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cAMP antagonizes ERK-dependent antiapoptotic action of insulin

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Insulin has antiapoptotic activity in various cell types. However, the signaling pathways underlying the antiapoptotic activity of insulin is not yet known. This study was conducted to determine if cAMP affects the antiapoptotic activity of insulin and the activity of PI3K and ERK in CHO cells expressing human insulin receptors (CHO-IR). Insulin-stimulated ERK activity was completely suppressed by cAMP-elevating agents like as pertussis toxin (Ptx) and cholera toxin (Ctx) after 4 h treatment. Insulin-stimulated PKB/Akt activity was not affected at all. Ptx treatment together with insulin increased the number of apoptotic cells and the degree of DNA fragmentation. Ctx or 8-br-cAMP treatment also increased the number of apoptotic cells and stimulated the cleavage of caspase-3 and the hydrolysis of PARP. Taken together, cAMP antagonizes the antiapoptotic activity of insulin and the main target molecule of cAMP in this process is likely ERK, not PI3K-dependent PKB/Akt. [BMB reports 2011; 44(3): 205-210]

INTRODUCTION

Insulin exerts a wide range of biological functions affecting carbohydrate, lipid, and protein metabolism as well as cell proliferation and differentiation (1). Insulin also has antiapoptotic activity in various cell types (2-4). We investigated the antiapoptotic activity of insulin and its related signaling pathways in CHO-IR cells (5, 6) and HepG2 liver cells (7). Apoptosis plays a pivotal role in diverse physiological and pathological processes including homeostatic maintenance of tissues and organs, autoimmune diabetes, and diabetic neuropathy (8, 9). Insulin binding to its receptor results in activation of intrinsic tyrosine kinase function and subsequent stimulation of signaling molecules, including insulin receptor substrate-1 (IRS-1), IRS-2, shc, PI3K, and ERK (1). Previous studies have suggested that ERK and PI3K play critical roles in the insulin signaling of antiapoptotic function (2-4). Indeed, the pharmacological suppression of PI3K and ERK inhibited the antiapoptotic action of insulin in HepG2 cells (5). The antiapoptotic action of insulin is also sensitive to the inhibition of farnesyltransferase or dominant negative mutation of H-Ras in CHO-IR cells (6).

GTP-binding proteins (G-proteins) can also play a role as signaling molecules for the insulin receptor. Studies have shown that the insulin receptor binds $Gi\alpha_2$, an inhibitory G-protein (10, 11). A series of studies have reported the significance of G-proteins in diabetic cardiomyopathy (12), the heart (13), and vascular smooth muscle (14). Several studies have also provided convincing evidence that insulin has nitric-oxide-dependent vasodilator activities (15, 16). Interestingly, insulin-stimulated cyclic GMP production was significantly reduced by Ptx and inhibitors of ERK in human umbilical vein endothelial cells (HUVECs) (17). Insulin-stimulated phosphorylation of ERK was completely abolished by Ptx. These results suggest that cAMP-dependent signaling components can control the antiapoptotic action of insulin.

The present study was conducted to examine the contribution of cAMP in the intracellular signaling pathways underlying the antiapoptotic action of insulin in CHO-IR cells. We provide evidence that the ERK-dependent antiapoptotic action of insulin is antagonized by cAMP.

RESULTS

To assess the effect of cAMP on insulin-induced phosphorylation of Akt and ERK, Ptx or Ctx were pretreated prior to insulin treatment to increase the intracellular cAMP levels by stimulating adenylate cyclase by inhibiting Gi protein or stimulating Gs protein, respectively. Insulin-induced phosphorylation of Akt or ERK was affected differently by Ptx. CHO-IR cells were serum-starved for 4 h and then pretreated with Ptx for four different durations (1 h, 2 h, 4 h, 8 h) before short-term insulin stimulation (100 nM, 5 min). Even though they were not affected earlier than 2 h, the amount of ERK phosphorylated by insulin was significantly reduced by prolonged pretreatments (app. 80% and 90% reduction after 4 h and 8 h, respectively) with Ptx (Fig. 1). Ptx pretreatment had no effect on the insulin-induced phosphorylation of Akt. Ctx pretreatment for 4 h also reduced the insulin-induced phosphorylation of

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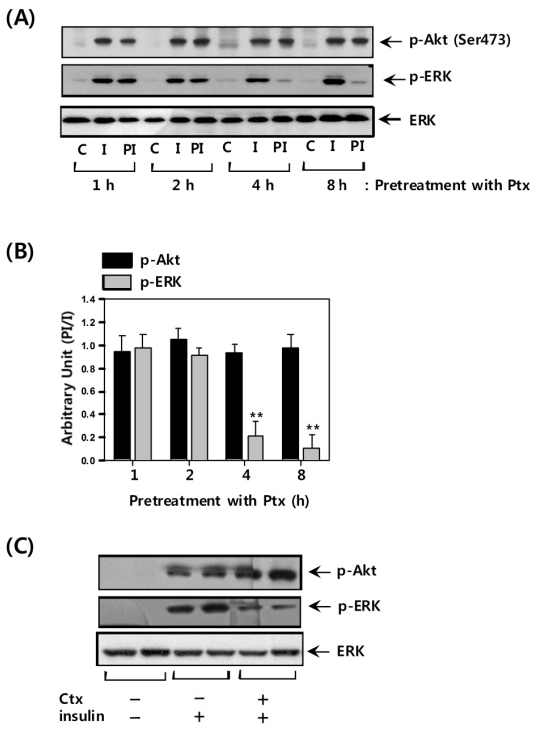

Fig. 1. Effect of Ptx and Ctx on insulin-induced phosphorylation of Akt and ERK. CHO-IR cells were serum-starved for 4 h and then pretreated with Ptx (200 μg/ml, 1-8 h) (A) or Ctx (1 mg/ml, 4 h) (C) before insulin stimulation (100 nM, 5 min). Cultured cells were homogenized and subjected to electrophoresis and immunoblot analysis (A). The data represent the mean ± S.E. of three independent observations (B). **P < 0.01 when compared to the group treated with Ptx for 1 h. C, control; I, insulin; PI, Ptx + insulin.

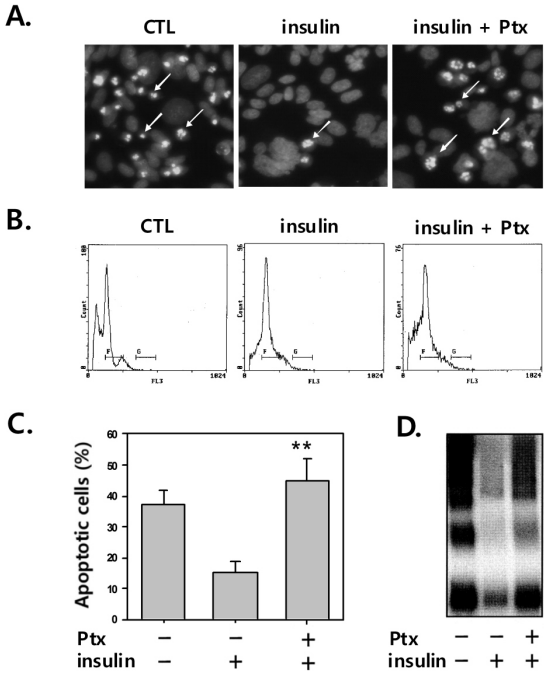

Fig. 2. Effect of Ptx on the antiapoptotic action of insulin. CHO-IR cells were serum-starved for 4 h, pretreated with Ptx (200 μg/ml, 4 h) and then further treated with insulin (100 nM) for an additional 24 h. The cells were then stained with H33342 (A) or subjected to flow cytometric analysis to measure the number of apoptotic cells (B) as described in the Materials and Methods. After staining the cells with H33342, the number of cells with apoptotic bodies within a restricted area was counted. Each bar represents the mean ± S.E. of three independent observations (C). Otherwise, genomic DNA was isolated from cultured cells and used to measure the degree of DNA fragmentation (D). **P < 0.01 when compared to the group treated with insulin-alone.

ERK, whereas it did not affect that of Akt (Fig. 1). We also tested whether Ptx pretreatment affects the insulin-induced phosphorylation of ERK in HepG2 cells that express endogenous insulin receptor molecules. Exactly like as in CHO-IR cells, Ptx pretreatment (4 h) blocked the insulin-induced phosphorylation of ERK whereas it did not affect that of Akt in HepG2 cells (results shown in the supplementary file).

Although Akt and ERK are expected to be important in exerting the apoptotic action of insulin, the role of cAMP in the antiapoptotic action of insulin is poorly understood. As shown in Fig. 2, Ptx significantly antagonized the antiapoptotic action of insulin. When CHO-IR cells were incubated in a serum-free medium for 24 h, a variety of apoptotic events were observed, but these events were effectively blocked by insulin. The results of three different experiments to determine the degree of apoptosis, staining of cells with H33342 (nuclear condensation and apoptotic body formation), flow cytometric analysis after propidium iodide staining (cells with subG1 DNA content), and measurement of DNA fragmentation (laddering) clearly demonstrated the antagonizing effect of cAMP on the antiapoptotic action of insulin (Fig. 2).

Next, we examined the mechanism of cAMP-induced blockade of the antiapoptitc action of insulin by analyzing the integrity of PARP and caspase-3. Pretreatment (4 h) of CHO-IR cells with Ctx, a cAMP-elevating bacterial toxin similar to Ptx, increased the number of apoptotic cells with subG1 DNA content in the presence of insulin for 24 h (Fig. 3B). Hydrolysis of intact PARP and caspase-3, a biochemical hallmark of apoptosis, was also increased by Ctx pretreatment (4 h) prior to the addition of insulin. Finally, the effect of 8-br-cAMP on the antiapoptotic action of insulin was evaluated. 8-br-cAMP was added to cultured cells at 4 h before insulin addition, which resulted in increased cleavage of caspase-3 and an increased number of apoptotic cells (Fig. 4).

The results showed that cAMP suppressed insulin-induced stimulation of ERK, but not of Akt, and that it antagonized the antiapoptotic action of insulin. Thus, ERK appears to be more closely linked with the antiapototic action of insulin than Akt

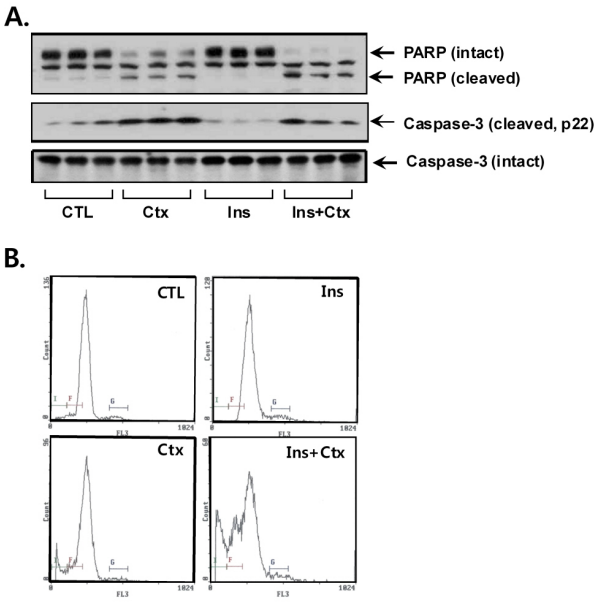

Fig. 3. Effect of Ctx on the antiapoptotic action of insulin. CHO-IR cells were serum-starved for 4 h and then pretreated with Ctx (1 mg/ml, 4 h) prior to insulin treatment (100 nM, 24 h). Cultured cells were homogenized and subjected to electrophoresis and immunoblot analysis (A) or flow cytometric analysis as described in the Materials and Methods (B).

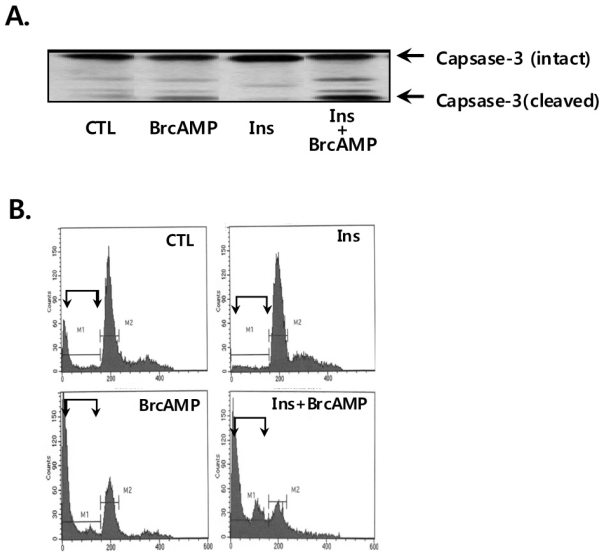

Fig. 4. Effect of 8-br-cAMP on the antiapoptotic action of insulin. CHO-IR cells were serum-starved for 4 h and then pretreated with 8-br-cAMP (100 μM, 4 h) before insulin treatment (100 nM, 24 h). Cultured cells were homogenized and then subjected to electrophoresis and immunoblot analysis (A) or flow cytometric analysis as described in the Materials and Methods (B).

because the cells were apoptotic, whereas Akt was still active in the presence of insulin and Ptx. To clarify this, PD98059, an inhibitor of the MEK-ERK cascade, was tested to determine if it could influence the antiapoptotic action of insulin. PD98059 suppressed insulin-induced phosphorylation of ERK and also led to a dose-dependent increase in DNA fragmentation that was suppressed by insulin (results shown in the supplementary file).

DISCUSSION

Although the insulin receptor tyrosine kinase is a standpoint of insulin signaling, it is not sufficient to explain the entire range of insulin action. Heterotrimetic G-proteins are linked to a number (>1,500) of cell surface receptors that are structurally and functionally coupled to effectors such as adenylate cyclase, phospholipases, and various ion channels (18). A number of observations have accumulated that describe the possible linkages between insulin receptor tyrosine kinase-derived signaling components and heterotrimetic G-proteins (19). G-proteins participate in insulin-stimulated translocation of glucose transporter GLUT4 in 3T3-L1 adipocytes (20, 21). In addition, Ptx can alter the action of insulin (22).

The main findings of the present study were as follows. First, cAMP suppressed insulin-induced stimulation of ERK, but not of Akt. Second, cAMP antagonized the antiapotoptic action of insulin. Third, the antiapoptotic action of insulin was blocked by pharmacological inhibition of ERK. The antiapoptotic action of insulin was sensitive to inhibition of farnesyltransferase and dominant negative mutation of H-ras in CHO-IR cells (6). Insulin-induced phosphorylation of Akt (ser473) was sensitive to the inhibition of farnesyltransferase, whereas insulin-induced activation of K-ras was not. Ras proteins are targets of isoprenylation, including farnesylation and geranylgeranylation; however, their sensitivity to inhibitors of farnesylation was quite different (23). Based on these observations, we suggested that the K-Ras/Raf-1/ERK cascade was not the main signaling route in the antiapoptotic action of insulin (6). Because H-ras is a potent activator of PI3K (24), suppression of H-ras can block PI3K and its downstream signaling molecules, including Akt. Indeed, the antiapoptotic action of insulin was sensitive to the inhibition of PI3K as well as ERK, and insulin-induced stimulation of ERK was sensitive to wortmannin, a PI3K inhibitor in HepG2 liver cells (5). However, in the present study, apoptosis was still observed in CHO-IR cells with active Akt after insulin stimulation in the presence of Ptx. Thus, Akt is an unlikely prerequisite to exert the antiapoptotic action of insulin, although PI3K stimulates Akt in response to insulin. PI3K can stimulate protein kinase C zeta (PKCζ) in response to insulin in rat adipocytes and muscles and insulin-induced activation of PI3K is sensitive to Ptx (22). Moreover, PKCζ can activate ERK upon EGF stimulation in tumor cells (25). Thus, the antiapototic action of insulin can be exerted through the following signaling cascades based on our observations and the results of previous

studies. Insulin stimulates H-ras, not K-ras, and subsequent activation of PI3K/PKCζ/ERK and cAMP can interfere with insulin-induced stimulation of PI3K. Moreover, several lines of suggestions are available.

Intracellular cAMP can increase protein-tyrosine phosphatase 1B (PTB1B) in adipocytes (26). Activation of PTP1B leads to a wide range of dephosphorylation at tyrosine residues of insulin receptor-stimulated signaling molecules including β-subunits of insulin receptors and insulin receptor substrates (IRSs). This is a typical desensitizing mechanism of insulin signaling based on receptors of tyrosine kinase activity. However, the role of PTP1B is wide and not specific enough to explain certain physiological responses in its state.

Gerits *et al*. (27) suggested that cAMP/PKA-dependent inhibition of ERK is mediated by raf-1, and that PKA-induced activation of ERK is interceded through B-raf. PKA and B-raf mediate ERK activation by thyrotropin (TSH) to stimulate thyroid cell stimulation (28).

Thus, cAMP can activate raf-1 to suppress ERK-1, leading to antagonization of the antiapoptotic action of insulin, whereas insulin activates Ras/B-raf signaling to activate ERK. Indeed, B-raf requires insulin-activated pathways for full antiapoptotic and proliferative activity in hematopoietic cells (29). PKA also directly induces serine/threonine phosphorylation, thereby reducing tyrosine phosphorylation of IRS-1 to suppress the differentiation of 3T3-L1 preadipocytes (30). In erythrocytes that possess the insulin receptors, PI3K and phopsphodiesterase 3B (PDE3B), insulin inhibits cAMP accumulation by activating PDE3B (31).

As discussed, cAMP and insulin receptor signaling generally antagonize each other rather than working together under a wide range of pathophysiological conditions. Taken together, the results of the present study suggest that cAMP antagonizes the ERK-dependent antiapoptotic action of insulin, although the direct target molecule of cAMP is not clear yet. Apoptosis is a well-recognized feature found in a number of organs in the pathological progress of diabetes. Insulin has potent antiapoptotic activity together with its various metabolic activities. The nature of insulin's action mechanism to suppress apoptosis is diverse and complex in a number of different target organs. This provides an additional framework to understanding the antiapoptotic action of insulin.

MATERIALS AND METHODS

Materials

Human recombinant insulin, PD98059, Ptx, Ctx, 8-br-cAMP, propidium iodide, H33342, Ham's F12 medium, and trypsin-EDTA solution were obtained from Sigma Chemical Corp. (St. Louis, MO), while FBS was obtained from Life Technologies Inc. (Rockville, MD). Monoclonal antibodies against phospho-ERK1/2 (E-4) and ERK2 (D-2) and polyclonal antibodies against phospho-Akt1 (ser473) and PARP (H-250) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and polyclonal antibodies against caspase-3 (intact) and cleaved caspase-3 (Asp175) were obtained from Cell Signal Tech. (Danvers, MA). Electrophoresis reagents, such as gels, Tris-glycine SDS running buffer, and poly (vinylidene difluoride) (PVDF) membrane, were obtained from Invitrogen (Carlsbad, CA).

Cell culture

CHO-IR cells (a generous gift from Dr. M. Bernier, NIA/NIH, Baltimore, MD, USA) were used in this study. CHO-IR cells were maintained in Ham's F-12 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS, and were grown in a humidified atmosphere of 5% CO_2 in air at 37°C.

Immunoblot analysis

Unless otherwise indicated, the cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin A). Equal amounts of protein were separated by SDS-PAGE on a 4-20% polyacrylamide gel and electrotransferred onto a PVDF membrane. The membrane was incubated in blocking buffer [5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween-20 (TBS-T)] for 1 h at room temperature, after which it was probed with different primary antibodies (1 : 1,000-1 : 5,000). After a series of washes, the membrane was further incubated with their respective horseradish peroxidase (HRP)-conjugated secondary antibodies (1 : 2,000-1 : 10,000). The signal was detected with the enhanced chemiluminescence (ECL) detection system (Intron, Korea).

Observation of apoptotic bodies

The degree of apoptosis was determined by staining cultured cells directly with H33342 (10 μg/ml), a cell membrane-permeable DNA-specific fluorescent dye. The fluorescent apoptotic bodies among the cell populations were observed under a fluorescent microscope equipped with a CoolSNAP-Pro-color digital camera (Media Cybernetics, Silver Spring, MD). The number of cells with apoptotic bodies within a restricted area of each group was counted.

Detection of apoptotic cells by flow cytometric analysis

The degree of apoptosis was also determined by measuring the number of cells with a DNA content less than 2N (subG1) by flow cytometry analysis after staining the cells with propidium iodide as originally described by Crissman and Steinkamp (32). The samples were analyzed with a Coulter EpicsTM cytometer (Beckman). Ten thousand events were collected for each sample. Both an excitation wavelength of 488 nm and a fluorescence emission wavelength of 580 nm were used.

DNA fragmentation

Internucleosomal DNA fragmentation analysis was conducted essentially as previously described (6). Briefly, pooled cellular DNA from adherent and detached cells was prepared using the Puregene Kit (Gentra Systems, Inc., Minneapolis, MN), and the purified DNA was then incubated with 20 μg/ml RNase A for 1 h at 37°C. Equal amounts of DNA from each sample (0.5 μg) were 3'-OH-labeled with 5 units of Klenow fragment of DNA polymerase I (New England Biolabs) and 0.5 μCi [α-^{32}P]-dCTP (3,000 Ci/mmol, Amersham Corp., Arlington Heights, IL) and electrophoresed on 6% (w/v) polyacrylamide gel. DNA was visualized by autoradiography of the dried gel using x-ray film and intensifying screens.

Statistical analysis

The results are presented as the mean ± SEM. The significance of the differences among groups was evaluated using a Student's t-test. A $P < 0.05$ was considered to be statistically significant.

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