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Protein kinase C-dependent antilipolysis by insulin in rat adipocytes

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Abstract

Recently, we have shown that protein kinase C (PKC) activated by phorbol 12-myristate 13-acetate (PMA) attenuates the β1-adrenergic receptor (β1-AR)-mediated lipolysis in rat adipocytes. Stimulation of cells by insulin, angiotensin II, and α1-AR agonist is known to cause activation of PKC. In this study, we found that lipolysis induced by the β1-AR agonist dobutamine is decreased and is no longer inhibited by PMA in adipocytes that have been treated with 20 nM insulin for 30 min followed by washing out insulin. Such effects on lipolysis were not found after pretreatment with angiotensin II and α1-AR agonists. The rate of lipolysis in the insulin-treated cells was normalized by the PKCα- and β-specific inhibitor Gö 6976 and PKCβ-specific inhibitor LY 333531. In the insulin-treated cells, wortmannin increased lipolysis and recovered the lipolysis-attenuating effect of PMA. Western blot analysis revealed that insulin slightly increases membrane-bound PKCα, βI, and δ, and wortmannin decreases PKCβI, βII, and δ in the membrane fraction. These results indicate that stimulation of insulin receptor induces a sustained activation of PKC-dependent antilipolysis in rat adipocytes.

Keywords: Adipocyte; Lipolysis; β-adrenergic receptor; PKC; PMA; Insulin; Wortmannin

1. Introduction

Adipose tissues have important functions in the regulation of energy balance. Adipocytes store excess energy supply as triglyceride droplets, resulting in the development of obesity. During fasting and exercise, triglycerides stored in the cells are hydrolyzed producing glycerol and free fatty acids, which are important oxidative fuels for other tissues such as liver, skeletal muscle, kidney, and the myocardium. Different hormones govern the use of energy in the triglyceride depots [1,2].

Rat adipocytes contain three β-adrenergic receptor (β-AR) subtypes: β1, β2, and β3. The three β-ARs are coupled with Gs protein and transmit an activation signal to adenylylcyclase, leading to an increase in cAMP. The resulting activation of protein kinase A (PKA) mediates activation of hormone-sensitive lipase (HSL), which hydrolyzes triglycerides stored in the cells [1,2]. The fact that the protein level of β2-AR is extremely low [3] and that lipolysis stimulated by norepinephrine and isoproterenol is not significantly affected by a β2-AR antagonist ICI 118551 [4] indicate a very minor role of the β2-AR signaling for lipolysis in rat adipocytes. β3-AR may represent the physiological receptor for high norepinephrine concentrations (100 nM), attained by sympathetic activity near adipose tissues in conditions such as fasting and cold exposure [5,6]. In rat adipocytes, norepinephrine at concentrations usually found in the circulation (1−25 nM) stimulates mainly the high-affinity β1-AR and thereby induces lipolysis [5]. In humans, circulating catecholamine stimulates the β1- and β2-ARs, which are critical determinants for the rate of lipolysis induced by submaximal exercise in subcutaneous adipose tissue [7] and lipolysis in response to insulin-induced hypoglycemia in skeletal muscle [8].

Abbreviations: β-AR, β-adrenergic receptor; α-AR, α-adrenergic receptor; PKA, protein kinase A; PKC, protein kinase C; PKB, protein kinase B; PDK, phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-triphosphate; HSL, hormone-sensitive lipase; PDE3B, phosphodiesterase 3B; IRS, insulin-receptor substrate; EGF, epidermal growth factor; RACK, receptor of activated C-kinase; C-KIP, PKC-interacting protein; PMA, phorbol 12-myristate 13-acetate; buffer A, 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 32.3 mM HEPES, pH 7.4, 2 mM glucose, 20 mg/ml BSA, and 200 nM adenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecylsulphate

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The rate of lipolysis is regulated not only by lipolytic hormones but also by antilipolytic hormones. Insulin is the most potent antilipolytic hormone in adipose tissues. It inhibits the activity of HSL by decreasing the cAMP level through phosphorylation and activation of phosphodiesterase 3B (PDE3B) [1]. PDE3B stimulation by insulin requires the activities of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB) [1,9,10]. Lipolysis is also inhibited by the activities of α2-AR and adenosine receptor that couple to Gi protein and inhibit adenylylcyclase, and by other activities of different effector molecules [1,2].

Using rat adipocytes, we have shown that phorbol 12-myristate 13-acetate (PMA) attenuates lipolysis induced by submaximally stimulating concentrations of isoproterenol and norepinephrine [4]. The decrease in lipolysis by PMA may be due to specific inhibition of the β1-AR system by the activity of protein kinase C (PKC). In this study, we attempted to characterize the signaling pathway of PKC-dependent antilipolysis in rat adipocytes. It was found that stimulation of insulin receptor substitutes for the action of PMA. The effect of insulin on lipolysis was blocked by PKC inhibitors and wortmannin.

2. Materials and methods

2.1. Materials

The β-AR agonists, isoproterenol and dobutamine, and the β1-AR antagonist, CGP 20712A, were purchased from Sigma (St. Louis, USA). Angiotensin II, phenylephrine, and methoxamine were from Wako Pure Chemical Industries (Osaka, Japan). The PKC inhibitors, Gö 6976 and LY 333531, were from Calbiochem (Darmstadt, Germany) and Alexis (San Diego, USA), respectively. PMA, wortmannin, and protease inhibitor cocktail were from Sigma. The rabbit anti-peptide antibodies recognizing PKCα, βI, and βII were purchased from Santa Cruz Biotechnologies (Santa Cruz, USA) and the rabbit anti-peptide antibodies to PKCδ, ε, and ζ were from Sigma.

2.2. Animals and adipocyte preparation

Male rats of the Charles River CD strain weighing 200−240 g (7 weeks old) were used. Animals were fed a standard commercial diet *ad libitum* and allowed free access to water. The light cycle was 08:00−20:00. Isolated adipocytes were prepared from the epididymal fat pads by the method previously described [4]. After collagenase digestion, cells were washed, suspended in Dulbecco's modified Eagle's medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 20 mg/ml bovine serum albumin (BSA), and 200 nM adenosine, and incubated at 37 °C for 4 h with gentle agitation. If necessary, cells were treated with insulin for the final 30 min of a 4-h incubation. Then, cells were washed with a buffered solution containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 32.3 mM HEPES, pH 7.4, 2 mM glucose, 20 mg/ml BSA, and 200 nM adenosine (buffer A). After being washed, the volume of packed adipocytes was determined.

2.3. Lipolysis and glycerol measurement

The packed adipocytes were diluted 10-fold with buffer A, and incubated at 37 °C for 20 min in the presence of pharmacological agents. The concentration of glycerol in the incubation mixture was measured to determine the rate of lipolysis. Lipolysis, as assessed by glycerol release, linearly increased for at least 40 min [11].

2.4. Western blot analysis

Adipocytes were washed three times with a buffered solution containing 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.5% protease inhibitor cocktail. Then, cells were processed for separation of the cytosol and membrane fractions as described previously [4].

The membrane fraction from adipocytes was mixed with the sample-loading buffer, and subjected to sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis followed by electrotransfer of protein onto a polyvinylidene difluoride membrane [4]. After incubation of the membrane with the primary and secondary antibodies, immunoreactive bands were detected by ECL Plus (Amersham, UK). Samples that were compared from each experiment were analyzed on the same immunoblot, and relative changes in the density of the bands were determined by scanning densitometry.

2.5. Statistics

Each experiment was performed in duplicates and repeated three times or more. Results were mean values ± SEM. In some cases, data were expressed as the percentage of the activity in control cells (taken as 100%). If necessary, data were examined by Student's *t* test to evaluate the statistical significance.

3. Results

3.1. Effects of insulin, angiotensin II, and α1-AR agonist on the lipolysis-attenuating effect of PMA

Rat adipocytes possess a PKC-dependent signaling pathway for antilipolysis [4]. To explore the mechanism of PKC signaling, adipocytes were treated with insulin, angiotensin II, and α1-AR agonist, which are known to activate the PKC pathway [2,12–17]. Adipocytes were treated with 20 nM insulin for 30 min, washed with an insulin-free buffer, and determined for the rate of lipolysis. As shown in Fig. 1, lipolysis stimulated by low concentrations of dobutamine (0.4 and 0.8 μM) and isoproterenol (8 and 16 nM) was lower in the insulin-treated cells than the control cells. PMA attenuated lipolysis stimulated by low concentrations of dobutamine and isoproterenol in the control cells, but not in the insulin-treated cells.

The concentration of insulin (20 nM) used for pretreatment of adipocytes was sufficient for the total inhibition of lipolysis induced by 0.4 and 0.8 μM dobutamine in rat adipocytes, and washing out insulin partially restored the lipolysis (Fig. 1). The restored lipolysis was again inhibited by insulin in the following assay of lipolysis. Thus, insulin causes a reversible and an irreversible inhibition of lipolysis.

Next, adipocytes were treated with angiotensin II and α1-AR agonists, phenylephrine and methoxamine, and lipolysis was induced in the presence and absence of PMA before washing out the agents. PMA was found to significantly attenuate lipolysis (Fig. 2). Methoxamine decreased lipolysis, but PMA further reduced it. The lipolysis-attenuating effect of PMA was detected in adipocytes that had been treated with angiotensin II, phenylephrine, and methoxamine for 30 min, and the agents were washed out (results not shown).

3.2. Effects of PKC inhibitor and β1-AR antagonist on lipolysis

In the following experiments, lipolysis was stimulated by 0.8 μM dobutamine, as the effect of insulin was apparent (Fig. 1). The results shown in Fig. 1 indicate that insulin may substitute for PMA and attenuate lipolysis. To assess the significance of PKC activity, the effects of the PKCα- and β-specific inhibitor