The protein phosphatases responsible for dephosphorylation of hormone-sensitive lipase in isolated rat adipocytes

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The levels of the cytosolic serine/threonine protein phosphatases (PP) in rat adipocyte extracts have been determined, by using both reference substrates and hormone-sensitive lipase (HSL) as substrates. Adipocytes contain significant levels of both PP1 and 2A (1.6 and 2.0 m-units/ml of packed cells respectively), with lower levels of PP2C and virtually no PP2B activity. PP2A and 2C exhibit similar degrees of activity against HSL phosphorylated at site 1, together accounting for 92% of the total. In contrast, site 2 is dephosphorylated predominantly by PP2A (over 50% of total activity), whereas PP1 and PP2C contribute approx. 20% and 30% respectively to the total phosphatase activity against that site. Total phosphatase activity in the adipocyte extracts was 2–3-fold higher against site 2 than against site 1. The possible significance of these findings to the regulation of HSL activity in adipose tissue *in vivo* is discussed.

INTRODUCTION

Control of adipose-tissue lipolysis by hormonal and neural effectors is mediated by regulation of the activity of hormone-sensitive lipase (HSL; EC 3.1.1.3) by reversible phosphorylation [1,2]. Phosphorylation of site 1 [3] ('regulatory site') of HSL by cyclic-AMP-dependent protein kinase (EC 2.7.1.37) results in activation of the enzyme [4,5], whereas phosphorylation of site 2 [3] ('basal site') which can be achieved *in vitro* by several protein kinases (e.g. AMP-activated protein kinase [6]), has no apparent direct effect on the enzyme activity *per se*, but inhibits subsequent phosphorylation of site 1, at least *in vitro* [6]. Site 2 is phosphorylated *in vivo* [3,7], but it is not yet clear under which conditions, if any, its extent of phosphorylation changes. Phosphopeptide-mapping studies from rat adipocytes pre-labelled with ^{32}P have demonstrated phosphorylation at site 1 in response to lipolytic hormones [7], but whether any prior dephosphorylation of site 2 is required remains to be established.

In eukaryotic cells four protein phosphatase (PP; EC 3.1.3.16) catalytic subunits, termed PP1, PP2A, PP2B and PP2C, account for most of the serine/threonine phosphatase activity towards substrates yet examined [8,9]. PP1, PP2A and PP2C have been shown *in vitro* to dephosphorylate a wide variety of phosphoproteins that are involved in a diverse range of cellular processes (reviewed in [8]); in contrast, PP2B displays comparatively narrow substrate specificity, most of these being proteins involved in the regulation of other kinases and phosphatases [10]. Only one study has examined the activity of purified protein phosphatases against HSL [11], and showed that PP2A and PP2C are major phosphatases acting against the two sites of HSL *in vitro*, whereas PP1 exhibited a lower activity and PP2B was completely inactive towards HSL. This study, however, was performed using non-physiological levels of HSL and purified preparations of protein phosphatases. On the basis of the levels of the different phosphatases reported to be present in the rabbit scapula fat [12], Olsson and Belfrage [11] concluded that PP2A was the major HSL phosphatase activity, whereas PP2C was unimportant in HSL regulation. In the present study, we have examined directly the dephosphorylation of HSL, phosphorylated at either site, using extracts of isolated rat adipocytes, and determined the relative levels of individual protein phosphatases acting against HSL.

MATERIALS AND METHODS

Materials

[γ-^{32}P]-ATP was obtained from ICN. Leupeptin, pepstatin A and antipain were from the Peptide Institute (Osaka, Japan). *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was from Sigma. Sephadex G-25 and Sephadex G-50 (superfine) were from Pharmacia. Dinonyl phthalate was from BDH. Centricon-30 microconcentrators were purchased from Amicon. Okadaic acid (OA) was obtained from Dr. Y. Tsukitani, Fujisawa Chemical Co., Tokyo, Japan, and both it and Inhibitor-2 (I-2) were generously provided by Professor P. Cohen (University of Dundee). AMP-activated protein kinase was kindly given by Dr. D. G. Hardie (University of Dundee). Other materials were as previously described [13].

HSL was purified from bovine perirenal adipose tissue essentially as described in [13], with the inclusion of chromatography on Mono S (HR 5/5 column; f.p.l.c. system, Pharmacia) with a linear 0–500 mM NaCl gradient at 1 ml/min for 60 min in place of heparin–Sepharose as the final purification step.

Phosphorylase kinase (EC 2.7.1.38) was purified from the post-glycogen pellet supernatant of a glycogen synthase preparation [14] as described in [15]. The active fractions from chromatography on Sepharose CL-4B were concentrated by vacuum dialysis and passed through a column of poly(L-lysine)–Sepharose (1 cm × 4 cm) equilibrated in 20 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/10% (v/v) glycerol to remove any contaminating phosphatases. The purified phosphorylase kinase

Abbreviations used: HSL, hormone-sensitive lipase; PP, protein phosphatase; OA, okadaic acid; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; PCV, packed cell volume; I-2, Inhibitor-2.

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was then concentrated by vacuum dialysis and stored in 20 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/30% glycerol at −20 °C.

Glycogen phosphorylase (EC 2.4.1.1) was purified from the run-through fractions of the DEAE-cellulose chromatography step from a glycogen synthase preparation [14] essentially by the method of Fischer and Krebs [16]. The glycogen phosphorylase was stored in 20 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/30% glycerol at −20 °C.

The catalytic subunit of cyclic-AMP-dependent protein kinase was purified from bovine heart as described in [17]. PP1 and PP2A catalytic subunits were purified as described in [18] from rabbit skeletal muscle.

Preparation of ^{32}P-labelled reference substrates

^{32}P-labelled phosphorylase, phosphorylase kinase and casein were all prepared to a specific radioactivity of approx. 1.0×10^6 d.p.m./nmol. Phosphorylase *b* (4.7 mg) was phosphorylated by incubation with 24 m-units of phosphorylase kinase in a total volume of 0.5 ml for 1 h at 30 °C in 50 mM Tris/HCl, pH 7.0, 0.1 mM $CaCl_2$, 10 mM Mg^{2+} and 0.2 mM [^{32}P]ATP. 1 unit of phosphorylase kinase activity incorporates 1 μmol of phosphate per min at 30 °C. Phosphorylase kinase (3 mg) was phosphorylated with 0.23 unit of the cyclic-AMP-dependent protein kinase in a total volume of 1 ml for 1 h at 30 °C in 50 mM Tris/HCl, pH 7.0, 10 mM Mg^{2+} and 0.2 mM [^{32}P]ATP (1 unit of cyclic-AMP-dependent protein kinase activity catalyses the incorporation of 1 μmol of ^{32}P/min into histone at 30 °C). Under these conditions the stoichiometry of phosphorylation for phosphorylase and phosphorylase kinase was 0.98 mol/mol and 2.3 mol/mol respectively. Excess [^{32}P]ATP was removed from these preparations by chromatography on Sephadex G-25 (0.5 cm × 20 cm) equilibrated in 20 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/10% glycerol, and the phosphoprotein peak fractions were concentrated on Centricon-30 microconcentrators. Both ^{32}P-phosphorylase and ^{32}P-phosphorylase kinase were stored in 50 mM Tris/HCl (pH 7.0)/30% glycerol at −20 °C and used within 1 week of preparation.

^{32}P-casein was prepared by incubating 10 mg of casein with 0.3 unit of cyclic-AMP-dependent protein kinase for 10–12 h at 30 °C in 0.5 ml of 50 mM Tris/HCl (pH 7.0)/0.1 mM EGTA/15 mM β-mercaptoethanol containing 10 mM Mg^{2+} and 0.2 mM ATP (1.0×10^6 d.p.m./nmol). The reaction was terminated by addition of both EDTA and sodium pyrophosphate to 10 mM each. After 10 min incubation on ice, excess ATP was removed by gel filtration in 50 mM Tris/HCl (pH 7.0)/0.1 mM EGTA/15 mM β-mercaptoethanol/5% glycerol as described in [19]. The ^{32}P-casein was stored at 4 °C and used within 1 week of preparation.

Preparation of ^{32}P-labelled HSL

HSL (~ 60 μg) was phosphorylated (2 h) with [γ-^{32}P]ATP as previously described [6] by using either the catalytic subunit of cyclic-AMP-dependent protein kinase (0.7 unit/ml, prephosphorylated with unlabelled ATP) or AMP-activated protein kinase (2 units/ml) to label site 1 and site 2 respectively. The reaction was terminated by addition of 10 mM EDTA and 10 mM sodium pyrophosphate, pH 7.0. Under these conditions the stoichiometry of phosphorylation of HSL was approx. 0.8 and 0.4 mol of phosphate/mol of HSL for site 1 and site 2 respectively. Residual [^{32}P]ATP was removed by centrifugal ultrafiltration using Centricon-30 microconcentrators, followed by washing with 3 × 2 ml 50 mM Hepes/0.1 mM benzamidine/1 mM dithiothreitol/50 mM NaCl/10 μg/ml leupeptin, pH 7.0. The alkyl polyoxyethylene ether detergent $C_{13}E_{12}$ (0.2%, v/v) was added to the phosphorylation incubations before these washes to help to stabilize HSL and to minimize its binding to the filter membranes. The ^{32}P-HSL was stored at 4 °C and used within 18 h of preparation.

Adipocyte isolation and preparation of extracts

Adipocytes were isolated by collagenase digestion of epididymal fat-pads [20] from 16 h-fasted Wistar rats (160–190 g body wt.), with modifications [21]. After digestion (45 min), the adipocytes were washed five times with Krebs–Ringer–Hepes buffer containing 200 nM adenosine and finally suspended to a concentration of 10% (v/v). The packed cell volumes (PCV) of the adipocyte suspensions were determined with a lipocrit centrifuge. Portions (400 μl) of the cell suspension were separated from the medium by centrifugation through dinonyl phthalate as described in [22], and the adipocytes were lysed by disruption (15 s vortex-mixing, 1 min sonication) in 100 μl of extraction buffer (50 mM Hepes, pH 7.0, 0.1 mM EGTA, 15 mM β-mercaptoethanol, 80 μg/ml antipain, 80 μg/ml pepstatin, 80 μg/ml leupeptin, 40 μM TPCK).

Phosphatase assays

Protein phosphatases in the adipocyte extracts were assayed by using as substrate 3 μM ^{32}P-glycogen phosphorylase [18], 1.6 μM ^{32}P-phosphorylase kinase [10], 2 μM ^{32}P-casein [19] or 0.23 μM ^{32}P-HSL. To compensate for the different stoichiometries of phosphorylation of the two sites on HSL, different amounts of HSL polypeptide were added containing the stated concentrations of phosphorylated HSL. The given concentrations refer to those of the phosphate groups present in each protein. Assays were carried out in 30 μl of 50 mM Tris/HCl, pH 7.0, containing 0.1 mM EGTA, 0.03% Brij-35, 0.1% β-mercaptoethanol and 1 mg/ml BSA essentially as described in [23], with the modification of using a 15 min incubation time to enable quantification of all four phosphatases within one experiment. The total phosphatase activity in all assays was restricted to less than 10% substrate dephosphorylation. Under these conditions the rate of substrate dephosphorylation was linear with time. PP1 was quantified as the activity inhibited by I-2, whereas PP2A was quantified as the activity inhibited by nanomolar concentrations of OA. The activities of PP2B and PP2C were estimated in the presence of micromolar OA (to inhibit PP1 and PP2A), and 0.2 mM Ca^{2+} and 10 mM Mg^{2+} respectively. When using rat adipocyte extracts in assays, final extract dilutions of 15–30-fold (phosphorylase kinase), 30–60-fold (site 1 ^{32}P-HSL/phosphorylase *a*/casein) and 90–120-fold (site 2 ^{32}P-HSL) were used. One unit of phosphatase activity catalyses the release of 1 μmol of [^{32}P]phosphate/min at 30 °C.

RESULTS

Quantification of the protein phosphatases present in adipocyte extracts

The exact concentrations of I-2 and OA necessary to inhibit PP1 and PP2A were determined in preliminary experiments using both extracts and the purified catalytic subunits of PP1 and PP2A as described in [23]. As shown in Figure 1, PP2A activity against phosphorylase *a* in adipocyte extracts was completely inhibited (≥ 90%) by 2 nM OA, whereas PP1 was completely inhibited by 2 μM OA or 0.2 μM I-2. Similar concentrations of OA and I-2 were necessary to inhibit the corre-

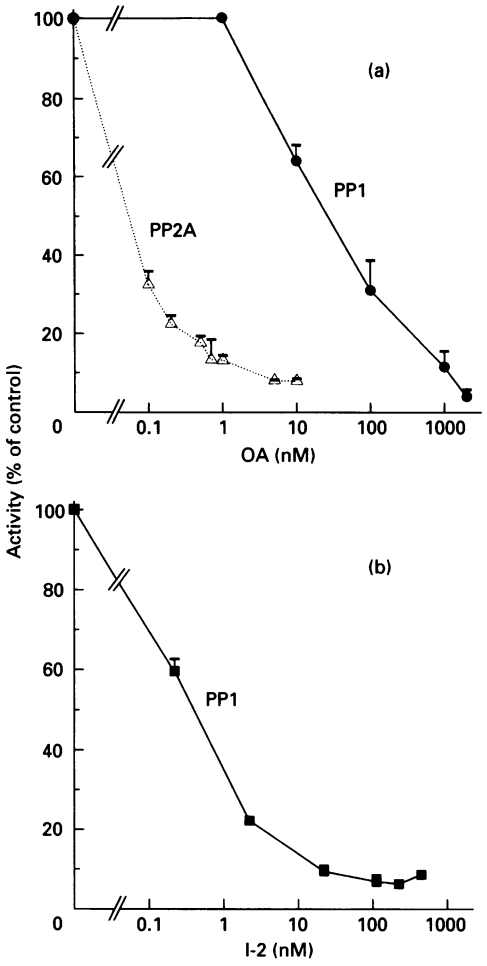

Figure 1 Effect of OA (a) and I-2 (b) on phosphorylase phosphatase activity in adipocyte extracts

Assays were performed at 120-fold dilution of the extracts in the absence of bivalent cations. In (**a**), the activity of PP2A was determined after 15 min preincubation with 0.4 μM I-2, the activity of PP1 being determined in the presence of at least 1 nM OA. In (**b**), the activity of PP1 was determined in the presence of 2 nM okadaic acid. Values are means $\pm$ S.E.M. ($n = 2$).

sponding purified catalytic subunits (results not shown). These concentrations of OA and I-2 were therefore used subsequently to quantify the phosphatases in adipocyte extracts.

The levels of the four protein phosphatases in crude adipocyte extracts were quantified by using the substrates phosphorylase *a* (for PP1 and PP2A), phosphorylase kinase (for PP2B) and casein (for PP2C). Approximately equal amounts of PP1 and PP2A activity was present within these extracts (1.6 and 2.0 m-units/ml of packed cells respectively). Lower but significant activity of PP2C was also present (0.24 m-unit/ml of packed cells), whereas less than 0.01 m-unit/ml of packed cells of PP2B activity was detected (Table 1).

Protein phosphatase activities in adipocytes against HSL

When the rate of dephosphorylation of HSL by the phosphatases in adipocyte extracts was determined under optimal conditions, HSL labelled at site 2 was dephosphorylated approx. 3-fold faster than HSL labelled at site 1 (0.43 ± 0.08 and 0.15 ± 0.01 m-unit/ml of PCV for site 2 and site 1 respectively).

Table 1 Quantification of protein phosphatases within adipocyte extracts

^{32}P-labelled phosphorylase *a*, phosphorylase kinase and casein were used to assay the protein phosphatases within adipocyte extracts as outlined in the Materials and methods section. Extracts were used at a final dilution of 30–60-fold, 15–30-fold and 15–60-fold in assays with phosphorylase, phosphorylase kinase and casein respectively. Values are means $\pm$ S.E.M.

Phosphatase	Reference substrate	Activity (m-units/ml of PCV)
PP1	Phosphorylase *a*	1.60 ± 0.35 ($n = 6$)
PP2A	Phosphorylase *a*	2.00 ± 0.20 ($n = 6$)
PP2B	Phosphorylase kinase	0.01 ± 0.005 ($n = 4$)
PP2C	Casein	0.24 ± 0.09 ($n = 4$)

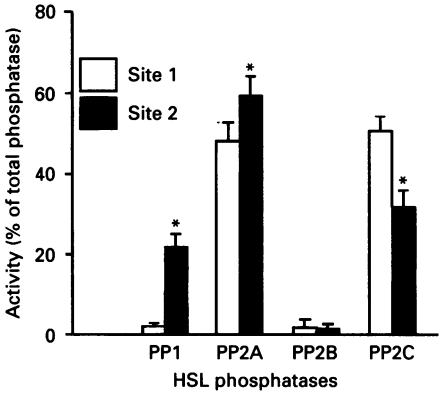

Figure 2 Activities of the protein phosphatases in rat adipocyte extracts acting against HSL phosphorylated at site 1 and site 2

^{32}P-HSL (2.0×10^6 d.p.m./nmol) labelled at either site 1 or site 2 was used to quantify the protein phosphatases in extracts of isolated adipocytes. The extracts were used at a final dilution of 30–60-fold (site 1) and 90–120-fold (site 2) and assayed in duplicate. The treatments used to assay the phosphatases selectively were as outlined in the Materials and methods section. Values are means $\pm$ S.E.M. ($n = 6$): $^*P < 0.005$ by Student's unpaired *t* test.

In preliminary experiments using ^{32}P-HSL as substrate, 0.5–1 nM OA was found to be necessary to inhibit over 90% of the PP2A activity. Addition of 0.4 μM I-2 was required to achieve > 90% inhibition of purified PP1 activity towards HSL in a standard assay. These inhibitor concentrations were therefore used in all further work.

The activities of the individual phosphatases in adipocyte extracts against HSL are shown in Figure 2. Despite the high levels of PP1 in the extracts, little PP1 activity against site 1 was detected (0.003 m-unit/ml of packed cells) (Figure 2). PP2A and PP2C were the only protein phosphatases that displayed activity towards HSL phosphorylated at site 1, with similar activities of each phosphatase being present (Figure 2). The high activity of PP2C towards HSL contrasts with the low abundance of this phosphatase within the extracts when assayed against casein (Table 1), indicating a relatively high PP2C specific activity for this site on HSL. The lack of PP2B activity in extracts towards HSL is consistent with the data with phosphorylase kinase as substrate, which indicated the virtual absence of this phosphatase within the adipocyte extracts. The sum of the individual activities for all four phosphatases for this site was $104 \pm 11.5\%$ (mean $\pm$ S.E.M., $n = 6$) of the total phosphatase activity observed in the absence of inhibitors and in the presence of bivalent cations.

HSL phosphorylated at site 2 was dephosphorylated by PP1, PP2A and PP2C in the adipocyte extracts. PP2A was the predominant phosphatase acting against this site (about 60% of total phosphatase activity). However, PP1 and PP2C also showed significant activity towards this site (21.9% and 31.9% respectively) (Figure 2). The individual phosphatase activities towards this site summed to 116 ± 13% of the total phosphatase activity assayed in the absence of phosphatase inhibitors and in the presence of bivalent cations.

DISCUSSION

The three protein phosphatases, PP1, PP2A and PP2C, were detected within rat adipocytes at significant levels when assayed against their reference substrates. Essentially no PP2B activity was detected in the rat adipocytes, either towards its acknowledged reference substrate ^{32}P-phosphorylase kinase [10] or towards HSL labelled in site 1 or 2. Phosphoprotein phosphatase 2B activity has previously been detected in rabbit scapula fat (partially brown adipose tissue) [12]; however, no values have been reported on the levels of this enzyme within white adipose tissue. Furthermore, purified PP2B has been shown to be inactive against HSL [11].

PP1 and PP2A appear to be the major phosphatase activities within rat adipocytes, as revealed by assays with phosphorylase *a* as substrate (2.00 ± 0.20 m-units/ml of PCV for PP2A, compared with 1.60 ± 0.35 m-units/ml of PCV for PP1). The relative levels of PP1 and PP2A determined here agree with the findings of a previous study [23]. PP2C is present within rat adipocytes at lower levels than for PP1 and PP2A; this quantification of PP2C in rat adipocytes has not been previously reported. A study using scapula fat [12] also demonstrated the presence of PP2C at a lower abundance than for PP1 and PP2A; however, as stated above, scapula fat partially contains brown adipose tissue, and therefore the two studies are not directly comparable.

PP1, PP2A and PP2C all have significant activity against HSL within rat adipocytes. The importance of PP2A-like phosphatases in the dephosphorylation of both sites of HSL and the lack of activity of PP2B are in agreement with previous observations [11]. It is not possible to say from the present data which isoform of PP2A [24] is responsible for the dephosphorylation of HSL. In contrast with the results of a previous study [11], PP2C appears to play a major role in the dephosphorylation of both sites of HSL within rat adipocytes. Selective dephosphorylation of site 2 may be possible by the action of PP1, as this phosphatase shows much greater activity towards site 2 (0.094 m-unit/mml of PCV) than to site 1 ($\sim$ 0.003 m-unit/ml of PCV).

Studies of the present type using tissue extracts, rather than those involving the use of purified phosphatase catalytic subunits as in [11], provide data with a greater physiological relevance, since studies of the latter type do not consider either the relative concentrations or the native states of the protein phosphatases within the cell. The concentration of HSL in rat adipocytes has been estimated to be about 0.5 μM [25]. The previous study used HSL at the sub-physiological concentration of about 25 nM [11], whereas in the present study 0.23 μM was used. The activities of PP2C reported here were determined in the presence of optimal concentrations (10 mM) of Mg^{2+}. However, $\sim$ 50% of this activity was still observed at the near-physiological Mg^{2+} concentration of 1 mM (results not shown), suggesting that PP2C would account for approx. 39% of the activity towards site 1 and 16% of the activity towards site 2 under physiological conditions.

The finding that PP2C is one of the major phosphatases acting towards HSL is of interest, and suggests that PP2C may play a specific role in the regulation of lipid metabolism. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and AMP-activated protein kinase (HMG-CoA reductase kinase) are the only known substrates of likely physiological significance for which PP2C represents the major phosphatase activity [12,26]. This may have major implications in the study of the regulation of lipid metabolism, since all three enzymes are involved in the regulation of cholesterol metabolism [27].

The total phosphatase activity against site 2 is significantly higher than that against site 1. In particular, the activity of PP1 is approx. 30-fold higher towards site 2 than site 1, although the activities of PP2A and PP2C are also higher towards site 2 than site 1 (4-fold and 2-fold respectively). A previous study [11] also suggested that the different phosphatases each showed a preference for site 2 over site 1, although this difference was not as pronounced as in the present work. Differences in the relative activities of the protein phosphatases towards site 1 and site 2 suggest that *in vivo* there is a considerable degree of selectivity by the protein phosphatases for the HSL substrate. The net dephosphorylation of HSL in adipocytes in response to insulin has been proposed to involve the action of protein phosphatases upon HSL [22]. Although the insulin-induced phosphorylation of I-2 in adipocytes [28] may represent one possible mechanism whereby hormones may influence the activity of HSL via protein phosphatases, this is unlikely to represent a major mechanism, in that PP1 does not represent a major fraction of the total HSL phosphatase activity. The effects of hormonal pre-treatment of adipocytes on the relative activities of the protein phosphatases towards the phosphorylation sites of HSL are currently under investigation.

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