes has been carried out with skeletal muscle, in which similar, but more pronounced, changes occur [5], and it seems likely that in this tissue insulin exerts translational control on protein synthesis at the level of peptide chain initiation [6, 7].

In the studies with liver [1–4], the cell-free systems which were used consisted of recombined ribosome and cell sap fractions. In such systems, most of the amino acid incorporation obtained represents elongation of existing nascent peptide chains, and the extent of initiation of new chains is small [8]. However, crude hepatic postmitochondrial supernatants (PMS)* are twice as active in amino acid incorporation as more highly fractionated systems [9], and the ribosomes in the crude system show some ability to re-initiate on to endogenous mRNA [10–12]. The data in the present report show that PMS preparations from livers of diabetic rats show a reduced ability to incorporate amino acids into protein. This difference between extracts from diabetic and control animals is almost completely abolished in the presence of inhibitors of peptide chain initiation.

* *Abbreviations:*
PMS : postmitochondrial supernatant;
Poly(I): polyinosinic acid;
ATA : aurintricarboxylic acid.

2. Materials and methods

Male, Porton-type Wistar rats were obtained from SACI, Braintree, UK, and were maintained on Oxoid pellets. Diabetes was induced in rats of 90–100 g body weight by intravenous administration of streptozotocin (kindly provided by Upjohn Ltd, Kalamazoo, Mich., USA) in citrate buffer, pH 4.5 at a dose of 100 mg/kg. The animals were used 5 days later, and were fed *ad libitum* throughout.

Livers were homogenised in 3 vol of TKM buffer (50 mM Tris, 100 mM KCl, 5 mM magnesium acetate, 6 mM mercaptoethanol) containing 0.25 M sucrose. PMS fractions were prepared by centrifugation of homogenates at 10 000 *g* for 10 min. Immediately before incubation, PMS samples were passed down columns of Sephadex G-25 (Pharmacia), equilibrated with TKM buffer, to remove endogenous amino acids. For assay of cell-free synthesis, PMS samples were diluted 1 in 3 with assay medium such that the final concentrations of components in the incubation mixture were as follows: 50 mM Tris–HCl (pH 7.6), 100 mM KCl, 5 mM magnesium acetate, 6 mM mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, creatine phosphokinase (50 μg/ml), [^{14}C]leucine (diluted with unlabelled leucine to a specific activity of 165 Ci/mol) or [^{14}C]phenylalanine (diluted with unlabelled phenylalanine to a specific activity of 167 Ci/mol) 0.42 μCi/ml, and the 19 other amino acids, unlabelled, at 30 μM each. After incubation at 37°C, samples were pipetted on to Whatman 3MM filter paper discs, and incorporation of radio-

activity into protein was assayed by the method of Mans and Novelli [13]. Counting efficiency was about 50%.

ATP, GTP, creatine phosphate, creatine phosphokinase and polyinosinic acid (Poly(I) were obtained from Boehringer Mannheim GmbH. Poly(I) solutions (in water) were heated to 90°C and cooled immediately before use. Aurintricarboxylic acid (ATA) was a gift from the late Professor A. Korner. L-[U-^{14}C]phenylalanine (490–513 Ci/mol) and L-[U-^{14}C]leucine (331 Ci/mol) were obtained from the Radiochemical Centre, Amersham, UK, and unlabelled amino acids from Sigma (London) Chemical Co. Cycloheximide was bought from Koch-Light Ltd., UK.

3. Results and discussion

PMS preparations from livers of diabetic rats were

Table 1

Influence of diabetes and insulin treatment on incorporation of amino acids into protein of postmitochondrial supernatants and the effect of inhibitors of peptide chain initiation.

Inhibitor	Treatment of animals			
	Normal	Diabetic		Insulin treated
Incorporation of phenylalanine (mol phe/mol ribosomes)	None	1.14	0.76	1.38
	Poly(I)	0.37	0.35	0.44
Incorporation of leucine (mol leu/mol ribosomes)	None	1.65	1.01	1.32
	Poly(I)	0.50	0.43	0.44
	ATA	1.02	0.88	0.97
Inhibition by poly(I) (%): Phenylalanine incorporation		67.5	53.9	68.1
Leucine incorporation		69.6	57.4	66.6

Incubations were carried out for 10 min under the conditions described in Materials and methods. Poly(I) was present at a concentration of 500 μg/ml, and ATA at 1×10^{-4} M. Each sample was prepared from the pooled livers of 4–6 animals. Insulin-treated diabetic animals were injected subcutaneously with protamine zinc insulin (4 units) twice daily for 2 days before the experiment, and with soluble insulin (5 units) 2 hr before death.

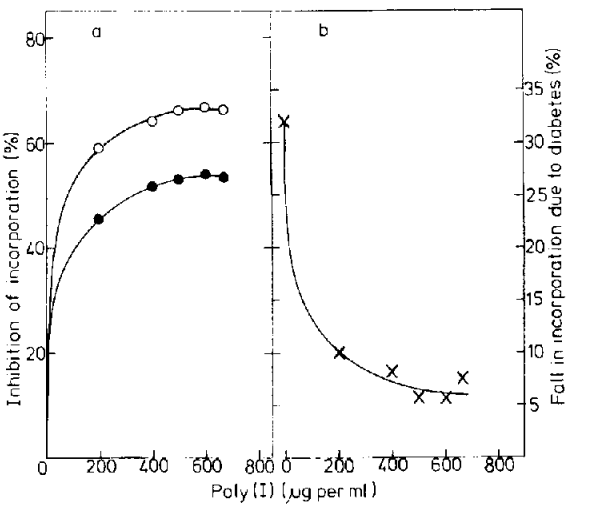

Fig. 1a). Inhibition of [^{14}C]phenylalanine incorporation into protein by postmitochondrial supernatant systems from normal and diabetic animals by increasing concentrations of poly(I). Samples were prepared and incubated as described in the Materials and methods section. Incubations were for 10 min. (o—o—o) PMS from normal rats; (•—•—•) PMS from diabetic rats. b) Effect of diabetes on protein synthesis in the PMS system in the presence of increasing concentrations of poly(I). Incubations were carried out as described above.

30–40% less active than samples from normal rats in incorporating [^{14}C]phenylalanine or [^{14}C]leucine into protein (table 1). This result was also obtained with [^{14}C]tyrosine (result not shown). Insulin treatment of the diabetic rats increased the protein synthetic activity of the PMS, in most cases restoring it completely to normal.

In order to see whether diabetes lowered activity by reducing the extent of chain initiation *in vitro*, incubations were carried out in the presence of the initiation inhibitors poly(I) and ATA. Under these conditions amino acid incorporation is due solely to completion of pre-existing nascent peptides. Poly(I) is thought to bind ribosomes available for initiation, thus preventing them from attaching to translatable messenger RNA's [14], and recent evidence shows that, in the reticulocyte lysate at least, this inhibitor does not affect rates of chain elongation [15]. ATA, on the other hand, acts by preventing attachment of 40 S ribosomal subunits to mRNA [16]. It can be seen from table 1 that in the presence of either inhibitor the reduction in incorporation due to diabetes was almost

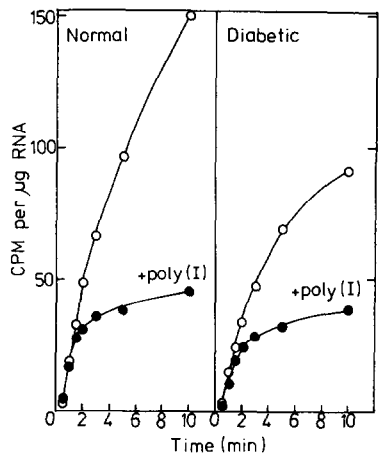

Fig. 2. Time course of incorporation of [^{14}C]leucine into protein in the presence and absence of poly(I) by PMS samples from (a) normal and (b) diabetic rats. Incubations were carried out as described in Materials and methods. Poly(I) was present at a final concentration of 500 µg/ml. (o—o—o) Poly(I) absent; (•—•—•) Poly(I) present.

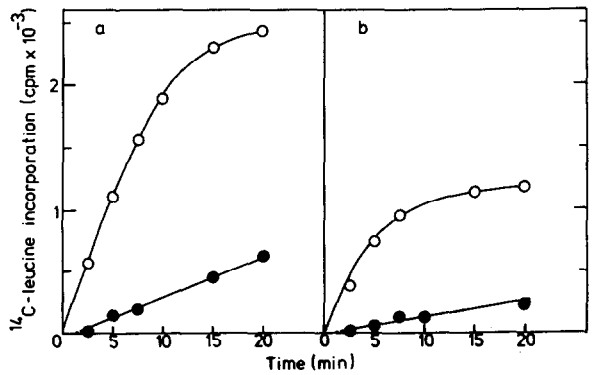

Fig. 3. Time course of incorporation of [^{14}C]leucine into total acid precipitable (o—o—o) peptide and into protein released from the ribosomes (•—•—•). PMS samples were incubated as described in Materials and methods. At appropriate intervals, samples were removed from the incubation mixture and rapidly mixed with an equal volume of 0.8 mM leucine containing 2% Triton X-100 and 200 µg/ml cycloheximide at 0°C. Radioactivity in protein was measured in samples of this mixture before and after removal of ribosomes by centrifugation at 157 000 *g* for 1.5 hr. a) PMS from normal rats; b) PMS from diabetic rats.

abolished. It is not clear why the extent of inhibition with ATA was less than that with Poly(I). Probably ATA was added at a suboptimal concentration, although that used was similar to the maximum found by others to inhibit initiation in this system without affecting elongation as well [12]. It is difficult to compare concentrations of this compound used in different laboratories, as preparations vary in their contamination with impurities. Moreover, the effective concentration of the inhibitor can be influenced by the concentration of protein present [17]. However, it is clear that the results with ATA confirm the conclusion that in this system the effect of diabetes is exerted primarily at the level of chain initiation.

Fig. 1(a) shows the responses of the system to increasing concentrations of Poly(I). At saturating concentrations of the polynucleotide (greater than 400 µg/ml), incorporation by extracts from normal or insulin treated rats was inhibited by 66–70%, and that from diabetic rats by just over 50% in a 10 min incubation. In the absence of new initiation each active ribosome completes the synthesis of half a nascent protein chain on average. Hence, in this time, 1.5 rounds of translation occur in the normal preparation and 1 round in the diabetic, in the absence of poly(I). In exhibiting saturation, poly(I) differs from initiation inhibitors known to affect peptide chain elongation at high concentrations [18, 19]. Fig. 1(b) shows how the difference in activity between extracts from normal and diabetic animals diminished as the concentration of poly(I) was increased. At a saturating concentration of the inhibitor the extent of incorporation by the diabetic samples was at least 90% of the control value and in some experiments was the same as the control.

Fig. 2 shows the time course of incorporation in the PMS system in the presence and absence of poly(I). In the absence of the inhibitor, the rate of incorporation of leucine during the first 2 min of incubation was 0.33 mol/mol ribosomes/min with the normal extract and 0.23 mol/mol ribosomes/min with the diabetic. This is comparable to the rate of protein synthesis reported for this system by Richardson et al. [9]. The inhibitory effect of poly(I) begins to be apparent after 1.5–2 min of incubation, suggesting that the average translation time for the proteins being synthesised is 3–4 min at 37°C, assuming that the ribosomes are evenly spaced along the messenger. There appears to be no difference in this respect between samples from normal and diabetic animals, suggesting that diabetes does not affect the rate of chain elongation in this

system. This suggestion is supported by estimations of the average translation time based on the kinetics of incorporation of radioactivity into total and released peptides, as described by other workers for cell culture [20] and tissue incubation [21] systems (fig. 3). The appearance of radioactivity in protein released from the ribosomes showed a lag of about 1.5 min before linear kinetics were obtained in PMS systems from either normal or diabetic rats, indicating an average translation time of about 3 min in each case. Unlike the situation with whole cells [20, 21], the rate of incorporation into released protein was considerably slower than the total incorporation rate throughout the period of active protein synthesis. It therefore appears that the termination and release process is somewhat impaired in the cell-free incubation system. However, sucrose gradient analysis of the incubation mixture shows that the polysomes disaggregate rapidly [22], indicating that "queueing" of ribosomes awaiting release from messenger RNA does not occur. Presumably nascent peptides remain attached to many ribosomes after they are released from the messenger RNA.

The observation that diabetes does not appear to affect the translation time in the PMS indicates that it has little or no effect on the rate of chain elongation in liver. Since the disease does result in a substantial reduction of protein synthesis in this system, one must conclude, by exclusion, that the effect is mainly on chain initiation. Thus the kinetic measurements confirm the conclusions of the inhibitor studies. Polysome profiles indicate that the rate of initiation relative to elongation is reduced *in vivo*, since the PMS from diabetic rats contains smaller polysomes [22]. Possible mechanisms for the effect on initiation include decreased availability of functional mRNA, an intrinsic defect in ribosomes from diabetic animals, which makes them less able to take part in initiation, or a decrease in availability or activity of initiation factors. Further work is required to distinguish between these alternatives.

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