REGULATION OF ADIPOSE TISSUE LIPOLYSIS: PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE IN INTACT RAT ADIPOCYTES

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1. Introduction

The rate-limiting step in the hydrolysis of adipose tissue triacylglycerols is catalyzed by the hormone-sensitive lipase [1]. It has been suggested that the activity of this enzyme is regulated by its phosphorylation, mediated by hormonal alterations of the intracellular concentration of cyclic AMP [2]. Although likely to be correct, this hypothesis has been based on rather indirect experimental evidence. It is, however, supported by our recent results: the identification of the hormone-sensitive lipase protein and the demonstration that the enzyme can be phosphorylated, and activated, in vitro with a cyclic AMP-dependent protein kinase [3,4]. These findings are not necessarily relevant for conditions in the living cell. To assert the physiological relevance of the phosphorylation of the isolated enzyme its phosphorylation must be demonstrated also in the intact adipocyte, i.e., under in vivo conditions.

We are studying mechanisms for the hormonal regulation of hormone-sensitive lipase, at the cellular and at the molecular level. Here we have incubated intact rat adipocytes with $^{32}P_i$ under conditions giving maximal rate of lipolysis (noradrenaline stimulation). Using a recently developed procedure for extensive purification of hormone-sensitive lipase at a preparative scale [5] we have isolated the enzyme from the adipocytes and shown that it is phosphorylated. In [6] we describe how exposure of the adipocytes to hormones alters the extent of phosphorylation of the enzyme.

Abbreviations: cyclic AMP, cyclic 3′,5′-adenosinemonophosphate; FFA, free fatty acids; Hepes, 2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethane sulfonic acid; PCV, packed cell volume; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis

2. Methods

2.1. *Adipocyte preparation, incubation with $^{32}P_i$ and monitoring FFA release*

Intact adipocytes (male Sprague-Dawley rats, 120–140 g) were prepared by collagenase digestion [7,8], amounts of cells given as PCV and their viability evaluated [7,8]. Cells from 25–35 rats were incubated in 25 ml Krebs-Ringer buffer with lower phosphate concentration (50 μM), containing 20 mM Hepes, 3.5% (w/v) bovine serum albumin and 5 mM glucose at pH 7.40 and 37°C under O_2 in a pH-stat titration apparatus used for continuous monitoring of FFA release [8]. Carrier-free [^{32}P]orthophosphate (The Radiochemical Centre, Amersham) was added to 125 μCi/ml final conc. After 40 min incubation noradrenaline (1 μg/ml final conc.) was added for an additional 5 min. FFA release rate increased 25-fold within 2 min of the addition of the hormone, showing the marked stimulation of hormone-sensitive lipase activity (cf. [6,8]).

2.2. *Isolation of hormone-sensitive lipase*

At the end of the incubation the adipocytes were separated from the bulk of $^{32}P_i$, within 5 min, by floating and resuspending twice in suspension medium containing: 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 2 mM ATP, and 0.5 μg/ml noradrenaline at 10°C. NaF was not added, since the partition of the enzyme between the floating fat and 110 000 × *g* supernatant (see below) was very sensitive to increased ionic strength [5], and it strongly

inhibited enzyme activity [3]. The cells were homogenized in 3 vol. ice-cold suspension medium in a Potter-Elvehjem type glass homogenizer. The fat was floated (1500 rev./min × 1 min), homogenized again in 1.5 vol. fresh suspension medium and the combined homogenates centrifuged 110 000 × *g* for 45 min at 4°C. The supernatant below the fat cake was removed by aspiration and filtered through glass-wool.

The hormone-sensitive lipase was isolated as will be detailed in [5]. In brief: the 110 000 × *g* supernatant was brought to pH 5.2 with 0.2 M acetic acid, the precipitate formed collected by centrifugation and suspended in 2.0 ml 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM dithioerythritol (pH 5.2, precipitate fraction). The hormone-sensitive lipase was solubilized in nonionic detergent, $C_{13}E_{12}$* and subjected to gradient sievorptive chromatography on QAE–Sephadex (Pharmacia, Uppsala). The pooled enzyme peak fractions were concentrated, and the detergent exchanged for C_8E_6 by chromatography on spheroidal hydroxylapatite (BDH) followed by adsorption chromatography on Ultrogel AcA 34 (LKB). Details will be given in the figure legends.

Hormone-sensitive lipase purified from epididymal fat pads was [^{32}P]phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase, purified from the same tissue, by incubation for 30 min at 37°C in 0.1 M Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithioerythritol and 0.1 mM [γ-^{32}P]-ATP [5].

2.3. *Analytical procedures*

ATP-citrate lyase activity was measured by the malate dehydrogenase-coupled procedure [9]. [^{32}P]Protein samples were precipitated and washed with trichloroacetic acid, extracted with diethyl-ether–ethanol [10] and dissolved with SDS under reducing conditions by sonication and heating [6]. Samples from adipocyte suspensions were first delipidated [6]. SDS–PAGE was according to [11] with modifications [6] in 14 cm slabgels with 8% acrylamide using phosphorylase *a* from rabbit muscle (94 000 daltons), human transferrin (76 700 daltons), bovine serum albumin (67 000 daltons), catalase from bovine liver (60 000 daltons), ovalbumin (43 000 daltons) and lactate dehydrogenase from rabbit muscle (34 000 daltons) as molecular weight markers. After kenacid blue (BDH) staining, gels were dried and autoradiographed on Osray M 3 films (Agfa-Gevaert). [^{32}P]Protein bands were quantitated by scanning densitometry at 633 nm (soft laser densitometer, model SL 504, Biomed Instr., Chicago, IL) of autoradiographs and calculation of relative peak areas. Protein was determined by a scaled-down version [12] of the Lowry method [13].

* The nonionic detergents used were polydisperse preparations of alkyl polyoxyethylene glycol. They are designated C_xE_y, where x = average number of alkyl chain carbons and y = oxyethylene units

3. Results

3.1. *Experimental outline*

The experiment was designed to isolate hormone-sensitive lipase from protein extracts of adipocytes, incubated with $^{32}P_i$, by a procedure developed for preparative-scale purification of the enzyme from adipose tissue [5], which results in an enzyme of >50% protein purity by SDS–PAGE (app. mol. wt 84 000) [5]. The presence of $^{32}P_i$ in enzyme, thus isolated from adipocytes, has been investigated for evidence for the phosphorylation of hormone-sensitive lipase in intact cells. Six similar experiments have been performed with the same general results.

3.2. *Initial fractionation of adipocyte proteins, precipitation of the lipase at pH 5.2 and gradient sievorptive chromatography on QAE–Sephadex*

The fat-depleted 110 000 × *g* supernatant contained part of the hormone-sensitive lipase from the adipocyte homogenate. Practically no enzyme was sedimented but part of it remained bound to the floated fat as indicated by pH-stat titration (presence of endogenous lipids made enzyme determination with radiolabeled substrate [14] impossible). The lipase activity of the 110 000 × *g* supernatant was almost quantitatively recovered in the pH 5.2 precipitate fraction with 1.5-fold increase of specific activity. Adipocyte protein extracts contained a large number of [^{32}P]phosphopeptides resolved by SDS–PAGE (fig.1, insert a). Most of these [^{32}P]phosphopeptides were also found in the pH 5.2 precipitate fraction (fig.1, insert b). A small 84 000 dalton [^{32}P]phosphopeptide band was present in both fractions. It was also found in samples from the isolated floating fat layer, but not from the 110 000 × *g* pellet and pH 5.2 supernatant (not illustrated).

Further fractionation of the hormone-sensitive

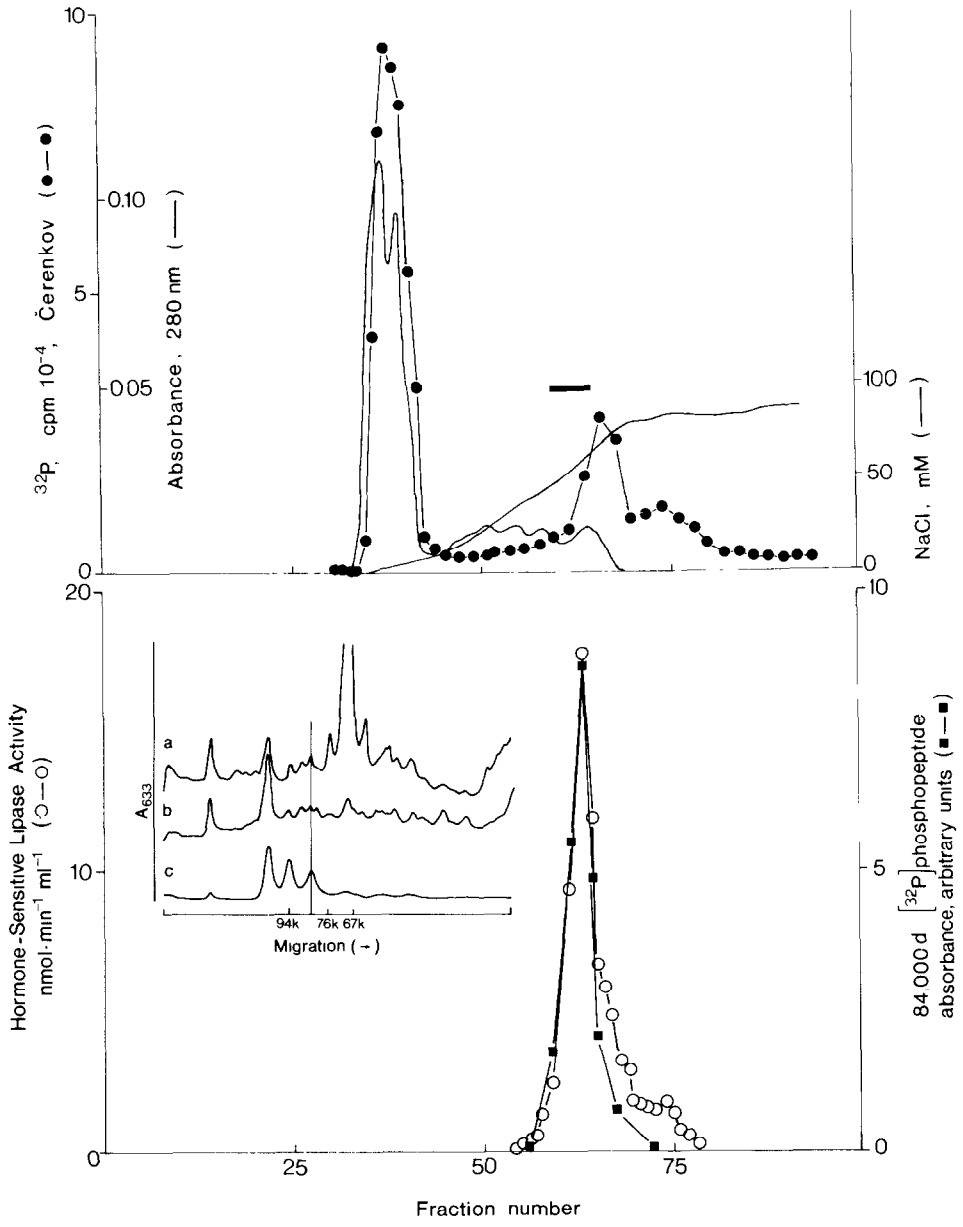

Fig.1. Gradient sievorptive chromatography on QAE–Sephadex of detergent-solubilized (pH 5.2) precipitate fraction. A QAE–Sephadex A-25 column (2.6 × 91 cm) was equilibrated at pH 8.05, 10°C in 40 mM Tris–HCl containing 20% (w/v) glycerol, 0.2 mM EDTA, 1 mM dithioerythritol and 0.2% $C_{13}E_{12}$ detergent. A 32 ml 0–85 mM NaCl linear gradient was applied immediately before the sample: 4.0 ml detergent-solubilized (pH 5.2) precipitate fraction obtained from 6.2 ml PCV of fat cells from 25 rats, containing 85 mM NaCl, 13% sucrose, 1 mM EDTA, 1 mM dithioerythritol and 6.3% $C_{13}E_{12}$. The column was eluted with 85 mM NaCl in the equilibrating buffer at 10°C at a flow-rate of 5.0 ml/h . cm^2. Fractions of (4.3 ml) were collected. Hormone-sensitive lipase activity was measured at 37°C with emulsified 1(3)-oleoyl-2-oleylglycerol as substrate [14]. [NaCl] and A_{280} were continuously monitored. The bar indicates the fractions pooled. ^{32}P radioactivity was measured as total Cerenkov radiation in the column fractions. [^{32}P]Phosphopeptide of 84 000 daltons was quantitated by scanning densitometry of autoradiographs from SDS–PAGE and calculation of the peak areas.

Insert: densitometric traces of autoradiographs of SDS–PAGE of: (a) adipocyte suspension, (b) pH 5.2 precipitate fraction; (c) pooled enzyme fractions from QAE–Sephadex chromatography. Molecular weight of marker proteins as indicated, $k = 10^3$. Migration position of 84 000 dalton [^{32}P]phosphopeptide indicated by vertical line.

lipase with gradient sievorptive chromatography on QAE–Sephadex gave an additional 15-fold purification (from 0.095–1.45 μmol fatty acid produced/min and mg protein) with 26% yield in the pooled fractions (51% total yield) (fig.1). The enzyme activity was eluted in the ascending slope of a ^{32}P radioactivity peak, which contained 3 major [^{32}P]phosphopeptides (fig.1, insert c). The two larger [^{32}P]phosphopeptides, 125 000 daltons and 94 000 daltons did not copurify with hormone-sensitive lipase activity. The 125 000 daltons [^{32}P]phosphopeptide was identified as ATP-citrate lyase (EC 4.1.3.8) by its apparent molecular weight [15,16] and enzyme activity (not illustrated). The 94 000 dalton [^{32}P]phosphopeptide had the same mobility (SDS–PAGE) as glycogen phosphorylase. The third [^{32}P]phosphopeptide, with app. mol. wt 84 000, closely paralleled hormone-sensitive lipase activity in consecutive column fractions over the enzyme activity peak (fig.1, bottom).

3.3. *Hydroxylapatite chromatography and adsorption chromatography on Ultrogel AcA 34*

Hydroxylapatite chromatography gave 8-fold concentrated enzyme with an increase of specific activity from 1.45–2.5 μmol fatty acid/min and mg protein. The 3 [^{32}P]phosphopeptides from the QAE–Sephadex chromatography were obtained in the pooled enzyme peak fractions from this step with the 84 000 dalton [^{32}P]phosphopeptide slightly enrichened (fig.2B,b). It was necessary to change the detergent at this stage for the adsorption of the enzyme to occur at the subsequent Ultrogel AcA 34 chromatography (cf. [5]).

At the latter step the enzyme adsorbed and was eluted with high phosphate concentration (present in the applied sample) (fig.2A). Since <0.1 mg protein was applied the A_{280} elution pattern could not be followed. However, SDS–PAGE on consecutive fractions indicated that practically all contaminating protein was eluted before hormone-sensitive lipase, with

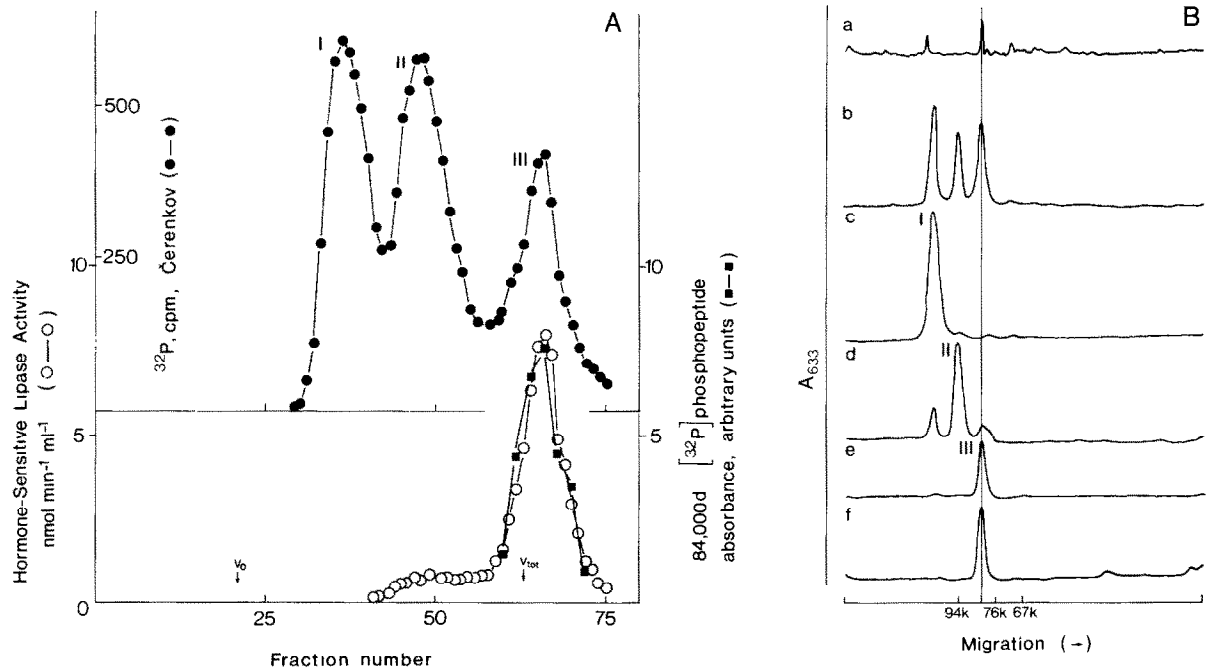

Fig.2. Chromatography on Ultrogel AcA 34 of pooled hormone-sensitive lipase from the hydroxylapatite chromatography step. A column (1.6 × 32 cm) of LKB Ultrogel AcA 34 was equilibrated in 5 mM potassium phosphate buffer (pH 7.0), 4°C, containing 30% (w/v) glycerol, 1 mM dithioerythritol, 0.15 M NaCl and 20 mM C_8E_6 detergent. A sample of pooled enzyme from the hydroxylapatite chromatography step was applied and eluted at 9 ml/h with the equilibrating buffer. Fractions of 1.0 ml were collected. (A) Total ^{32}P in fractions, hormone-sensitive lipase activity and 84 000 dalton [^{32}P]phosphopeptide, determined as in fig.1.(B):(a) densitometric trace of protein-stained gel after SDS–PAGE of pooled hormone-sensitive lipase peak fractions from Ultrogel AcA 34 chromatography of two separate enzyme preparations from 30 and 35 rats, respectively; densitometric traces of autoradiographs on SDS–PAGE from (b) pooled enzyme from hydroxylapatite chromatography; (c) peak I; (d) peak II; (e) peak III; (f) in vitro [^{32}P]phosphorylated, purified hormone-sensitive lipase.

some minor contaminants in the ascending slope of enzyme activity. Densitometric trace of protein stain from SDS–PAGE of enzyme peak fractions pooled from 2 preceding experiments indicated a protein purity of ~35% for the hormone-sensitive lipase 84 000 dalton polypeptide (fig.2B,a). Overall recovery of enzyme activity compared to the 110 000 × *g* supernatant was 6%. A peak of ^{32}P-radioactivity (III) was eluted with the enzyme activity, well resolved from 2 other peaks, which were due to [^{32}P]ATP-citrate lyase (I) and, mainly, the 94 000 dalton [^{32}P]phosphopeptide (II) (fig.2). Peak III ^{32}P radioactivity was almost entirely due to the 84 000 dalton [^{32}P]phosphopeptide (fig.2B,e). This [^{32}P]phosphopeptide had the same mobility (SDS–PAGE) as purified, in vitro [^{32}P]phosphorylated, hormone-sensitive lipase (fig.2B,f). The identity was verified by the approximately constant relation between enzyme activity and ^{32}P radioactivity in the 84 000 dalton polypeptide over the enzyme activity peak (fig.2A).

4. Discussion

These results show that hormone-sensitive lipase is phosphorylated in intact rat adipocytes. The copurification of the 84 000 dalton [^{32}P]phosphopeptide with enzyme activity over several isolation steps, to an enzyme protein purity of 35%, is strong evidence for its identification with hormone-sensitive lipase. The [^{32}P]phosphorylation of the enzyme occurred within the fat cells and not during the isolation procedure since, when the cells were broken, the ATP in the homogenisation medium immediately diluted the [γ-^{32}P]ATP in the adipocytes. Moreover, [^{32}P]phosphorylated enzyme was obtained when cells were extracted with SDS solution, within seconds of separation from the incubation medium [6].

[^{32}P]Hormone-sensitive lipase seemed to be the main constituent of the 84 000 dalton [^{32}P]phosphopeptide band obtained by SDS–PAGE of adipocyte proteins. This [^{32}P]phosphopeptide band was only obtained from fractions containing enzyme; the only significant amount not used in the isolation of the enzyme was found in the floating fat layer.

The demonstration that hormone-sensitive lipase can be phosphorylated in the intact cell is required for, but does not prove, a physiological role for this process in the hormonal regulation of enzyme activity. The effects of hormones on the phosphorylation will be described in [6].

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