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Effect of G protein-coupled signaling pathways on insulin receptor tyrosine kinase signaling pathways and insulin's anti-apoptotic function

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Abstract

Although the activation of receptor tyrosine kinase (RTK) is crucial to execute a number of physiological response by insulin, the roles of signaling pathways derived by G protein-coupled receptors (GPCRs) are unravelled. We previously showed that insulin suppressed apoptotic cell death through signaling pathways including stimulation of PI3 kinase and ERK. The aim of the present study was to examine whether the activation of cAMP-dependent signaling pathways might affect insulin's anti-apoptotic function and RTK-dependent signaling components including ERK and Akt/PKB upon insulin stimulation in CHO-IR cells. cAMP-dependent signaling pathways were stimulated by treatments with cholera toxin (CTX, an activator of Gs protein), pertussis toxin (PTX, an inhibitor of Gi protein) or 8-bromo-cAMP (a cell membrane-permeable analogue of cAMP) together with - or without insulin in CHO-IR cells. Activities of ERK and Akt/PKB were analyzed by immunoblotting experiments using specific antibodies against activated ERK and Akt/PKB proteins. Apoptotic cell death was evaluated with fluorescence microscopy after H33342 staining and with cell cycle analysis with flow cytometer after propidium iodide staining. Addition of CTX as well as PTX suppressed ERK activity stimulated by insulin treatment. However, insulin-induced Akt/PKB activity was more increased by the addition of PTX or CTX. 8-bromo-cAMP treatment also led to a suppression of ERK activity and an additional increase in Akt/PKB activity stimulated by insulin. Regardless of an additional increase in Akt/PKB activity, insulin's antiapoptotic function was blocked by activations of cAMP-dependent signaling pathways.

Taken together, the present study showed that the activation of cAMP-dependent signaling pathways regulated downstream steps of RTK after

binding of insulin to its receptor in different manners, down-regulating the ERK activity and up-regulating Akt/PKB activity in CHO-IR cells. From the blockade of insulin's anti-apoptotic function even with the up-regulation of Akt/PKB activity by cAMP pathways, it is suggested that insulin's anti-apoptotic function is not totally dependent on Akt/PKB activity or, at least, is regulated at any downstream signaling steps of Akt/PKB activation.

CONTENTS

ABSTRACT i

CONTENTS iii

LIST OF FIGURES v

I. Introduction 1

II. Material and Methods 4

1. cell culture
2. LDH assay
3. MTT assay
4. western blot
5. flow cytometric analysis
6. H33342 staining
7. statistical analysis

III. Results 8

1. Effect of the elevated cAMP on insulin

signaling pathways.
(1) Effect of PTX on insulin signaling pathways
(2) Effect of CTX on insulin signaling pathways
(3) Effect of 8-bromo-cAMP on insulin signaling pathways
2. Effect of CTX, PTX and cAMP on insulin's anti-apoptotic activity
(1) Effect of PTX and CTX on cell cytoxicity
(2) Effect of CTX and 8-bromo-cAMP on cell viability

IV. Discussion 18

V. Reference 20

LIST OF FIGURES

Figure 1. Effect of PTX on insulin signaling pathways 10

Figure 2. Effect of CTX on insulin signaling pathways 11

Figure 3. Effect of 8-bromo-cAMP on insulin signaling pathways 12

Figure 4. Effects of CTX and PTX on cell cytotoxicity.............15

Figure 5. Effects of CTX and cAMP on PARP hydrolysis and caspase-3 activity 16

Figure 6. Effects of CTX and 8-bromo-cAMP on the nuclear condensation 17

Figure 7. Effect of CTX and cAMP on the intracellular DNA content 18

introduction

Receptor protein-tyrosine kinases (RTKs) is the largest family of enzyme-linked receptor, which phosphorylate their substrate proteins on tyrosine residues. This family includes the receptors for most polypeptide growth factors, such as insulin, epidermal growth factor (EGF), nerve growth factor (NGF), and other growth factors *(Malbon 2004)*. RTKs have an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. Activation of the receptor is achieved by ligand binding to the extracellular domain, which induces dimerization of the receptor *(Schlessinger 2000)*. Receptors are then able to autophosphrylate tyrosine residues outside the catalytic domain, which form SH2 or phosphotyrosine binding (PTB) sites. The SH2 and PTB domains serve as docking sites for the recognition and recruitment of SH2 domain-containing proteins or adaptor proteins *(Gavi et al., 2006)*. G protein-coupled receptors (GPCRs) is the largest family of cell surface molecules involved in signal transmission *(Gavi et al., 2006)*. GPCRs preferentially couple to heterotrimeric G proteins that are grouped into four classes, known as Gαq/11, Gαi/o, Gαs and Gα12/13 *(Simon et al., 1991).* The binding of ligands to the extracellular domain of G protein-coupled receptors send a signal into the interior of the cell, and the G protein induce the synthesis of a small, diffusible molecule, as we known, one of the most commonly used molecules is cyclic AMP (cAMP). cAMP is synthesized from ATP by adenylyl cyclase, an enzyme of the plasma membrane that is always under hormonal control. cAMP plays a important roles in controlling the proliferation, survival, and differentiation of a wide variety of animal cells *(Gavi et al., 2007).*

GPCRs'- and RTKs'- signaling pathways are involved in a variety of physiological and pathological conditions. Possible cross-talk between RTKs and G-proteins was first proposed from the observation that experimentally-induced diabetes leads to the loss of Gi-protein expression in liver *(Houslay et al., 1987)*. Targeted elimination of G-αi2 in liver, skeletal muscle and white adipose tissue of transgenic mice was found to induce frank insulin resistance *(Houslay et al., 1987)*. Whereas expression of a constitutively active mutant of G-αi2 in skeletal muscle, liver and adipose tissue markedly enhanced the glucose-tolerance of trangenic mice and activated translocation of the insulin-sensitive GLUT4 glucose transporter to the cell surface *(Song et al., 2001)*. However, in the molecular terms, the exact nature of the cross-talk between G protein-coupled signaling pathways and insulin receptor tyrosine signaling pathways are not understood yet *(Malbon, 2004)*.

Insulin is the most potent anabolic hormone known and is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis. Insulin is secreted by the beta cell of pancreatic islets of Langerhans in response to increased circulating levels of glucose. *(Pessin and Saltiel, 2000).* Insulin exerts its biological effects by binding to their respective transmembrane receptors (RTK) *(Ullrich et al., 1985).* One of the earliest steps in signal transduction by insulin is the extensive phosphorylation of IRS-1. Tyrosyl-phosphorylated IRS-1 then interacts with numerous SH2 domain-containing proteins, including PI3'-kinase and the guanine-nucleotide exchange factor Grb2/SOS *(Parrizas et al., 1997).* PI3'-kinase then initiates phospholipid turnover, and Grb2/SOS activation results in initiation of the MAP kinase signal transduction cascade by sequential phosphorylation and activation of

proto-oncogenes Ras and Raf and the MAPK/ERK kinases *(Parrizas et al., 1997)*.

It has been shown that insulin protects different mammalian cells from apoptosis through the activation of insulin receptors. For instance, insulin promotes rat retinal neuronal cell survival in a P70S6K-dependent manner *(Wu et al., 2004).* and the antiapoptotic mechanism is used by the insulin receptor in embryo fibroblasts *(Prisco et al., 1999)*. Moreover, the anti-apoptotic function of insulin and its related signaling pathways was found in Chinese hamster ovary cells expressing wild-type human insulin receptors (CHO-IR) *(Kang et al., 2003)*

However, little is known about the significance of non-RTK signaling components like as GPCR-coupled signaling pathway in the suppression of apoptotic cell death by insulin. The present study investigated whether the activation of cAMP-dependent signaling pathways might affect insulin's anti-apoptotic function and activities of RTK-dependent signaling components including ERK and Akt/PKB upon insulin stimulation in CHO-IR cells.

Material and Methods

1. Reagents

Insulin, F-12 cell culture medium, pertussis toxin (Gi protein inhibitor), cholera toxin (Gs protein activator) and 8-bromo-cAMP (a cell membrane-permeable analogue of cAMP) was obtained from Sigma (st. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, USA), lactate dehydrogenase (LDH) cytotoxicity kit was obtained from Takara (Otsu, Shiga Japan) and MTT cytotoxicity kit and Hoechst 33342 (a DNA-specific fluorescent dye) from Sigma(st. Louis, MO, USA). Antibodies against to phospho-AKt (rabbit polyclonal IgG Ser473), phospho-ERK1/2 (mouse monoclonal IgG E-4),PARP (rabbit polyclonal IgG H-250) and caspase-3 (rabbit polyclonal IgG, H-277) were obtained from Santa Cruz (Santa Cruz, CA, U.S.A)

2. Cell culture

Chinese hamster ovary cells overexpressing human insulin receptor molecules (CHO-IR) cells were kindly provided by Dr. M. Bernier (National Institute on Aging, Baltimire, MD, USA) and maintained in F-12 medium supplemented with 10% FBS, streptomycin (100μg/ml) and penicillin (100U/ml) at 37°C atmosphere and 5% CO_2. Confluent cells were washed twice with Dubelcco's Phosphate-buffered Saline (D-PBS) before a 4 h incubation in serum-free medium before pretreatments with various inhibitors.

3. Cytotoxicity assays

3.1 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate (Fernandez et al.,2006). The release of LDH from CHO-IR cell was used to detect cytotoxicity. Briefly, culture medium was centrifuged at 12,000 rpm for 3 min at room temperature to ensure accumulation of cells. The cell-free culture medium (50μl) was collected and then incubated with 50 μl of the reaction mixture of cytotoxicity detection kit (Takara) in a 96-well microwell plate for 30 min at room temperature. The optical density at 490 nm wavelength was then measured by using an ELISA plate reader (Sunrise, TECAN AUSTRIA). Percent cytotoxicity was determined relative to the control group.

3.2 MTT 〔3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide〕 assay

MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membrane, thus resulting in its accumulation within healthy cells. Briefly, 500μl MTT solution (1mg/mL) was added into each well and cultured for 30 min at 37°C. Then supernatant was removed and 2-propanol (500μl) was added to each well. The optical density at 570 nm wavelength was then measured by using an ELISA plate reader (Sunrise, TECAN, AUTRIA).

4. Western blot analysis

CHO-IR cells were preincubated in serum-free medium for 4 h, and then stimulated with insulin (100ng/ml) in the present of PTX, CTX or 8-bromo-cAMP for an additional 4 h. Cells were collected by scripping and washed twice with ice-cold D-PBS. Collected cell pellets were lysed in a lysis buffer 〔50mM Tris-HCl (PH7.5), 150mM NaCl, 1% nonidet P-40, 2mM EDTA〕supplemented with inhibitors for various proteases and phosphatases and kept on ice for 15 min. Cell lysates were centrifuged at 15,000 rpm at 4 °C for 15 min and the supernatants were stored at -20 °C until use. Protein concentration was determined with BCA™ protein assay reagent (Pierce, U.S.A). Aliquots of the lysates (10-15μg of protein) were separated on a 4-20% Tris-Glycine polyacrylamide gel (Invitrogen, carlsbad, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) with a glycine transfer buffer〔96mM glycine, 12mM Tris-HCl(PH8.8), 20% MeOH(v/v)〕. After blocking the nonspecific site with 1% Non-fat Dry Milk (SANTA CRUZ, CA, USA) in 10% tween-20 in Tris-borate-buffered saline (TBS-T), the membrane was then incubated with specific primary antibodies (1:1,000-1:500) at 4 °C for overnight. The membrane was washed with TBS-T 2-3 times, further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000- 1:2500) at room temperature for 30 min. The immunoactive protein were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (iNtRON, Korea).

5. Hoechst 33342 staining

Hoechst 33342 nucleic acid stain is a popular cell-permeant nuclear

counterstain that emits blue fluorescence when bound specifically to double stranded DNA (dsDNA). This dye is often used to distinguish condensed nuclei in apoptotic cells. After treatments, CHO-IR cells were incubated with Hoechst 33342 (10μg/ml) for 30 min and photographed with a fluorescent microscope (Olympus IX-51,Japan) equipped with digital camera (DP71, Olympus, Japan).

6. Flow cytometric analysis

Apoptotic cells can be recognized by flow cytometry through their diminished stainability with the DNA-specific fluorochrome PI, in which the hydrodiploid population can be quantified by DNA content frequency histograms. Briefly, CHO-IR cells was treated with insulin for 24 h after pretreatment CTX and 8-bromo-cAMP. After treatments, cells was collected and centrifuged at 5,000 rpm at 4 °C for 5 min. then washed twice with ice-cold D-PBS. Cells were fixed with 70% alcohol for 30 min, centrifuged at 1,000 rpm at room temperature for 3 min and the collected cells were washed once with ice-cold D-PBS. Cells were incubated with 1ml PI (50μg/ml) solution and then subjected to the flowcytometric analysis to measure the number of cells with subdiploid (subG1) DNA content.

7. Statistical analyses

Student's t-test and one-way ANOVA were used to determined the statistical significance of difference between for a variety of experimental and control groups. P-values less than 0.05 were considered statistically significant.

Results

1. Effect of the elevated cAMP on insulin signaling pathways

Although the significance of receptor tyrosine kinase in insulin receptor signaling pathways is widely understood, it is uncertain whether G protein-mediated cAMP generation can affect insulin's signaling cascades. Therefore, the present study examined the effects of elevated cAMP on PI3 kinase activity and ERK activity which is stimulated by insulin in CHO-IR cells. CHO-IR cells were pretreated with CTX or PTX to stimulate adenylate cyclase, otherwise, pretreated with a cell membrane-permeable cAMP analogue (8-bromo-cAMP) for 30 min before insulin treatment.

CHO-IR cells were incubated in serum-free medium for 4 h and pretreated with CTX (1000ug/ml), PTX (200ng/ml) or 8-bromo-cAMP before insulin (100nM) treatment. Insulin-stimulated ERK activity was markedly suppressed by the addition of PTX (Fig. 1), at least, with 4 h pretreatment. However, insulin-stimulated Akt activity was more augmented by PTX pretreatment for 2 h. These results suggest that elevated cAMP by inhibition of Gi may positively regulate insulin-induced Akt stimulation, however, negatively regulate insulin-induced ERK stimulation. In order to support this possibility, the effect of stimulation of Gs protein by CTX pretreatment on ERK/Akt activities was investigated (Fig. 2). Pretreatment with CTX (4h) suppressed insulin-stimulated ERK activity but more increased insulin-stimulated Akt activity. Furthermore, pretreatment with 8-bromo-cAMP for 30 min together with insulin also suppressed

ERK stimulation by insulin but augmented Akt stimulation by insulin in a dose-dependent manner (Fig. 3). These results confirm the notion that cAMP elevation mediated by G protein can cross-talk insulin receptor tyrosine kinase signaling cascades by suppressing ERK activity but augmenting Akt activity.

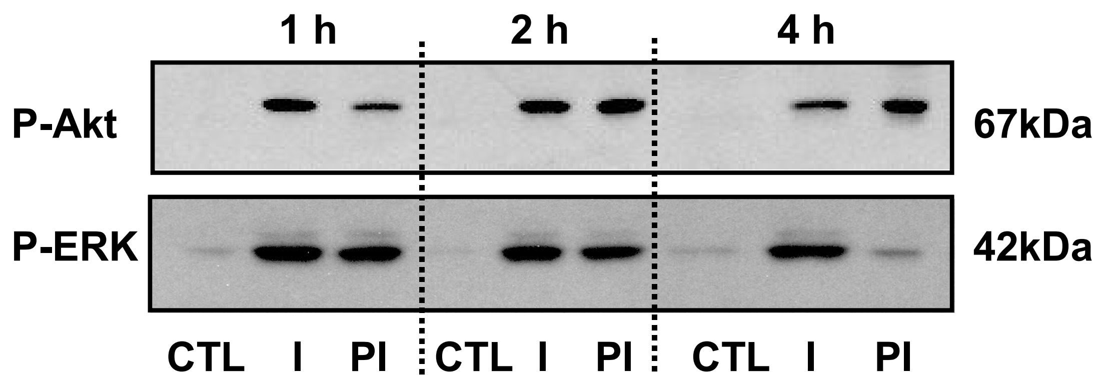

Fig.1. Effect of PTX on insulin signaling pathways. CHO-IR cells (1×10^6 cells/ml) were serum-starved for 4 h and then pretreated with PTX (200μg/ml) for indicated times before insulin (100nM) treatment for 5 min. Whole cell lysates were prepared and the protein levels were determined by western blotting as described in 'Materials and Methods'.

CTL, control; I, insulin; PI, PTX+insulin

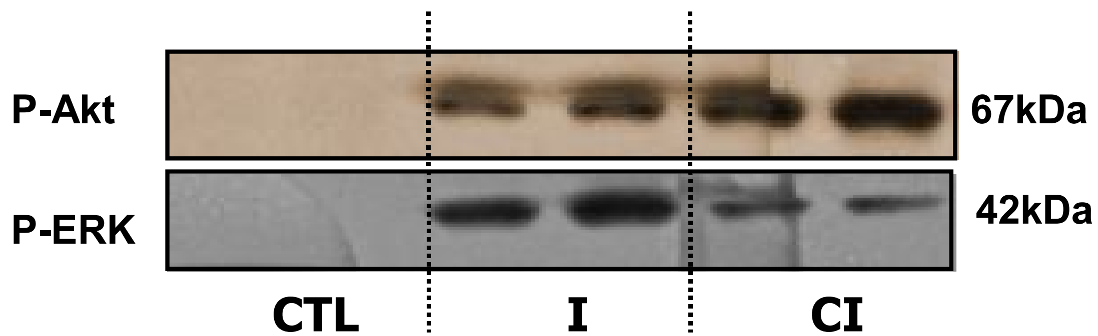

Fig.2. Effect of CTX on insulin signaling pathways. CHO-IR cells (1×10^6 cells/ml) were serum-starved for 4 h and then pretreated with CTX (1000 μg/ml) for an additional 4 h before insulin (100 nM) treatment for 5 min. Whole cell lysates were prepared and the protein levels were determined by western blotting as described in 'Materials and Methods'.

CTL, control ; I, insulin ; CI, CTX+insulin

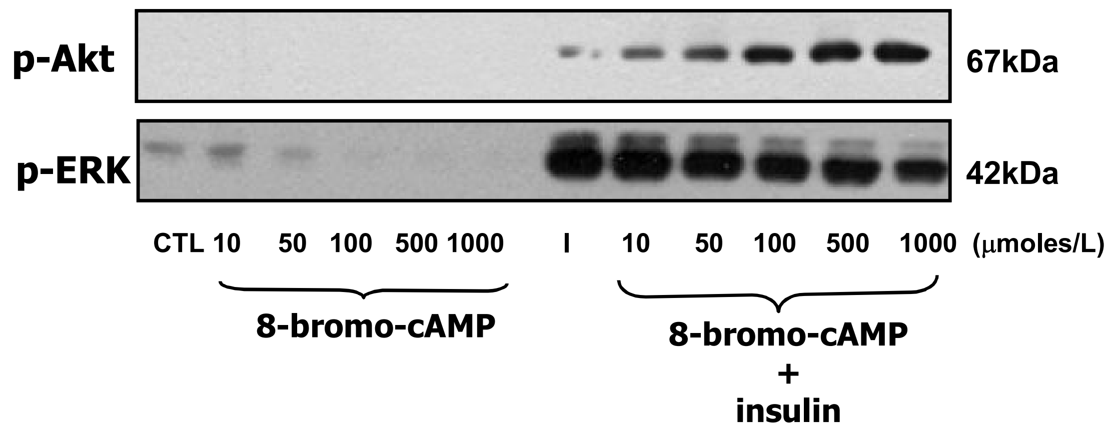

Fig.3. The effect of 8-bromo-cAMP on insulin signaling pathways. CHO-IR cells (1×10^6 cells/ml) were serum-starved for 4 h and then pretreated with 8-bromo-cAMP (10-1000 μmoles/l) for 30 min before insulin (100 nM) treatment for 5 min. Whole cell lysates were prepared and the protein levels were determined by western blotting as described in 'Materials and Methods'.

CTL, control ; I, insulin

2. Effect of CTX, PTX and 8-bromo-cAMP on insulin's anti-apoptotic activity

2.1.Effect of CTX and PTX on the cellular viability

We have showed that insulin can suppress the apoptotic cell death through receptor tyrosine kinase-coupled signaling pathways in CHO-IR cells *(Kang et al., 2003)*. However, little is known about the significance of G protein-mediated cAMP production in insulin's antiapoptotic function. The present study showed that insulin receptor tyrosine kinase signaling pathways (ERK and Akt stimulation) were influenced by reagents increasing intracellular cAMP content. From these results, we further investigated whether the elevation of intracellular cAMP production by PTX or CTX treatment can affect insulin's anti-apoptotic activity. Trearment of CHO-IR cells with insulin (100 nM) lowered the LDH release ($P<0.05$) into the culture medium when compared to serum-free control, however, combination of PTX or CTX increased the LDH release compared to the insulin-alone ($P<0.05$). MTT activity increased by insulin treatment, was decreased by the addition of PTX or CTX, respectively.

2.2 Effect of CTX and 8-bromo-cAMP on the biochemical events of apoptosis

Although, previous results showed that PTX or CTX induced cytotoxicity in the presence or absence of insulin, the nature of cytotoxicity needs to be clarified whether it should be necrotic- or apoptotic cell death. The caspase family of cysteine proteases plays a pivotal role in mediating apoptosis through the proteolysis of specific targets that include PARP, the nuclear lamins and caspase-dependent DNase *(Kang et al., 2003)*. Thus, the effects of CTX or

8-bromo-cAMP on the PARP hydrolysis or the proteolytic activation of caspase-3 were evaluated in CHO-IR cells (Fig. 6). Insulin's activity of suppressing PARP hydrolysis and caspase-3 cleavage was inhibited by CTX or 8-bromo-cAMP. To further confirm this finding , cells were stained with a DNA-specific fluorescent dye H33342, to observe the degree of nuclear condensation, which is an apoptotic phenomenon. The number of apoptotic cells with condensed nuclei was increased by the addition of CTX and 8-bromo-cAMP together with insulin, compared to the insulin-alone. Moreover, the degree of apoptosis was determined with flow cytometric analysis measuring DNA content of each cell and counting the number of events below diploid (<2N) DNA content. The number of apoptotic cells having less 2N DNA content was increased by the addition of CTX and 8-bromo-cAMP together with insulin, compared to the insulin-alone (Fig. 7).

From these results, the elevation of intracellular cAMP content acts as a negative regulator to lead an apoptotic cell death which is suppressed by one of the insulin receptor tyrosine kinase pathways.

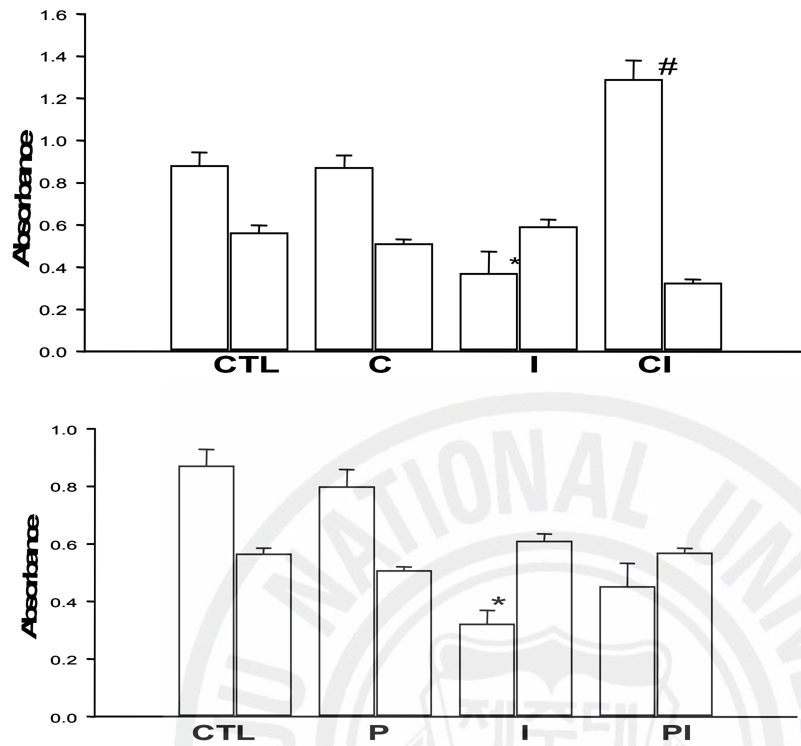

Fig.4. The effects of CTX and PTX on cytotoxicity. CHO-IR cells (3×10^5 cells/ml) were serum-starved for 4 h and pretreated with CTX (1000ng/ml) and PTX (200ng/ml) for 4 h before insulin (100 nM) treatment for 24 h. The cytotoxicity were determined with LDH assay and MTT assay as described in 'Material and Methods'. CTL, control ; I, insulin ; C, CTX ; CI, CTX+insulin ; P, PTX ; PI, PTX+insulin

* P<0.05 compared to the control.

# P<0.05 compared to the insulin-alone.

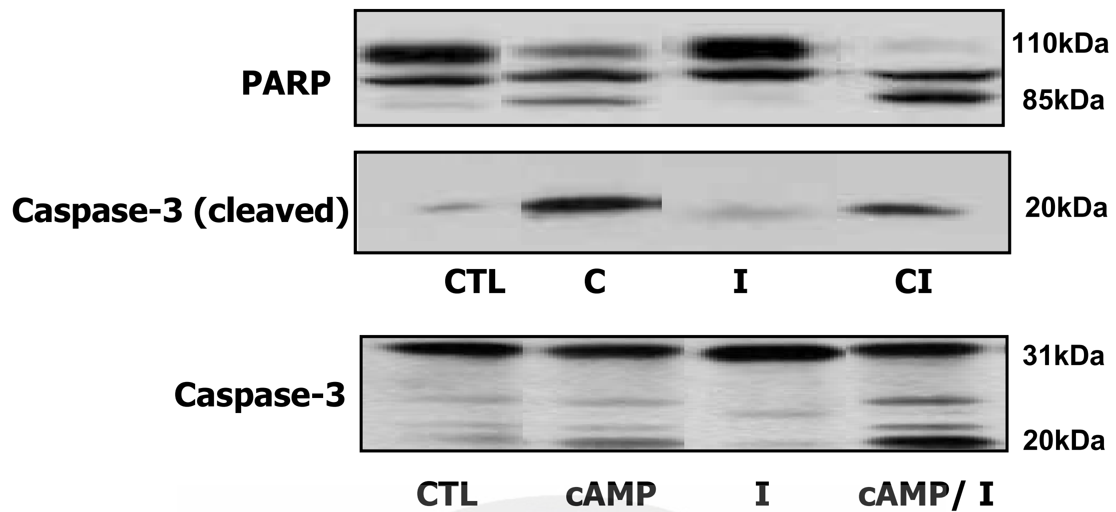

Fig.5. Effects of CTX and cAMP on PARP hydrolysis and caspase-3 activity. CHO-IR cells (1×10^6 cells/ml) were serum-starved (4 h) and pretreated with CTX (1 mg/ml) or 8-bromo-cAMP (100 μmols/ml) for 4h then treated insulin (100 nM) for 24 h. Whole-cell lysates were prepared and protein level was determined by western blotting as described in 'Material and Methods'.

CTL, control ; I, insulin ; cAMP, 8-bromo-cAMP ; C, CTX ; CI, CTX+insulin

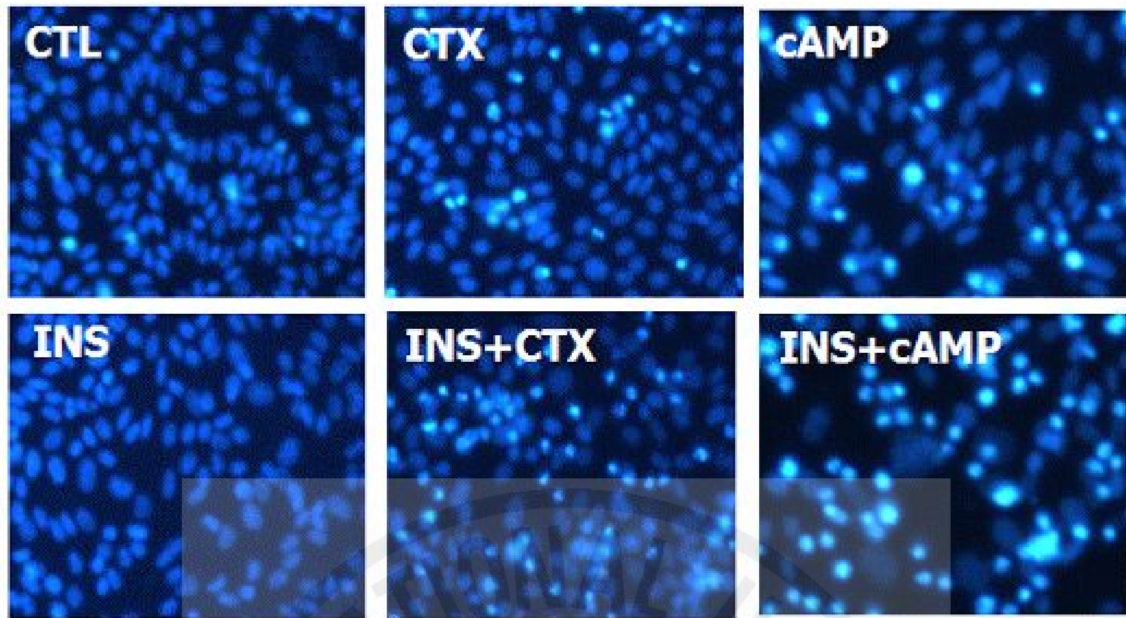

Fig.6. Effect of CTX and 8-Bromo-cAMP on the nuclear condensation

CHO-IR cells (3×10^5 cells/ml) were pretreated with CTX (1 mg/ml, 4 h) or 8-bromo-cAMP (100 μmol/ml, 30 min), then treated with insulin (100 nM) for an additional 24 h. Cultured cells were stained with H33342 and photographed as described in 'Materials and Methods'.

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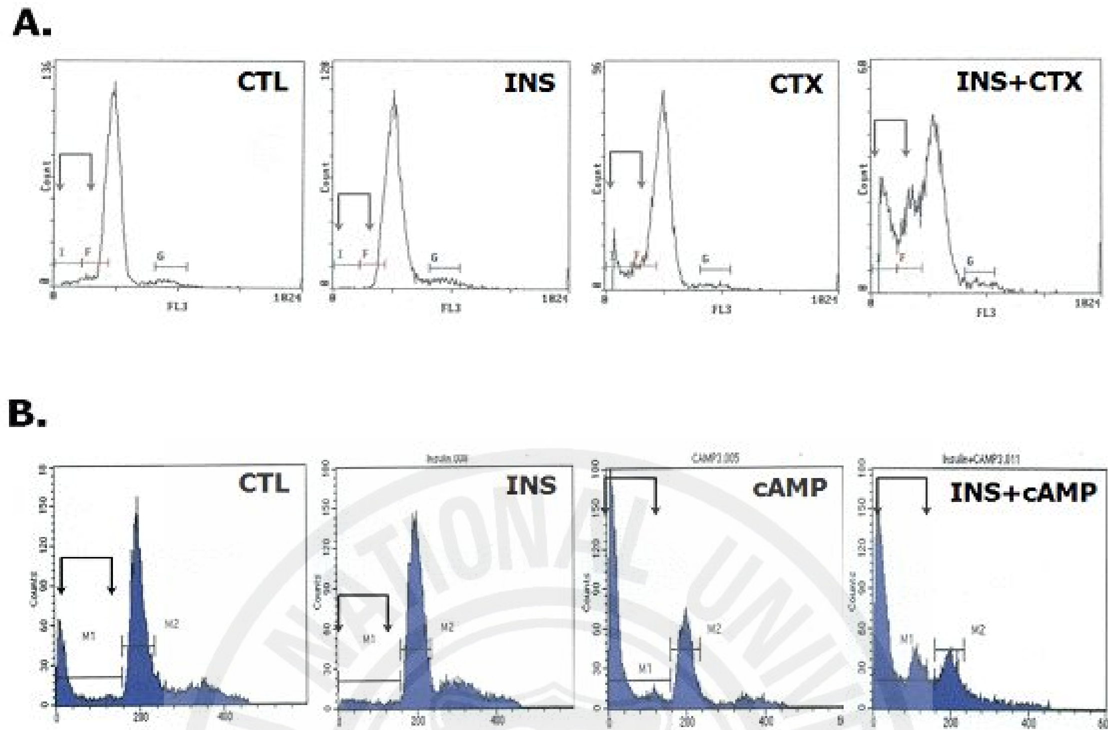

Fig.7. Effect of CTX and cAMP on the intracellular DNA content

CHO-IR cells (1×10^{6} cells/ml) were pretreated with CTX (1 mg/ml, 4 h) (A) or with 8-bromo-cAMP (100 μmoles/ml, 30 min), then treated insulin (100nM) for an additional 24 h. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis as described in 'Material and Methods'.

Discussion

In the present study, we examined the effect of G protein-mediated cAMP production on insulin receptor tyrosine kinase signaling pathways and insulin's anti-apoptotic activity in CHO-IR cells. Our results showed that the elevation of intracellular cAMP content by CTX or PTX treatment suppressed ERK activity which was stimulated by insulin but more augmented Akt activity which was stimulated by insulin. Insulin is a potent survival factor in different cell systems *(Bertrand et al., 1998, Diaz et al., 1999, Rampalli and Zelenka 1995)*, In the present study, insulin also inhibited the apoptosis which was induced by growth factor depletion (serum-starvation) from culture medium in CHO-IR cells. This insulin's anti-apoptotic activity was suppressed by pretreatment with CTX or 8-bromo-cAMP. We also examined the effect of PTX on insulin's anti-apoptotic activity with western blot analysis against PARP or activated caspase-3 and flow cytometric analysis. Although the PTX affected the activities of Akt and ERK which are important mediators of insulin's anti-apoptotic activity, PTX less affected the insulin's anti-apoptotic activity.

Interestingly, while CTX inhibited the insulin's anti-apoptotic activity, it also enhanced insulin-stimulated glucose uptake (data not shown). Insulin stimulates glucose uptake by promoting a rapid translocation of the insulin-responsive isoform of the glucose transporter from intracellular vesicles to the plasma membrane *(Welsh et al., 2005)*. It is known that insulin, through the activation of its receptor tyrosine kinase, leads to the stimulation of the phosphoinositide 3-kinase (PI3K). the results in the production of PtdIns(3,4,5) in the plasma membrane, which leads to the activation of protein kinase B (Akt). Akt have been

implicated in insulin-stimulated GLUT4 translocation *(Watson et al., 2004)*. In the present study, CTX more increased Akt activity which was stimulated by insulin. This result raises a possibility of G-protein mediated cAMP-dependent pathway(s) to cross-talk with insulin receptor tyrosine kinase pathways, thereby potentiating cellular glucose transport.

Diverse GPCRs ligands can activate P^{42} and P^{44} MAPK in several cellular systems *(Watson et al., 2004)*. One of the best-characterized signaling components that regulate the activation of MAPKs is cAMP *(Yehia et al., 2001)* cAMP play an opposite role in the regulation of MAPKs depending on cell and receptor type *(Yehia et al., 2001).* cAMP inhibits the growth of fibroblasts cells, smooth muscle cells, and adipocytes at least in part, by blocking the binding of Raf-1 to Ras, thus blocking the MAPK pathway *(Yehia et al., 2001)*. In the present study, the modulation of G protein-coupled receptor by CTX or PTX decreased the activity of ERK which was stimulated by insulin. 8-bromo-cAMP also decreased ERK activity which was stimulated by insulin in a dose-dependent manner in CHO-IR cells. 8-bromo-cAMP also suppressed the insulin's anti-apoptotic activity. Because we did not measure the activity of upstream protein of ERK, the relationship between the reduced activity of ERK and the inhibition of insulin's anti-apoptotic activity remains to be addressed.

Akt/PKB is a well-known key player to facilitate cell survival and to prevent apoptotic cell death *(New et al., 2007)*. In the present study, the insulin's anti-apoptotic activity was inhibited by treatment with CTX or 8-bromo-cAMP, however, the activity of AKt/PKB which was stimulated by insulin was more augmented by CTX or 8-bromo-cAMP. This result suggests that insulin's anti-apoptotic function is not totally dependent on Akt/PKB activity or, at least, is regulated at any downstream signaling steps of Akt/PKB activation.

Taken together, results of the present study showed that cAMP-dependent signaling pathways may regulate downstream steps of insulin receptor tyrosine kinase signaling pathways in different manners, down-regulating the ERK activity and up-regulating Akt/PKB activity in CHO-IR cells and suppress the insulin's anti-apoptotic activity.

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요 약

인슐린에 의한 여러 가지 생물학적 반응과정에서 receptor tyrosine kinase (RTK)의 활성화가 중요한 역할을 하고 있지만 G 단백질 매개 수용체 (GPCRs)에 의한 신호전달계통의 기전은 아직 명확하지 않다. 이전의 연구에서 인슐린은 PI3 kinase 및 ERK 신호전달경로를 통하여 세포사멸을 억제하는 것이 밝혀진 바 있다. 따라서 본 연구의 목적은 CHO-IR 세포에서 cAMP 의존적 신호전달 경로의 활성화가 인슐린에 의한 세포사멸 억제 기능 및 ERK와Akt/PKB를 포함한 RTK 의존적 신호전달경로에 미치는 영향을 알아보고자 하였다. CHO-IR 세포에서 cAMP 의존적 신호전달 경로를 활성 시키기 위하여 cholera toxin (CTX, Gs 단백질의 활성유도체), pertussis toxin (PTX, Gi 단백질 억제제) 및 8-bromo-cAMP (세포막 투과성 cAMP 유사물질)을 단독 혹은 인슐린과 병합하여 처리하였다. ERK 와 Akt/PKB의 활성도는 그들에 특이하게 결합하는 항체를 사용하여 immunoblot 실험방법으로 측정하였다. 세포사멸은 세포를 H33342로 염색 후 현미경으로 관찰하거나 또는 propidium iodide로 염색 후flow cytometer로 세포주기를 분석하는 방법으로 측정하였다. CTX 및 PTX 처리에 따른 세포내 camp 농도의 증가는 CHO-IR 세포에서 인슐린 자극에 의한 ERK 활성을 억제하였지만 Akt/PKB의 활성은 더욱 증가시켰다. 8-bromo-cAMP도 마찬가지로 ERK의 활성을 억제하였고 반대로 Akt/PKB의 활성을 증가시켰다. 하지만 Akt/PKB 활성의 증가와 관계없이 cAMP 의존적 신호전달 경로의 활성화는 인슐린에 의한 세포사멸 억제기능을 억제하였다. 결론적으로 cAMP 의존적 신호전달 경로의 활성화는 CHO-IR 세포에서 인슐린이 인슐린 수용체와 결합 후 진행되는 RTK 의존적 신호전달 경로에 대하여 ERK 활성을 억제하거나 또는 Akt/PKB 활성을 증가시키는 등 서로 다른 방식을 통하여 조

절한다. 또한 cAMP 의존적 신호전달 경로의 활성화가 Akt/PKB 활성의 증가와 관계없이 인슐린의 세포사멸 억제기능에 대한 억제효과는 인슐린의 세포사멸 억제기능이 부분적으로 Akt/PKB 활성에 의존한다거나 혹은 적어도 Akt/PKB 활성 후 다음 단계의 신호전달 경로에서 조절을 받고 있다는 것을 설명한다.

감사의 글

2년 동안의 대학원 생활동안 많은 것들을 배웠습니다. 힘들기도 행복하기도 했던 그 사간동안 많은 분들의 도움으로 저의 부족한 면을 재울 수 있었던 것 같습니다. 물론 아직도 많은 부분에서 부족하다는 것을 알고 있고 더욱더 발전해나가야 할 것이라고 생각해 봅니다.

학위 과정 중에 모든 면에서 부족했던 저를 잘 이끌어 주시고 항상 웃는 모습으로 많은 가르침을 주신 박덕배 교수님께 감사의 말씀을 드립니다. 이후에도 많은 가르침을 부탁드리고 항상 건강하세요. 그리고 논문의 수행과 완성이 있기까지 애정과 관심을 보여주신 강희경 교수님, 현진원 교수님께도 감사의 말씀을 드립니다. 그리고 대학원 과정동안 늘 한결같이 학문의 길을 이끌어 주신 조문제 교수님, 이영기 교수님, 강현욱 교수님, 고영상 교수님, 정영배 교수님, 고관표 교수님, 유은수 교수님, 이근화 교수님, 은수용 교수님, 김수영 교수님, 진심으로 감사드립니다. 그리고 많은 도움이 주신 대학원생들 감사의 말을 전합니다.

끝으로 나의 소중한 친구들에게 고마움을 느끼고 사랑의 마음을 전하며, 이 논문을 바칩니다.