Dephosphorylation of perilipin by protein phosphatases present in rat adipocytes

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Abstract By incubating ^{32}P-labelled adipocytes, and extracts from these cells, in the presence or absence of specific inhibitors, we evaluated the contribution of protein phosphatases PP1, PP2A and PP2C, to the dephosphorylation of perilipin, an acutely hormone-regulated adipocyte phosphoprotein. Under conditions to completely inhibit PP2A activity, perilipin phosphatase activity in extracts remain unaffected, but PP1 inhibition results in abolition of perilipin phosphatase activity. Inhibition of PP1 (and 2A) in intact adipocytes stimulated lipolysis and increased phosphorylation of perilipin. No involvement of PP2C was found. Hence, PP1 constitutes the predominant if not sole perilipin phosphatase in adipocytes.

Key words: Lipolysis; Perilipin; Protein phosphorylation; Protein phosphatase; Adipocyte

1. Introduction

The rate-limiting step of adipose tissue lipolysis is catalysed by hormone-sensitive lipase (HSL), an enzyme that is acutely regulated by hormones that elevate cAMP and activate cAMP-dependent protein kinase (PKA) (reviewed in [1,2]). It has been widely accepted that on stimulation of lipolysis in adipocytes by catecholamines [3], specific serine phosphorylation of the 'regulatory site' (Ser-563) or site 1 of HSL by PKA occurs [4]. A further 'basal site' (Ser-565), or site 2 of HSL, is phosphorylated in vivo [4], but it is not yet clear under which conditions, if any, its extent of phosphorylation changes. Subsequently two further PKA phosphorylation sites, Ser-659 and Ser-660, have been discovered [5]. Insulin, which exerts anti-lipolytic effects by both cAMP-dependent and -independent mechanisms not yet fully understood [6], inhibits HSL via dephosphorylation of the enzyme, possibly, in part, by stimulating a phosphatase activity [7]. However, it is evident that lipolytic regulation is not due to HSL reversible phosphorylation alone, as there exists a discrepancy between the degree of activation of HSL in vitro (2–3-fold) and of the extraordinary stimulation of lipolysis observed in the intact cell (50–100-fold) [8,9]. This discrepancy may be underlined, at least in part, by the discovery that on lipolytic stimulation, HSL alters its subcellular localisation [10], and has been shown to translocate onto the lipid droplet [11].

Perilipins are proteins tightly associated with the surface of lipid storage droplets in adipocytes, and are highly phosphorylated by PKA on lipolytic stimulation, in concert with the activation of HSL (reviewed in [12]). Current evidence suggests that the perilipins not phosphorylated by PKA provide a barrier against hydrolysis by neutral lipid lipases, and this barrier is removed upon phosphorylation by PKA ([13], C. Londos, unpublished data).

In response to insulin, the degree of perilipin phosphorylation is decreased, and it has been suggested that insulin, in addition to lowering cAMP levels, may also stimulate a perilipin phosphatase activity [14]. In a previous study from this laboratory, it was shown that adipocytes contain significant levels of both protein phosphatase 1 (PP1) and 2A (PP2A) (1.6 and 2.0 mU/ml packed cells respectively), with lower levels of PP2C and virtually no PP2B activity. PP2A and PP2C were shown to exhibit similar degrees of activity against HSL phosphorylated at the regulatory site, whereas the basal site is dephosphorylated predominantly by PP2A (over 50% of total activity), with PP1 and PP2C constituting the rest of the activity against this site [15]. There is, as yet, no published information on the phosphatases that may be acting on the two recently discovered phosphorylation sites on HSL.

In the present study we examine directly the dephosphorylation of perilipin, both in intact adipocytes and in extracts of lipolytically stimulated cells, in the presence or absence of various specific inhibitors. The identified perilipin phosphatase activity, namely PP1, has previously been shown to be activated in adipocytes in response to insulin [16], and we discuss the possibility that insulin may be mediating some of its anti-lipolytic effects via stimulation of PP1 and dephosphorylation of perilipin.

2. Materials and methods

2.1. Materials

[^{32}P]Orthophosphate was purchased from ICN. The protease inhibitors pepstatin, leupeptin and antipain were from the Peptide Institute, Osaka, Japan. Collagenase was from Worthington Biochemical Corporation, NY, USA. Purified catalytic subunits of protein phosphatases 1 and 2A, and inhibitor 2 (I-2) were purchased from Boehringer Mannheim, Lewes, UK. Okadaic acid (OA) was obtained from Calbiochem, and all other chemicals were from Sigma, Poole, UK. Anti-perilipin NH_2-terminal antibody was raised in rabbits as described previously [17]. Male Wistar rats, fed ad libitum on a diet of standard laboratory chow, were raised in house at the Hannah Research Institute, or the Comparative Biology Centre at the University of Newcastle upon Tyne.

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Abbreviations: HSL, hormone sensitive lipase; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; OA, okadaic acid; I-2, inhibitor 2; SDS-PAGE, sodium dodecyl sulphate polyacrylamide electrophoresis

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2.2. Adipocyte isolation

Adipocytes were isolated by collagenase digestion of epididymal fat pads from 180–200 g male Wistar rats killed by cervical dislocation, as described in [18]. All manipulations of adipocytes were performed in reduced phosphate (50 μM KH_2PO_4) Krebs-Ringer solution at 37°C, buffered with 25 mM HEPES pH 7.4 containing 2.5 mM $CaCl_2$, and 1% bovine serum (reduced phosphate KRH or red. P_i KRH). 200 nM adenosine was included to suppress cAMP production and stimulation of cAMP-dependent protein kinase activity [19]. Following isolation, cells were shaken at 37°C for 1 h, to allow the intracellular cAMP to return to basal levels. Cells were then washed in BSA-free red. P_i KRH supplemented with 200 nM adenosine. The packed cell volume (PCV) of the final suspension was determined by aspirating small aliquots into capillary haematocrit tubes and spinning in a microhaematocrit centrifuge.

2.3. Estimation of glycerol release

Aliquots of 20 μl were withdrawn from agitated incubations of cells at 200 μl cells/ml PCV, and added to 200 μl GPO-Trinder reagent (Sigma Diagnostics). This procedure was a modification of the method in [20]. Glycerol release is expressed as nmol glycerol/ml PCV.

2.4. ^{32}P Labelling of adipocytes

Adipocytes (200 μl PCV/ml) were loaded with [^{32}P]P_i, by incubating the cells in BSA free red. P_i KRH supplemented with 200 nM adenosine and 200 μCi/ml [^{32}P]P_i for 1 h in a shaking water bath at 37°C. ^{32}P-Labelled adipocytes were either incubated under 'basal' conditions, lipolytically stimulated with 100 nM isoproterenol and 1 U/ml adenosine deaminase or incubated in the presence of various concentrations of OA, for the indicated length of time.

2.5. Preparation of ^{32}P-labelled adipocyte extracts

Adipocytes were allowed to float to the surface and the infranatant containing the [^{32}P]P_i was removed by aspiration with a syringe. The remaining adipocytes were lysed with ice-cold 50 mM Tris-HCl buffer pH 7.4 containing 225 mM sucrose, 1 mM EDTA, 1 mM benzamidine, 1 μg/ml of pepstatin, 1 μg/ml leupeptin, 1 μg/ml antipain, (buffer B). For the preparation of extracts in which perilipin phosphatase activity was to be estimated, buffer B was supplemented with 3% Triton N-101. For the analysis of the phosphorylation state of perilipin in the intact cell, buffer B was supplemented with 1% Triton N-101 and 50 mM NaF to inhibit phosphatase activity in the extract. The lysate was then vortexed vigorously and centrifuged at 13 000 × *g* for 5 min at 4°C. Solubilised protein extract was then aspirated from under the solidified lipid fraction and kept on ice.

2.6. Estimation of perilipin dephosphorylation in extracts

^{32}P-Labelled extracts containing 3% Triton, prepared for the estimation of perilipin phosphatase activity, were diluted 1:3 in buffer B containing inhibitors or other additions to give the final concentrations indicated in the text. These diluted extracts were then incubated for up to 120 min at 37°C with shaking, and samples were removed into an equal volume of Laemmli sample buffer for SDS-PAGE. ^{32}P-Labelled extracts containing 1% Triton and 50 mM NaF prepared from intact cells treated with or without inhibitors were added immediately to an equal volume of Laemmli sample buffer for SDS-PAGE.

2.7. SDS-PAGE and Western blot analysis

SDS-PAGE was performed [21] using a Tris/glycine buffer system with Hoeffer mini-gel apparatus. For Western blotting, protein samples subjected to SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes, probed with anti-perilipin antibodies, and the amount of perilipin determined by Enhanced Chemiluminescence reagents from Amersham, UK. Proteins subjected to SDS-PAGE were stained with Coomassie blue R, and phosphoproteins were visualised by exposure to Fuji film. The loss of ^{32}P from the prominent 66 kDa band was estimated by digitalisation with a Fuji BAS-1500 phosphorimager.

3. Results

3.1. Characterisation of perilipin phosphatase activity in adipocyte extracts

Preliminary experiments confirmed the presence of a predominant 66 kDa phosphoprotein in ^{32}P-labelled lipolytically stimulated adipocyte extracts as reported previously [14]. The band was identified as perilipin by Western blotting with an antibody raised against a perilipin NH_2-terminal recombinant peptide (Fig. 1). Furthermore, the radioactive polypeptide co-purified with perilipin (detected by Western blotting) through several purification steps (not shown). Incubation of extracts at 37°C resulted in a significant loss of ^{32}P label from this band, over a time course of up to 120 min. It was estimated that the rate of loss of ^{32}P label from perilipin was approximately linear over the first 40 min of incubation under these conditions, which was the maximal length of incubation used in subsequent experiments. There was negligible proteolysis of extract proteins under these conditions as estimated by Coomassie blue R staining of the polyacrylamide gels (not shown). Furthermore, immunoreactivity of the 66 kDa band did not decrease during this period, indicating that proteolysis of perilipin was not occurring (Fig. 1). Thus, this loss of radiolabel from the 66 kDa polypeptide was attributed to the dephosphorylation of perilipin, due to an endogenous phosphatase activity present in adipocyte extracts.

3.2. Identification of protein phosphatases acting on perilipin

Over the 40 min of incubation, endogenous perilipin phosphatase activity accounted for the loss of approximately 44% of the ^{32}P label from perilipin. This was classified as 100% endogenous activity in subsequent experiments. On addition of 50 mM NaF, a potent non-specific phosphatase inhibitor, all phosphatase activity was abolished. (This observation also constitutes indirect evidence that no proteolysis is taking place.) Similarly, addition of OA, a specific inhibitor of PP1 and PP2A, to a concentration of 1 μM almost entirely eliminated the perilipin phosphatase activity, 99% of the ^{32}P label remaining incorporated in perilipin. The inclusion of Mg^{2+} (required for the activation of PP2C), in the presence of 1 μM OA, caused no significant change of perilipin phosphatase activity over that in the presence of 1 μM OA alone. Further, the inclusion of Mg^{2+} alone in the incubation buffer resulted in no statistically significant difference in endogenous perilipin phosphatase activity, which indicates that PP2C is not a significant perilipin phosphatase. The degree of perilipin

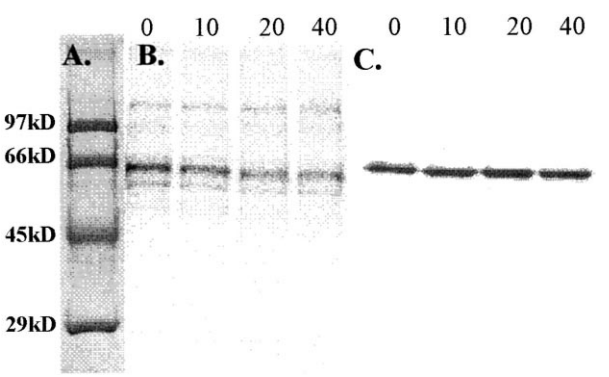

Fig. 1. Identification of 66 kDa phosphoprotein in adipocyte extracts as perilipin. A: Protein molecular weight markers. B: Phosphorimage of extract from ^{32}P-labelled adipocytes in the absence of any additions, after 0, 10, 20, and 40 min of incubation at 37°C. C: Western blot of extract from adipocytes after 0, 10, 20, and 40 min of incubation at 37°C, probed with an affinity-purified antibody raised against an NH_2-terminal peptide of perilipin A (all 10% SDS gels).

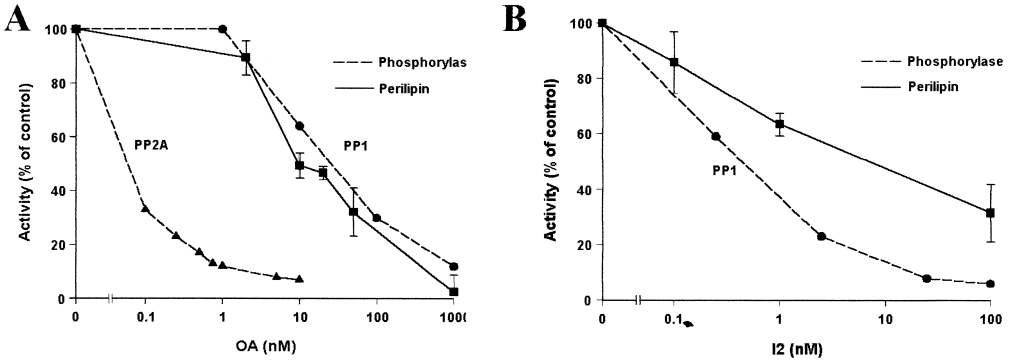

Fig. 2. Effects of OA and I-2 on the perilipin phosphatase activity of adipocyte extracts. ^{32}P-Labelled adipocyte extracts were prepared as described in Section 2, and incubated for 40 min at 37°C (A) in the presence of increasing concentrations of OA or (B) in the presence of increasing concentrations of I-2. After 40 min of incubation extracts were subjected to SDS-PAGE, and phosphorimaged as described in Section 2. Results are presented as a percentage of the endogenous activity in the absence of inhibitors, ± S.E.M. (A, $n = 2$–7; B, $n = 3$). Data are compared against the effects of the two inhibitors on PP1 and PP2A phosphatase activity in adipocyte extracts as in Wood et al. [15], using phosphorylase *a* as a substrate.

dephosphorylation under these various conditions is shown in Table 1.

The inclusion of OA, a specific inhibitor of PP1 and PP2A, with IC_{50} values of 15 nM and < 0.1 nM respectively, had an inhibitory effect on the endogenous perilipin activity present in the extracts. The addition of 2 nM OA, a concentration at which the activity of PP2A would be expected to be over 90% inhibited [15], had no significant effect on perilipin dephosphorylation. Further increases to the OA concentration, resulted in a decrease in the perilipin phosphatase activity in a dose-dependent manner, with an IC_{50} of about 12 nM. Finally, as noted in Section 3.1, inclusion of OA at a concentration of 1 μM resulted in the almost total abolition of perilipin phosphatase activity (Fig. 2A).

Similarly, on increasing the concentration of I-2, a specific inhibitor of PP1, a dose-responsive decrease in the perilipin phosphatase activity was observed, with an IC_{50} of about 10 nM. On addition of 100 nM I-2, approximately 70% of the endogenous activity was lost (Fig. 2B). In Fig. 2A,B, the inhibitory effects of OA and I-2 on perilipin phosphatase activity are compared to the effects previously observed in this laboratory, on the phosphorylase *a* phosphatase activity of PP1 and PP2A. These activities were estimated by the release of ^{32}P from ^{32}P-labelled phosphorylase *a* by the endogenous PP1 and PP2A present in adipocyte extracts.

These findings indicate that PP1 is responsible for perilipin dephosphorylation in adipocyte extracts. This is supported by further incubations of ^{32}P-labelled extracts, in which the activity of each of these phosphatases was increased by the inclusion of additional purified subunits of the phosphatases. Inclusion of an excess of the catalytic subunit of PP1 (PP1c) resulted in an observed increase of phosphatase activity against perilipin, in a dose-responsive manner. No statistically significant increase in perilipin phosphatase activity was observed on inclusion of an equivalent excess of PP2Ac (data not shown).

3.3. *Effect of OA on lipolytic rate and the phosphorylation state of perilipin in intact adipocytes*

Since the cell is permeable to OA it can be used to inhibit phosphatases in the intact adipocyte. It has been shown previously [22] that incubation of adipocytes with 1 μM OA, which is sufficient to inhibit PP1 and 2A but not 2B and 2C, increases dramatically the phosphorylation state of many proteins. In this study, the effect of OA on the phosphorylation of perilipin in the intact adipocyte was investigated (Fig. 3). Cells incubated in the absence of OA or isoproterenol remained in basal lipolytic state, with minimal glycerol release. Inclusion of 1 μM OA resulted in a significant

Table 1
Perilipin dephosphorylation in adipocyte extracts

Incubation conditions	Loss of ^{32}P label from perilipin (%)
No additions (endogenous)	43.7 ± 2.1 (100)
50 mM NaF	−0.8 ± 2.8 (−1.9 ± 6.5)
1 μM OA	1.1 ± 2.8 (2.4 ± 6.4)*
1 μM OA+20 mM Mg^{2+}	6.1 ± 2.4 (13.9 ± 5.4)*
20 mM Mg^{2+}	39.7 ± 1.7 (90.8 ± 4.0)

Extracts were incubated for 40 min at 37°C, with or without additions shown. Data are presented as means ± S.E.M. ($n = 4$–10). Numbers in parentheses refer to loss of ^{32}P label as percentage of control. *Not significantly different, $P > 0.1$.

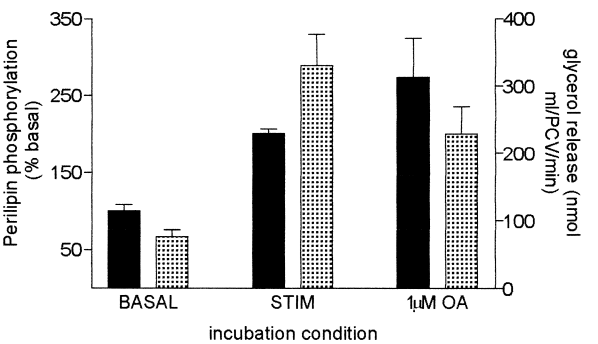

Fig. 3. Effect of OA on glycerol release and phosphorylation of perilipin in intact adipocytes. ^{32}P-Labelled adipocytes were prepared as described in Section 2, and incubated for 10 min at 37°C either under basal conditions, in the presence of 100 nM isoproterenol and 1 U/ml ADA (stimulated), or in the presence of 1 nM or 1 μM okadaic acid. Solid bars represent the glycerol release from intact adipocytes over the 10 min of incubation, assayed as described in Section 2, expressed as nmol of glycerol released per ml of packed cells per minute. Hatched bars represent the phosphorylation state of perilipin after the 10 min of incubation, estimated by phosphorimage analysis of ^{32}P-labelled perilipin after SDS-PAGE, expressed as percentage of perilipin phosphorylation under basal conditions. Both sets of data are presented as mean ± S.E.M. ($n = 4$).

increase in glycerol release ($P < 0.05$), representing a 3.4-fold increase in lipolysis. This compares to a 4.5-fold increase in lipolysis by stimulation with 100 nM isoproterenol ($P < 0.05$).

Phosphorimage analysis of extracts from these cells revealed that inclusion of 1 μM OA resulted in a significant increase ($P < 0.05$) in the phosphorylation state of perilipin, 2.7-fold over that under basal conditions, which compares with a 1.9-fold increase in the phosphorylation of perilipin by stimulation with isoproterenol (Fig. 3). These findings support the role of PP1 in being responsible for dephosphorylation of perilipin and indicate that 2B and 2C are not involved. Lower levels of OA were without significant effect on lipolysis or perilipin phosphorylation suggesting PP2A is not involved, although due to the uncertainty over the intracellular concentration of PP2A in adipocytes, this cannot be firmly established.

4. Discussion

In contrast to the amount known about perilipin phosphorylation by cAMP-dependent kinase in response to catecholamines [14], relatively little is known about the mechanism by which it is dephosphorylated. Londos et al. first showed that insulin counteracted the isoproterenol effect of ^{32}P incorporation into perilipin, by a mechanism independent of insulin's ability to lower cAMP levels [6]. Thus, it has been suggested that insulin mediates its effects, at least in part, by activating a specific serine phosphatase. Although perilipin has several putative phosphorylation sites as identified from the primary sequence [23], the phosphorylation state of specific sites have not been studied. Hence, only the global phosphorylation state, and subsequent dephosphorylation can be studied.

The evidence presented here indicates that dephosphorylation of perilipin in adipocytes is mediated via PP1. The inclusion in extracts of OA at concentrations which would be expected to inhibit $>90\%$ of PP2A activity, leaving that of PP1 unaffected, had no effect on the dephosphorylation of perilipin. Thus it would seem that PP2A plays no significant part in the dephosphorylation of perilipin. Furthermore, increasing the concentration of OA to levels which would be expected to increasingly inhibit the activity of PP1, resulted in inhibition of perilipin phosphatase activity. Inclusion of OA at 1 μM, a concentration at which PP1 and PP2A activity is fully inhibited, resulted in the abolition of the perilipin phosphatase activity in adipocyte extracts. 1 μM OA was also found to increase the phosphorylation state of perilipin in intact adipocytes in concert with the stimulation of lipolysis, offering evidence that inhibition of PP1 (or PP2A) activity increases perilipin phosphorylation, presumably on those sites phosphorylated by PKA, and mimics the stimulation of lipolysis by PKA in vivo.

More evidence for the role of PP1 was gathered by incubating extracts in the presence of I-2, a specific cytosolic inhibitor of PP1 [24]. The addition of increasing concentrations of I-2 resulted in the decrease of perilipin phosphatase activity in a concentration-dependent manner. On comparison of the I-2 inhibitory effects on the perilipin phosphatase activity with those previously observed in this laboratory, we observe a K_i for I-2 of about 80% that previously determined for I-2, estimated by release of ^{32}P from ^{32}P-labelled phosphorylase *a* by PP1 in adipocyte extracts, and purified PP1c. This shortfall in the ability of I-2 to inhibit perilipin phosphatase activity compared with PP1 may be a function of the protocol used or substrate specificity, i.e. perilipin as opposed to phosphorylase *a*. It has been noted previously that I-2 can have different effects on the kinetics of dephosphorylation of distinct substrates of PP1 [25]. No evidence was obtained for a significant contribution of PP2C to the dephosphorylation of perilipin.

Several researchers have reported that PP1 in 3T3-L1 adipocytes is stimulated by the action of insulin [26,27], but whether this is via the mechanism reported in skeletal muscle has yet to be determined [28]. The data presented from this study are supported by the observations of Misek and Saltiel who, in studying the insulin-mimicking effects of glycosyl phosphatidylinositols, showed that these compounds inhibited isoproterenol stimulated lipolysis in intact rat adipocytes, and blocked the phosphorylation of a 70 kDa phosphoprotein in the cytosol of these cells, via the activation of a PP1 activity [29]. However, only a small proportion of this phosphoprotein was found in the cytosol, being almost entirely lipid-associated, and is likely to correspond to perilipin, although there is no evidence in direct support of this. Taken together, the data are consistent with PP1 being involved in the insulin-mediated dephosphorylation of perilipin, in that it has been demonstrated that PP1 can be activated by insulin in intact adipocytes [16], that perilipin can be dephosphorylated by PP1 (present work) and that perilipin is dephosphorylated in response to insulin [14]. Definitive proof, however, will need to come from a cell model lacking PP1 activity.

Evidence to date indicates that HSL and perilipin are phosphorylated and dephosphorylated in parallel under a variety of conditions. However, the current and previous data indicate that the regulation of the phosphorylation state of the two proteins differ in that the phosphorylation state of perilipin can apparently be accounted for by two proteins, namely PKA and PP1, whereas HSL is subject to control by additional kinases and phosphatases, the specific role of which has yet to be clarified.

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