NOREPINEPHRINE CONTROL OF ENDOGENOUS POLYPEPTIDE PHOSPHORYLATION IN WHITE FAT CELLS

Raymond COUNIS, Sylvie MONGONGU, Michel PIERRE+, Jacques E. LOEB+ and Marian JUTISZ
Laboratoire des Hormones Polypeptidiques, CNRS, 91190 Gif sur Yvette and *+Institut de Recherches Scientifiques sur le Cancer BP no. 8, 94800 Villejuif, France*

Received 11 September 1978
Revised version received 29 September 1978

1. Introduction

Increasing evidence exists that cyclic AMP mediates most hormone effects in many different tissues through regulation of the activity of protein kinases [1–3]. Multiple physiological effects observed in fat cells, including increase in lipid and glycogen metabolism [4,5], result from the hormone-induced change in protein substrate phosphorylation [6–9]. We have attempted to investigate how a single stimulus can result in a multiplicity of effects, by examining the action of norepinephrine on phosphorylation reactions in both intact adipocytes or in adipocyte cell subfractions.

This paper reports characterization in adipocytes of a number of polypeptides phosphorylated consequently to the action of norepinephrine. The phosphorylations can be observed only in conditions where hormone effects were prevented from rapid reversal.

2. Materials and methods

2.1. *Chemicals*

Collagenase (*Clostridium histolyticum*) was obtained from Worthington. [^{32}P]Orthophosphate, carrier free, was purchased from the CEA (Saclay). Nucleotides were supplied by Boehringer (Mannheim) L-(−)-norepinephrine bitartrate was a gift from Boehringer, Badrial Lab. (Reims). X-ray films were from Sakura and from Kodak (RP royal X-O-mat). All other reagents used were A-grade.

Male Wistar rats (CF strain, 160–180 g) were given a standard laboratory diet and free access to water. Fat cells were dissociated from epididymal adipose tissue as in [10] with minor modifications consisting in collagenase concentration (360 U/g adipose tissue) dissociation period (30–40 min) and presence of 20 mM Hepes in Krebs-Ringer buffer (pH 7.4).

2.2. *In situ phosphorylation of endogenous fat cell polypeptides*

Isolated fat cells, first submitted to a 45 min preincubation at 37°C in Krebs-Ringer–Hepes containing 3% bovine serum albumine (BSA), were then centrifuged, washed and transferred in a fresh thermoequilibrated medium consisting of Krebs-Ringer–Hepes–BSA, minus P_i. [^{32}P]Orthophosphate (60 μCi/ml) was added and fat cell suspension was incubated for 15 min at 37°C in order to label intracellular ATP. Aliquots were then treated with norepinephrine (2 μM) or without (controls) and incubation was further carried out for 10 min.

At the end of incubation period, suspensions were centrifuged and cells were washed 3 times at room temperature with a solution containing 0.25 M sucrose, 0.01 M Tris–HCl (pH 7.4) and 1 mM EDTA. In order to prevent hormone-induced phosphorylations from a rapid reversal (see below), stimulated fat cells were washed with the medium containing 2 μM norepinephrine.

Adipocytes, freed from bovine serum albumin and non-incorporated labelled orthophosphate, were resuspended in cold washing buffer containing 10 mM

EDTA and all subsequent steps, including homogenization and subcellular fractionation [11] were performed at 4°C. Under these conditions, both protein kinase and phosphoprotein phosphatase were blocked (not shown). Finally, proteins were estimated as in [12] and precipitated with 10% trichloroacetic acid (TCA).

2.3. *Electrophoretic analysis of polypeptides*

Proteins precipitated with 10% TCA were dissolved in the sample buffer in [13] and dialyzed overnight against the same buffer.

Electrophoresis in presence of dodecylsulfate (SDS) was then performed as in [13] using either 10% or 15% polyacrylamide. Protein staining was achieved with Coomassie blue. Gels were then submitted to 0.5 N $HClO_4$ for 30 min at 90°C [14], washed to remove hydrolyzed products and finally hardened in 10% acetic acid. Autoradiography was performed by applying X-ray film to the dehydrated gel for 3–15 days, and the autoradiographs were then scanned densitometrically. In some cases, gels were counted for radioactivity by scintillation method using dissolved gel slices.

2.4. *Glycerol estimation*

Lipolysis was estimated by measurement of glycerol

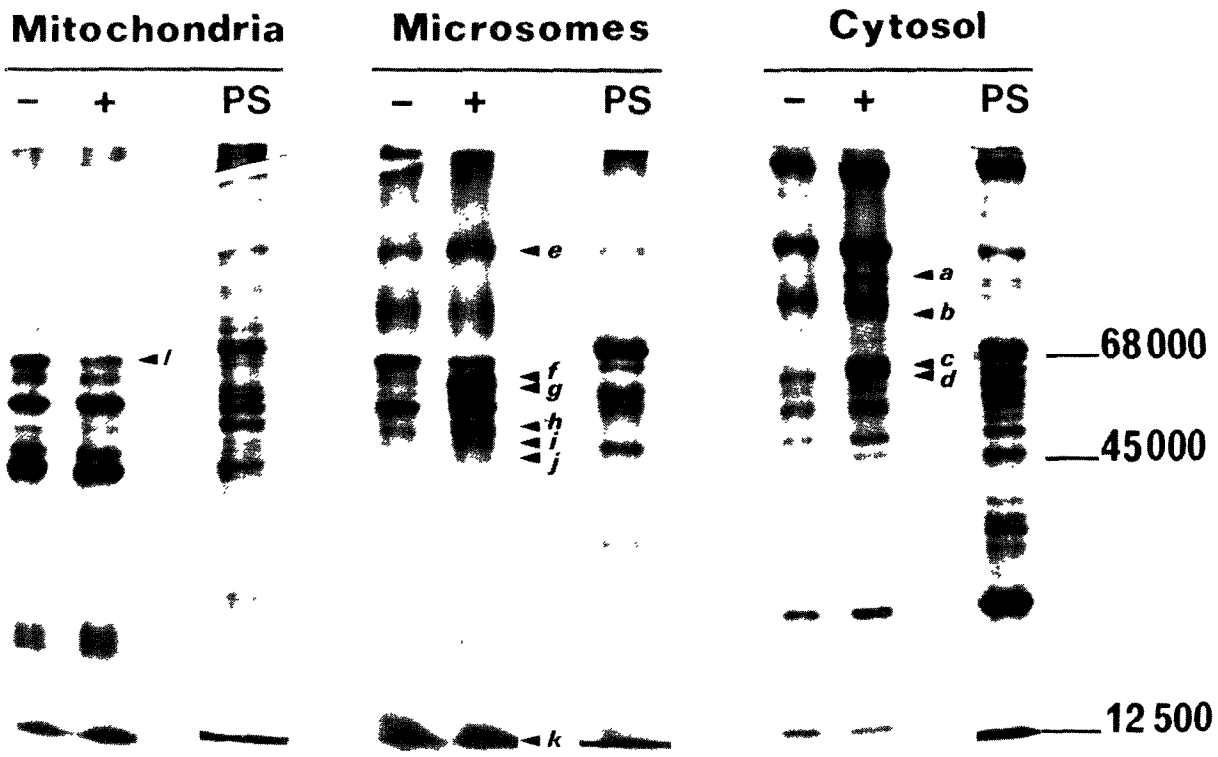

Fig.1. Effect of norepinephrine on phosphorylation of endogenous adipocyte polypeptides in intact cells. Adipocytes were incubated as in section 2. Aliquots containing 40 μg protein of cytosol, microsomes and mitochondria from control (–) and norepinephrine-stimulated (+) cells were subjected to 10% polyacrylamide SDS–gel electrophoresis. A photograph mounting shows side by side autoradiographs and protein staining pattern (PS). Marker proteins were bovine serum albumin (68 000), ovalbumin (45 000) and cytochrome *c* (12 500). Arrows indicate polypeptides where phosphorylation was reproducibly modified by norepinephrine. The apparent molecular weights were determined from a standard curve obtained by using the following marker proteins: RNA polymerase (165 000, 155 000, 39 000), phosphorylase *a* (92 000), bovine serum albumin, ovalbumin, chymotrypsinogen A (25 000), trypsin inhibitor from soybean (21 500) and cytochrome *c*. Results (mol. wt) follow. Cytosol: (*a*) 102 000, (*b*) 81 000, (*c*) 66 000; (*d*) 63 000. Microsomes: (*e*) 124 000; (*f*) 66 000; (*g*) 63 000; (*h*) 49 000; (*i*) 47 000; (*j*) 43 000; (*k*) 12 500. Mitochondria: (*l*) 70 000.

production [15]. Results are expressed as μmol glycerol released/g fat cell lipids [16] as a function of incubation time.

3. Results

Autoradiography following gel electrophoresis allowed visualization of the intracellular polypeptides phosphorylated consequently to the norepinephrine stimulation of intact adipocyte. Thus, the phosphorylation of 10 different polypeptides in mitochondria, microsomes and cytosol reveals to be under hormonal control (fig.1). These polypeptides were distributed through the 3 checked subcellular fractions of adipocytes, 4 in cytosol (designated *a, b, c, d,* with respective mol. wt 102 000, 81 000, 66 000 and 63 000) 7 in microsomes (designated *e, f, g, h, i, j, k,* with respective mol. wt 124 000, 66 000, 63 000, 49 000, 47 000, 43 000 and 12 500) and 1 in mitochondria (designated *l,* with mol. wt 70 000). Only two pairs of phosphorylated substrates were observed both in microsomes and cytosol with identical molecular weights (polypeptides *f* and *c,* mol. wt 66 000; polypeptides *g* and *d,* mol. wt 63 000).

Most of the norepinephrine effects consisted of an increase in polypeptide phosphorylation. However, mitochondria contained a unique mol. wt 70 000 polypeptide (*l*), the phosphorylation of which was significantly decreased (-60%) by norepinephrine.

Removal of norepinephrine by extensive cell washings completely reversed the lipolysis induced with the hormone after a 10 min incubation period (fig.2). Further addition of norepinephrine to these cells, reinitiated their lipolytic response suggesting that the hormone was dissociated from its receptor sites by the successive washings. Figure 3 shows that cessation of hormonal stimulus by removing norepinephrine from the washing medium, resulted in the rapid reversal of hormone-induced phosphorylation of polypeptide substrates. If the initial hormone concentration was maintained in the washing medium, dephosphorylation was prevented. Table 1 shows % change in phosphate incorporation due to norepinephrine stimulation, as compared with the control groups. Reproducibility of the obtained values was largely improved when phosphorylated polypeptides

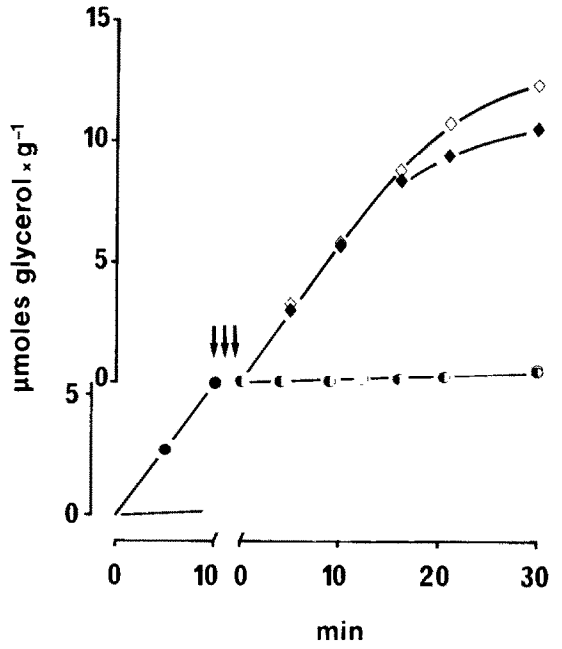

Fig.2. Effect of cell washing on the hormone-induced lipolysis. Adipocytes were incubated 10 min in presence of norepinephrine 2 μM (● - ●) (62 mg cellular lipids/ml Krebs-Ringer–Hepes + 3% BSA). Aliquots were subjected to 3 extensive washings (arrows) at room temperature with the incubation medium without norepinephrine and thereafter, cells were submitted to a further incubation either in presence (◇ - ◇) or not (◐ - ◐) of norepinephrine 2 μM. Controls were run simultaneously, with adipocytes in the presence (◆ - ◆) or absence (○ - ○) of norepinephrine. Experimental protocol:

Symbol	Presence of norepinephrine		
	1st incn. (10 min)	washings	2nd incn.
○ - ○	–	–	–
◆ - ◆	+	+	+
◇ - ◇	+	–	+
◐ - ◐	+	–	–

were prevented from the washing-induced tendency to return to basal values. A unique exception was however observed in a microsomal protein band of mol. wt 124 000 (fig.3, polypeptide *e*) which entirely retained the incorporated phosphate in spite of washing with a hormone-free medium.

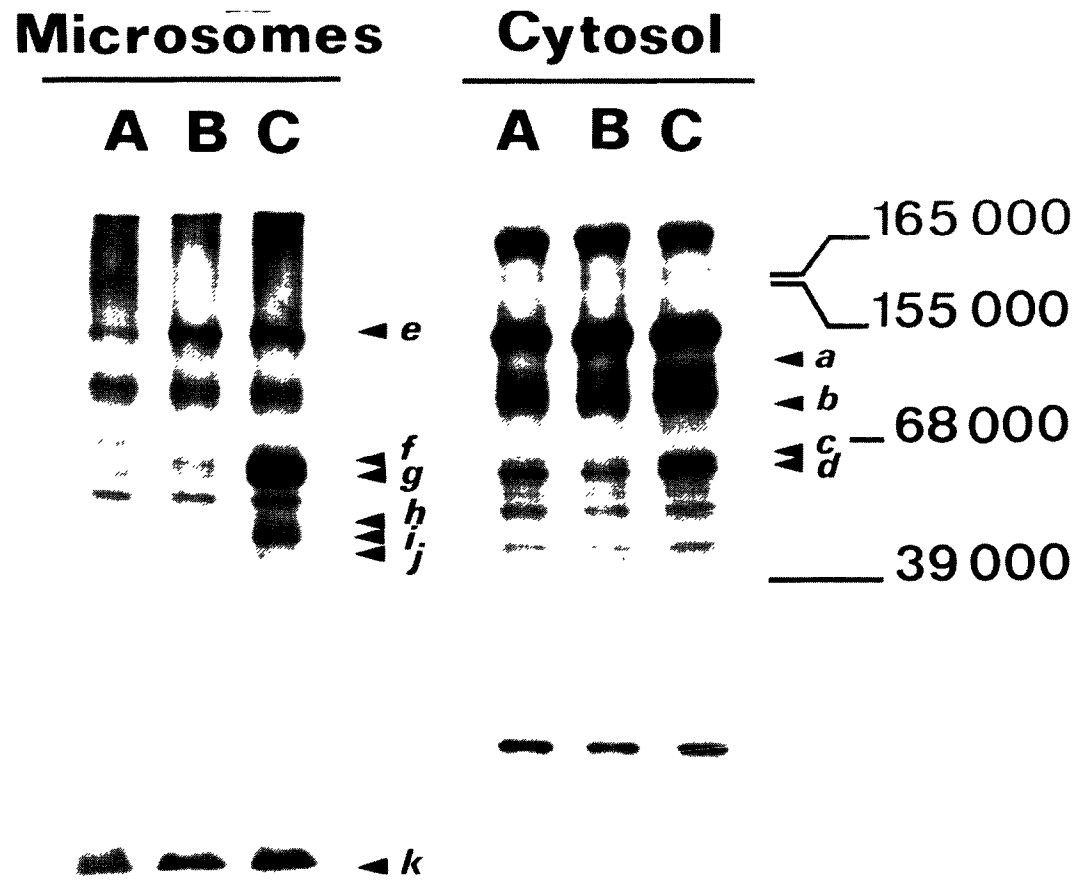

Fig.3. Effect of cell washing on norepinephrine-induced phosphorylation of endogenous adipocyte polypeptides. Adipocytes were preincubated, labelled and finally exposed to norepinephrine or not as in section 2.2. At the end of the stimulation period, aliquots of norepinephrine-stimulated adipocytes were washed either in absence (B) or in presence of 2 μM norepinephrine (C). Controls (A) were incubated and washed in absence of hormone. Subsequent steps were as described in legend of fig.1; only microsome and cytosol fractions were prepared and 40 μg protein of either fraction were analyzed by gel electrophoresis. No difference was seen in protein staining patterns of either A, B, C groups. Protein markers were RNA polymerase from *E. coli* (α-subunit, mol. wt 39 000, β-subunit, mol. wt 155 000, β'-subunit, mol. wt 165 000) and BSA (mol. wt 68 000).

4. Discussion

Ten polypeptides are shown here to be concerned with norepinephrine-controlled phosphorylation in intact cells in either mitochondria, microsomes and cytosol. Comparing these results with [17–19], analogies and discrepancies appeared in either number, nature (molecular weight) or, when determined [19], subcellular location of the polypeptide targets. Methodologies employed for protein analysis, phosphoprotein revealing and, as shown in this study, cell washing, may be a source of alterations in observed phosphorylations. Indeed, successive dilutions of hormone in cell medium resulted in a rapid dephosphorylation of phosphorylated polypeptides. Reversal of norepinephrine-induced phosphorylations was in good agreement with the stoppage of lipolysis (the criterion used for evaluation of hormonal effect), initially due to cessation of adenylate cyclase activation. Cyclic AMP was in fact able to reproduce in cell-free conditions norepinephrine effects obtained in intact adipocytes: ^{32}P incorporation into proteins

Table 1
Effect of norepinephrine on phosphorylation of endogenous polypeptides in intact cells

Phosphopolypeptide (mol. wt × 10^{-3})	% Change in ^{32}P incorporation due to 2 μM norepinephrine		
	Mitochondria	Microsomes	Cytosol
124		120 ± 8[c]	
102			116 ± 5[c]
81			117 ± 11[c]
70	(−) 60 ± 8[b]		
66		207 ± 7[c]	350 ± 18[c]
63			100 ± 6[b]
49		157 ± 7[c]	
47		175 ± 10[c]	
43		175 ± 15[b]	
12.5		52 ± 10[a]	

[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$

Conditions for incubation of fat cells are reported in fig.1 legend. Autoradiographs were scanned densitometrically. Values are means ± SEM of 3 experiments

of microsomes and cytosol prepared from unstimulated adipocytes, using [γ-^{32}P]ATP as substrate for protein kinases, was shown to be stimulated with 5 μM cyclic AMP which increased labeling of polypeptides having identical molecular weights as in the case of intact cells in the presence of norepinephrine.

Thus versatile adaptability is ensured by high turnover of both cyclic AMP (through adenylate cyclase and phosphodiesterase activities) and protein substrate phosphorylations (through cyclic AMP-dependent protein kinase and phosphoprotein phosphatase activities).

However, the mol. wt 124 000 polypeptide band (polypeptide *e*) present in microsomal fraction, which was phosphorylated via norepinephrine-induced mechanism, appeared not to undergo dephosphorylation after cessation of cell stimulation. This fact suggests the existence of at least one delayed action of norepinephrine in the adipocyte. We only localized the mol. wt 124 000 phosphopolypeptide *e* in the microsomal fraction as a result of hormone stimulation of intact cells, and not in other fractions. Further investigation showed that this phosphopolypeptide was neither an integral constituent of microsomal membranes (detergent action, not shown), nor a structural ribosomal protein due to its high molecular weight [20,21]. This phosphopolypeptide, probably tightly associated with ribosomes, may be in our opinion, rather concerned with the regulation of protein synthesis. This hypothesis has been reinforced [22,23].

The nature of the endogenous substrates thus remains at present unknown in absence of additional experiments. Disregarding other effects, there is evidence that lipolytic hormones modulate the activity of a number of enzymes in white fat cells including triglyceride lipase, phosphorylase kinase, phosphorylase, glycogen synthetase [5,6] and probably also the enzymes of the lipogenic pathway [9,24]. Conceivably the hormone-controlled phosphopolypeptides observed in the present study should be in close relation with the latter enzyme systems. However, identification of substrates through their molecular weights (or size) is only indicative, and, for the time being, no other information is available as to their nature.

Acknowledgement

The experimental work described in this paper was supported by a grant from the Centre National de la Recherche Scientifique (ATP no. 2398).

References

[1] Kuo, J. F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. USA 64, 1349–1355.
[2] Langan, T. A. (1973) Adv. Cyclic Nucl. Res. 3, 99–153.
[3] Walsh, D. A. and Ashby, C. D. (1973) Recent Prog. Horm. Res. 29, 329–359.
[4] Vaughan, M. and Steinberg, D (1965) in: Handbook of Physiology, sec. 5: Adipose Tissue (Renold, A. E. and Cahill, G. H. eds) pp. 335–347, Am. Physiol. Soc. Washington DC.
[5] Steinberg, D. (1976) Adv. Cyclic. Nucl. Res. 7, 157–198.
[6] Rubin, C. S. and Rosen, O. M. (1975) Ann. Rev. Biochem. 44, 831–887.
[7] Huttunen, J. K., Steinberg, D. and Mayer, S. E. (1970) Biochem. Biophys. Res. Commun. 41, 1350–1356.
[8] Huttunen, J. K. and Steinberg, D. (1971) Biochim. Biophys. Acta 239, 411–427.
[9] Killilea, S. D., Brandt, H. and Lee, E. Y. C. (1976) Trends Biochem. Sci. 1, 30–33.
[10] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[11] Angel, A. and Sheldon, H. (1965) Ann. NY Acad. Sci. 131, 157–168.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Laemmli, U. K. and Favre, H. (1973) J. Mol. Biol. 80, 575–599.
[14] Schneider, W. C. (1945) J. Biol. Chem. 161, 293–303.
[15] Wieland, O. (1957) Biochem. Z. 329, 313–319.
[16] Dole, V. P. and Meinertz, H. (1960) J. Biol. Chem. 235, 2595–2609.
[17] Benjamin, B. and Singer, I. (1975) Biochemistry 14, 3301–3309.
[18] Forn, J. and Greengard, P. (1976) Arch. Biochem. Biophys. 176, 721–733.
[19] Avruch, J., Leone, G. R. and Martin, D. (1976) J. Biol. Chem. 251, 1511–1515.
[20] Loeb, J. E. and Blat, C. (1970) FEBS Lett. 10, 105–108.
[21] Cawthon, M. L., Bitte, L. F., Krystosek, A. and Kabat, D. (1973) J. Biol. Chem. 249, 275–278.
[22] Meyuhas, O., Reshef, L., Gunn, J. M., Hanson, R. W. and Ballard, F. J. (1976) Biochem. J. 158, 1–7.
[23] Benne, R., Edman, J., Traut, R. R. and Hershey, J. W. B. (1978) Proc. Natl. Acad. Sci. USA 75, 108–112.
[24] Correze, C., Nunez, J. and Gordon, A. (1977) Mol. Cell Endocrinol. 9, 133–144.