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Calyculin and okadaic acid promote perilipin phosphorylation and increase lipolysis in primary rat adipocytes

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Abstract

Lipolysis is primarily regulated by protein kinase A (PKA), which phosphorylates perilipin and hormone-sensitive lipase (HSL), and causes translocation of HSL from cytosol to lipid droplets in adipocytes. Perilipin coats lipid droplet surface and assumes to prevent lipase access to triacylglycerols, thus inhibiting basal lipolysis; phosphorylated perilipin facilitates lipolysis on PKA activation. Here, we induced lipolysis in primary rat adipocytes by inhibiting protein serine/threonine phosphatase with specific inhibitors, okadaic acid and calyculin. The incubation with calyculin promotes incorporation of ^{32}Pi into perilipins, thus, confirming that perilipin is hyperphosphorylated. The lipolysis response to calyculin is gradually accompanied by increased accumulation of phosphorylated perilipin A in a concentration- and time-responsive manner. When perilipin phosphorylation is abrogated by the addition of N-ethylmaleimide, lipolysis ceases. Different from a considerable translocation of HSL upon PKA activation with isoproterenol, calyculin does not alter HSL redistribution in primary or differentiated adipocytes, as confirmed by both immunostaining and immunoblotting. Thus, we suggest that inhibition of the phosphatase by calyculin activates lipolysis via promoting perilipin phosphorylation rather than eliciting HSL translocation in adipocytes. Further, we show that when the endogenous phosphatase is inhibited by calyculin, simultaneous PKA activation with isoproterenol converts most of the perilipin to the hyperphosphorylated species, and induces enhanced lipolysis. Apparently, as PKA phosphorylates perilipin and stimulates lipolysis, the phosphatase acts to dephosphorylate perilipin and attenuate lipolysis. This suggests a two-step strategy governed by a kinase and a phosphatase to modulate the steady state of perilipin phosphorylation and hence the lipolysis response to hormonal stimulation.

Keywords: Lipolysis; Perilipin; Okadaic acid; Calyculin; Phosphorylation; Dephosphorylation; Protein phosphatase; Hormone-sensitive lipase; Adipose triglyceride lipase; Lipid droplet; Isoproterenol

1. Introduction

Adipocyte lipolysis is the lipase-catalyzed hydrolysis of triacylglycerol to glycerol and free fatty acids, which is physiologically stimulated by catecholamines through elevating cAMP and activating cAMP-dependent protein kinase A (PKA) [1,2]. PKA phosphorylates hormone-sensitive lipase (HSL) and causes translocation of HSL from the cytosol to the lipid droplets in adipocytes [3–5], an action that enhances triacylglycerol breakdown [1,2]. Although important, HSL is not the only lipase for triacylglycerol hydrolysis because adipocytes derived from HSL null mice retain ~ 50% of residual lipase activity and are responsive to adrenergic lipolytic stimulation [6]. An adipose

Abbreviations: AGTL, Adipose triglyceride lipase; fDMEM, Phenol red-free and serum-free Dulbecco's modified Eagle's medium; HSL, Hormone-sensitive lipase; PCV, Packed cell volume; PKA, cAMP-dependent protein kinase A; PPase, Protein serine/threonine phosphatase; PP1, protein phosphatase-1; PP2A, Protein phosphatase-2A; SDS-PAGE, SDS polyacrylamide gel electrophoresis

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triglyceride lipase (ATGL) was recently identified to be abundant on the cytosol and the lipid droplets in adipocytes and is predominantly responsible for the initial step of triacylglycerol hydrolysis [7,8].

Besides the catalytic lipases, perilipins are a family of structural proteins that coat the surface of lipid droplets in adipocytes [9,10] and are proposed to modulate lipolysis as triacylglycerol substrate-associated regulator [1,2,5]. Perilipin A is the major isoform and perilipin B is less abundant and a shorter variant. Both proteins arise from alternatively spliced transcripts and share a common N-terminal domain of 1 to 405 amino acids [11,12]. A lower level of adipocyte perilipin is associated with higher lipolytic activity and higher circulating concentrations of free fatty acids in human obese individuals [13]. The perilipin deficiency leads to an increased level of basal lipolysis but an attenuated response to β-adrenergic-stimulated lipolysis in adipocytes derived from perilipin-null mice [14]. By contrast, the expression of ectopic perilipin A in fibroblasts inhibits basal lipolysis in the absence of PKA stimulation [5,15–17]. Upon PKA activation, perilipin A is phosphorylated at up to six serine residues [1,2]. This phosphorylation ensures lipolysis mediated by either HSL [5,15,17] or non-HSL lipase [5,15,16] in the reconstituted fibroblasts. Further, a recent study revealed that the phosphorylation of perilipin A is required for the translocation of HSL to the lipid droplets during PKA-stimulated lipolysis [5]. These observations suggest that perilipin A functions as both a suppressor of basal lipolysis and a necessary enhancer of PKA-stimulated lipolysis [1,2].

Though the control of lipolysis has been long focused on cAMP and cAMP-regulated protein kinase, PKA, the results of three studies implied that protein serine/threonine phosphatase (PPase) might also participate in the lipolytic cascade [18–20]. A 1997 study provided the first clue that adipocyte lipolysis can be stimulated by specific PPase-1 (PP1) and -2A (PP2A) inhibitors, okadaic acid and calyculin [18]. Later, in adipocytes and the cell-free system consisting of isolated endogenous lipid droplets and HSL, okadaic acid was found to stimulate lipolysis, an action that did not result from increased activity of HSL but was attributable in part to a translocation of HSL and a change in the surface physicochemical character of lipid droplets [19]. However, this finding was not directly examined. As well, okadaic acid was found to inhibit perilipin dephosphorylation in vitro in adipocyte extracts and increase lipolysis in adipocytes [20]. Nonetheless, the molecular basis by which inhibition of PPase activates lipolytic response in intact fat cells has still not been resolved.

In this study, we induced lipolysis by PPase inhibition with calyculin and okadaic acid in primary rat adipocytes. Incubation with calyculin promoted the incorporation of ^{32}Pi into perilipins, thus confirming that perilipin is hyperphosphorylated. We show that lipolysis activated by calyculin is gradually accompanied by an accumulation of phosphorylated perilipin A but is not a consequence of accelerated redistribution of HSL from the cytosol to the lipid droplets in adipocytes. As perilipin phosphorylation is abrogated by the addition of N-ethylmaleimide, lipolysis also ceases, thus, suggesting that the phosphorylation of perilipin rather than the translocation of HSL plays an important role for the lipolytic action on inhibition of the PPase with calyculin. Further, we show that when the phosphatase is inhibited by calyculin, simultaneous PKA activation with isoproterenol converts most of the perilipin A to the hyperphosphorylated species, therefore resulting in an enhanced lipolysis. These data suggest that the endogenous phosphatase participates in the lipolytic regulation in cooperation with PKA by acting to modulate the steady state of the phosphorylation perilipin A.

2. Materials and methods

2.1. Materials

Calyculin A was purchased from Cell Signaling Technology (Beverly, MA). Okadaic acid, N-ethylmaleimide, phenol red-free Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose, and horseradish peroxidase conjugated lgG, were from Sigma (St. Louis, MO). Polyclonal antibodies against rat perilipin [21] or rat HSL [4] were generous gifts from C. Londos at the U.S. National Institutes of Health. Nitrocellulose membrane, prestained protein molecular weight markers, and SuperEnhanced chemiluminescence detection reagents were from Applygen Technologies Inc. (Beijing, China).

2.2. Isolation and culture of primary rat adipocytes

Adipocytes were isolated from epididymal fat pads of Sprague–Dawley rats (160–200 g) [22]. The fat pads were minced and digested in 5 ml Krebs–Ringer solution containing 25 mM HEPES, pH 7.4, 1 mg/ml type I collagenase and 1% defatted bovine serum albumin (BSA). After incubation for 40 min at 37 °C in a water bath shaken at 100 cycles/min, cells were filtered through a nylon mesh and washed 3 times in pre-warmed phenol red-free and serum-free DMEM (fDMEM). Adipocytes floating in the tube were centrifuged at 200×*g* for 3 min. Packed adipocytes were diluted in 1% BSA-fDMEM to generate a 10% (v/v) cell suspension. The packed cell volume (PCV) of the final suspension was determined according to the reported method [23]. Prior to the treatment, the fat cells were incubated for 1 h at 37 °C and shaken at 30 cycles/min, to restore intracellular cAMP level to a basal level. We observed that incubation of adipocytes in fDMEM but not in Krebs–Ringer solution reduced variability of cell and basal lipolysis.

2.3. Lipolysis assays

A total of 50 μl of packed adipocytes was suspended in 500 μl fDMEM (10% PCV) and treated as described. The culture medium was collected and heated at 70 °C for 10 min, to inactivate residual lipases. Glycerol released in the medium served as an index of lipolysis and was determined by use of a colorimetric assay (GPO Trinder reaction) from the absorption at 490 nm [24]. Lipolysis data were expressed as micromolecules of glycerol released per milliliter PCV.

2.4. Preparation of cytosolic and fat cake fractions from primary rat adipocytes

Following treatment, the culture media was assayed for glycerol. The fat cells were packed by centrifuging the cells for 3 min at 200×*g*, and homogenized in ice-cold fractionation buffer (50 mM Tris–HCl, pH 7.4, 255 mM sucrose, 1 mM EDTA, 0.1 mM sodium orthovanadate [Na_3VO_4], and 50 mM sodium fluoride [NaF]), as described [3,25]. The cell lysate was incubated on ice for 15 min and then centrifuged at 20,000×*g* for 30 min at 4 °C. The solidified fat cake of intracellular lipid droplets floated on top of the tube. The cytosolic fraction was localized below the layer of the fat cake. The fractions were collected, mixed with concentrated Laemmli sample buffer (62 mM Tris–HCl, pH 6.8, 5% SDS, 1% beta-mercaptoethenol, 0.1 mM Na_3VO_4, 50 mM NaF, and 15% glycerol in final) and adjusted equivalently against PCV [3]. The samples were heated to 95 °C for 5 min and cleaned at 12,000×*g* for 10 min, prior to loading on SDS-PAGE.