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Protein kinase CβI interacts with the β1-adrenergic signaling pathway to attenuate lipolysis in rat adipocytes

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ABSTRACT

We have shown previously that insulin attenuates β1-adrenergic receptor (β1-AR)-mediated lipolysis via activation of protein kinase C (PKC) in rat adipocytes. This antilipolysis persists after removal of insulin and is independent of the phosphodiesterase 3B activity, and phorbol 12-myristate 13-acetate (PMA) could substitute for insulin to produce the same effect. Here, we attempted to identify the PKC isoform responsible for antilipolysis. Isolated adipocytes were treated with high and low concentrations of PMA for up to 6 h to degrade specific PKC isoforms. In the PMA-treated cells, the downregulation profiles of PKC isoforms α and βI, but not βII, δ, ε, or ζ, correlated well with a decrease of lipolysis-attenuating effect of PMA. After rats fasted for 24 h, adipocyte expression of PKC isoform α increased, while expression of PKCδ decreased. Fasting did not change the potency of PMA to attenuate lipolysis, however. The lipolysis-attenuating effect of PMA was blocked by the PKCβI/βII inhibitor LY 333531, but not by the PKCβII inhibitor CGP 53353 or the PKCδ inhibitor rottlerin. These data suggest that PKCβI interacts with β1-AR signaling and attenuates lipolysis in rat adipocytes.

1. Introduction

Adipose tissues are important for the regulation of energy balance. Adipocytes store an excess supply of energy as triglyceride droplets. During fasting and exercise, these triglycerides are hydrolyzed to produce glycerol and free fatty acids, which are important oxidative fuels for other tissues such as liver, skeletal muscle, kidney, and the myocardium [1]. Defects in triglyceride metabolism may contribute to the development of obesity [2]. Chronic elevation in plasma free fatty acid levels is closely linked to type 2 diabetes and cardiovascular complications [3]. Thus, an accurate regulation of lipolysis to maintain a normal level of plasma free fatty acids is essential to the prevention of these disorders [1–3].

The cAMP-dependent pathway is one of the best-understood mechanisms for the activation of lipolysis in adipocytes [1]. Catecholamines are potent lipolytic agents, stimulating the β-adrenergic receptor (β-AR) and leading to an increase in intracellular cAMP. The rise in cAMP levels activates protein kinase A (PKA), which then phosphorylates cytosolic hormone-sensitive lipase (HSL) and the lipid droplet-associated protein, perilipin [1]. This phosphorylation causes an increase in the hydrolysis of triglyceride by HSL and adipose triglyceride lipase, which works in concert with HSL [4]. Phosphorylation of perilipin by PKA may be a critical step for the induction of lipolysis [5].

Insulin negatively regulates lipolysis by stimulating phosphodiesterase 3B (PDE3B) activity [1]. The binding of insulin to its receptor triggers the activation of the receptor tyrosine kinase, which phosphorylates insulin-receptor substrates (IRSs). IRSs act as scaffolding proteins for several Src homology 2-containing proteins that recognize the IRS-phosphorylated tyrosine residues. Upon binding to the IRS, phosphatidylinositol 3-kinase (PI3K) initiates a series of events, including the activation of protein kinase B (PKB) [6]. PKB acts as an insulin-sensitive PDE3B kinase [1,6,7]. Phosphorylation and activation of PDE3B promotes degradation of cAMP and reduction in PKA activity [1].

Insulin provokes rapid changes in phospholipid metabolism and thereby generates biologically active lipids that serve as intracellular signaling factors. Conventional, novel, and atypical protein kinase Cs (cPKCs, nPKCs, and aPKCs) that are activated by this insulin signaling cascade have key roles in the regulation of insulin's metabolic effects [8,9]. We have previously shown that insulin interacts with the β1-AR signaling pathway via activation of PKC in rat adipocytes [10]. Isolated adipocytes that were treated with insulin, followed by an insulin washout, exhibited attenuated lipolysis when induced with the β1-AR agonist dobutamine. The PI3K inhibitor wortmannin blocked insulin's effect on lipolysis, while the PDE3B inhibitor cilostamide had no effect

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Abbreviations: β-AR, β-adrenergic receptor; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PI3K, phosphatidylinositol 3-kinase; HSL, hormone-sensitive lipase; PDE3B, phosphodiesterase 3B; IRS, insulin-receptor substrate; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecylsulphate; buffer A, Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, pH 7.4, 20 mg/ml BSA, and 200 nM adenosine; buffer B, 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 2.6 mM CaCl2, 1.2 mM MgSO4, and 32.3 mM HEPES, pH 7.4, 2 mM glucose, 20 mg/ml BSA, and 200 nM adenosine; buffer C, 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 1 mM EGTA

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[10]. Phorbol 12-myristate 13-acetate (PMA) could substitute for the action of insulin [10,11]. The PKC inhibitors, GF 109203X, Gö 6976 and LY 333531, blocked the antilipolytic effects of both insulin and PMA [10,11]. In this study, we attempted to identify the PKC isoform responsible for antilipolysis in rat adipocytes. Isolated adipocytes were treated with PMA under various experimental conditions to induce downregulation of PKCs. Alternatively, the cellular expression of PKCs was altered by allowing the rats to fast for 24 h prior to the preparation of isolated adipocytes. The PKC isoform present in these adipocytes was measured and correlated with the lipolysis-attenuating effect of PMA. We also examined the effect of PMA in the presence of specific PKC inhibitors. In all, the data obtained suggested that PKCβI is a negative regulator of the β1-AR-mediated lipolysis.

2. Materials and methods

2.1. Materials

The β1-AR agonist dobutamine, the PKCβII inhibitor CGP 53353, the PKCδ inhibitor rottlerin, and PMA were purchased from Sigma (St. Louis, USA). The PKCα/β inhibitor Gö 6976 and the PKCβI/βII inhibitor LY 333531 were obtained from Calbiochem (Darmstadt, Germany) and Alexis (San Diego, USA), respectively. The rabbit anti-peptide antibodies recognizing PKCs α, βI, and βII were purchased from Santa Cruz Biotechnologies (Santa Cruz, USA) and the rabbit anti-peptide antibodies to PKCs δ, ε, and ζ were from Sigma. Anti-rabbit IgG linked with horseradish peroxidase was from Calbiochem.

2.2. Downregulation of PKCs with PMA and modified expression of PKCs with fasting

The epididymal fat pads were collected from male rats of the Charles River CD strain (7 weeks old), and the adipocytes were isolated as described previously [10,11]. To degrade PKC, adipocytes were treated with 0.1 to 2 μM PMA 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 (buffer A) at 37 °C for up to 6 h.

To examine the effect of fasting, food was withheld from the rats for 24 h, starting at 08:00. Control rats were fed a standard commercial food *ad libitum*. Free access to water was given to both feeding and fasting rats. The light cycle was 08:00–20:00. Rats were euthanized by decapitation at 08:00, and isolated adipocytes were prepared. The cells were then incubated in buffer A with gentle agitation at 37 °C for 5 h.

2.3. Lipolysis

Prior to measurement of lipolysis, adipocytes were washed three times 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 B). Packed cells obtained with the final centrifugation were diluted 10-fold with buffer B. Aliquots (0.25 ml) of the cell suspension were mixed with an equal volume of buffer B containing the β1-AR agonist dobutamine and other pharmacological agents, and incubated at 37 °C for 30 min [10,11]. After incubation, the concentration of glycerol in the mixture was measured to determine the rate of lipolysis [12].

2.4. Western blot analysis

Adipocytes were washed five times with a buffered solution containing 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 1 mM EGTA (buffer C). Packed cells obtained with the final centrifugation were added buffer C containing 2% sodium dodecylsulphate (SDS) and 0.5% protease inhibitor cocktail, kept at 60 °C for 30 min, and centrifuged at 15,000 *g* for 10 min. The resulting supernatants were mixed with the sample-loading buffer [11]. Aliquots containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. After electrotransfer of protein onto a polyvinylidene difluoride membrane, the membrane was incubated with the primary and secondary antibodies. Immunoreactive bands were detected by ECL Plus [11]. Samples from each experiment were analyzed on the same immunoblot, and relative changes in band density were determined by scanning densitometry [10].

2.5. Statistics

Each experiment was repeated three times or more. Results are shown as mean ± SEM. If necessary, statistical significance was assessed with a Student's *t* test.

3. Results

3.1. Dobutamine-induced lipolysis

The β1-AR agonist dobutamine causes a dose-dependent increase in lipolysis, with maximal lipolysis occurring upon addition of 16 μM dobutamine [11]. With submaximal concentrations of dobutamine (0.4–1.6 μM), the lipolysis-attenuating effect of PMA is apparent and inhibition of lipolysis by the β1-AR antagonist CGP 20712A is clearly detected [11]. Here, lipolysis was induced with 0.8 and 1.6 μM dobutamine to assess the effect of PMA. The rate of lipolysis in untreated adipocytes (control cells) was 13.0 ± 1.3 and 16.4 ± 1.1 μmol glycerol/ml packed cells/h (mean ± SEM, $n = 3$) for 0.8 and 1.6 μM dobutamine, respectively. To evaluate the effect of PMA in multiple sets of experiments, lipolysis rates were expressed as a percentage of the control.

3.2. Effect of PMA-induced downregulation of PKC on the lipolysis-attenuating effect of PMA

PMA treatment activates cPKCs and nPKCs, but not aPKCs. Upon prolonged treatment, these PMA-responsive PKCs are proteolytically degraded [13,14]. This PMA-induced downregulation has been used to study the role of specific types of PKCs during insulin signaling and other biological functions [8,14–17]. In our previous study [11], we found that treating rat adipocytes with 2 μM PMA for 6 h decreases cellular expression of PKCs α, βI, βII, δ, and ε and abolishes the lipolysis-attenuating effect of PMA. In this study, the rate of downregulation was examined during early timepoints of PMA treatment. As shown in Fig. 1-A and B, western blots on total cell lysates revealed a marked decrease in

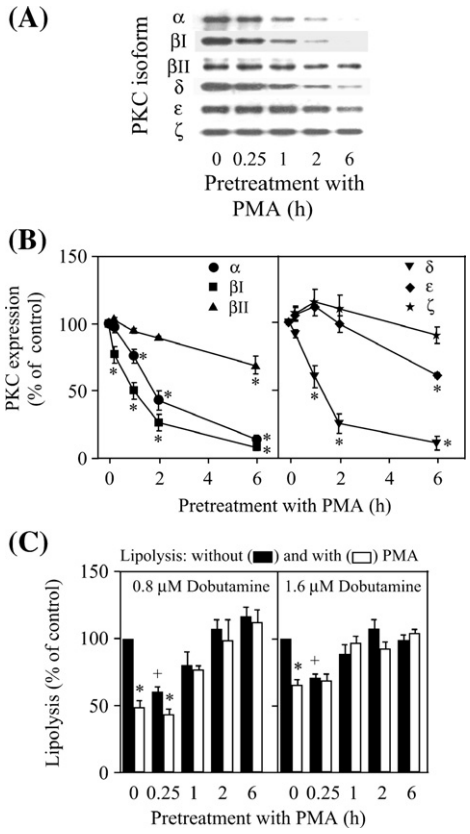

Fig. 1. PKC expression and lipolysis-attenuating effect of PMA in adipocytes pretreated with PMA. (A) Adipocytes were incubated in buffer A at 37 °C without PMA for 6 h, with 2 μM PMA for the final 0.25, 1, and 2 h of a 6 h incubation, and with 2 μM PMA over the period of a 6 h incubation. After incubation, the cells were washed, and the total cell lysates were subjected to western blot analysis with antibodies recognizing PKCs α, βI, βII, δ, ε, and ζ. The results shown are a representative from three experiments. (B) Immunoblots obtained in (A) were scanned and quantified by a densitometer. The results are expressed as mean ± SEM of three experiments and were expressed as the percentage of control cells (pretreated without PMA). *$p < 0.05$ or less, compared to control. (C) Adipocytes pretreated as in (A) were washed with buffer B, and were treated with 0.8 and 1.6 μM dobutamine in the presence and absence of 0.8 μM PMA. The results are expressed as mean ± SEM of seven experiments and were expressed as the percentage of lipolytic activity determined without PMA in control cells. *$p < 0.05$ or less, compared to no PMA. +$p < 0.05$ or less, compared to control cells.