The inactivation of rat adipocyte Mg^{2+} -dependent phosphatidate phosphohydrolase by noradrenaline

Christopher H. K. CHENG* and E. David SAGGERSON
Department of Biochemistry, University College London, Gower Street,
London WC1E 6BT, U.K.

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The inactivation of rat adipocyte Mg^{2+} -dependent phosphatidate phosphohydrolase by noradrenaline [Cheng & Saggerson (1978) *FEBS Lett.* **87**, 65–68; Cheng & Saggerson (1978) *FEBS Lett.* **93**, 120–124] persists for at least 40 min in crude defatted homogenates kept at 0°C or 20°C, but is diminished at 37°C. The effect of noradrenaline persists through the isolation of post-105 000 g supernatants and is then stable in these preparations at 0°C and 37°C. Inclusion of albumin (10–20 mg/ml) in homogenization buffers abolishes the effect of noradrenaline. The effect of noradrenaline is not removed by dialysis of extracts or by raising the concentrations of Mg^{2+} or phosphatidate in assays.

When rat adipocytes are incubated with noradrenaline followed by freeze-stopping, homogenization and rapid assay, it is found that phosphatidate phosphohydrolase activity is decreased (Cheng & Saggerson, 1978*a,b*). This effect is confined to the Mg^{2+} -dependent form of the enzyme. At present the mechanism underlying this hormonal effect is unknown.

In the present study it is shown that inactivation of the enzyme resulting from noradrenaline treatment of the cells is persistent and stable under certain circumstances and can still be observed when the enzyme is assayed in post-105 000 g supernatants. Some aspects of the kinetics of the noradrenaline-inactivated enzyme have been investigated and attempts have been made to reverse this inactivation.

Materials and methods

Animals and chemicals were obtained and treated as described by Cheng & Saggerson (1978*a*).

Adipocytes were obtained by collagenase treatment of rat epididymal adipose tissue pieces by the method of Rodbell (1964) with minor modifications (Saggerson & Tomassi, 1971).

Adipocytes equivalent to one-half of a fat-pad were incubated in 4 ml of Krebs–Ringer bicarbonate (Krebs & Henseleit, 1932) containing fatty-

acid-poor albumin (40 mg/ml) and 5 mM-glucose for 30 min as described by Cheng & Saggerson (1978*a*). The cells were recovered by centrifugation, freeze-stopped in liquid N_2 , subjected to Ultra-Turrax homogenization in ice-cold 0.25 M-sucrose medium containing 10 mM-Tris/HCl buffer, pH 7.4, 1 mM-EDTA and 1 mM-dithiothreitol, and the resultant extracts were briefly centrifuged to obtain defatted homogenates as described by Cheng & Saggerson (1978*a*). Alternatively, adipocytes equivalent to four and a half fat-pads were incubated in 36 ml of the same Krebs–Ringer bicarbonate-based medium for 30 min, followed by centrifugation for approx. 15 s at 200 g_{av} . The resulting infranatant was removed by aspiration and the cells were transferred to 9 ml of ice-cold 0.25 M-sucrose medium containing 10 mM-Tris/HCl buffer, pH 7.4, 1 mM-EDTA and 1 mM-dithiothreitol, in a glass tube. The cells were broken by swirling on a vortex mixer for 1 min (Martin & Denton, 1970) followed by centrifugation at 3000 g_{av} for 1 min in a Sorvall RC2B centrifuge at 2–4°C. The infranatant below the resulting fat-layer was re-centrifuged at 105 000 g_{av} for 1 h. The resulting clear supernatant (avoiding the small amount of fat floating on the surface) was collected and used for enzyme assays. Phosphatidate phosphohydrolase (EC 3.1.3.4) was assayed with 1.4 mM-sodium phosphatidate by the method of Jamdar & Fallon (1973) as described previously (Cheng & Saggerson, 1978*a*). Total phosphatidate phosphohydrolase activity was measured with 2.5 mM- $MgCl_2$ present and Mg^{2+} -dependent phos-

* Present address: Hormone Research Laboratory, University of California, San Francisco, CA 94143, U.S.A.

phatidate phosphohydrolase activity was calculated by subtraction from this of the small amount of Mg^{2+} -independent phosphatidate phosphohydrolase measured in the absence of $MgCl_2$. Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Cheng & Saggerson (1978a). Lactate dehydrogenase activity was not affected by treatment of adipocytes with noradrenaline and therefore was used as a reference enzyme to correct for any variability in the recovery of adipocytes from incubations or any incompleteness in the breakage of cells on homogenization. Phosphatidate phosphohydrolase activities are expressed as phosphatidate phosphohydrolase/lactate dehydrogenase activity ratios, where phosphatidate phosphohydrolase activity is measured as nmol of P_i released/min and lactate dehydrogenase activity as μ mol of NADH oxidized/min.

Results and discussion

Persistence of inactivation of phosphatidate phosphohydrolase by noradrenaline

As found previously (Cheng & Saggerson, 1978a,b), exposure of adipocytes to noradrenaline for a relatively brief period (30 min in this case) resulted in a substantial decrease in Mg^{2+} -dependent phosphatidate phosphohydrolase activity measured in crude extracts obtained after freeze-stopping and disrupting the cells with an Ultra-Turrax homogenizer (Fig. 1). Standing these homogenates in ice for up to 40 min resulted in no alteration in phosphatidate phosphohydrolase activity, the noradrenaline effect being stable under these conditions (results not shown). However, phosphatidate phosphohydrolase activity rapidly decreased at 37°C in extracts from untreated cells (Fig. 1). This loss of activity at 37°C was less apparent in extracts from noradrenaline-treated cells and therefore the effect of noradrenaline was diminished. In other experiments (results not shown) it was found that phosphatidate phosphohydrolase activities in extracts from either control or noradrenaline-treated cells were stable at 20°C for up to 40 min. These findings suggest that the temperature-sensitive generation of some inhibitory factor(s) occurs in crude homogenates. Furthermore, it is possible, though by no means certain, that unincubated extracts from noradrenaline-treated cells already contain such factor(s).

When adipocytes were incubated, followed by relatively gentle disruption and then centrifugal fractionation, the inactivation of Mg^{2+} -dependent phosphatidate phosphohydrolase brought about by noradrenaline treatment was still apparent when the enzyme was assayed in post-105 000g supernatants. In these extracts phosphatidate phosphohydrolase activities, and hence the noradrenaline

Fig. 1. Stability of Mg^{2+} -dependent phosphatidate phosphohydrolase activity in Ultra-Turrax homogenates. Adipocytes were incubated with (●) or without (○) $0.5 \mu M$ noradrenaline for 30 min, freeze-stopped, homogenized and briefly centrifuged as described in the Materials and methods section. Before enzyme assays, extracts were incubated for the indicated times at 37°C. Values are means for two separate experiments. The bars indicate the range of values.

effect, were stable at both 0°C and 37°C (results not shown).

These findings imply that the inhibitory factor(s) generated in crude extracts originate either from particulate cell components or from small fat-droplets that may not be completely removed by brief centrifugation of the homogenates.

The effect of albumin

When fatty-acid-poor albumin was incorporated into homogenization media (Fig. 2), Mg^{2+} -dependent phosphatidate phosphohydrolase activity in defatted homogenates from control cells was decreased ($P < 0.05$ with 10 and 15 mg of albumin/ml and $P < 0.02$ with 20 mg of albumin/ml). On the other hand, albumin increased phosphatidate phosphohydrolase activity in extracts from noradrenaline-treated cells. At albumin concentrations of 10–20 mg/ml in homogenization media, the effect of noradrenaline was abolished. Since in all experiments 50 μ l portions of tissue extracts were taken for assay of phosphatidate phosphohydrolase in final volumes of 0.5 ml, carry-over of albumin into the assay did not exceed 2 mg/ml. Inclusion of albumin up to this concentration in assays of defatted

Fig. 2. Effect of albumin in homogenization media on Mg^{2+} -dependent phosphatidate phosphohydrolase activity in Ultra-Turrax homogenates

Adipocytes were incubated with (●) or without (○) $0.5 \mu M$ -noradrenaline for 30 min, freeze-stopped, homogenized in $0.25 M$ -sucrose containing $10 mM$ -Tris/HCl buffer (pH 7.4), $1 mM$ -EDTA, $1 mM$ -dithiothreitol and the indicated concentration of fatty-acid-poor albumin and then briefly centrifuged as described in the Materials and methods section. The values are means for three separate experiments. The bars indicate $\pm$ S.E.M.

homogenates prepared in albumin-free homogenization media did not appreciably alter the assay of phosphatidate phosphohydrolase activity or abolish the persistent effect of $0.5 \mu M$ -noradrenaline (results not shown). Similarly albumin up to $2 mg/ml$ did not appreciably affect the assay of phosphatidate phosphohydrolase in post- $105\,000g$ supernatants. However, addition of albumin at $20 mg/ml$ to the assays decreased the phosphatidate phosphohydrolase/lactate dehydrogenase activity ratio from 35.7 to 14.5 in extracts from control cells and from 16.2 to 8.5 in extracts from $0.5 \mu M$ -noradrenaline-treated cells. Moller *et al.* (1977) have also observed inhibition of adipocyte Mg^{2+} -dependent phosphatidate phosphohydrolase on addition of albumin to the assays. In other experiments (results not shown) it was found that breakage of cells in media containing albumin at $20 mg/ml$ followed by preparation of post- $105\,000g$ supernatants resulted in lower phosphatidate phosphohydrolase activities than in samples prepared in parallel in the absence of albumin. With albumin present no difference in phosphatidate phosphohydrolase activity could be seen between extracts from control and $0.5 \mu M$ -noradrenaline-treated cells. However, the stability of phosphatidate phosphohydrolase normally observed in high-speed supernatants at $37^\circ C$ was lost when albumin was present

and decreases in phosphatidate phosphohydrolase activity were observed in both control and noradrenaline-treated extracts. It is concluded that the presence of relatively low concentrations of albumin during the preparations of adipocyte extracts can effectively abolish the effect of noradrenaline on phosphatidate phosphohydrolase activity. It is possible that albumin may bind some inhibitory factor(s) present in the noradrenaline-treated cells. To exert these effects albumin must be present early in the preparation of the extracts. These experiments are complicated by the fact that albumin has an inhibitory effect on phosphatidate phosphohydrolase, which cannot be explained in terms of direct inhibition of the phosphatidate phosphohydrolase assay.

The effect of dialysis

Dialysis against the normal homogenization medium or against this medium containing fatty-acid-poor albumin ($20 mg/ml$) did not reactivate phosphatidate phosphohydrolase in high-speed supernatants from $0.5 \mu M$ -noradrenaline-treated cells (two experiments, results not shown).

The effect of mixing extracts from control and noradrenaline-treated cells

Post- $105\,000g$ supernatants were prepared from control and from $0.5 \mu M$ -noradrenaline-treated cells, mixed in various proportions (ranging from three parts noradrenaline-treated cells + one part control cells to three parts control cells + one part noradrenaline-treated cells) and kept at $0-4^\circ C$ for 30 min before assay of Mg^{2+} -dependent phosphatidate phosphohydrolase. The resultant activities in these mixtures were not appreciably different from those predicted by summing the appropriate proportions of the two contributing activities. These findings appear to show that noradrenaline-treated samples do not contain any free factor(s) that can decrease the control phosphatidate phosphohydrolase activity. Alternatively control samples do not contain any factor(s) capable of restoring the noradrenaline-inactivated activity. The same results were obtained when post- $105\,000g$ supernatants were mixed and incubated for up to 40 min at $37^\circ C$ or when defatted homogenates of control and noradrenaline-treated cells were mixed and kept at $0^\circ C$ or $20^\circ C$ for up to 40 min.

Effect of noradrenaline treatment on Mg^{2+} and phosphatidate dependence

Pretreatment of cells with $0.5 \mu M$ -noradrenaline resulted in Mg^{2+} -dependent phosphatidate phosphohydrolase activities (at $2.5 mM$ - Mg^{2+}) in post- $105\,000g$ supernatants that were lower than the control activity by approx. 50% at all tested concentrations of phosphatidate ($0.05-2.0 mM$). Since

Mg²⁺-independent phosphatidate phosphohydrolase activity is not affected by noradrenaline (Cheng & Saggerson, 1978a), the effect of noradrenaline on total phosphatidate phosphohydrolase was less apparent at very low Mg²⁺ concentrations. However, above 1.5 mM-Mg²⁺, the effect of noradrenaline was essentially the same at all tested concentrations of Mg²⁺ up to 6.5 mM (results of two experiments). There was no tendency for the noradrenaline effect to be diminished when phosphatidate or Mg²⁺ were increased to concentrations higher than those normally used (i.e. 2.5 mM-Mg²⁺ and 1.4 mM-phosphatidate).

General discussion

A relatively rapid effect of noradrenaline on the adipocyte leading to inactivation of Mg²⁺-dependent phosphatidate phosphohydrolase is clearly established both in the present study and in previous studies (Cheng & Saggerson, 1978a,b). The two most likely ways of achieving this modulation of activity would seem to be either through a covalent modification type of mechanism or by tight binding of some (presumably hydrophobic) regulatory ligand(s) to the enzyme. Such factor(s) might arise as a result of the lipolytic action of noradrenaline. Fatty acids added to incubations of adipocytes are not particularly effective in mimicking the action of noradrenaline (Cheng & Saggerson, 1978b), but other hydrophobic ligands could be involved. The finding that the noradrenaline effect is abolished simply by inclusion of albumin in homogenization media argues against a covalent modification type of mechanism. It is presumed that albumin acts either to remove inhibitory factor(s) from the enzyme or to prevent their interaction with the enzyme taking place. The latter possibility might appear more likely since subsequent addition of albumin to assays does not abolish the noradrenaline effect. However, it is possible that albumin is ineffective in this respect under the conditions of the assay. At present it is not

known whether the enzyme is already in contact with inhibitory factor(s) in the intact noradrenaline-treated adipocyte or whether these are only brought together by the act of homogenization. In this respect it is interesting that equally large persistent effects of noradrenaline are seen when vigorous homogenization with an Ultraturrax homogenizer is employed or when the cells are disrupted gently. If the interaction of the enzyme with inhibitory factor(s) is solely an artefact occurring on cell breakage, then phosphatidate phosphohydrolase activity may be a faithful indicator of the cell content of such factor(s). If, on the other hand, the effect of noradrenaline reflects a genuine physiological event in the intact cell, it is necessary to establish conditions for reversal of this effect once it has occurred. These possibilities require further work.

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References

- Cheng, C. H. K. & Saggerson, E. D. (1978a) *FEBS Lett.* **87**, 65–68
- Cheng, C. H. K. & Saggerson, E. D. (1978b) *FEBS Lett.* **93**, 120–124
- Jamdar, S. C. & Fallon, H. J. (1973) *J. Lipid Res.* **14**, 517–524
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Martin, B. R. & Denton, R. M. (1970) *Biochem. J.* **117**, 861–877
- Moller, F., Green, P. & Harkness, E. J. (1977) *Biochim. Biophys. Acta* **486**, 359–368
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
- Saggerson, E. D. & Tomassi, G. (1971) *Eur. J. Biochem.* **23**, 109–117