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Adipose-tissue Mg^{2+}-dependent phosphatidate phosphohydrolase

Control of activity and subcellular distribution *in vitro* and *in vivo*

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1. The subcellular distribution of Mg^{2+}-dependent phosphatidate phosphohydrolase in rat adipocytes between a soluble and a membrane-bound fraction was measured by using both centrifugal fractionation and a novel Millipore-filtration method. 2. The relative proportion of the phosphohydrolase associated with the particulate fraction was increased on incubation of cells with noradrenaline or palmitate. Insulin on its own decreased the proportion of the phosphohydrolase that was particulate and abolished the effect of noradrenaline, but not that of palmitate. The effect of noradrenaline on phosphohydrolase distribution was rapid, the effect being maximal within 10 min. Noradrenaline exerted this effect with a similar concentration-dependence to its lipolytic effect. Inclusion of albumin in homogenization buffers decreased the proportion of the phosphohydrolase that was particulate, but did not abolish the effect of noradrenaline. 3. There was limited correlation between the proportion of the phosphohydrolase that was particulate and the measured rate of triacylglycerol synthesis in adipocytes incubated under a variety of conditions. 4. Starvation, streptozotocin-diabetes and hypothyroidism decreased the specific activities of the phosphohydrolase and glycerolphosphate acyltransferase in homogenates from epididymal fat-pads. Restoration of these activities in the diabetic state was seen after administration of insulin over 2 days or, in the short term, within 2 h after a single administration of insulin. Administration of thyroxine over 3 days caused restoration of these activities in the hypothyroid state. Starvation and diabetes increased the proportion of the phosphohydrolase found in the microsomal fraction. This change was not seen when albumin was present in homogenization buffers. 5. The possible role of fatty acids as regulators of the intracellular translocation of the phosphohydrolase, together with the role of this enzyme in the regulation of triacylglycerol synthesis in adipose tissue, is discussed.

INTRODUCTION

The Mg^{2+}-dependent phosphatidate phosphohydrolase (PPH) of adipose tissue appears to be under various forms of control, implying a possible role for this enzyme in the regulation of triacylglycerol synthesis in this tissue. In the long term adipocyte PPH activity is decreased through starvation and aging, and is increased in obesity and with increasing adipocyte size (Moller *et al.*, 1977; Belfiore *et al.*, 1978; Jamdar *et al.*, 1981, 1984; Jamdar & Osborne, 1982). In the short term adipocyte PPH activity is decreased by lipolytic agents *in vitro* (Cheng & Saggerson, 1978*a*,*b*, 1980; Cheng *et al.*, 1980) and *in vivo* (Lawson *et al.*, 1981). These effects are opposed by insulin (Cheng & Saggerson, 1978*b*). Although some studies have been conducted with the liver enzyme, there is no conclusive evidence to suggest that PPH is regulated by a phosphorylation/dephosphorylation mechanism (Berglund *et al.*, 1982; Butterwith *et al.*, 1984*b*). Adipose-tissue Mg^{2+}-dependent PPH is found in both soluble and membrane-bound forms (Jamdar & Fallon, 1973; Moller *et al.*, 1977; Saggerson *et al.*, 1980), and it has been suggested (Moller & Hough, 1982) that it represents an example of an 'ambiquitous' enzyme (Wilson, 1980). Moller *et al.* (1981) have shown that incubation of adipocytes with lipolytic agents increases the PPH activity in the microsomal fraction and decreases that in the cytosol. Likewise, association of adipocyte cytosolic PPH with microsomes (microsomal fractions) is altered in a cell-free system by various cations (Moller & Hough, 1982). Translocation of PPH between cytosol and endoplasmic reticulum has also been demonstrated in hepatocytes (Cascales *et al.*, 1984; Butterwith *et al.*, 1984*b*; Pittner *et al.*, 1985) and in a cell-free system obtained from liver (Martin-Sanz *et al.*, 1984, 1985; Hopewell *et al.*, 1985). Fatty acids and their CoA thioesters appear to be important in this regard in the hepatic system, and it has been suggested (Brindley, 1985) that the cytosolic PPH in liver represents an inactive reserve of enzyme which, when translocated to the endoplasmic reticulum, enables cells to respond to an increased fatty acid supply by increased synthesis of glycerolipids. It is not known whether this mechanism is applicable in adipose tissue, where fatty acid supply notionally could be increased both by insulin (through increasing lipoprotein lipase activity and stimulating fatty acid synthesis) and by lipolytic hormones.

In the present study we have further investigated the effect of the lipolytic agent noradrenaline on the intracellular translocation of PPH in adipocytes, together with the effect of insulin on this phenomenon. We have also attempted to ascertain the involvement of fatty acids in the process and to correlate changes in PPH distribution with measured changes in flux through the triacylglycerol-synthesis pathway. In addition, we present the first evidence that the subcellular distribution of

Abbreviations used: GPAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); PPH, phosphatidate phosphohydrolase (EC 3.1.3.4)

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adipose tissue PPH can be altered *in vivo*, together with studies of the effects of diabetes and hypothyroidism on PPH activity in this tissue.

MATERIALS AND METHODS

Chemicals

These were obtained as described by Bates & Saggerson (1979). In addition, egg phosphatidylcholine, DL-propranolol, noradrenaline, streptozotocin, sodium palmitate, L-thyroxine and bovine albumin were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Before use, albumin was subjected to a defatting procedure (Chen, 1967) with minor modifications (Saggerson, 1972). Phospholipase D was isolated from cabbage by the method of Davidson & Long (1958). Phosphatidic acid was prepared by the action of phospholipase D on egg phosphatidylcholine, followed by extraction and conversion of the product into the sodium salt (Ansell & Hawthorne, 1964) and determination of purity and yield as described by Cheng & Saggerson (1978*a*). Before use in cell incubations, sodium palmitate was associated with fatty acid-poor albumin (Evans & Mueller, 1963) and the fatty acid content standardized by the method of Itaya & Ui (1965). [U-^{14}C]Glucose was from Amersham International (Amersham, Bucks., U.K.), and bovine insulin (six times recrystallized) was from Boots Co. (Nottingham, U.K.). Protamine zinc insulin was from Weddel Pharmaceuticals, London E.C.1, U.K.

Animals

These were male Sprague–Dawley rats bred at University College London and maintained on a 13 h-light/11 h-dark cycle with light from 06:00 to 19:00 h. Normally animals were taken for experimentation when aged 5–6 weeks (160–180 g), except for hypothyroid rats and their age-matched euthyroid controls. These were selected when aged approx. 4 weeks (80–100 g) and then maintained for a further 4 weeks. The hypothyroid state was induced over this period by giving an iodine-deficient diet together with 0.01% (w/v) 6-n-propyl-2-thiouracil in the drinking water. This procedure causes severe depression of the plasma concentrations of thyroid hormones, and the animals cease to grow after approx. 3 weeks (Chohan *et al.*, 1984; Saggerson & Carpenter, 1986). Thyroxine-treated hypothyroid rats were injected intraperitoneally with L-thyroxine (20 μg/kg), which has been reported to act as a physiological replacement dose in thyroidectomized rats (Reichlin *et al.*, 1970). The injections were repeated 24 and 48 h afterwards and the rats killed after a further 24 h. Thyroxine (2.5 mg/ml) was dissolved in 1 M-NaOH, diluted to the required concentration in 10 mM-potassium phosphate buffer (pH 7.4) containing 0.15 M-NaCl, and injected at 0.2 ml/rat. As it has been reported that propylthiouracil inhibits extrathyroidal conversion of thyroxine into tri-iodothyronine (Oppenheimer *et al.*, 1972), these rats were transferred to normal diet and drinking water immediately after the first thyroxine injection. It was noticeable that the rats started to grow again during the 3 days of thyroxine treatment. Diabetes was induced by a single subcutaneous injection of streptozotocin (100 mg/kg) dissolved in 0.2 ml of 50 mM-sodium citrate buffer (pH 4.0) containing 0.15 M-NaCl. After 2 days animals were selected as diabetic if showing as strongly positive 'Clinistix' test for urinary glucose (> 0.5% glucose, w/v). These animals lost weight and showed marked hyperglycaemia, as described by Chatzipanteli & Saggerson (1983). The 2-day-insulin-treated diabetic rats received a subcutaneous injection of protamine zinc bovine insulin (20 units/kg) at approx. 10:00 h on the second day after streptozotocin. This treatment was repeated after 24 and 48 h and the rats were killed 3–4 h after the final injection. The 2 h-insulin-treated diabetic animals received only the first insulin injection at approx. 10:00 h and were killed 2 h later.

Isolation of adipocytes from epididymal adipose tissue

These were obtained by collagenase treatment of epididymal-fat-pad pieces (Rodbell, 1964) and washed twice with Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (10 mg/ml). The cells were then suspended as a stock in the same medium (cells equivalent to two-thirds of a fat pad/ml).

Incubation of adipocytes

Samples (1.0 ml) of the stock cell suspension (approx. 10^7 cells) were added to 25 ml silicone-treated flasks and incubated with shaking (70 cycles/min) at 37 °C in a final volume of 4 ml of Krebs–Ringer bicarbonate containing 1.27 mM-Ca^{2+}, fatty acid-poor albumin (40 mg/ml), adenosine deaminase (4 munits/ml) and 5 mM-glucose. The flask contents were continuously gassed with O_2/CO_2 (19:1). Incubation times and other additions to flasks are indicated in the legends to individual Tables and Figures.

Preparation of defatted homogenates of adipocytes

Adipocytes were separated from incubation media by centrifugation for 20 s at 200 $g_{av.}$ and the underlying medium was removed by aspiration. The cells were transferred to a glass tube in ice containing 0.25 M-sucrose, 10 mM-Tris/HCl buffer (pH 7.4), 1 mM-EDTA and 1 mM-dithiothreitol (sucrose medium) or, where indicated, sucrose medium containing fatty-acid-poor albumin at 10 mg/ml (sucrose/albumin medium). The tube contents were agitated for 1 min on a vortex mixer (Martin & Denton, 1970) and the resultant homogenate was then left on ice for 5 min. Portions of the defatted homogenates were then aspirated from under the floating bulk fat with a Pasteur pipette.

Subcellular fractionation of adipocytes by centrifugation

Defatted homogenates were prepared from the pooled adipocytes of six incubation flasks in 9 ml of sucrose medium. These were then centrifuged at 2–4 °C in an SS-34 rotor ($r_{av.}$ = 10.8 cm) of a Sorvall RC5-B Superspeed centrifuge by accelerating the centrifuge at 20000 $g_{av.}$, maintaining that field for 1 min and then decelerating with the brake on (integrated field-time = 30200 g-min). The pellet (mitochondrial + nuclear fractions) was resuspended in 2.0 ml of sucrose medium. The supernatant was centrifuged at 2–4 °C for 1 h at 105000 $g_{av.}$ in a Beckman model L ultracentrifuge fitted with a 50 Ti rotor. The high-speed supernatant was then aspirated, avoiding the top layer of floating fat, and the walls of the tube were gently washed with sucrose medium to remove excess supernatant. The microsomal pellet was resuspended in 2.0 ml of sucrose medium.

Subcellular fractionation of adipocytes by filtration

Defatted homogenates were prepared from the pooled adipocytes of two incubation flasks by using 3 ml of sucrose medium; 1.5 ml of each defatted homogenate was transferred to a 2 ml plastic syringe fitted with a Swinnex filter assembly containing a Millipore pre-filter (pore size 1.2 μm) separated by a rubber O-ring from a Millipore filter of 0.3 μm pore size. The homogenate was forced rapidly, but smoothly, through the filter assembly with the aid of the plunger, and the filtrate was collected on ice. Further details of this method are given in the Results and discussion section.

Subcellular fractionation of whole epididymal fat-pads

Rats were killed by a blow on the head followed by cervical dislocation. One fat-pad was rapidly removed, cooled in ice-cold sucrose medium, weighed and immediately homogenized in 8 ml of ice-cold sucrose medium in a glass Potter–Elvehjem homogenizer with a Teflon pestle (eight passes at 400 rev./min, with radial clearance of 0.2 mm). During this procedure the contralateral fat-pad was also removed, weighed and homogenized in 8 ml of sucrose/albumin medium. The use of sucrose or sucrose/albumin medium for the first homogenization was alternated for each animal. Homogenates were centrifuged at 2–4 °C by accelerating at 3000 $g_{av.}$, maintaining that field for 1 min and then decelerating with the brake on (integrated field-time = 4500 g-min). A 6 ml portion of the infranatant was separated from the floating fat and the pellet (mainly nuclei, blood and cell debris) and centrifuged at 105000 g for 1 h as above. The resulting pellet (mitochondrial and microsomal fractions) was resuspended in 1 ml of sucrose medium. The remainder of the 3000 $g_{av.}$ supernatant was saved for enzyme assays.

Analytical methods

PPH was assayed as phosphate release from an aqueous dispersion of phosphatidate. The method is a minor modification of that of Jamdar & Fallon (1973) as described by Cheng & Saggerson (1978*a*). Samples of tissue extracts (50 or 75 μl) were assayed at 37 °C in a final volume of 0.5 ml containing 100 mM-Tris/maleate buffer (pH 6.8), 1 mM-dithiothreitol, 0.1 mM-EGTA and 1.4 mM-sodium phosphatidate; 2.5 mM-$MgCl_2$ was present in assays of the Mg^{2+}-dependent PPH and was absent from assays of the Mg^{2+}-independent activity. Mg^{2+}-dependent activity was calculated by subtraction of the Mg^{2+}-independent activity from total PPH activity. After 30 min, the reaction was terminated by addition of 0.5 ml of 10% (w/v) trichloroacetic acid, precipitated protein was removed in a bench centrifuge and P_i in the supernatant was measured by the method of Ames & Dubin (1960).

GPAT was assayed radiochemically at 30 °C by measuring the incorporation of [^{14}C]glycerol-3-phosphate into butanol-soluble products (Saggerson *et al.*, 1980). Assays were performed for 7 min in a final volume of 1.0 ml containing 100 mM-Tris/HCl buffer (pH 7.4), 0.7 mM-dithiothreitol, fatty-acid-poor albumin (1.75 mg/ml), 1 mM-[U-^{14}C]-*sn*-glycerol 3-phosphate (0.4 μCi/μmol), 100 μM-palmitoyl-CoA and 100 μl of defatted homogenate. These conditions are such that the microsomal activity will predominate over the mitochondrial GPAT activity by a factor of at least 6:1 (Rider & Saggerson, 1983).

$NADP^+$–cytochrome *c* reductase (EC 1.6.2.4) and lactate dehydrogenase (EC 1.1.1.27) were assayed spectrophotometrically at 25 °C by the methods of Phillips & Langdon (1962) and Saggerson (1974) respectively.

After incubation of adipocytes with [U-^{14}C]glucose, cell lipids were extracted into hexane (Dole, 1956). The hexane extracts were treated as described by Saggerson & Tomassi (1971) and incorporation of ^{14}C into acylglycerol glycerol was measured (Saggerson & Greenbaum, 1970).

Glycerol was assayed enzymically in deproteinized samples of adipocyte incubation media by the method of Garland & Randle (1962). Non-esterified fatty acids in incubation media were measured as described by Cheng & Saggerson (1978*a*).

Protein was measured by the method of Lowry *et al.*

Table 1. Changes on incubation of adipocytes with noradrenaline

Adipocytes were incubated for 30 min with or without 0.5 μM-noradrenaline, followed by centrifugal fractionation of defatted homogenates. The values are means ± S.E.M. for five separate experiments: [a] $P < 0.01$ for effects of noradrenaline.

	Treatment of cells	
	Control	With noradrenaline
Activities in defatted homogenates (nmol/min per unit of lactate dehydrogenase)		
Mg^{2+}-dependent PPH	54 ± 4	52 ± 4
Mg^{2+}-independent PPH	4 ± 1	4 ± 1
Activities in microsomal fractions (nmol/min per mg of protein)		
Mg^{2+}-dependent PPH	51 ± 9	103 ± 10[a]
Mg^{2+}-independent PPH	4 ± 1	5 ± 1
Glycerol accumulation in incubation media (mM)	0.02 ± 0.00	0.55 ± 0.06[a]
Non-esterified fatty acid in microsomal fraction (μmol/mg of protein)	0.31 ± 0.05	0.42 ± 0.04

Table 2. Determination of intracellular distribution of PPH by centrifugal fractionation

The measurements were made in the same experiment as Table 1 and are means ± S.E.M. for five experiments: [a] $P < 0.001$ for effects of noradrenaline.

Fraction	Treatment of cells	Percentage distributions of recovered activities			
		Mg^{2+}-dependent PPH	Mg^{2+}-independent PPH	$NADP^+$–cytochrome *c* reductase	Lactate dehydrogenase
Nuclear + mitochondrial	Control	4 ± 0	71 ± 3	28 ± 3	5 ± 0
	Noradrenaline	11 ± 0[a]	69 ± 2	26 ± 1	4 ± 1
Microsomal	Control	21 ± 1	29 ± 3	63 ± 3	2 ± 0
	Noradrenaline	40 ± 1[a]	31 ± 2	65 ± 1	1 ± 0
105000 *g* supernatant	Control	75 ± 1	0	9 ± 1	94 ± 1
	Noradrenaline	50 ± 1[a]	0	10 ± 1	94 ± 1
Total particulate	Control	25 ± 1	100	91 ± 1	6 ± 0
	Noradrenaline	51 ± 1[a]	100	90 ± 1	6 ± 0

(1951), with fatty-acid-poor bovine serum albumin as a standard. The DNA content of adipocytes was determined by the method of Switzer & Summer (1971).

Statistical methods and presentation of data

Statistical significance was assessed by Student's *t* test. Bars in Figures represent S.E.M. Where these are not shown, they lie within the area of the symbol.

RESULTS AND DISCUSSION

Effect of noradrenaline on subcellular distribution of PPH in adipocytes

In previous studies (Cheng & Saggerson, 1978*a*,*b*), incubation of adipocytes for 10–60 min with noradrenaline resulted in significant decreases in Mg^{2+}-dependent PPH activity. These changes were seen if cells were freeze-stopped in liquid N_2 and the enzyme was assayed immediately after vigorous disruption of the cells with an Ultra-Turrax tissue disintegrator. The mechanism of this inactivation is not understood, but a covalent-modification type of mechanism is unlikely, since the effect of noradrenaline is abolished if the cells are homogenized in media containing albumin (Cheng & Saggerson, 1980). Table 1 shows that, if cells are gently broken by vortex-mixing without freeze-stopping and the homogenates are then left in ice to allow separation of the bulk fat (see the Materials and methods section), no effect of noradrenaline is apparent. It is presumed either that this procedure allowed relaxation of the activity before assay or that the mode of cell breakage is crucial to the observation of this effect. Similar absence of any hormonal effect on total PPH activity was apparent in other experiments (see Tables 4 and 5 and Figs. 1 and 2). As a check, inactivation of PPH on exposure of cells to noradrenaline was observed in parallel experiments (results not shown) if the freeze-stop/rapid-assay procedure was used, confirming the results of Cheng & Saggerson (1978*a*,*b*).

Table 1 shows that treatment of cells with noradrenaline significantly increased PPH activity in the microsomal fraction. This change was confined to the Mg^{2+}-dependent activity, confirming the findings of Moller *et al.* (1981). Since there was no change in the total homogenate activity, it is presumed that this represents a redistribution or 'translocation' of activity between soluble and particulate compartments and that the stable decrease in PPH activity in 105000 *g* supernatants from noradrenaline-treated cells (Saggerson *et al.*, 1979; Cheng & Saggerson, 1980) can now be interpreted in the same way.

Table 2 shows that centrifugal fractionation of adipocyte homogenates resulted in distribution patterns for lactate dehydrogenase, $NADP^+$–cytochrome *c* reductase and Mg^{2+}-independent PPH similar to those found by Saggerson *et al.* (1980). The change in subcellular distribution of Mg^{2+}-dependent PPH mainly between the soluble and microsomal fractions is apparent, and it is clear that noradrenaline had no effect on the distribution of the other three activities. Two differences between the present study and that of Moller *et al.* (1981) are apparent. First, in the control state, the ratio of Mg^{2+}-dependent/Mg^{2+}-independent PPH activities was approx. 13:1 (Table 1) compared with 2:1 (Moller *et al.*, 1981). Second, it may be calculated that Moller *et al.* (1981) only observed approx. 11% and 19% of the measured Mg^{2+}-dependent PPH in microsomal fractions without or with noradrenaline treatment respectively. By contrast, these values were approximately doubled in the present study (Table 2). It is possible that breakage of cells by vortex-mixing (see the Materials and methods section) may dislodge less membrane-associated PPH than in more vigorous homogenization procedures (see Moller *et al.*, 1981). In addition, the higher salt concentration used in homogenizaton buffers in this study (10 mM- rather than 1 mM-Tris/HCl) might also cause an increase in the proportion of the enzyme that is particulate (Moller & Hough, 1982).

We wished to examine the subcellular distribution of the enzyme simultaneously under a variety of cell incubation conditions. Because of the large number of samples, each containing a relatively small quantity of cells, subcellular fractionation by centrifugation was not

Table 3. Determination of intracellular distribution of PPH by Millipore-filtration fractionation

Adipocytes were incubated for 30 min with or without 0.5 μM-noradrenaline, followed by filtration of the defatted homogenates or subsequently prepared 105000 *g* supernatants. The values are means ± S.E.M. for four separate experiments, with the same cell preparations as used in Tables 1 and 2: percentage recovery indicates the proportion of an enzyme activity recovered in the 0.3 μm filtrate. [a] $P < 0.01$, [b] $P < 0.001$ for effects of noradrenaline.

	Treatment of cells	
	Control	With noradrenaline
Filtration of defatted homogenate		
Recovery of Mg^{2+}-dependent PPH (%)	78 ± 4	55 ± 1[a]
Recovery of $NADP^+$–cytochrome *c* reductase (%)	37 ± 8	28 ± 4
Calculated % of particulate PPH	35 ± 4	63 ± 2[b]
Filtration of 105000 *g* supernatant		
Recovery of Mg^{2+}-dependent PPH (%)	79 ± 2	78 ± 1
Recovery of lactate dehydrogenase (%)	97 ± 5	100 ± 5

particularly suitable and the method of fractionation by filtration was developed. Besides enabling the processing of a larger number of samples, this method proved to be extremely rapid. Ideally the method should permit passage through the filter of all 'soluble' enzyme, but should retain all 'particulate' material. In reality the 0.3 μm filter was a compromise that allowed passage of virtually all lactate dehydrogenase activity (taken thereafter as 100%) together with a reasonable flow rate, but with incomplete retention of the microsomal marker $NADP^+$–cytochrome *c* reductase (Table 3). Treatment of adipocytes with noradrenaline significantly increased retention by the filter of Mg^{2+}-dependent PPH without significantly altering the retention of $NADP^+$–cytochrome *c* reductase. In subsequent experiments (e.g. Table 5, Figs. 1 and 2) the percentage retention of the microsomal marker varied between 82 and 72% but was not significantly changed by prior treatments of the incubated cells that resulted in redistribution of PPH. In all of these experiments, the fractions of the total Mg^{2+}-dependent PPH in a defatted homogenate that are 'particulate' (P) or 'soluble' (S) were calculated by solution of eqns. (1) and (2):

$$P + S = 1 \quad (1)$$

$$\text{Fractional recovery of PPH} = S + P \times (\text{fractional recovery of } NADP^+\text{–cytochrome } c \text{ reductase}) \quad (2)$$

On this basis Table 3 shows that the filtration method resulted in calculations of the percentage of particulate PPH that were approx. 10% higher than those in paired centrifugal fractionations (cf. Tables 2 and 3). Possible explanations for this discrepancy are: first, some of the filtered $NADP^+$–cytochrome *c* reductase may be 'soluble' rather than 'particulate'; second, the substantial hydrostatic pressures on centrifugation for 1 h at 105000 *g* may dislodge some membrane-associated PPH; third, 21–22% of the 'soluble' PPH in 105000 *g* supernatants was retained by the 0.3 μm filter (Table 3). This would be sufficient to explain the small discrepancy between the two methods, and it is noteworthy that Butterwith *et al.* (1984*a*) have observed aggregated forms of the enzyme on gel filtration of PPH derived from rat liver high-speed supernatants. In summary, the filtration method appears to give a rapid, reproducible, method for detecting changes in location or aggregation of the enzyme. The criteria for judging 'particulate' versus 'soluble' enzyme may be slightly different from those used with centrifugal fractionation.

The filtration method was used to investigate the time- and concentration-dependence of the noradrenaline effect. With 1 μM-noradrenaline this was extremely rapid (Fig. 1*a*), with the maximum change relative to the control being reached in 10 min. This is more rapid than the activity decrease reported by Cheng & Saggerson (1978*a*). Using freeze-stop methods, Cheng & Saggerson (1978*b*) showed that incubation of cells for 30 min with noradrenaline decreased the total PPH activity by approx. 40%. If this decrease was equally expressed in the cytosolic and membrane-bound enzyme, the translocation phenomenon would be sufficient to 'buffer' the particulate activity almost exactly. If an increase in non-esterified fatty acid is a major effector of this change (see the Introduction), it does not relate quantitatively to extracellular accumulation of fatty acid, which is apparent at 10 min but continues to rise linearly thereafter (Fig. 1*b*). However, it is noteworthy that intracellular fatty acid concentration rises more rapidly than the extracellular concentration at early times after addition of noradrenaline (Angel *et al.*, 1971*a*,*b*; Heindel *et al.*, 1974). Fig. 2 shows that the intracellular redistribution of PPH has a very similar noradrenaline dose-dependence to the stimulation of lipolysis by the hormone: 0.13 μM- and 0.08 μM-noradrenaline resulted in half-maximal effects on PPH translocation and glycerol release respectively. Furthermore, consideration of data from individual experiments showed a highly significant linear correlation between the change in the percentage of particulate PPH in this experiment (Fig. 2) and the change in glycerol release relative to the controls ($r = 0.865$, $P < 0.001$, $n = 24$).

Effect of albumin on subcellular distribution of PPH in adipocytes

Bovine serum albumin (10 mg/ml) should cause substantial removal of fatty acids and displacement of PPH from liver microsomes (Hopewell *et al.*, 1985) and abolishes inactivation of adipocyte PPH by noradrenaline in freeze-stop experiments (Cheng & Saggerson, 1980). Table 4 shows that vortex-mixing of cells in sucrose medium containing albumin (10 mg/ml) resulted in a small decrease in the total measured activity of PPH; a similar effect was noted by Cheng & Saggerson (1980).

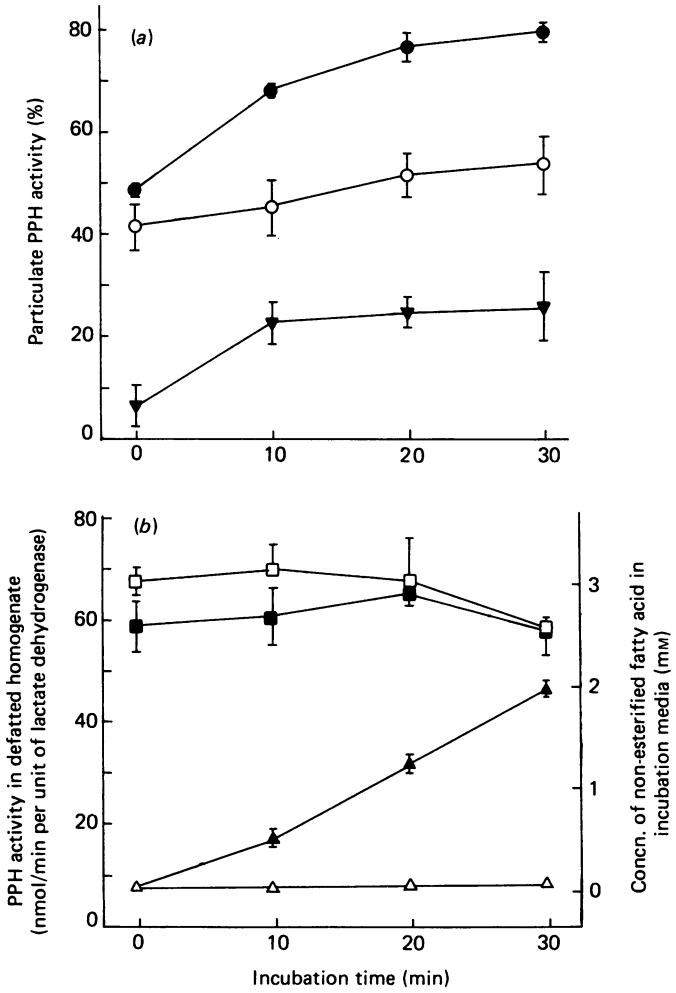

Fig. 1. Time course of the effect of noradrenaline on the intracellular distribution of Mg^{2+}-dependent PPH

Incubations were with (black symbols) or without (white symbols) 1 μM-noradrenaline ($n = 4$ throughout). (*a*) ●, ○, calculated % of particulate PPH by using Millipore-filtration fractionation; ▼, change in % distribution of PPH with noradrenaline. (*b*) ■, □, PPH activity in defatted homogenates; ▲, △, non-esterified fatty acid accumulation in incubation media.

Accompanying this, albumin caused a substantial decrease in the percentage of particulate PPH. This finding would not be at variance with a possible role of intracellular fatty acids in controlling the intracellular distribution of the enzyme. However, the effect of noradrenaline to increase the particulate proportion of the activity by approx. 25% was still apparent, suggesting that noradrenaline may exert other effects on the system other than those mediated by fatty acids. If these other effects were mediated by cyclic AMP, the response is different from that in liver, where at low fatty acid concentrations cyclic AMP (Butterwith *et al.*, 1984*b*) or glucagon (Pittner *et al.*, 1985) decrease the proportion of membrane-associated PPH. It is noteworthy that direct demonstration in a cell-free system of translocation of PPH between cytosolic and microsomal fractions in response to long-chain fatty acids or their CoA thioesters has not as yet been demonstrated with adipose tissue, whereas this is clearly demonstrable in liver (Martin-Sanz *et al.*, 1984; Hopewell *et al.*, 1985). Using similar methodology to Martin-Sanz *et al.* (1984) with recombined adipocyte cytosol and microsomes, we have not been able to demonstrate such an effect (S. J. Taylor & E. D. Saggerson, unpublished work). It is also noteworthy that the fatty acid content of microsomes was only slightly and non-significantly increased after incubation of cells with noradrenaline (Table 1).

Relationship between subcellular distribution of PPH and rate of triacylglycerol synthesis

It has been proposed by Brindley (1985) that in liver the cytosolic PPH represents an inactive reservoir of activity that becomes metabolically functional when it translocates to the endoplasmic reticulum. If this event is of major regulatory significance for the rate of conversion of phosphatidate into diacylglycerol (and hence triacylglycerol), there should be correlation in different short-term states between the percentage of particulate PPH activity and the rate of flux through the pathway. This is testable in adipocytes, where incorporation of [^{14}C]glucose into acylglycerol glycerol can be readily measured and where the process is stimulated by insulin (Saggerson, 1972; Sooranna & Saggerson, 1975). Furthermore, availability of fatty acids generally appears to be a limiting factor for triacylglycerol synthesis by incubated adipocytes such that the process is considerably stimulated directly by palmitate and, to a lesser extent, indirectly by addition of lipolytic agents (Saggerson, 1972, 1985). In the presence of noradrenaline there is a time-dependent accumulation of fatty acid (see Fig. 1) and a time-dependent inactivation of enzymes in the triacylglycerol synthesis pathway (Cheng & Saggerson, 1978*a*; Rider & Saggerson, 1983; Hall & Saggerson, 1985; Saggerson, 1985). As a consequence the rate of triacylglycerol synthesis under these conditions will be a complex variable with time. We therefore wished to obtain an estimate of flux through the pathway as close as possible to the time of sampling the cells for measurement of PPH activity. This was found to be feasible by injecting 1 μCi of [U-^{14}C]glucose into cell incubations 25 min after starting incubations with 5 mM unlabelled glucose. At 30 min cells were sampled, thereby permitting PPH activity measurements to be related to rates of triacylglycerol synthesis in the previous 5 min. In three separate experiments (results not shown), rates of glucose incorporation into acylglycerol glycerol was found to be linear over the period 5, 10, 15 and 20 min after injection of the label in the presence and absence of insulin, with an intercept on the time axis of approx. 30 s in both cases. Furthermore, enzymic assays of incubation medium glucose showed that none of the tested states shown in Table 5 resulted in significant depletion of the 5 mM unlabelled glucose before addition of the label; thereafter specific radioactivity of glucose over the 5 min test period was the same in all cases.

Table 5 shows that both propranolol and insulin abolished the change in PPH location brought about by noradrenaline. Propranolol also totally blocked the lipolytic effect of noradrenaline, whereas insulin caused a partial inhibition of lipolysis. Presumably in part owing to stimulation of glucose transport by insulin, with resultant elevation of glycerol 3-phosphate concentrations (Denton *et al.*, 1966; Saggerson & Greenbaum, 1970), and in part to the lipolytic provision of fatty acids, cells

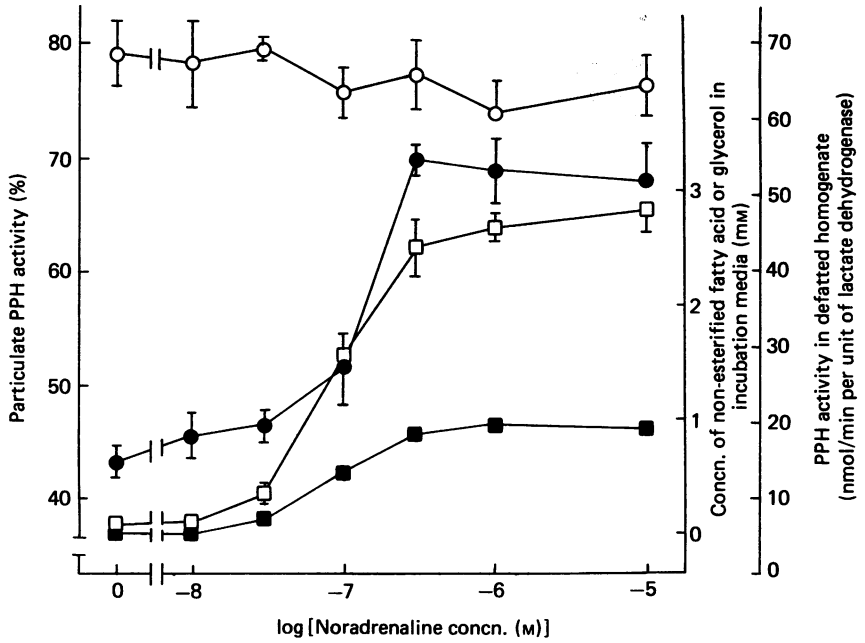

Fig. 2. Concentration-dependence of the effect of noradrenaline on the intracellular distribution of Mg^{2+}-dependent PPH

Incubations were for 30 min: $n = 5$, except for 30 nM-noradrenaline where $n = 4$. ○, PPH activity in defatted homogenates. ●, Calculated % of particulate PPH by using Millipore-filtration fractionation. □, Non-esterified fatty acid accumulation in incubation media. ■, Glycerol accumulation in incubation media.

Table 4. Effect of albumin in extraction buffer on intracellular distribution of PPH

Adipocytes were incubated for 30 min with or without 0.5 μM-noradrenaline, followed by preparation of defatted homogenates in sucrose medium or sucrose/albumin. Subcellular fractionation was by the filtration method. The values are means ± S.E.M. for four separate experiments: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ for effects of noradrenaline; $^{d}P < 0.05$, $^{e}P < 0.02$, $^{f}P < 0.01$ for effects of albumin.

Albumin (1%) in extraction buffer	Measurement	Treatment of cells: Control	Treatment of cells: With noradrenaline (0.5 μM)
Absent	Mg^{2+}-dependent PPH in defatted homogenate (nmol/min per unit of lactate dehydrogenase)	68 ± 5	68 ± 2
	Calculated % of particulate PPH	39 ± 2	64 ± 2[b]
	Glycerol accumulation (mM)	0.01 ± 0.00	0.38 ± 0.01[c]
Present	Mg^{2+}-dependent PPH in defatted homogenate (nmol/min per unit of lactate dehydrogenase)	52 ± 4[d]	57 ± 2[f]
	Calculated % of particulate PPH	14 ± 3[f]	40 ± 6[ae]
	Glycerol accumulation (mM)	0.01 ± 0.00	0.37 ± 0.01[c]

incubated with insulin + noradrenaline had 7-fold increased pathway flux, but with no significant change in PPH localization relative to the control. Insulin alone doubled pathway flux in the presence or absence of added palmitate, but significantly decreased the percentage of particulate PPH in the absence of palmitate. This is opposite to the situation in liver, where insulin increases the proportion of membrane-associated PPH when fatty acids were only present in low amounts (Pittner *et al.*, 1985). Incubation of cells with palmitate elicited a 1180 ± 160% increase in pathway flux, compared with a 620 ± 200% increase in this with noradrenaline. By contrast, although the percentage of particulate PPH was increased by palmitate (by 19 ± 2% over the control), this change was less than that seen with noradrenaline (29 ± 3% increase over the control). In the presence of palmitate, insulin now had no significant effect on the intracellular distribution of PPH, whereas the pathway flux was doubled. Therefore Table 5 shows that, although there are situations in which the percentage of particulate PPH and flux through the pathway appear to increase in tandem, there are others in which flux can be increased with no change or even a decrease in the percentage of particulate PPH.

Table 5. Comparison of intracellular distribution of Mg^{2+}-dependent PPH activity with rates of triacylglycerol synthesis

Values for both sets of experiments are means ± S.E.M. for four separate measurements. Adipocytes were incubated for 25 min, at which time 1 μCi of [U-^{14}C]glucose was added to some incubation flasks. All incubations were terminated at 30 min, followed by either determination of ^{14}C incorporation into acylglycerol glycerol or subcellular fractionation by the filtration method. [a] $P < 0.05$, [b] $P < 0.02$, [c] $P < 0.01$, [d] $P < 0.001$ versus the control values.

Additions to incubations	Glycerol accumulation in incubation media (mM)	PPH/lactate dehydrogenase activity ratio in defatted homogenate	Calculated % of particulate PPH	Triacylglycerol synthesis (μg-atoms/5 min per mg of DNA)
None	0.02±0.00	53±3	44±3	0.36±0.08
Propranolol (10 μM)	0.02±0.00	51±3	46±2	0.25±0.05
Noradrenaline (0.5 μM) +propranolol (10 μM)	0.02±0.00	52±2	43±2	0.30±0.05
Noradrenaline (0.5 μM)	0.61±0.04[d]	50±3	72±2	2.14±0.79
Noradrenaline (0.5 μM) +insulin (3 nM)	0.15±0.01[d]	53±2	50±2	2.64±0.49[b]
None	—	57±5	46±1	0.49±0.05
Insulin (1.5 nM)	—	57±5	36±3[a]	1.14±0.06[d]
Palmitate (3 mM)	—	53±4	64±3[d]	5.67±0.65[c]
Palmitate (3 mM)+ insulin (1.5 nM)	—	53±5	70±3[d]	11.97±0.75[d]

Table 6. Measurements of Mg^{2+}-dependent PPH and GPAT in fractions of whole fat-pads prepared in sucrose medium

The values are means ± S.E.M. for the numbers of experiments indicated in parentheses. Activities were measured in 3000 *g* supernatants. The % of particulate PPH is the proportion of this activity that sediments at 105000 *g*. Bracketed body wts. indicate these at start of starvation or streptozotocin treatment, time of hormone administration and time of death as appropriate. [a] $P < 0.05$, [b] $P < 0.02$, [c] $P < 0.01$, [d] $P < 0.001$ for comparisons with the control or the euthyroid groups as appropriate; [f] $P < 0.02$, [g] $P < 0.01$, [h] $P < 0.001$ for effect of insulin treatment of diabetic rats or thyroxine treatment of hypothyroid animals.

Condition	Body wt. (g)	Fat-pad wt. (mg)	Mg^{2+}-dependent PPH activity (nmol/min per mg of protein)	Mg^{2+}-dependent PPH activity (% particulate)	GPAT activity (nmol/min per mg of protein)
Control (6)	169±2	257±8	33.5±2.6	10.5±1.1	7.81±0.51
2-day starved (9)	174±3 145±3	141±16	16.9±2.4[d]	17.6±2.2[b]	4.88±1.00[a]
Diabetic (7)	176±1 162±3	143±15	17.2±1.9[d]	23.4±3.9[c]	4.92±0.76[c]
2-day-insulin-treated diabetic (6)	173±1 166±4 186±3	202±28	34.8±5.1[g]	9.6±1.7[g]	11.94±0.96[ch]
2 h-insulin-treated diabetic (6)	172±2 157±1	167±25	24.7±1.0[bg]	15.7±0.7[c]	10.00±0.83[ah]
Euthyroid (6)	264±7	648±68	32.4±2.2	16.3±0.9	8.87±0.32
Hypothyroid (9)	160±4	322±10	7.3±0.9[d]	14.2±2.8	6.88±0.57[c]
3-day-thyroxine-treated hypothyroid (7)	152±4 161±3	341±34	19.7±2.5[ch]	—	8.74±0.32[f]

Changes *in vivo* in subcellular distribution of PPH and in PPH and GPAT activities

The measurements are presented in Table 6 (tissues homogenized in sucrose medium) and Table 7 (paired tissues homogenized in sucrose/albumin medium); the values for percentage of particulate PPH obtained with whole fat-pads were appreciably lower than with adipocytes. Although a small portion of the particulate activity is lost on centrifugation at 3000 *g* for 1 min after homogenization (see the Materials and methods section), we mainly attribute this difference to differences in homogenization procedure. We are unaware of any previous studies of alteration of adipose tissue PPH

Table 7. Measurements of Mg^{2+}-dependent PPH in fractions of whole fat-pads prepared in sucrose/albumin medium

Homogenates were made from the contralateral pads to those used in Table 6. Activities were measured in 3000 *g* supernatants. Lactate dehydrogenase was measured in sucrose medium and sucrose/albumin medium homogenates and hence could be used as a reference enzyme to permit calculation of these activities per mg of protein. [a]$P < 0.02$, [b]$P < 0.01$, [c]$P < 0.001$ for comparisons with the control or euthyroid groups as appropriate; [d]$P < 0.02$, [e]$P < 0.01$ for effect of insulin treatment of diabetic rats; [f]$P < 0.05$, [g]$P < 0.02$, [h]$P < 0.01$, [i]$P < 0.001$ for effect of inclusion of albumin in homogenization media (compared with values in Table 6).

Condition	Mg^{2+}-dependent PPH activity	
	(nmol/min per mg of protein)	(% particulate)
Control	26.9±2.7	9.3±1.1
48 h starved	14.3±1.4[b]	6.8±0.9[i]
Diabetic	16.1±2.6[a]	13.9±3.1
48 h-insulin-treated diabetic	32.3±4.0[e]	4.7±1.1[adf]
2 h-insulin-treated diabetic	17.5±1.3[ah]	10.5±1.6[g]
Euthyroid	31.3±3.3	23.0±1.3[h]
Hypothyroid	7.5±2.5[c]	10.4±1.3[c]

subcellular distribution with physiological state. Furthermore, there appear to be no previous studies of adipose-tissue PPH and GPAT activities in diabetes and hypothyroidism. In accord with previous studies of PPH (Moller *et al.*, 1977; Jamdar & Osborne, 1982) and GPAT (Angel & Roncari, 1967; Aas & Daae, 1971; Jamdar & Osborne, 1982), both activities decreased in starvation. Diabetes had very similar effects to starvation. Both diabetes and starvation significantly increased the percentage of particulate PPH, but this effect was abolished by inclusion of albumin in homogenization buffers. Insulin replacement for 2 days restored PPH and GPAT activities to values equivalent to, or even higher than, the control values and also decreased the percentage of particulate PPH to the control value (Table 6). The net effect in these states is again a 'buffering' of the actual activity of the particulate form of PPH, which is 3.5, 3.0, 4.0 and 3.3 nmol/min per mg of protein in the control, starved, diabetic and 2-day-insulin-treated diabetic states respectively. However, this does not sustain pathway flux in starvation since adipocytes from starved animals show decreased rates of incorporation of [^{14}C]glucose into acylglycerol glycerol or of [^{14}C]palmitate into triacylglycerol (Saggerson, 1972; Harper & Saggerson, 1976). Changes in total PPH may therefore be more indicative of the capacity of the pathway.

When extracts were prepared in sucrose/albumin media, the percentage of particulate PPH in tissues from the 2-day-insulin-treated diabetics was significantly below the control value. Thus, both *in vivo* and *in vitro* (Table 5) insulin decreases the percentage of particulate PPH. The effect of albumin (compare Tables 6 and 7) suggests that fatty acids might be involved in promoting translocation of PPH to particulate fractions in diabetes and starvation, but it is apparent (Table 7) that insulin must have some separate effect on this process.

A noteworthy and unexpected finding was that GPAT and PPH activities were totally and partially restored respectively 2 h after a single insulin administration to the diabetic animals (Table 6). When sucrose/albumin media were used, this restoration of PPH was abolished (Table 7), suggesting that albumin removed an activator of the enzyme. The restoration of GPAT activity by insulin in the short term was still evident in the presence of albumin (results not shown). These rapid actions of insulin are not understood at present, but merit further study. This short-term insulin treatment did not decrease the percentage of particulate PPH to the control value (Table 6).

Euthyroid control animals were 2–3 weeks older than the other control group, but their fat-pads had comparable specific activities of GPAT and PPH. However, it was noteworthy that the 8-week-old animals had significantly higher values for the percentage of particulate PPH ($P < 0.001$ with or without albumin in the medium). In fact albumin significantly increased the percentage of particulate PPH in the 8-week-old group of animals (Table 7). In the hypothyroid state PPH and GPAT activities were decreased, by 77% and 22% respectively. When extracts were prepared in sucrose/albumin medium it was also evident that the percentage of particulate PPH was decreased in hypothyroidism, suggesting a role for thyroid hormones in the distribution of the enzyme that is independent of fatty-acid-mediated effects. Thyroxine replacement therapy for 3 days restored GPAT and partially restored PPH activity (Table 6).

It is apparent that adipose-tissue GPAT and PPH activities respond differently to changes in hormonal and nutritional state compared with those in the liver. The decreases in activity reported here for starvation, diabetes and hypothyroidism may be contrasted with the increases in hepatic Mg^{2+}-dependent PPH in starvation (Kinnula *et al.*, 1978; Mangiapane *et al.*, 1973; Vavrecka *et al.*, 1969) and diabetes (Murthy & Shipp, 1979; Whiting *et al.*, 1977; Woods *et al.*, 1982) or with glucagon (Pittner *et al.*, 1985), and the absence of change or small increase in hepatic microsomal GPAT observed in diabetes (Whiting *et al.*, 1977; Bates & Saggerson, 1977; Dang *et al.*, 1984) or hypothyroidism (Dang *et al.*, 1985).

Conclusion

This study demonstrates hormonal effects that change the subcellular distribution of PPH in adipocytes both *in vivo* and *in vitro*. In some instances these changes can be related to alterations in the availability of fatty acids. However, we have also observed situations where all or some of the changes caused by noradrenaline, insulin or thyroid hormones do not appear to relate to this. In

addition, although increased association of PPH with cellular membranes may be occurring in some instances in states with increased triacylglycerol-synthesis pathway flux, there is no strict relationship between these events. It is apparent that there may be a complex interplay of factors dictating the control of PPH and its role in the regulation of adipose-tissue triacylglycerol synthesis.

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