eIF-2 INITIATION FACTOR ACTIVITY IN POSTRIBOSOMAL SUPERNATANT OF HYPERTROPHYING RAT DIAPHRAGM

Ugo CARRARO and Claudia CATANI

National Research Council Unit for Muscle Biology and Physiopathology, Institute of General Pathology, University of Padova, via Loredan 16, 35100 Padova, Italy

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1. Introduction

Unilateral sectioning of the phrenic nerve causes a transitory hypertrophy of the denervated hemidiaphragm, followed by atrophy [1]. This seemingly unusual manifestation of the loss of innervation is commonly attributed to the chronic, periodic stretching of the denervated fibers by contraction of the still functional contralateral hemidiaphragm [2,3]. During the hypertrophy there is a considerable accumulation of proteins. A significant increase in the rate of protein synthesis was observed in vivo as soon as 1 day after nerve section [4,5]. The enhanced rates of protein synthesis arise at least in part from the extra availability of ribosomes, in fact increased content and synthesis of RNA have been observed in the early stages of hypertrophy [6]; moreover the hypertrophy of denervated diaphragm shows a coordinated enhancement of the activity of polysomes and of the capacity of cell sap to support incorporation of amino acids, although the ribosomes did not show a significantly different polysomal profile [7,8]. On the other hand the rate of disaggregation of the polysomes after in vitro incubation of denervated muscle appear to be slower than that of control preparations [8].

Since a role of initiation factors in the control of ribosome activity has been suggested to explain these results, we have measured in the hypertrophying hemidiaphragm the first step in the initiation of protein synthesis, i.e., the formation of the ternary complex between eukaryotic initiation factor 2, guanosine triphosphate and the aminoacylated form of the methionine-accepting tRNA which functions as the initiator tRNA (eIF-2–GTP–met-tRNA$_f$ complex). This reaction appears to become rate limiting for protein synthesis in heme-deficient reticulocytes and in the presence of low levels of double-stranded RNA or oxidized glutathione. This control operates to a varying extent in different cells, and may be induced or repressed causing increased or decreased peptide chain initiation (reviewed [9]). Reduced activity of eIF-2 has been reported in muscle from starved rats [10].

2. Materials and methods

Left unilateral phrenicotomy was performed under ether anesthesia by removal of a section of nerve using an intercostal or cervical approach on adult male Wister rats (180–200 g body wt).

eIF-2–GTP–met-tRNA$_f$ complex formation in postribosomal supernatant was carried out essentially as in [10]. Hemidiaphragm muscles from 2 or 3 rats were removed from anaesthetized rats, blotted, weighed, pooled and homogenized in 9 vol. solution (2°C) containing 0.25 M KCl, 10 mM Tris (pH 7.4) (at 20°C) and 3 mM dithiothreitol. Following centrifugation of the homogenate for 80 min in a Beckman 75 Ti rotor at 60 000 rev./min, the upper 2/3rds of the supernatant were collected. Ternary complex formation was performed incubating (30°C) 0.02 ml postribosomal supernatant in 0.15 ml total vol. containing 90 mM KCl, 20 mM Tris (pH 7.5), 3 mM dithiothreitol, 1.33 mM GTP, 30 μg bovine serum albumin, 10 μM $CaCl_2$, 14 μM $MgCl_2$ and 330 μM EDTA. $MgCl_2$, $CaCl_2$ and EDTA were added to the incubation mixture to buffer the endogenous divalent metal cations in order to prevent the effect on the ternary complex formation [10] of variable amounts of Mg^{2+}, arising from possible variable contamination

of muscle postribosomal supernatants with extracellular fluids. Reactions were started by the addition of a solution containing 2 pmol [^{35}S]met-tRNA$_f$ (spec. act. 80 000 dpm/pmol). After 15 min the reactions were stopped by addition of 5 ml ice-cold buffer containing 90 mM KCl, 20 mM Tris (pH 7.5), 5 mM $MgCl_2$. This solution was filtered through a Millipore membrane (HAWP, pore size 0.45 μm) pre-soaked under vacuum in the buffer solution. The filters were then washed 3 times with 5 ml same cold buffer, dried and counted in a toluene based scintillator.

The amount of [^{35}S]met-tRNA$_f$ which remained acylated after 15 min incubation was estimated by spotting 5 μl incubation mixture onto filter paper discs and counting the washed discs in a toluene based scintillator [10].

Rabbit liver tRNA, prepared as in [11] was charged using *Escherichia coli* aminoacyl-tRNA synthetases, which charge only the initiator form of eukaryotic met-tRNA [12]. Aminoacyl-tRNA synthetase was purchased from Miles Labs., L-[^{35}S]methionine (spec. act. 1100 Ci/mmol) from The Radiochemical Centre, Amersham.

The RNA content of the tissue was determined as in [13], using yeast tRNA as standard. $E^{1\%}_{1\,cm} = 210$ was used to convert the A_{260} into mg RNA.

3. Results and discussion

Table 1 shows that the left hemidiaphragm weighs less than the right and the hypertrophy after denervation must therefore be expressed as % difference from unoperated left hemidiaphragm [14]. After 1 day and 2 days the weight of denervated tissue had increased by 11% and 18%, respectively.

The RNA content expressed per gram muscle in the diaphragm denervated for 1 day showed a slight increase by comparison with controls, while after 2 days the increase was appreciable and statistically significant. The RNA concentrations shown in table 1 are higher than those in [6]. The overestimation may arise in part from the RNA used as standard (see section 2), and probably from the acid-soluble products of protein released during alkaline treatment, that cause errors in ultraviolet measurement of RNA [13]. On the other hand it is known that diaphragm has a RNA concentration higher than the skeletal muscle [15].

We confirm that the activity of the eIF-2 initiation factor could be estimated in the postribosomal supernatant of the hypertrophying diaphragm as a nitrocellulose filter bound complex with [^{35}S]-met-tRNA$_f$ and GTP. The complex formation required initiator tRNA and GTP, and it was inhibited by aurintricarboxylic acid (ATA). The initiator tRNA could not be replaced by L-[^{35}S]methionine and deacylated tRNA in our assay conditions, while the non-hydrolyzable GTP analog, GMP–P-(CH_2)-P, supported 90% of full activity (see table 2).

The data in table 3 show that 2 days of denervation enhanced binding activity of postribosomal supernatant whether expressed per gram of muscle or

Table 1
Change in weight and RNA content of the left hemidiaphragm during the early stages of its transitory hypertrophic response to denervation

Condition of hemidiaphragm	Muscle weight			Total RNA		
	mg	% Increase		mg/g muscle	% Increase	
Control right	170 ± 3	(56)		3.84 ± 0.18	(15)	
Unoperated left	154 ± 5	(20)		4.01 ± 0.27[c]	(5)	
Denervated left						
1 day	171 ± 5[a]	(14)	+ 11	4.26 ± 0.24[c]	(4)	+ 11
2 days	183 ± 5[b]	(22)	+ 18	4.76 ± 0.37[d]	(6)	+ 24

[a] $p < 0.05$ versus unoperated left by Student's t-test
[b] $p < 0.001$ versus unoperated left
[c] p difference not statistically significant versus control right
[d] $p < 0.02$ versus control right

Each value is the mean ± SEM with the number of observations in parenthesis

Table 2
Characteristics of supernatant-dependent binding of initiator tRNA to filters

Incubation condition	[^{35}S]met-tRNA$_f$ bound	
	cpm/filter	% Inhibition
Complete	683	
+ ATA	120	82
–GTP + GMP–P-(CH_2)-P	494	11
–[^{35}S]met-tRNA$_f$ + L-[^{35}S]methionine	0	100
–[^{35}S]met-tRNA$_f$ + L-[^{35}S]methionine + tRNA	0	100

Postribosomal supernatant was prepared from normal diaphragm. As indicated aurintricarboxylic acid, ATA (60 μM), GMP–P-(CH_2)-P (1.33 mM), L-[^{35}S]methionine (3.24 nM, spec. act. 0.2 μCi/pmole) and rabbit liver tRNA (37 μg) were present from the start of incubation

corrected for total RNA content of the hemidiaphragm. Increased activity in supernatant from denervated hemidiaphragm did not appear to result from decreased dilution of [^{35}S]met-tRNA$_f$ by endogenous initiator tRNA or from diminished rates of deacylation of substrate tRNA. In fact after denervation very little change of free methionine was seen in the hypertrophying diaphragm [16]. Furthermore the determination of eIF-2 activity in postribosomal supernatant was in our conditions absolutely dependent on the addition of pre-charged [^{35}S]met-tRNA$_f$ (see table 2). The RNA content of the postribosomal supernatant was 0.374 ± 0.033 mg/g muscle in control hemidiaphragms and 0.432 ± 0.049 mg/g muscle in 2 days denervated hemidiaphragms (means ± SEM of 7 obs.). Methionine acceptance activity of initiator tRNA, measured as in [12] using a large excess of *Escherichia coli* aminoacyl-tRNA synthetases and limiting amount of tRNA, was 7.05 and 6.61 pmol amino acid/nmol tRNA prepared from control and 2 days denervated postribosomal supernatants. As shown in table 3 the addition of tissue extracts from control and operated diaphragms reduced the amount of [^{35}S]met-tRNA$_f$ which remained acylated during incubation. However the extent of deacylation was similar with each tissue source. Thus the increased concentration of tRNA in postribosomal supernatant of 2 days hypertrophying diaphragm, causing a larger dilution of substrate [^{35}S]met-tRNA$_f$, would reduce the differences between control and denervated preparations. Therefore, the effect of denervation on eIF-2 initiation factor may be even greater than the differences we have observed.

Though the in vivo rate of protein synthesis [4,17] and the concentration of RNA [6] increase within 24 h of denervation, at this time we are not able to see a statistically significant difference in binding activity between control and operated hemidiaphragms (see table 3).

On the other hand, since 2 days after denervation the RNA content of the hypertrophying hemidia-

Table 3
Change in eIF-2 initiator factor and deacylase activities of postribosomal supernatant from the left hemidiaphragm during the early stages of its transitory hypertrophic response to denervation

Condition of hemidiaphragm	eIF-2 activity			Deacylase activity	
	[^{35}S]met-tRNA$_f$ (pmol/g muscle)		% Increase	Acid-insoluble radioactivity after 15 min incubation (% of that present at t = 0)	
Control right	2.52 ± 0.24	(20)		84 ± 2	(16)
Unoperated left	2.33 ± 0.42[a]	(7)		86 ± 3	(6)
Denervated left					
1 day	3.15 ± 0.30[a]	(6)	+ 25	91 ± 3	(6)
2 days	5.55 ± 1.47[b]	(7)	+ 120	85 ± 3	(4)

[a] Difference not statistically significant versus control right
[b] p = 0.01 versus control right

The amount of [^{35}S]met-tRNA$_f$ which remained acylated after 15 min incubation in the absence of postribosomal supernatant was 94 ± 2 (11) % of that present at t = 0. Each value is the mean ± SEM with the no. obs. in parenthesis

phragm had increased by 24% while the eIF-2 activity had more than doubled, such an increase does not reflect merely the enhanced content of ribosomal machinery in the hypertrophying hemidiaphragm. Thus the increased activity of eIF-2 accounts at least in part for the enhancement of protein synthesis in hypertrophying diaphragm but evidently such an increase is not a primary event. The mechanism by which eIF-2 activity is modified during the denervation hypertrophy and the possible role of the other initiation factors as a primary control of protein synthesis remains to be determined.

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References

[1] Sola, O. M. and Martin, A. W. (1953) Am. J. Physiol. 172, 324–332.
[2] Feng, T. P. and Lu, D. X. (1965) Sci. Sin. 14, 1772–1784.
[3] Vandenburg, H. and Kaufman, S. (1979) Science 203, 265–268.
[4] Turner, L. V. and Garlick, P. J. (1974) Biochim. Biophys. Acta 349, 109–113.
[5] Goldberg, A. L. and Dice, J. F. jr (1974) Ann. Rev. Biochem. 43, 835–869.
[6] Manchester, K. L. and Harris, E. J. (1968) Biochemistry J. 108, 177–183.
[7] Turner, L. V. and Manchester, K. L. (1973) Biochim. Biophys. Acta 299, 612–620.
[8] Manchester, K. L. (1974) Biochemistry 13, 3062–3068.
[9] Revel, M. and Groner, Y. (1978) Ann. Rev. Biochem. 47, 1079–1126.
[10] Rannels, D. E., Pegg, A. E. and Rannels, S. R. (1976) Biochem. Biophys. Res. Commun. 72, 1481–1489.
[11] Rogg, H., Wehrly, W. and Staehelin, T. (1969) Biochim. Biophys. Acta 195, 13–15.
[12] Stanley, W. M. jr (1974) Methods Enzymol. 29, 530–546.
[13] Blobel, G. and Potter, V. R. (1968) Biochim. Biophys. Acta 166, 48–57.
[14] Stewart, D. M. (1968) Am. J. Physiol. 214, 1139–1146.
[15] Margreth, A. and Novello, F. (1964) Exp. Cell Res. 35, 38–51.
[16] Turner, L. V. and Manchester, K. L. (1973) Biochim. Biophys. Acta 320, 352–356.
[17] Buse, M., McMaster, J. and Buse, J. (1965) Metabolism 14, 1220–1232.