A DOCTORAL DISSERTATION

The Nature of Antiapoptotic Action Mechanism of Insulin

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Graduate School
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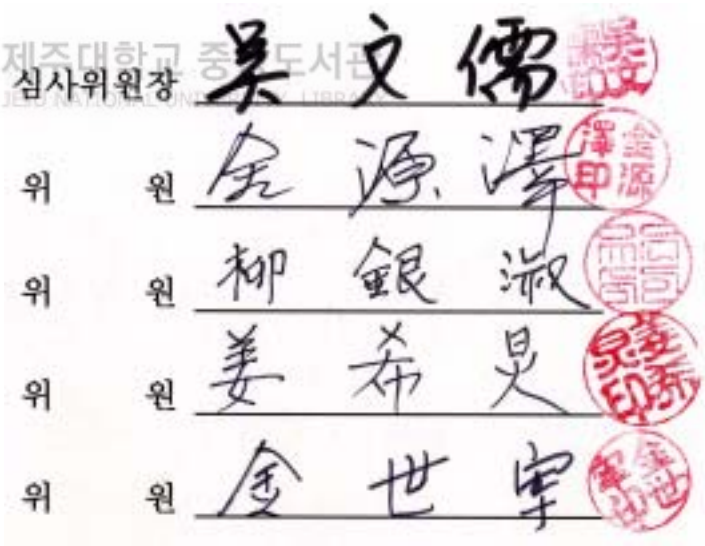

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The Nature of Antiapoptotic Action Mechanism of Insulin

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(Supervised by Professor **Se Jae Kim**)

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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Abstract

Insulin has well-known activities in controlling energy metabolism, cellular proliferation and biosynthesis of functional molecules to maintain a biological homeostasis. Recently several studies have suggested that insulin may protect cells from apoptosis, a programmed cell death, in different cell lines. However, little is known about the nature of its antiapoptotic mechanism. With these facts as backgrounds, the present study investigated the mechanism by which insulin can protect cells from apoptosis using two different cell lines, chinese hamster ovary cells which express human insulin receptors (CHO-IR cells) and hepatoblastonema cells that expresses intrinsic insulin receptors (HepG2). Intracellular DNA contents, the degrees of nuclear condensation, or poly(ADP-ribose) polymerase (PARP) hydrolysis were measured to verify the occurrence of apoptotic events. Caspase-3 activity and reactive oxygen species (ROS) accumulation within cells were also measured. Western blot analysis was performed to identify signaling molecules activated in response to insulin.

In CHO-IR cells, removal of serum from culture medium (serum-starvation) resulted in a marked apoptotic cell death. However, addition of insulin to culture medium protected cells from apoptotic death induced by serum starvation. Moreover, serum starvation also increased caspase-3 activity, known as one of apoptotic cascades, which were blocked by the addition of insulin. Generation of ROS

was also increased by serum starvation and insulin suppressed such an increase of ROS. Although addition of nitric oxide (NO) donor to culture medium did not induce apoptosis, an elevation of intracellular cGMP content by adding a cGMP analogue or an inhibitor of cGMP-specific phosphodiesterase suppressed insulin's antiapoptotic activity.

The present study further examined which signaling pathways are important in exerting insulin's antiapoptotic function. To this purpose, various inhibitors of key steps in signaling cascades activated by growth factors have been widely used. Among diverse signaling cascades stimulated by insulin, inhibition of extracellular signal-regulated protein kinase (ERK), phosphatidylinositol-3'-kinase (PI3 kinase) and Gi protein suppressed insulin's antiapoptotic function whereas inhibition of p21Ras, one of upstream component for activating ERK, did not affect.

It was further investigated whether insulin's antiapoptotic function shown in CHO-IR cells may be occurred in a genuine human cell line, HepG2 cells. Like as in CHO-IR cells, insulin protected HepG2 cells from apoptotic death induced by serum starvation. ERK and PI3 kinase played an important role in exerting insulin's antiapoptotic function. Suppression of ROS generation and inhibition of caspase-3 stimulation were also induced by insulin in HepG2 cells.

Taken together, the present study suggest that insulin plays an important role in protecting cells from apoptotic death by decreasing

oxidative stress resulted from ROS generation, and by suppressing key steps of apoptotic cascades including caspase-3 stimulation. In this process, several signaling molecules like as ERK, PI3 kinase and Gi protein may play important roles to exert insulin's antiapoptotic function.

Key words : insulin, apoptosis, antiapototic activity, CHO-IR, HepG2, ROS, caspase-3, PI3-kinase, ERK

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Introduction

1. General features of apoptosis

Programmed cell death (PCD) or apoptosis, is one of different cell death types including necrosis. It has been discovered that apoptosis can be an important part of animal development (Clarke and Clarke, 1996). Since the first observation of apoptosis had been reported during amphibian metamorphogenesis (Vogt, 1842), normal cell death was discovered to occur in both invertebrates and vertebrates (Glucksmann 1951; Clarke and Clarke, 1996). The term PCD or apoptosis was initially used to describe the cell deaths that occur in predictable places and at predictable times during development, to emphasize that the deaths are somehow programmed into the developmental plan of the organism (Lockshin and Williams, 1965). Besides needs for normal development, apoptosis is a crucial step to maintain normal homeostasis in multicellular animals. Multicellular animals often need to get rid of cells that are in excess or potentially dangerous. Therefore, apoptosis allows the organism to tightly control cell numbers and tissue size, and to protect itself from harmful cells like as damaged or genetically mutated cells that threaten homeostasis. Necrosis is a pathological process caused by the progressive degradable action of enzymes that is generally associated with severe cellular trauma, finally leading the uncontrolled cell lysis, and ultimately cell death. Necrosis and

apoptosis are the two different types of cell death showing the distinct morphologic differences (Kerr *et al.,* 1972). Cells dying by necrosis become rapidly swollen. They increase in size because of an accumulation of sodium and water within the cytosol. Soon thereafter, the mitochondria become progressively enlarged and the normal folding of the mitochondrial cristae is lost. Finally, the plasma membrane of the cell is ruptured and the cytosolic contents leak from the cell, causing a inflammatory reaction and injury to neighboring cells. On the contrary, cells undergoing apoptosis become progressively small and rapidly lose cell-to-cell and cell-to-matrix adhesion. Moreover, plasma membrane remains structurally intact and nuclear chromatin becomes condensed, nuclear membrane disappears and finally, the condensed chromatin fragments into small pieces (Majno and Joris, 1995; Earnshaw, 1995). The terminal stages of apoptosis are characterized by the cell disintegrating into "apoptotic bodies", which are comprised of pieces of condensed chromatin and a small amount of cytosol containing normal appearing mitochondria which are surrounded by the relatively intact plasma membrane (Lieberthal and Levine, 1996). The nuclear changes associated with apoptosis are results from endonuclease-directed DNA fragmentation (Wyllie et al., 1984; Enari *et al.,* 1998). The DNA fragments into nucleosomal-sized fragments that are multiples of the size of a nucleosome (app. 200bp), showing "ladder" pattern when electrophoresed on agarose gel, which is a highly reliable biochemical marker of apoptosis (Lieberthal and Levine, 1996). In necrosis, while DNA also eventually becomes degraded, the fragments generated are

not of uniform size so that electrophresis of DNA from a necrotic cell appears as a smear rather than a ladder. Apototic bodies are rapidly phagocytosed and degraded by macrophages as well as by surrounding epithelial cells (Lieberthal and Levine, 1996, 1998). Phagocytosis of apoptotic cells and apoptotic bodies provides an efficient mechanism for the removal of dead cells without incurring any of the surrounding tissue inflammation and injury that is associated with necrosis (Kerr *et al.,* 1972).

2. Factors causing apoptosis

Apoptosis has been known to be essential for normal embryonic development and maintenance of normal cellular homeostasis. Recently it has been recognized that the same biochemical pathway can be induced by different forms of stress and cellular injury. In general, the mechanism of cell death induced by cytotoxic events is determined by the severity of injury, with severe insults causing metabolic collapse and necrosis, and milder insults causing apoptosis (Thompson, 1995; Lieberthal *et al.*, 1996). ATP depletion has been suggested to induce apoptosis or necrosis by the remained amount of ATP in mouse proximal tubular (MPT) cells of kidney (Lieberthal *et al.,* 1998). From that study, when ATP levels were reduced to 25-70% of control levels all cells died by apoptosis whereas died by necrosis when ATP levels were reduced to below 15% of control values. The severity of hypoxia in the induction of apoptosis also has been demonstrated in different organs like as heart (Gottlieb *et al.,* 1994) and

brain (Martinou *et al.*, 1994). Increased production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide and the hydroxyradical, has been demonstrated to cause apoptosis as well as necrosis. Like as other stresses, exposure to excess amount of ROS causes necrosis while less severe oxidative stress cause apoptosis. Antioxidants and ROS scavengers can prevent apoptosis in a number of situations (Jacobson, 1996). The signaling pathways in which ROS participate are quitely diverse and affect many signaling molecules with a positive or negative effect on apoptosis, including PI3 kinase, NF-kB, and the Map kinase family members (Finkel, 1998). In addition, several of the signaling pathways initiated by loss of growth factors are at least partially modulated by oxidative stress (Bae *et al.*, 1997; Chen *et al.*, 1995). DNA damage may also contribute the death of some cells associated with the administration of chemotherapeutic agents like cisplatin (Lieberthal *et al.*, 1996). When DNA damage cannot be repaired, the tumor suppressor gene p53 is activated and induces apoptosis, thereby preventing the survival of mutated and potentially transformed cells. One of other important apoptotic triggers that usually do not cause necrosis are those induced by receptor-ligand interactions. The best characterized receptor family that induces apoptosis is the tumor necrosis factor (TNF) receptor superfamily, of which Fas (CD95) is the most prominent member (Nagata, 1997). Cytokines such as interleukin-1 also induce receptor-mediated apoptosis (Thijs and Thijs, 1998).

3. Survival factors and apoptosis

All cells constitutively express all the components of the apoptotic pathway necessary for apoptosis. The constant presence of "survival factors", which constantly prevent the activation of the apoptotic pathway, are required by all cells if they are to remain viable (Raff, 1992). If "survival factors" are deficient or absent, cells will automatically undergo apoptosis. Two distinct classes of survival signals have been described; soluble factors such as growth factors and cytokines (Raff, 1992), and the adhesion of cells to matrix and to neighboring cells (Meredith et al., 1993). Among different growth factors, epidermal growth factor (EGF) (Lieberthal *et al.,* 1998b), insulin-like growth factor (IGF-1) and lysophosphatidic acid (LPA) (Levine *et al.,* 1997) and insulin (Lee-Kwon *et al.*, 1998; Park *et al.,* 2000) have been identified to play an antiapoptotic role. This effect of growth factors has generally been ascribed to the proliferative effect of these hormones, but may also be due to inhibition of apoptotic pathway.

4. Insulin and apoptosis

Insulin has a variety of functions like as glucose uptake, glycogen biosynthesis, inhibition of lipolysis and stimulation of cellular proliferation. Insulin also can protect cells from apoptosis mediated by growth factor removal in different cell lines (Bertrand *et al*., 1998; Diaz *et al*., 1999; Rampalli *et al*., 1995). Recently the insulin's anti-apoptotic function and its related signaling pathways have been demonstrated in chinese hamster ovary cells expressing wild type human insulin receptors (CHO-IR) (Lee-Kwon *et*

al., 1998; Park *et al*., 2000). Although a number of molecules involved in the prevention or induction of apoptosis have been identified, the mechanisms by which insulin participate in this process remain largely unknown.

Reactive oxygen species (ROS) have been implicated as important pathologic mediators in many clinical disorders (Cross *et al*., 1987), including type II Diabetes Mellitus (Wolff *et al*., 1991; Nourooz-Zadeh *et al*., 1995). In addition, oxidative stress disrupts insulin-induced signaling events such as insulin receptor tyrosine phosphorylation, IRS-1 phosphorylation and activation of phosphatidylinositol 3-kinase (PI3 kinase) (Hansen *et al*., 1999) as well as redistribution of insulin receptor substrate-1 (IRS-1) and PI3 kinase in adipocytes (Tirosh *et al*., 1999). However, conflicting results on the effect of H_2O_2 on insulin signaling are also available. H_2O_2 mimics the stimulatory effects of insulin on glucose transport and lipid synthesis in adipocytes (Mukherjee *et al*., 1978; May and de Haen, 1979) and insulin transiently generates H_2O_2 via NADPH oxidase, which is integral to activation of the insulin signaling cascades in adipocytes (Mahadev *et al*., 2001). Insulin-induced generation of H_2O_2 reversibly inhibits protein-tyrosine phosphatase 1B (PTP1B) thereby enhances the early insulin action cascades (Mahadev *et al*., 2001b). So far, there is no general agreement by which oxidative stress affects insulin signaling and its physiological functions, or by which insulin modulates ROS generation.

5. Purpose of the present study

Following insulin binding, the insulin receptors undergo activation of their intrinsic tyrosine kinase function and subsequent stimulation of signaling molecules (Virkamaki *et al*., 1999). Recently it was reported that insulin delayed apoptosis induced by serum starvation (Lee-Kwon *et al.*, 1998), by a mechanism that is dependent on short-lived farnesylated proteins in CHO-IR cells (Park *et al.,* 2000). However, intracellular signaling cascades are highly divergent and numerous signaling proteins are differently involved to accomplish any specific function(s). Moreover, there is little evidence whether insulin really acts as a survival factor in physiological cell systems expressing intrinsic insulin receptors. With these facts as backgrounds, the present study was carried out in order to investigate whether insulin exerts an antiapoptotic activity in HepG2 cells as well as CHO-IR cells. Furthermore, it was also investigated which signaling steps or intracellular biochemical events are important to mediate insulin's antiapoptotic activity.

Materials and Methods

Materials

Human recombinant insulin, PD98059, SB202190, the fluorogenic caspase-3 substrate, Ac-DEVD-AMC, and the caspase inhibitor, z-DEVD-fmk, propidium iodide, 2', 7'-dichlorofluorescin diacetate (H_2DCFDA) were obtained from Calbiochem (La Jolla, CA). Wortmannin, Dulbecco's modified Eagle's medium (D-Mem), D-PBS, trypsin-EDTA solution were obtained from Sigma Chemical Corp. (St. Louis, MO), and FBS from Life Technologies Inc. (Rockville, MD). Monoclonal antibodies against phospho-ERK1/2 (E-4), phospho-JNK (G-7), phospho-p38MapK (D-8), ERK2 (D-2) and polyclonal antibodies against phospho-Akt1 (ser473), PARP (H-250) were from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoresis reagents, such as gels, Tris-glycine SDS running buffer, and poly(vinylidene difluoride)(PVDF) membrane were from Novex Corp. (San Diego, CA).

Cell Culture

HepG2, a hepatoblastonema cell line used in this study was obtained from Korean Cell Line Bank (Seoul, Korea) and grown in D-Mem containing 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal

bovine serum (FBS), and maintained in a humidified atmosphere of 5% CO_2 in air at 37 ℃. Chinese hamster ovary cells expressing wild- or mutated human insulin receptors were kindly provided by Dr. Masato Kasuga (Kobe University, Kobe, Japan) and grown Ham's F-12 medium containing FBS and antibiotics and maintained at same conditions adopted for maintaining of HepG2 cells. CHO-IR cells expressing mutated human insulin receptors are as follows; CHO-IR/*ΔSOS,* lacking the mSOS1-Ras interaction by the mutation of mSOS1 protein. Two days after plating in 35-mm tissue culture dishes, cells were serum-starved for 24 h (HepG2) or 4 h (CHO-IR) and then further incubated under different conditions by experimental purposes. Cells were directly subjected to experiments, or quickly frozen in liquid nitrogen and stored at 70 ^{o}C until analysis.

SDS-PAGE and immunoblotting

After appropriate treatments, the fluid was removed, and the dishes were rapidly frozen in liquid nitrogen. 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 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin A). Same amount of proteins were separated by SDS-PAGE on 4-20% polyacrylamide gel and electrotransferred onto 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 and then probed with different primary antibodies (1:1,000 1:5,000). After a series of washes, the membrane was further incubated with different horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000 1:10,000). The signal was detected with enhanced chemiluminescence (ECL) detection system (Intron, Korea).

MTT assay

This measure of mitochondrial function was performed as described previously (Parrizas *et al.*, 1997) with cells seeded on 24-well plates. Following treatments, the medium was removed from the wells, and 200 ㎕ of MTT reagent (Sigma) at a concentration of 1 mg/ml in RPMI-1640 medium without phenol red was added to each well. After 1 h incubation at 37 °C, the cells were lysed by addition of 1 volume of isopropanol and shaking for 20 min. Absorbance of converted dye was measured at wavelength of 570-690 nm.

DNA laddering

Cells were lysed in lysis solution (Puregene, Gentra Systms, Inc., Minneapolis, MN) and incubated overnight at room temperature prior to the addition of 20 ㎍/ml Rnase A for 1 h at 37 °C. The samples were deproteinized, followed by DNA precipitation with isopropanol. The concentration and purity of DNA were determined spectrophotometrically

by measuring UV absorbance ratio at 260 over 280 nm. Equal amounts of DNA from each sample (1 μg) were 3'-OH-labeled with 5 U Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) and 0.5 μCi [α-^{32}P]dCTP (~3000 Ci/mmol, Amersham Corp., Arlington Heights, IL) in the presence of 10 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. After 10 min incubation at room temperature, the reaction was terminated by the addition of 10 mM EDTA. The samples were electrophoresed on 6% polyacrylamide gel. Following electrophoresis, the gel was fixed in a solution composed of 15% methanol and 5% glacial acetic acid, dried, and analyzed by autoradiography using x-ray film and intensifying screens. In addition, the radioactivity associated with the DNA fragments was quantitated by electronic autoradiography with a Packard InstantImager (Meriden, CT).

Determination of caspase-3 activity

After treatments with reagents, cells were collected, lysed in 0.5 ml of ice-cold caspase assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA) for 15 min. After centrifugation at 12,000 x g for 15 min at 4 ^{o}C, aliquots of supernatant were incubated with 10 μM Ac-DEVD-AMC for 3 h at 37 ^{o}C. The fluorescence from the cleaved product was detected with Spectrafluor multiwell fluorescence reader (Tecan, Austria) at the wavelengths of 360 nm and 465 nm for excitation and emission, respectively.

Detection of apoptotic cells with flow cytometric analysis and H33342 staining

The degree of apoptosis was determined by measuring the number of cells showing below the G1 DNA content from flow cytometric analysis after staining of cells with propidium iodide as originally described by Crissman and Steinkamp (1973). Briefly, after treatments, floated cells were collected by pipetting, and attached cells were trypsinized. Both of collected cells were washed twice with D-PBS and fixed in ice-cold ethanol. The cells were incubated with 50 μg propidium iodide in D-PBS and 20 μg/ml Rnase A for 30 min at room temperature. The samples were analyzed with a Coulter EpicsTM cytometer (Beckman). Ten thousands of events were collected for each sample. An excitation wavelength of 488 nm and a fluorescence emission of 580 nm were used. Otherwise, cells were stained with a DNA-specific fluorescent dye (H33342) then observed under a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, MD) to examine the degree of nuclear condensation.

Measurement of ROS

H_2DCFDA, a cell-permeable fluorogenic probe that is useful for the detection of ROS, was used to measure the degree of ROS accumulation within cells. Cultured cells were briefly washed once with D-PBS, further

incubated in D-PBS containing 10 μM H_2DCFDA for 10 min at 37 ^{o}C. The fluorescence intensity was measured with Spectrafluor multiwell fluorescence reader (Tecan, Austria) at 485 nm and 535 nm wavelengths for excitation and emission respectively, under constant conditions to allow quantitative comparisons of relative fluorescence intensity from cells with different treatments. Fluorescent cell images were instantly captured under a fluorescent inverted microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, MD).

p38 Map Kinase assay

p38Map kinase activity was measured as described (Heidenreich and Kummer, 1996) with some minor modifications. Confluent cells grown in 35-mm dishes were switched to serum-free medium containing 0.1 % BSA and then treated with various factors, as described in the figure legends, followed by lysis at 4 ^{o}C for 15 min in 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM β -glycerophosphate, 5 mM sodium pyrophospate, 50 mM NaF, 1% Triton X-100, 1 mM orthovanadate, 0.15 mM Pefablock SC, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 nM okadaic acid, 10 nM tautomycin, and 10 nM cypermethrin. Equal amounts of protein from clarified lysates were immunoprecipitated with a rabbit polyclonal anti-p38 Map kinase antibody (C-20, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 90 min at 4 ^{o}C after which protein G-plus/protein-A agarose was added for another 90 min.

The immune complexes were washed twice in lysis buffer containing 0.5 M NaCl, followed by two additional washes with kinase buffer [20 mM HEPES (pH 7.4), 1 mM EGTA, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, and 0.2 mM orthovanadate]. The kinase reaction was performed by resuspending the pellet in 20 μl of kinase assay buffer containing 0.1 mg/ml recombinant GST-ATF fusion protein (generous gift from Dr. Roger J. Davis, University of Massassuchetts, Worcester, MA), 25 μg/ml cAMP-dependent protein kinase inhibitor peptide (UBI), 20 μM [α-^{32}P]-ATP (10 cpm/fmol), and 1 mM dithiothreitol for 15 min at 22 ^{o}C. The reaction was stopped by addition of Laemmli sample buffer, and the samples were analyzed by one-dimensional 4-12% SDS-PAGE under reducing conditions and autoradiography.

In vitro binding of active Ras to GST-RBD

GST-RBD was kindly provided by Dr. Johannes L. Bos (Utrecht University, Netherlands) and was used to determine the relative amount of active GTP-bound p21Ras, as previously described (de Rooij and Bos, 1997). Ras is a small GTPase that cycles between an inactive GDP-bound and an active GTP-bound form. Thus, the minimal Ras-binding domain (RBD) of Raf1 (a.a. 51-131) can be used to detect an active GTP-bound Ras by measuring the degree of active Ras-RBD binding. This novel method is based on the observation that RBD binds RasGTP in vitro with a Kd of 20 nM whereas the affinity between RBD and RasGDP is three

orders of magnitude lower. The prokaryotic expression vector (pGEX-2T) containing sequences for a fusion protein of glutathione S-transferase (GST) and RBD was transformed into *E. coli* VL21 cells. These transformed cells were grown in LB medium and the induction of GST-RBD expression was carried out by addition of 0.2 mM isopropyl-thiogalactopropyranoside (IPTG). After 4 h, cells were pelleted by centrifugation, suspended in PBS and then disrupted in B-PER reagent (PIERCE, Rockford, IL). The cell lysate was centrifuged at 5000 x g for 5 min at 4 ^{o}C and Triton X-100 was added to the supernatant to reach a final concentration of 1% (w/v), After centrifugation at 12,000 x g for 10 min at 4 ^{o}C, the supernatant was mixed with glycerol (10% final, w/v), aliquoted, and stored at - 80 ^{o}C until use. Crude GST-RBD extract was incubated with glutathione-bound agarose 4B beads (Pharmacia, Uppsala, Sweden) for 30 min at 4 ^{o}C. The beads were recovered by centrifugation and washed twice with lysis buffer II [25 mM Hepes, pH 7.5, 150 mM NaCl, 1% (w/v) NP-40, 0.25% (w/v) sodium deoxycholate, 10% (w/v) glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. For GST-RBD assay, cultured cells were lysed in 0.5 ml of lysis buffer II on ice, centrifuged at 10,000 x g for 20 min, and the clarified lysates incubated with precoupled GST-RBD beads for 30 min at 4 ^{o}C. The beads were pelleted by centrifugation and washed three times in lysis buffer II before solubilization in Laemmli sample buffer. The samples were separated on a 16% SDS-PAGE gel under reducing conditions, and immunoblotted with anti-panRas monoclonal antibody (2.5 μg/ml).

Statistical analysis

Statistical analysis was performed using an analysis program, StatViewR (Abacus Concepts, Berkely, CA, USA). The student's *t*-test was used to analyze the difference between control and experimental groups.

Result

1. Antiapoptotic activity of insulin in CHO-IR cells

1.1 Insulin protects CHO-IR cells from apoptosis induced by serum starvation

Recently insulin can protect cells from apoptosis mediated by growth factor deprivation in different cell lines (Diaz *et al.,* 1999; Bertand *et al.,* 1998; Rampalli *et al.,* 1995). Although a number of molecules involved in the prevention or induction of apoptosis have been identified, the mechanisms by which insulin participates in this process remain largely unknown. Thus, CHO cells expressing the human insulin receptors (CHO-IR) were used to evaluate the importance of different signaling steps activated by insulin in this process.

The ability of insulin in CHO-IR cells was studied initially after incubation for 24 h in the absence or in the presence of insulin at the indicated concentrations. Cells were stained with a DNA-specific fluorescent dye, H33342, to observe the degree of nuclear condensation, which is an apoptotic phenomenon (Fig. 1). A number of cells incubated in serum-free medium (i.e. growth factor deprivation) showed highly condensed nuclei. Insulin treatment clearly decreased the number of cell with condensed nuclei (Fig. 1). The effect of insulin showed a concentration-

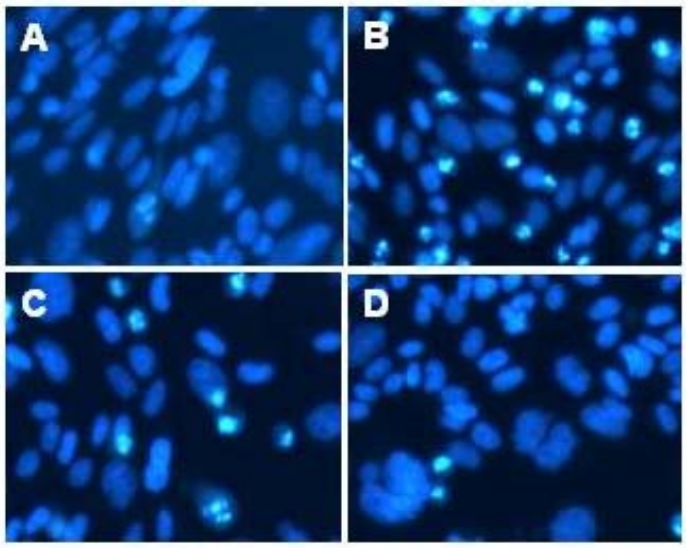


Figure 1. Insulin's antiapoptotic activity in CHO-IR cells (the degree of nuclear condensation). CHO-IR cells were plated into 35 mm culture dishes at a density of 1 x 10^6 cells at 1 day before experiments. Cells were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (D-PBS) twice and then incubated in serum-free Ham's F-12 medium for 3 h before insulin or fetal bovine serum (FBS) treatment. After 24 h of treatment, the membrane-permeable, DNA-specific fluorescent dye, H33342 (10 ㎍/ml medium at final) was directly added to cultured cells. The fluorescent image of nuclei of cells were observed and photographed under inverted fluorescent microscope equipped with a CoolSNAP-Pro digital camera. Condensed, fragmented nuclei are shown in apoptotic cells.

dependent pattern. The degree of apoptosis was also determined with flow cytometric analysis measuring DNA content of each cell and counting the number of events below diploid (2N) DNA content (Fig. 2). Additionally, the DNA laddering assay was then used to assess the presence of internucleosomal DNA fragmentation, one of the hallmark of apoptotic death (Wyllie, 1993). Fig. 3 showed that insulin reduced the degree of DNA fragmentation that were generated in response to serum withdrawal. These results suggest that insulin can protect CHO-IR cells from apoptosis induced by serum starvation.

1.2 Insulin blocks caspase-3 activity

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 (Stennicke & Salvasen 1998; Nagata, 2000). The function of caspase-3 activity has been described (Porter & Janicke, 1999) as being involved in the execution of apoptosis in a tissue and cell type, or death stimulus-specific manner. From the results shown in Fig. 4, serum starvation may induce apoptosis via mechanisms including caspase-3 activation. Serum starvation sharply elevated caspase-3 activity when compared with the presence of 10% FBS in the medium. Caspase-3 activity was undetectable where z-DEVD-fmk (10 μmol), a potent cell-permeable caspase-3 inhibitor, was added, demonstrating the specific nature of this assay (Fig. 4A). And insulin also

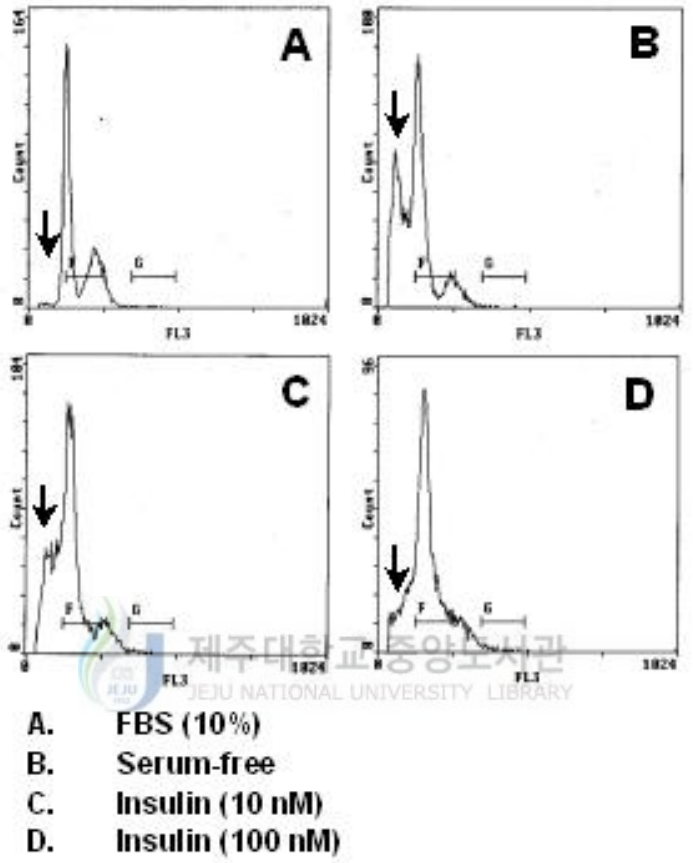


Figure 2. Insulin's antiapoptotic activity in CHO-IR cells (intracellular DNA content measured by flow cytometry). CHO-IR cells were plated into 35 mm culture dishes at a density of 1 x 10^6 cells at 1 day before experiments. Cells were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (D-PBS) twice and then incubated in serum-free Ham's F-12 medium for 3 h before insulin or fetal bovine serum (FBS) treatment (24 h). The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis as described in "Material and Methods". Each arrow indicates the peak of population of apoptotic cells having sub G1 (< 2N) DNA content per cell.

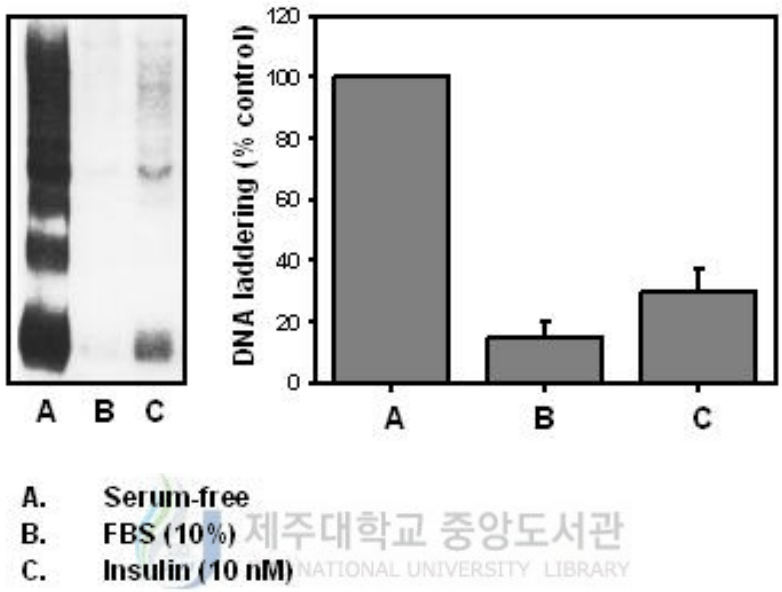


Figure 3. Insulin's antiapoptotic activity in CHO-IR cells (DNA laddering). CHO-IR cells were plated into 35 mm culture dishes at a density of 1 x 10^6 cells at 1 day before experiments. Cells were washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (D-PBS) twice and then incubated in serum-free Ham's F-12 medium for 3 h before insulin or fetal bovine serum (FBS) treatment (24 h). Total genomic DNA was isolated and same amount (1 mg) from each sample was 3'-OH-labeled with [*a*-32P]dCTP. After electrophoresis on a 6% polyacrylamide gel, the radioactivity of each lane was counted, otherwise, exposed to a x-ray film for autoradiography.

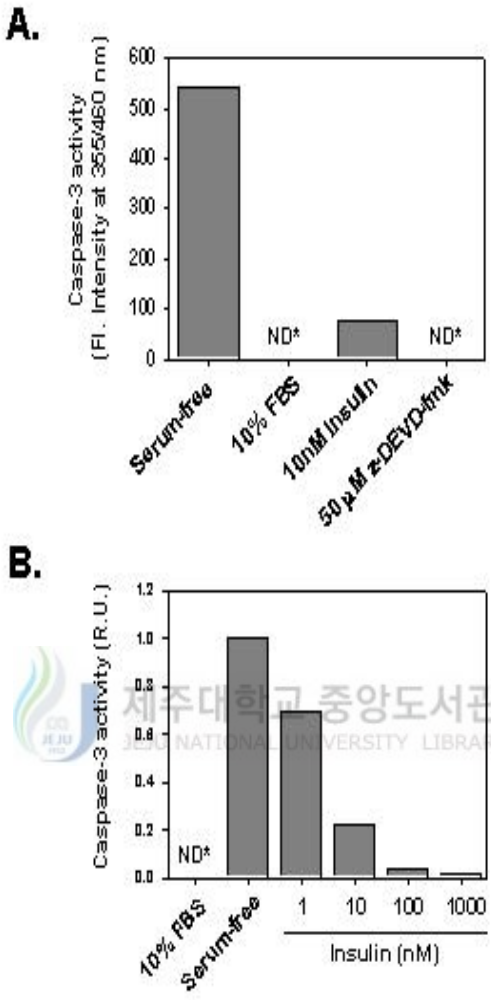


Figure 4. Inhibition of caspase-3 activity by insulin in CHO-IR cells. Confluent cells were washed twice with D-PBS, and serum-starved for 3 h. Cells were then pretreated with z-DEVD-fmk (10 mM), a caspase-3 inhibitor for 30 min before insulin (A; 10 nM, B; 1-1000 nM) or FBS (10%) addition. After 18 h treatment, cells were lysed and an aliquot of each lysate with equal amount of protein was incubated with 10 mM (final) Ac-DEVD-AMC for 3 h at 37 ˚C. Each bar is a representative of two independent experiments. ND*, non-detected.

suppressed caspase-3 activity by a concentration-dependent manner (Fig. 4B).

1.3 Insulin receptor-specific signaling

Activation of insulin receptor (IR) tyrosine kinase is essential for many of the biological actions of insulin. The diagram of insulin receptor-induced signaling cascades are showin in Figure 5. The ligand binding receptor initiates intracellular signals by stimulating tyrosine phosphorylation of endogenous substrates [e.g., insulin receptor substrate (IRS) and Shc proteins]. Tyrosine phosphorylation of IRS-1 allows compartmentalization of signaling molecules, including members of the class I phosphatidylinositol 3-kinase (PI3-kinase) family (Backer *et al.*, 1992). Insulin induces also the formation of Shc-Grb2-Sos complex that enables p21Ras activation (Ouwens *et al.*, 1994; Sasaoka *et al.*, 1994).

Several lines of evidence suggest that insulin receptor tyrosine kinase activity may be involved in most of the actions of insulin. To assess the requirement for kinase function of the antiapoptotic protection, CHO/neo cells lacking insulin receptor expression and CHO-IR cells were used. Fully confluent cells were serum-starved for 3h and further incubated in the presence of different doses of insulin for 10 minutes. Cells were lysed and subjected to immunoblotting using different antibodies against phosphorylated active signaling molecules. The phosphorylaton of tyrosine (PY) was increased by insulin treatment in CHO-IR cells, showing the

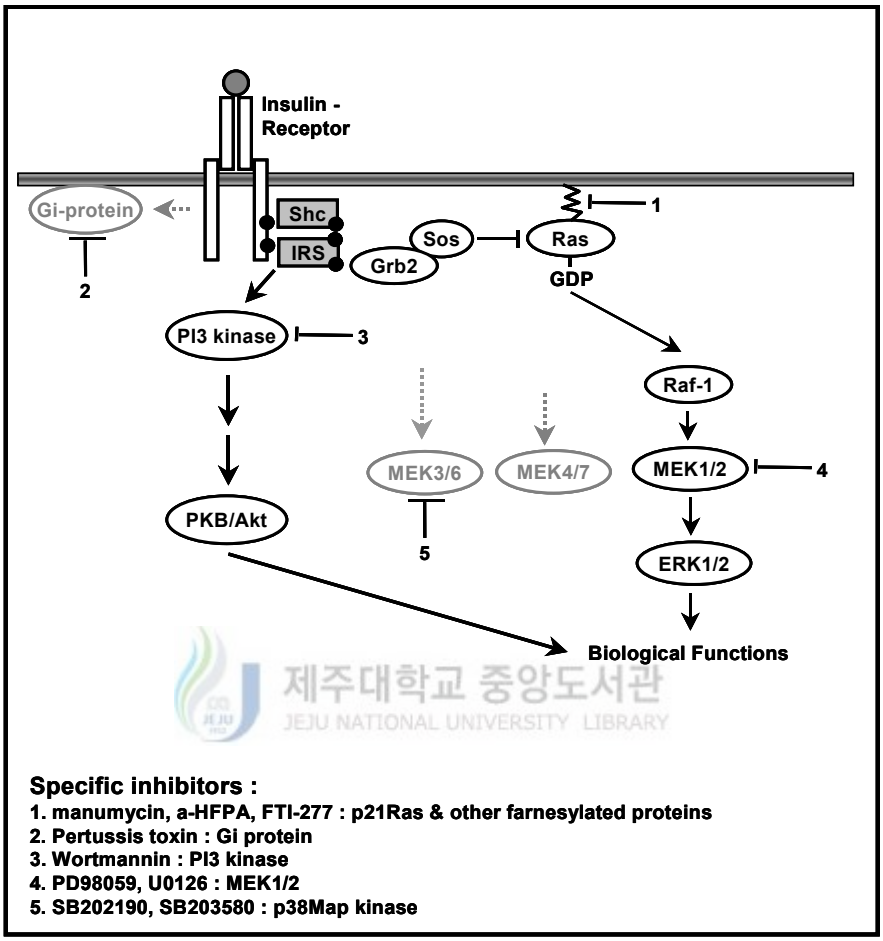


Figure 5. Diagram of insulin receptor-induced signaling cascades. After insulin binding to α-subunits of its receptor, β-subunits were phosphorylated at a number of tyrosine residues (autophosphorylation) with intrinsic receptor tyrosine kinase (RTK) activity of β-subunits. Although PI3 kinase-Akt cascade and p21Ras-raf-MEK-ERK cascade are well-known signaling cascades derived by insulin-receptor binding, a number of different signaling molecules have known to be activated by insulin.

dose-dependent manner (Fig. 6). Phosphorylation of Akt showed similar activation in CHO-neo and CHO-IR cells, but phosphorylation of ERK was stimulated only in CHO-IR cells. These results show the insulin receptor-specific signaling nature of CHO-IR cells, not of CHO/neo cells.

1.4 Effect of Ras on insulin's antiapoptotic function

The Ras-dependent signaling pathways appears to couple the signaling events that begin at the plasma membrane to the nuclear events. Insulin is one of the major stimulants of the Ras signaling pathway. The biological importance of the Ras pathway has been studied extensively with the use of inhibitors of protein farnesylation inhibitors (FTIs) and dominant negative mutants of Ras (Wiese *et al.,* 1995; Sasaoka *et al.*, 1994; Jhun *et al.*, 1994; De Meyts *et al.*, 1996).

Protein farnesylation is a regulated posttranslational modification that allows attachment of a number of proteins, including p21Ras, Rho, Rac and others. By inducing the activity of the enzyme farnesyl protein transferase, insulin increases the pool of membrane-associated p21Ras and promotes GTP loading on Ras (Goalstone *et al*., 1996; Goalstone *et al*., 1997). It has been documented that manumycin, a selective protein FTI, blocks the antiapoptotic protection exerted by insulin in IR-expressing CHO cells maintained the absence of growth factors (Lee-Kwon *et al.*, 1998). To assess whether manumycin can inhibit insulin-stimulated Ras function, active p21Ras protein was detected by a GST-RBD assay together with

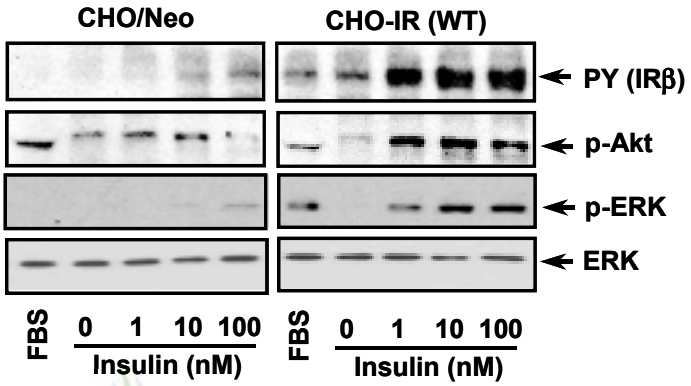


Figure 6. Comparison of signaling events stimulated specifically by insulin in CHO/neo and CHO-IR cells. Fully confluent cells were serum-starved for 3 h and further incubated in the presence of different doses of insulin for 10 min. Cells were lysed and immunoblotting was carried out using different antibodies against phosphorylated active signaling molecules. for 10 min. Cells were lysed and subjected to immunoblotting using different antibodies against phosphorylated active signaling molecules. CHO/neo, CHO cells lacking insulin receptor expression; PY (IRβ), tyrosine-phosphorylated β-subunit of insulin receptor; Immunoblot of ERK protein is an internal standard of each group.

Western blot analysis. The GST-RBD (Ras binding domain) assay was used to determine the relative amount of active GTP-bound p21Ras (de Rooij and Bos, 1997). The GST-RBD fusion protein contains the minimal Ras binding domain of Raf-1 (amino acids 51-131). The confluent cells were pretreated with different inhibitors (manumycin, *α*-HFTA, FT1-277) of farnesylation of proteins including p21Ras for 1 h after serum starvation (3 h), then further treated with insulin for 10 min (A) or 18 h (Fig. 7B & 7C). Figure 7A showed that inhibition of protein farnesylation by different FTIs does not suppress p21Ras activation by insulin. However, manumycin inhibited insulin's antiapoptotic function with DNA laddering assay (Fig. 7B) and flow cytometric assay (Fig. 7C). These results indicate that p21Ras activity is not required for the insulin's antiapoptic function in CHO-IR cells whereas inhibition of farnesylation of proteins other than p21Ras may suppress the insulin's antiapoptotic protection.

1.5 Effect of Ras on antiapoptotic function of insulin in CHO-IR/*ΔSOS* cells

The importance of p21Ras activation in the survival function of insulin was examined further by using CHO-IR/*ΔSOS* cells (Fig. 8), expressing a transdominant negative mutant of the Ras-specific exchange factor, mSOS1. mSOS is the mammalian homologue of the Drosophila gene, son-of-sevenless, which becomes bound to the plasma membrane and thereby localized close to membrane-bound p21ras (Christian *et al.*, 1995).

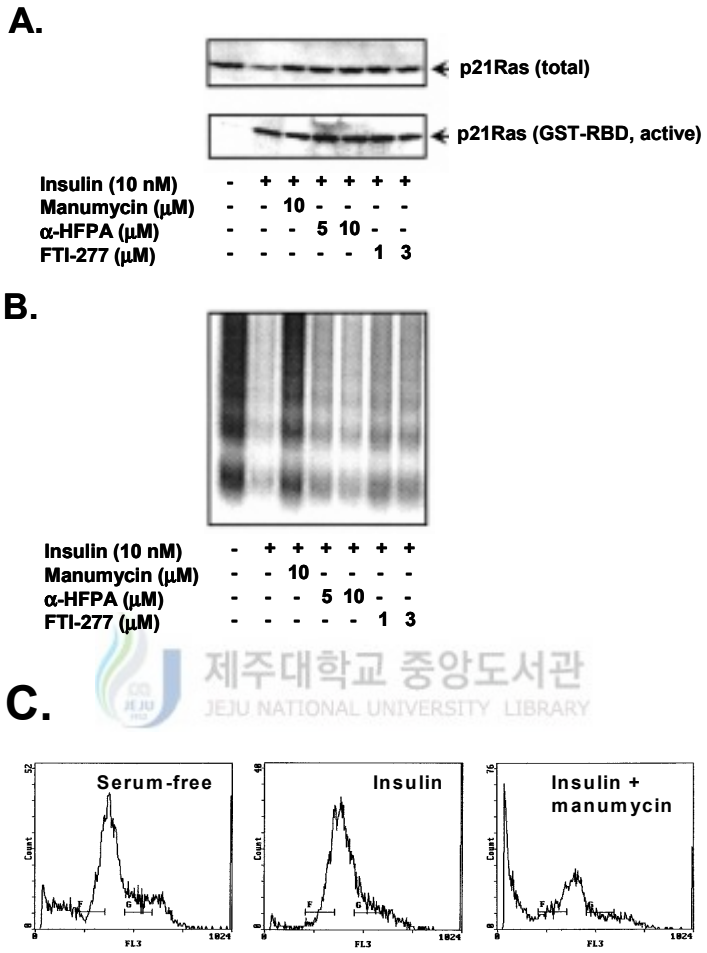


Figure 7. NO demand of p21Ras activity for the insulin's antiapoptotic function in CHO-IR cells. Confluent cells were pretreated with different inhibitors of farnesylation of proteins including p21Ras for 1 h after serum starvation (3 h), then further treated with insulin for 10 min (A) or 18 h (B, C). p21Ras activation by insulin was detected using GST-RBD assay (A) and the degree of apoptosis in different groups was determined by DNA laddering assay (B) or flow cytometric assay as described in "Materials and Methods".

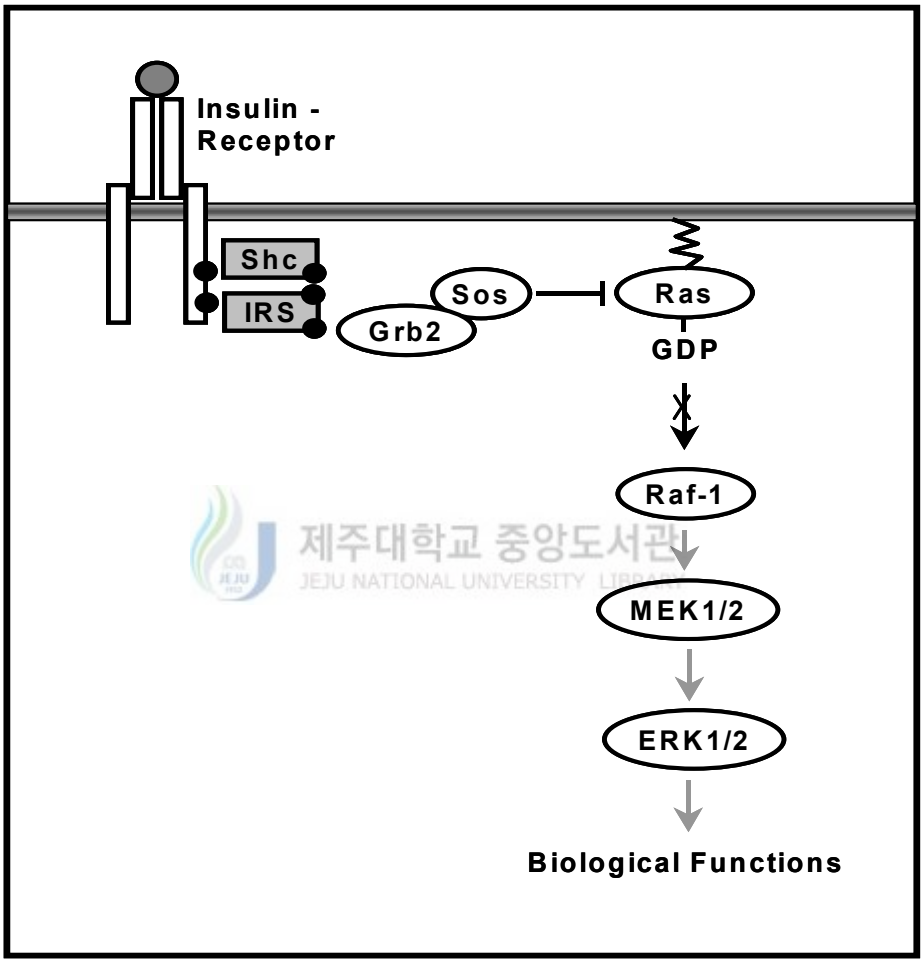


Figure 8. CHO-IR/*ΔSOS*, CHO-IR cells lacking intrinsic p21Ras activity. p21Ras isn't activated in CHO-IR/*Δ SOS* cells because they express a deletion mutant of mSOS1 protein that lacks the guanine nucleotide exchange domain of p21Ras.

GST-RBD assay showed that insulin does not activate p21Ras in CHO-IR/*ΔSOS* (Fig. 9A). Any significant differences in p21Ras activation by insulin were not shown between CHO-IR wild type and CHO-IR/*ΔSOS* cells (Fig. 9B). However, manumycin inhibited the insulin's antiapoptotic function in both cell types. The result supports the notion that p21Ras activity is not required for the insulin's antiapoptotic function in CHO-IR cells whereas inhibition of farnesylation of proteins other than p21Ras may suppress the insulin's antiapoptotic protection.

1.6 Effect of PI3-kinase inhibition on insulin receptor-specific signaling

Activation of the insulin receptor increases PI3-kinase activity, whose function has been associated with the anti-apoptotic signaling in various cell types (Yao and Cooper, 1995; Minshall *et al.*, 1996). However, in CHO-IR cells, the activation of PI3-kinase by insulin did not play a role in protecting cells from apoptosis induced by serum starvation because insulins anti-apoptotic protection was not altered by wortmannin or LY294002 (Lee-Kwon *et al.*,1998), two chemically unrelated inhibitors of PI3-kinase. In the present study, wortmannin (an inhibitor of PI3-kinase) blocked the phosphorylation of Akt stimulated by insulin, and PD98059 (an inhibitor of MEK1/2) suppressed the activation of ERK by insulin (Fig. 10). However, CHO-IR cells pretreated with higher dose (100 nM) of wortmannin were apoptotic to a large extent even in the presence of insulin (Fig. 11, 12). The protective effect of insulin was also markedly

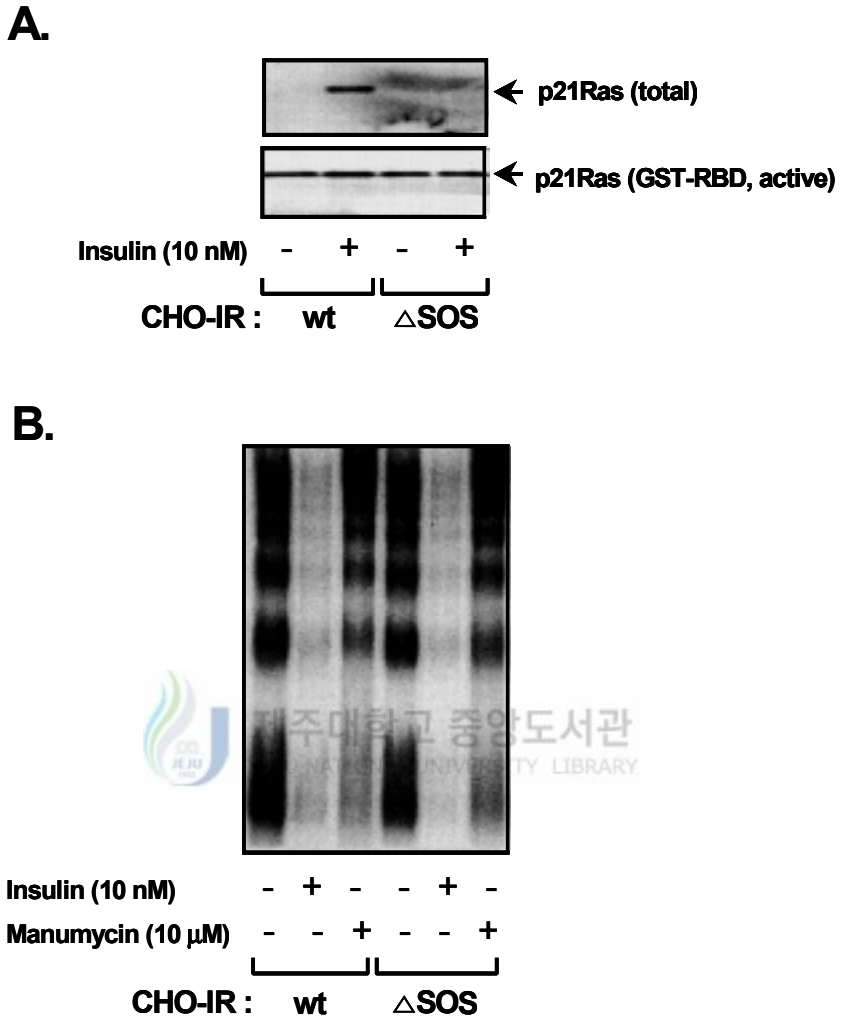


Figure 9. Insulin's antiapoptotic activity in CHO-IR/*ΔSOS* cells. Fully grown CHO-IR/*ΔSOS* cells and CHO-IR cells were serum-starved for 3 h and pretreated with manumycin (B) for 1 h before insulin addition. GST-RBD assay (A) was performed to compare the degree of p21Ras activation between two different cell lines. DNA laddering assay (B) was performed after 18 h of insulin treatment.

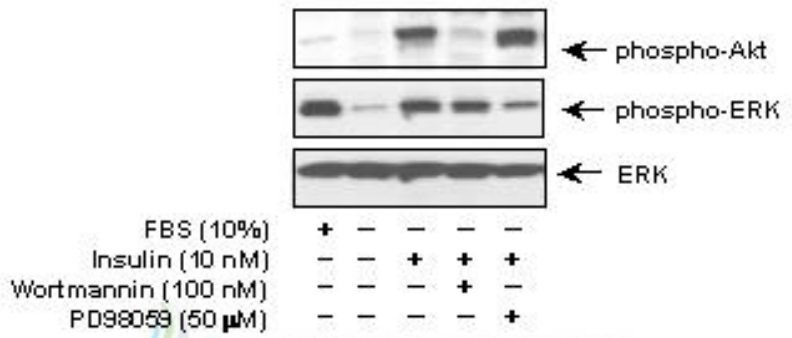


Figure 10. Specific inhibition of insulin receptor-specific signaling cascades by different inhibitors in CHO-IR cells. CHO-IR cells were serum-starved for 3 h and then pretreated with wortmannin (PI3 kinase inhibitor) or PD98059 (MEK1/2 inhibitor) before insulin or FBS treatment (10 min). Cells were lysed and immunoblotting was carried out using different antibodies against phosphorylated active signaling molecules. Immunoblot of ERK protein is an internal standard of each group.

blocked by PD98059 treatment (Fig. 11, 12).

1.7 Inhibition of PI3-kinase and ERK block caspase-3 activity

Caspase-3 activity was measured 24 h after insulin (100 nM) treatment in the absence or presence of inhibitors as indicated (Fig. 13). Serum starvation sharply elevated caspase-3 activity compared with the presence of 10% FBS in the medium. Insulin markedly suppressed caspase-3 activity induced by serum starvation. Treatment with inhibitors (manumycin, PD98059, wortmannin) suppressed insulin's antiapoptotic function. This result means that insulin-mediated survival pathway is dependent on the activation of PI3-kinase and ERK in CHO-IR cells.

1.8 Insulin receptor-independent activation of ERK1/2 by LPA

Lysophosphatidic acid (LPA) promotes growth, differentiation, survival and motility in many different cell types. Therefore LPA has been suggested to play a central role in a broad range of physiological and patho-physiological processes, including vascular and neuronal function and cancer. Three closely related G-protein-coupled cell-surface receptors mediate some of these effects, but assigning specific functions to particular receptor subtypes has been challenging and several lines of evidence indicate that other LPA signaling mechanisms might exist (Celine *et al*., 2003). LPA is potent bioactive lipid mediators with specific and multiple

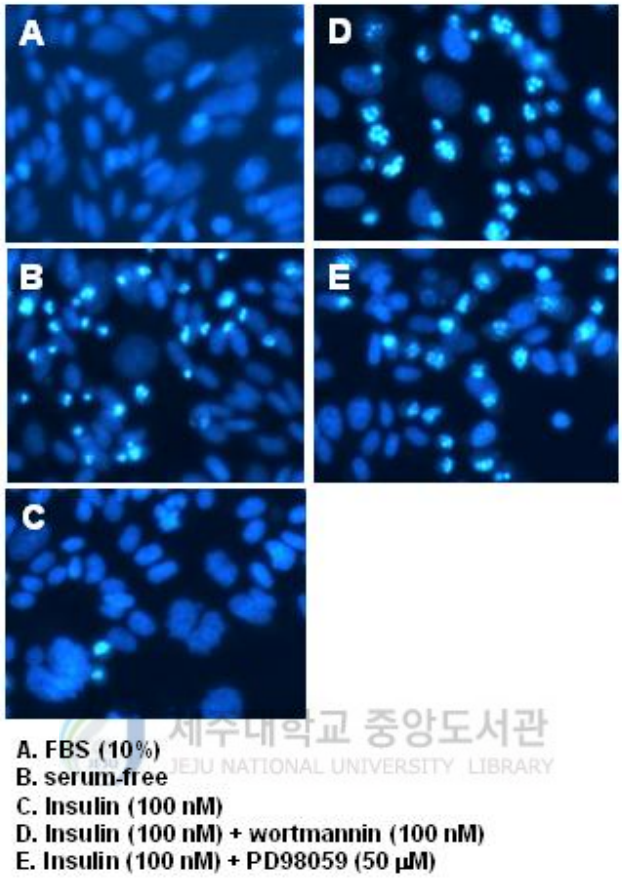


Figure 11. Suppression of insulin's antiapoptotic activity by inhibition of PI3 kinase as well as MEK1/2 (nuclear condensation and fragmentation). CHO-IR cells were serum-starved for 3 h and then pretreated with wortmannin (PI3 kinase inhibitor) or PD98059 (MEK1/2 inhibitor) before insulin or FBS treatment (18 h). After treatment, the membrane-permeable, DNA-specific fluorescent dye, H33342 (10 μg/ml medium at final) was directly added to cultured cells. The fluorescent image of nuclei of cells were observed and photographed under inverted fluorescent microscope equipped with a CoolSNAP-Pro digital camera. Condensed, fragmented nuclei are shown in apoptotic cells.

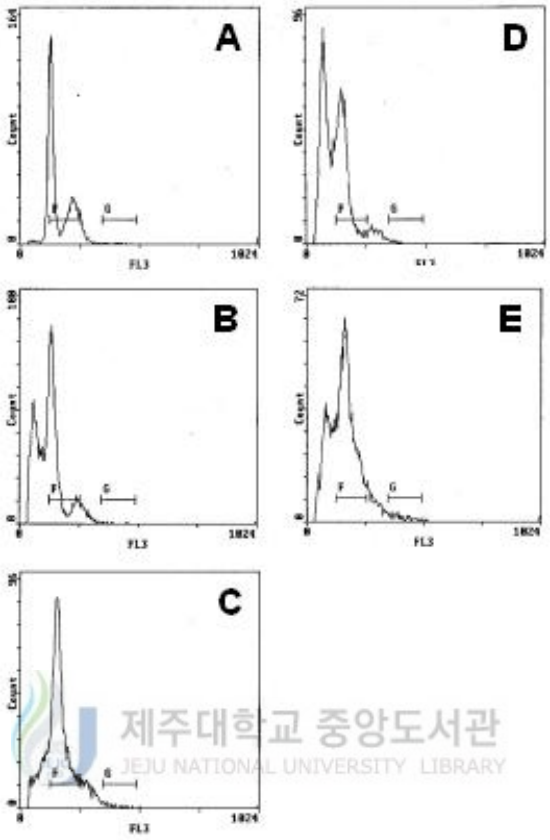
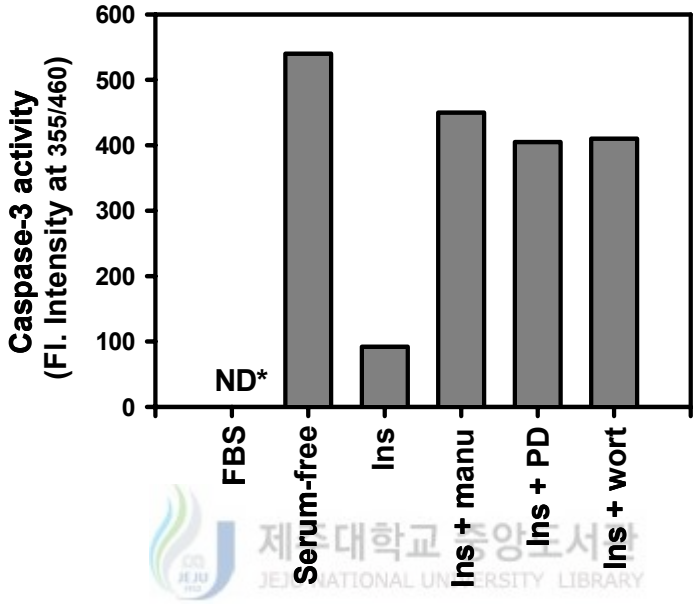


Figure 12. Suppression of insulin's antiapoptotic activity by inhibition of PI3 kinase as well as MEK1/2 (Flow cytometric analysis). CHO-IR cells were serum-starved for 3 h and then pretreated with wortmannin (PI3 kinase inhibitor) or PD98059 (MEK1/2 inhibitor) before insulin or FBS treatment (18 h). The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis as described in "Material and Methods". The first peak of each panel indicates the peak of population of apoptotic cells having sub G1 (< 2N) DNA content per cell.



FBS (10%); Ins (insulin, 10 nM); manu (manumycin, 10 μM); PD (PD98059, 50 μM); wort (wortmannin, 100 nM)

Figure 13. Increase of caspase-3 activity by inhibition of PI3 kinase, MEK1/2 or protein farnesylation. CHO-IR cells were serum-starved for 3 h and then pretreated with wortmannin (100 nM, PI3 kinase inhibitor), PD98059 (50 μM, MEK1/2 inhibitor) or manumycin (10 μM, protein farnesylation inhibitor) before insulin or FBS treatment (18 h). Cells were lysed, an aliquot was used to measure caspase-3 activity as described in "Material and Methods". Each bar is a mean of two repeated experiments. ND*, non-detected.

cellular effects. They bind to distinct specific G protein-coupled receptors in the plasma membrane (Goetzl *et al.*, 1998; Hla *et al.,* 2001). LPA receptor subtypes that are able to couple to one or multiple heterotrimeric G proteins (Hla *et al.*, 2001).

LPA is known to stimulate ERK1/2 activity via pathways independent of receptor tyrosine kinase signaling (Fig. 14). In the present study, LPA did not blocked the insulin's antiapoptotic function in CHO-IR/*ΔSOS* cells, which lacks the receptor tyrosine kinase activity (Fig. 15). Therefore, ERK activation *per se* does not play a role in protecting cells from apoptosis.

1.9 Effect of Gi protein on insulin's antiapoptotic activity

G-protein-coupled receptors constitute one of the most important families of membrane receptors through which cells communicate with each other and organisms adapt to changes in the internal and external environments. Heterotrimeric GTP binding protein (G-protein), which binds seven-helix transmembrane receptors, plays important physiological roles in mediating specific functions of many hormones, neurotransmitters, and chemokines (Bourne *et al.*, 1991). Ligand-receptor binding leads to the different members, Giα and Gsα, inhibitory- and stimulatory Gα, respectively. It has been reported that pertussis toxin (Ptx) suppresses the different members, Giα and Gsα, inhibitory- and stimulatory Gα, respectively. It has been reported that pertussis toxin (Ptx) suppresses the function of inhibitory

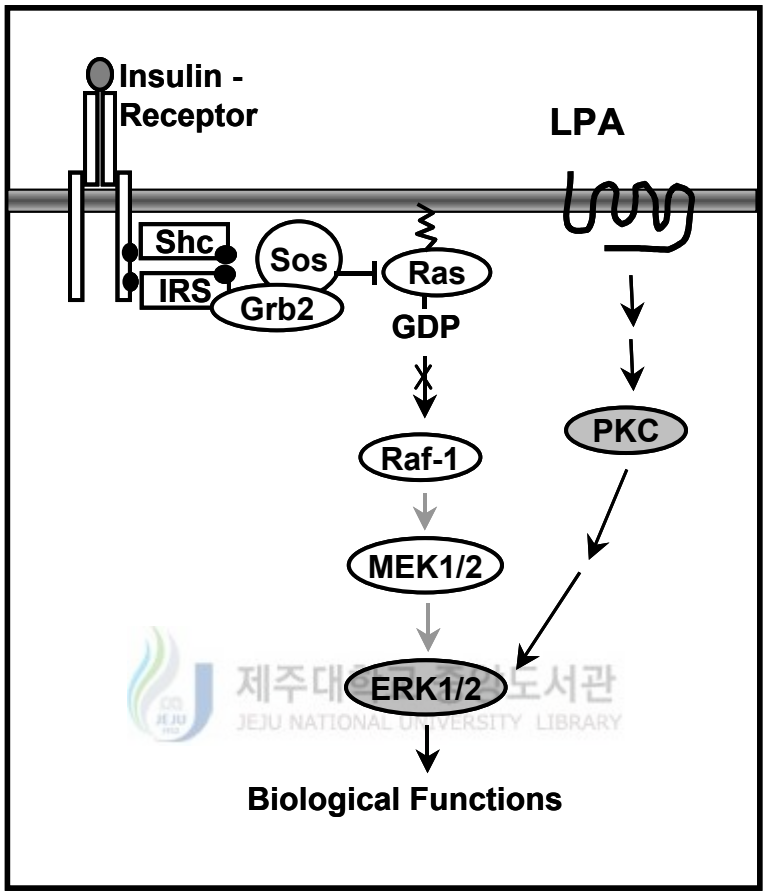
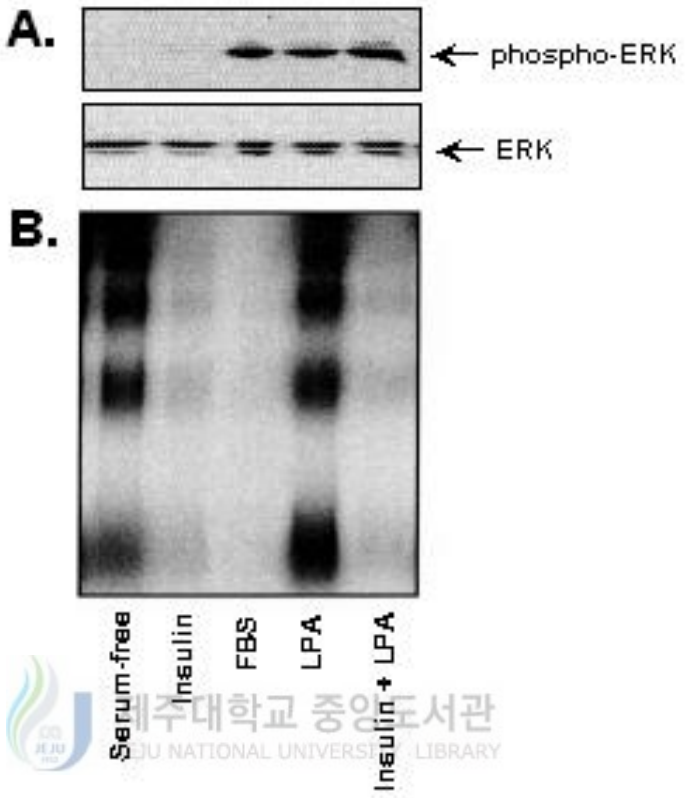


Figure 14. Diagram of insulin-independent activation of ERK1/2 by lysophosphatidic acid (LPA) in CHO-IR/*ΔSOS* cells. LPA is known to stimulate ERK1/2 activity via pathways independent of receptor tyrosine kinase signaling.



Insulin (10 nM); FBS (10%); LPA (lysophosphatidic acid, 2 μM)

Figure 15. ERK activation *per se* without playing a role in protecting cells from apoptosis in CHO-IR/*ΔSOS* cells, lacking the insulin-dependent ERK activation pathway. CHO-IR/*ΔSOS* cells were fully grown, serum-starved and further treated FBS, insulin and/or LPA for 10 min (A) or 18 h (B). Activation of ERK was determined from immunoblot assay for active, phosphorylated ERK (A) and the degree of apoptosis was determined by DNA laddering assay (B) as described in "Material and Methods". FBS (10%), insulin (10 nM), LPA (2 μM).

trimeric guanine nucleotide binding protein (Gi protein). Previously, it has been reported that insulin signaling pathways were involved with pertussis toxin (an inhibtior Giα)- or cholera toxin (a stimulator of Gsα)-sensitive processes (Zeng and Houslay, 1995; Standaert *et al.*, 1995), and that insulin may decrease serine phosphorylation of Giα-2 in rat hepatocytes (Morris *et al.*, 1995). Yoshimori et al., showed that Ptx inhibited insulin-induced glucose uptake. Ptx-sensitive Gi-proteins are substrates for ADP-ribosylation catalyzed by Ptx (Yoshimori *et al.,* 2000).

CHO-IR cells or CHO-IR/⊿SOS cells were serum-starved for 3 h and pretreated with pertussis toxin (100 ng/ml) for 1 h before insulin (10 nM) addition. After 18 h treatment, cells were harvested, then subjected to experiments to measure the degree of nuclear condensation and fragmentation using H33342 staining, intracellular DNA content with flow cytometric analysis or DNA laddering as different markers of apoptosis induction (Fig. 16). Consistently, pertussis toxin blocked the insulin's antiapoptotic protection of CHO-IR cells. This result suggests that Ptx-sensitive Giα protein may participate in the insulin-mediated protection of CHO-IR cells from apoptosis.

1.10 Insulin suppresses NO production in CHO-IR cells

During the last decade, there has been an explosion in interest for understanding the involvement of nitric oxide (NO) or other reactive oxygen species (ROS) in a wide variety of both physiological and

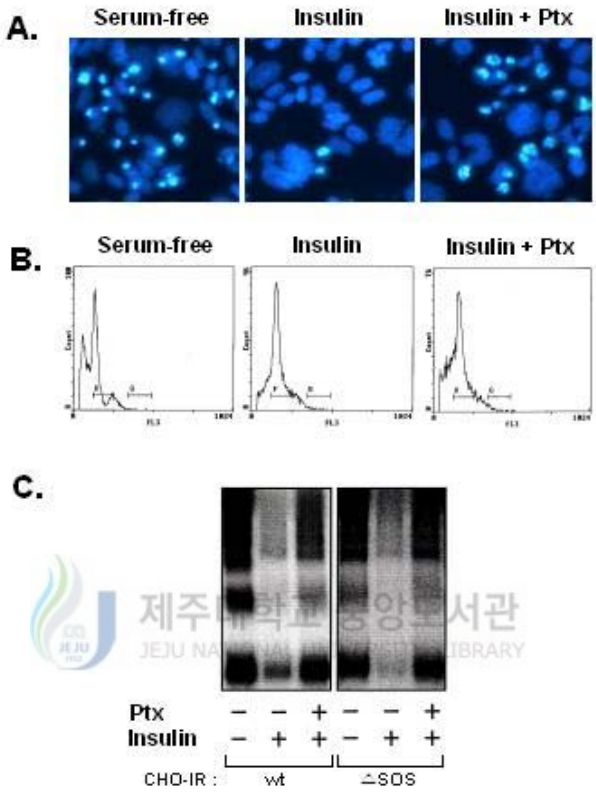
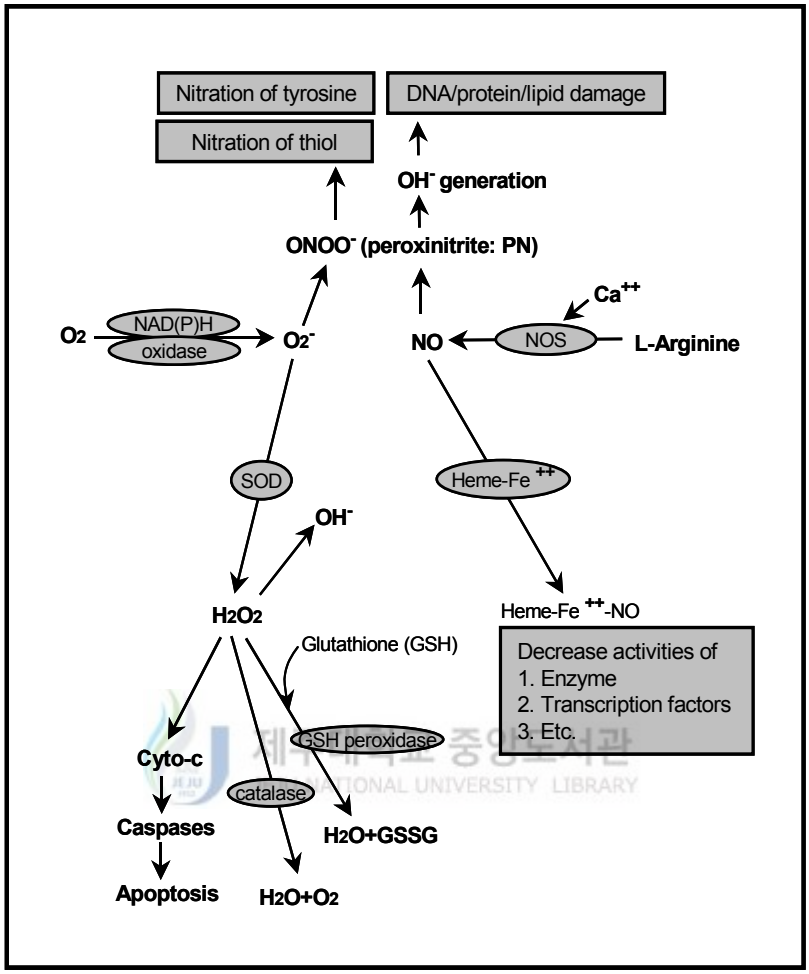


Figure 16. Role of Gi protein in mediating insulin's antiapoptotic activity. CHO-IR or CHO-IR/ *SOS* cells were serum-starved for 3 h and pretreated with Pertussis toxin (Ptx, 100 ng/ml) for 1 h before insulin (10 nM) addition. After 18 h treatment, cells were harvested, then subjected to experiments to measure the degree of nuclear condensation and fragmentation using H33342 staining (A), intracellular DNA content with flow cytometric analysis (B) or DNA laddering (C) as different markers of apoptosis induction.

pathophysiological processes (Fig. 17). The roles of ROS in apoptosis are well documented. 2-ethyl-2-thiopseudourea (ETU) is highly specific and potent inhibitors of iNOS over both eNOS or neuronal NOS (nNOS). Inhibition of iNOS by ETU effectively suppressed PARP hydrolysis and nuclear condensation in HepG2 cells treated with As_2O_3 (Kang *et al.*, 2003). In this present study, CHO-IR cells were serum-starved, further treated with insulin (10 nM) or ETU for 18 h. After treatment, culture medium was collected and used for NO measurement by Griess reaction and cells were subjected to DNA laddering assay. Insulin decreased NO production (Fig. 18A), but the treatment with ETU failed to induce DNA fragmentation (Fig. 18B). We suggest, therefore, insulin decreases NO production, but such a decrease of NO production *per se* does not protect CHO-IR cells from apoptosis. This suggestion was further supported by a result that the change of NO content within CHO-IR cells by treatment with NO donor (SNP) or NO scavenger (PTIO) is not enough to affect apoptosis (Fig. 19).

To demonstrate that a biological response is mediated by this free radical species, various compounds that could inactivate NO have been used to prevent the effects of NO (Feelisch, 1998). In large part, the use of the so-called NO scavengers has developed as a complement to the application of NO donors, rather than authentic NO, in most studies of NO-related biological effects. Among compounds of this class phenyltetramethylimidazolineoxyl-oxides (PTIO) and especially its water-soluble derivative carboxy-PTIO have become very popular NO



1. Superoxide (O_2^-) or hydrogen peroxide (H_2O_2) is relatively unreactive ROS towards biological molecules.
2. Hydroxy radical (OH-) is highly reactive ROS.
3. DPI, inhibitiors against NADPH oxidase as well as nitric oxide synthase (NOS).
4. PDTC, an inhibitor of superoxide dismutase (SOD).
5. NAC, hydrogen peroxide scavenger by mimicking GSH.

Figure 17. Illustration of oxidative stress and its effects on biological functions. Living cells have capacity to maintain the balance of oxidized- and reduced environments by producing or scavenging reactive oxygen species (ROS) like as superoxide, nitric oxide or hydrogen peroxide. Excessive production of ROS may affect various harmful modifications of intracellular substances, leading to cellular damages.

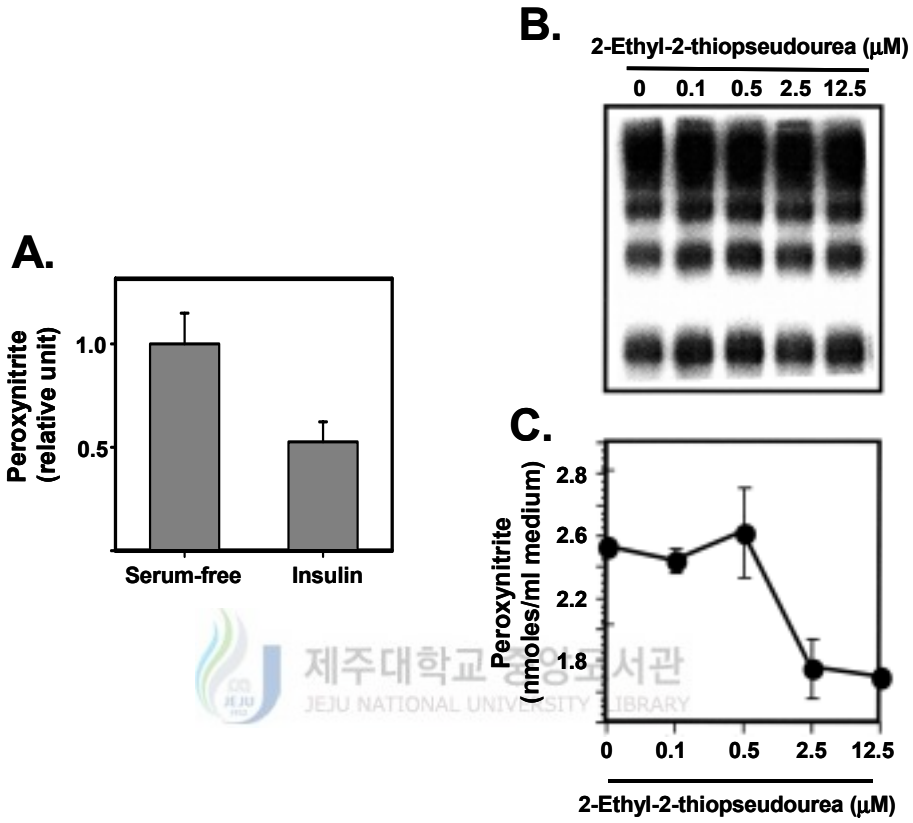


Figure 18. Suppression of NO production by insulin or an iNOS inhibitor CHO-IR cells were serum-starved, further treated with insulin (10 nM) (A) or 2-ethyl-2-thiopseudourea (an inhibitor of inducible nitric oxide synthase, iNOS) for 18 h. After treatment, culture medium was collected and used for NO measurement by Griess reaction (A, C) as described in "Material and Methods". Otherwise, cells were subjected to DNA laddering assay (B) after treatment with different doses of 2-ethyl-2-thiopseudourea.

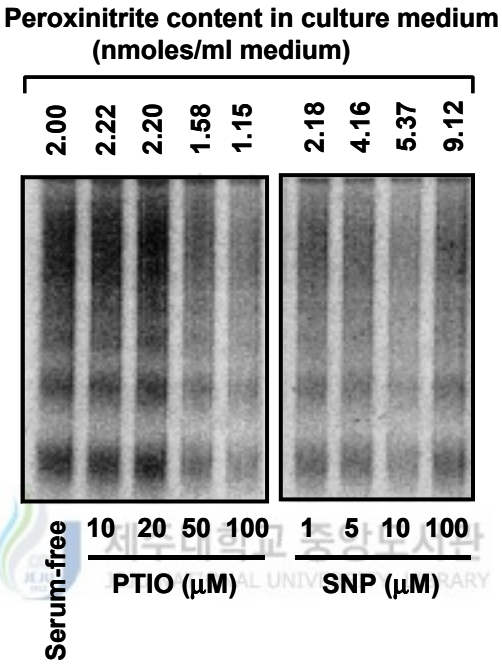


Figure 19. Activation of NO-itself is not enough to induce apoptosis in CHO-IR cells. Serum-starved CHO-IR cells were incubated in the presence of different doses of NO scavanger (carboxy-2-phenyl-4, 4, 5, 5-tetra methyl imidazoline-1-oxyl-3-oxide, PTIO) or NO donor (sodium nitroprusside, SNP) as indicated in figure for 18 h. Collected medium was subjected to Griess reaction for the measurement of NO content and cells were subjected to DNA laddering assay. Each numerical value represents the concentration of NO in medium.

scavengers and have been increasingly used in the field of NO related studies (Pfeiffer *et al.*, 1997). Sodium nitroprusside (SNP) is a commonly used vasodilator which acts as a NO targeting many proteins either by directly binding heme-containing proteins such as soluble guanylyl cyclase (sGC), nitrosylation of thiol residues, nitration of tyrosine or oxidizing DNA and proteins (Balligand & Cannon, 1997). In culture of neonatal cardiomyocytes, SNP and 8-Br-cGMP induce apoptosis (Wu *et al.*, 1997), whereas NO functions as either pro- or antiapoptotic effector in chick embryonic heart cells (Stefanelli *et al.*, 1999). 8-Br-cGMP (a potent analogue of cGMP) and dipyridamole (DP, a specific inhibitor of cGMP-specific phosphodiesterase, PDE5) were treated with insulin for 18 h as indicated, and harvested cells were subjected to DNA laddering assay (Fig. 20 & 21). 8-Br-cGMP inhibits insulin's antiapoptotic function by a concentration-dependent manner (Fig. 20), DP also shows similar results (Fig. 21). These results indicate that cGMP-PKG may inhibit insulin's antiapoptotic function.

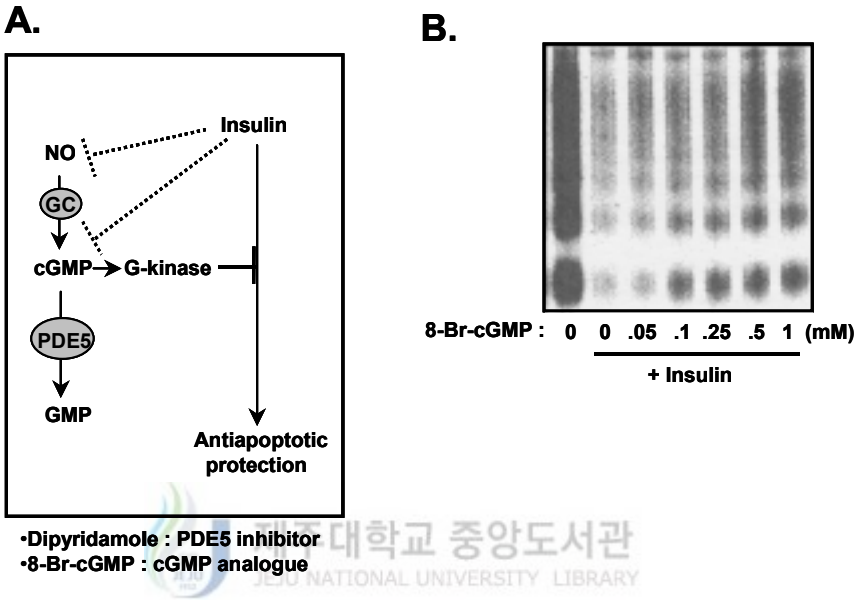


Figure 20. cGMP-PKG as inhibitor of insulin's antiapoptotic function. **A.** Diagram of protein kinase G (PKG) stimulation via NO-cyclic GMP (cGMP) pathway and reagents to modulate cGMP content. Dipyridamole (DP, a specific inhibitor of cGMP-specific phosphodiesterase, PDE5); 8-bromo-cGMP (a potent analogue of cGMP). **B.** Serum-starved CHO-IR cells were treated with different doses of 8-Br-cGMP together with insulin for 18 h as indicated, and harvested cells were subjected to DNA laddering assay.

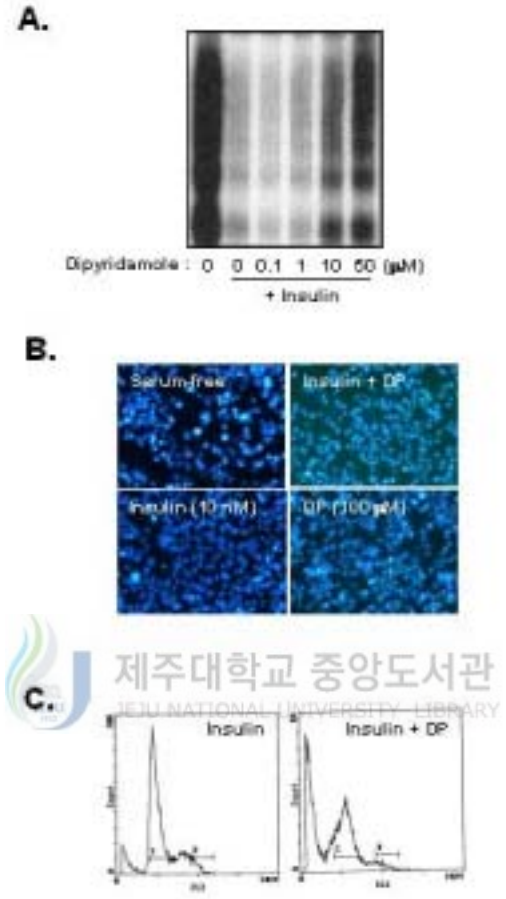


Figure 21. cGMP as inhibitor of insulin's antiapoptotic function in CHO-IR cells. CHO-IR cells were serum-starved for 3 h, then incubated with insulin and/or dipyridamole, a reagent increasing the basal content of cGMP by blocking hydrolysis of cGMP for an additional 18 h. Harvested cells were subjected to DNA laddering assay (A), observation of nuclear condensation and frgmentation (B), or measurement of intracellular DNA content with flow cytometric analysis (C), respectively.

2. Antiapoptotic activity of insulin in HepG2 cell

2.1 Insulin protects HepG2 cells from apoptosis

Results from our present studies using CHO-IR cells showed insulin's antiapoptotic function. However, it is not yet clear whether insulin really plays an important role in protecting cells or tissues constructing body from apoptosis because CHO-IR cell line is an experimental cell model which expresses excess numbers of insulin receptor molecules than any physiological cell models. Thus, it was adopted other physiological cell model expressing insulin receptor molecules and used to investigate insulin's antiapoptotic function.

To examine the involvement of insulin as a survival factor, HepG2 cells were used. HepG2 is a hepatoblastonema cell line that expresses intrinsic insulin receptors. HepG2 cells were incubated in serum-free medium for 24 h and then washed with D-PBS twice, then treated with 100 nM insulin for an additional 18 h for caspase-3 assay or for
48 h for the determination of apoptotic events, nuclear condensation and fragmentation (H33342 staining). 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 (Fig. 22B). From the overall experiments to test occurrence of apoptotic events, insulin blocked apoptosis induced by serum starvation in HepG2 cells (Fig. 22A). It was further investigated the effect of insulin on the caspase-3

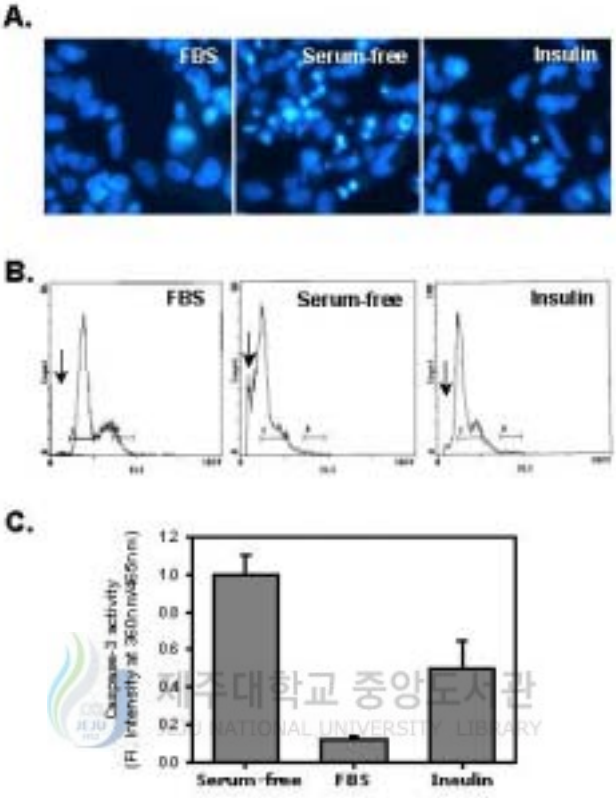


Figure 22. Insulin action protecting HepG2 cells from apoptosis. HepG2 cells were serum-starved overnight, then treated with FBS (10%) or insulin (100 nM) for an additional 18 h (C) for caspase-3 assay or for 48 h for the determination of apoptotic events, nuclear condensation and fragmentation (H33342 staining) (A) or intracellular DNA content (flow cytometric analysis) (B) as described in "Material and Methods". Each bar in (C) is a mean ± S.E. of 3 separated experiments.

activity. Insulin markedly suppressed caspase-3 activity induced by serum starvation (Fig. 22C). Serum starvation sharply elevated caspase-3 activity compared with the presence of 10% FBS in the medium.

2.2 Insulin delays apoptosis induced by oxidative stress in HepG2 cells

Like as deprivation of growth factors (serum starvation) from culture media, oxidative stress is also an important apoptotic inducer. To test whether oxidative stress can induce apoptotic death of HepG2 cells and whether insulin can protect cells from apoptosis induced by an oxidative stress, the effect of H_2O_2 (1 mM) was added to culture medium with or without insulin. The degree of apoptosis was measured with flow cytometry analysis measuring DNA content of each cell and counting the number of events below diploid (<2N) DNA content (Fig. 23). The percentages of apoptotic cells were 23.2 ± 3.5% (control, serum-free) and 4.1% ± 0.7% (100 nmol/l insulin) respectively. The effect of H_2O_2 (1 mmol/l) was also examined in order to determine whether an additional oxidative stress accelerates an apoptotic process induced by serum starvation. The addition of H_2O_2 significantly increased the percentage of apoptotic cells (35.3 ± 4.2%) compared to control groups ($P<0.05$). Insulin treatment also significantly decreased (8.9 ± 2.6%, $P<0.01$) the degree of apoptosis compared to H_2O_2-alone group. 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 (Fig. 23). A

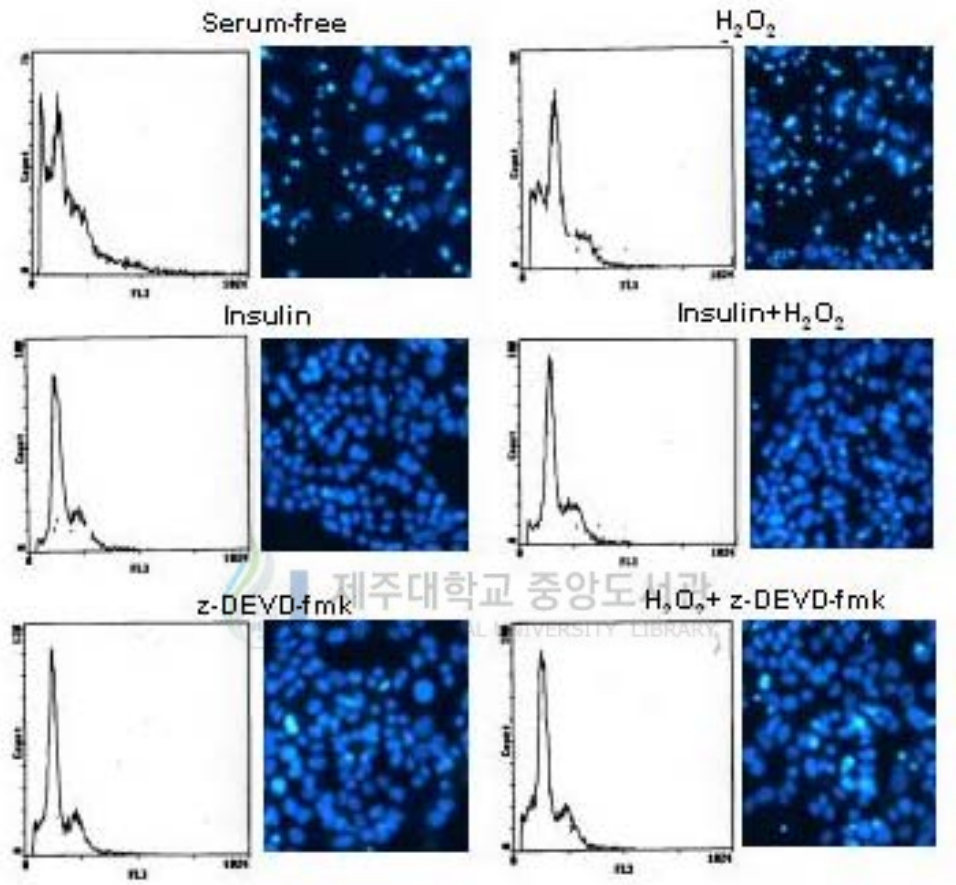


Figure 23. Insulin action blocking apoptosis induced by serum starvation or H_2O_2 addition. HepG2 cells were serum-starved (24 h), preincubated with 10 μM z-DEVD-fmk (30 min), and further incubated with 100 nM insulin and 1 mM H_2O_2 for an additional 48 h. The degree of apoptosis is represented as the intracellular DNA content measured by flow cytometric analysis and the photographs showing cells with highly condensed nuclei stained with H33342.

number of cells incubated in serum-free medium or treated with H_2O_2 for 48 h showed highly condensed nuclei. Insulin treatment clearly decreased the number of cells with condensed nuclei in both groups. We also investigated whether the activation of caspase-3 is associated with the apoptotic process induced by serum starvation or oxidative stress. z-DEVD-fmk, a cell permeable caspase-3 inhibitor, effectively protected cells from apoptosis by serum starvation or H_2O_2 treatment from results of flow cytometric analysis and H33342 staining. The degree of proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), a target molecule of caspases, was paralleled with dose of H_2O_2 treated (Fig. 24A). The integrity of PARP was increased by insulin treatment compared with serum starvation. H_2O_2-induced PARP cleavage was also effectively blocked by insulin (Fig. 24C). These results suggest that insulin can protect HepG2 cells from apoptosis induced by serum starvation or oxidative stress.

2.3 Insulin suppresses ROS production in HepG2 cells

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 (CADD) (Stennicke & Salvasen, 1998; Nagata, 2000). It was examined whether insulin affects intracellular ROS accumulation (Fig 25). H_2O_2 (1 mM) was also added together with insulin to determine whether insulin can diminish the amount of intracellular ROS increased by H_2O_2 addition. From the microscopic

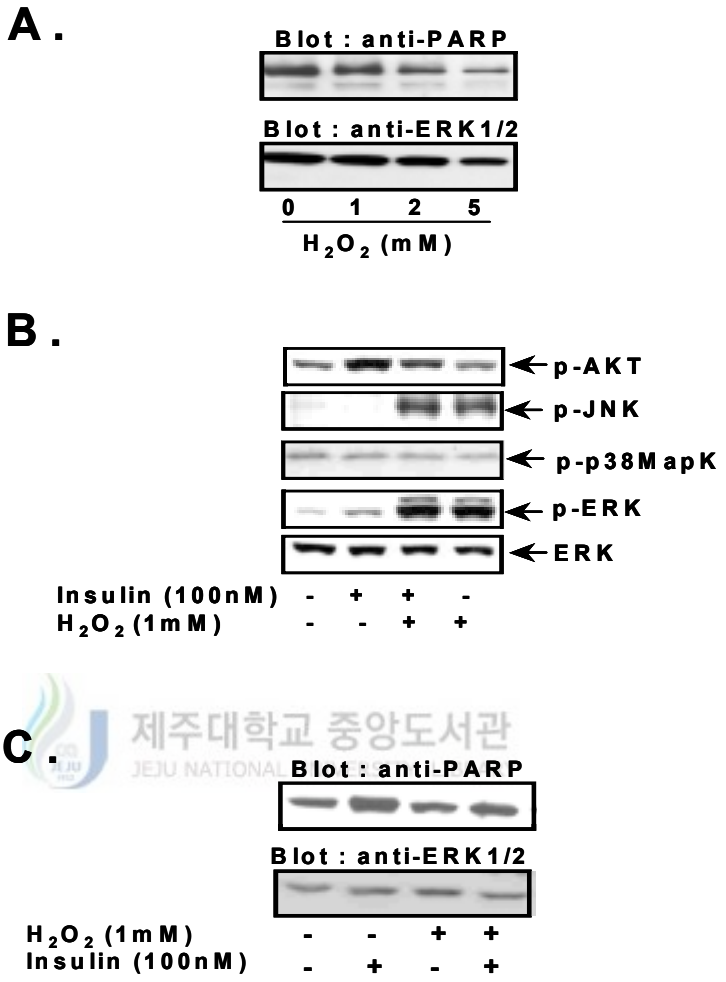


Figure 24. Induction of PARP cleavage and ERK1/2 stimulation by H_2O_2. HepG2 cells were serum-starved for 24 h and treated with H_2O_2 and/or insulin for 10 min (B) or for 48 h (A, C). Collected cells were lysed and subjected to acrylamide gel electrophoresis and immunoblotting for poly(ADP-ribose) polymerase (PARP), active-, phospho-Akt and activated mitogen activated protein kinases(Map kinases) including c-jun NH_2-terminal kinase (JNK), p38Map kinase and ERK. Intact ERK is an internal standard (A-C).

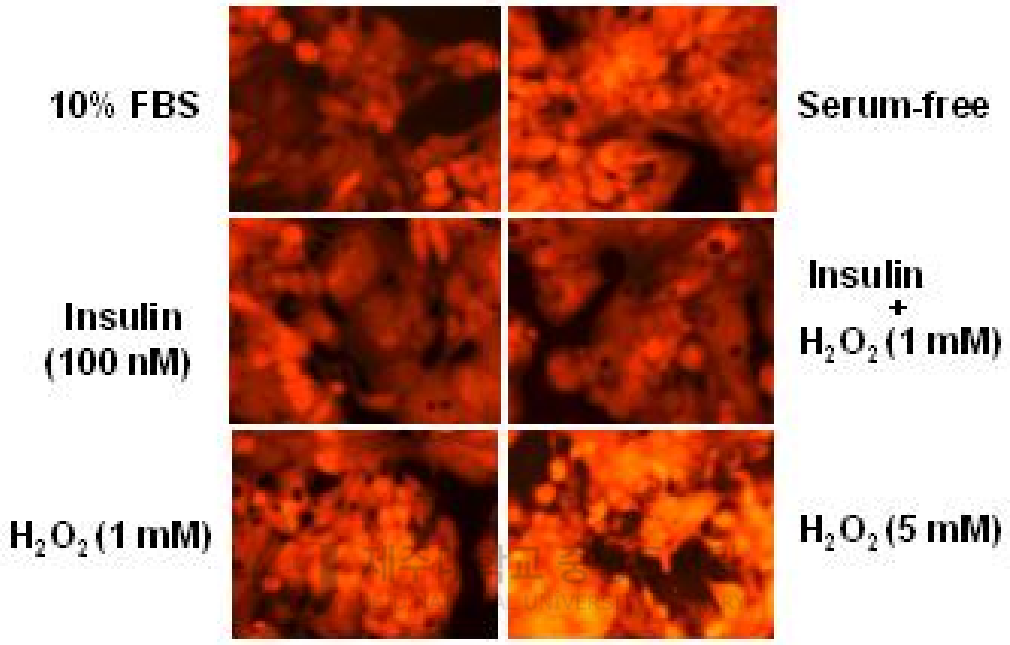


Figure 25. Insulin as a suppressor ROS production induced by serum starvation or H_2O_2 addition. Cells were serum-starved for 24 h and further treated with insulin and/or H_2O_2 for an additional 3 h. Each panel shows the DCF fluorescence from cells imaged with a CoolSNAP-Pro digital camera attached to the inverted fluorescent microscope.

observation, the intensity of fluorescence from DCFH oxidized by intracellular ROS was increased where cells were incubated for 3 h in serum-free medium or in medium containing H_2O_2 (1-5 mM). Insulin lowered both of the intensities of fluorescence elicited by serum starvation and H_2O_2 addition.

2.4 Insulin's antiapoptotic function is sensitive to protein farnesylation but insensitive to inhibition of Gi protein in HepG2 cells

Farnesylation is a regulated posttranslational modification that allows attachment of a number of proteins, including p21Ras, to the plasma membrane. By inducing the activity of the enzyme farnesyl protein teansferase, insulin increases the pool of membrane-associated p21Ras and promotes GTP loading on Ras (Goalstone & Draznin, 1997; Goalstone *et al.*, 1997). It has been recently documented that manumycin, a selective protein farnesylation inhibitor (FTI), blocks the antiapoptotic protection exerted by insulin in IR-expressing CHO cells maintained in the absence of growth factors (Lee-Kwon *et al.,* 1998).

HepG2 cells were pretreated with manumycin (10 μM), an analogue of farnesyl diphosphate, pertussis toxin (100 ng/ml) before insulin treatment. Manumycin blocked the ability of insulin (Fig. 26), but not pertussis toxin. So these results indicate that insulin's antiapoptotic function is sensitive to inhibition of protein farnesylation but insensitive to inhibition of Gi protein in HepG2 cells.

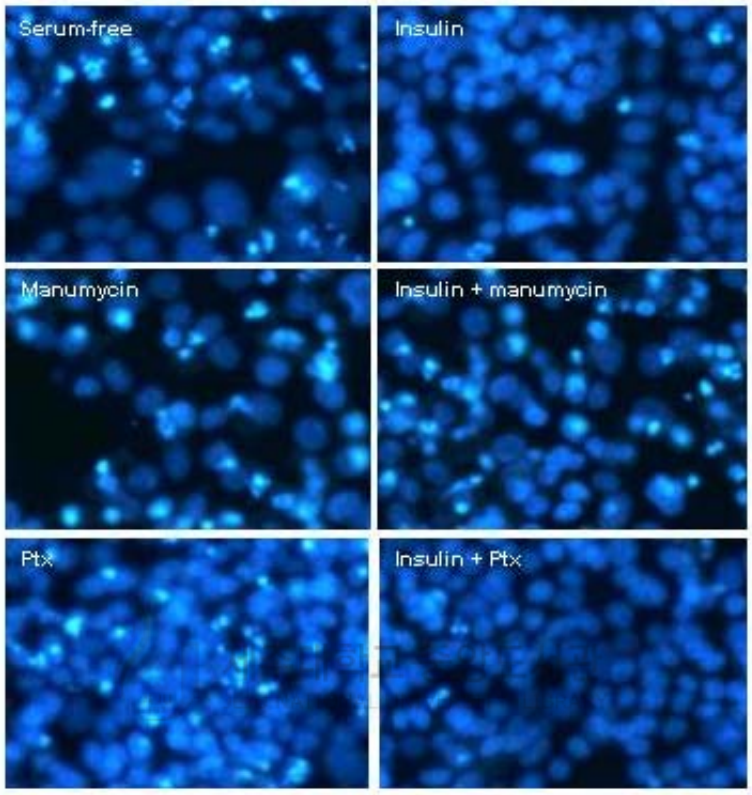


Figure 26. Insulin's antiapoptotic function sensitive to inhibition of protein farnesylation but insensitive to inhibition of Gi protein in HepG2 cells. HepG2 cells were serum-starved for 24 h, pretreated with pertussis toxin (Ptx, 100 ng/ml) or manumycin (10 μM) before insulin (100 nM) treatment. After 48 h of insulin treatment, cells were stained with H33342 as described in "Materials and Methods".

2.5 Inhibition of PI3 kinase and ERK blocks insulin's antiapoptotic activity

Activation of the insulin receptor increases PI3 kinase activity, whose function has been associated in the antiapoptotic signaling in various cell types (Yao & Cooper, 1995; Minshall *et al.*, 1996). However, in CHO-IR cells, the activation of PI3 kinase by insulin did not play a role in protecting cells from apoptosis induced by serum starvation because the insulin's antiapoptotic protection was not altered by wortmannin or LY294002 (Lee-Kwon *et al.*, 1998), two chemically unrelated inhibitors of PI3 kinase. In the present study, however, HepG2 cells pretreated with 100 nM wortmannin were apoptotic to a large extent even in the presence of insulin (Fig. 28). A specific inhibitor of MEK1, PD98059, was also used to assess the role of ERK protection against apoptosis. The protective effect of insulin was markedly blocked by 50 μM PD98059 (Fig. 28) and the insulin stimulation of ERK activity was also completely blocked (Fig. 27). Unexpectively, insulin stimulation of ERK activity was clearly suppressed by wortmannin whereas Akt stimulation by insulin was unaffected by PD98059 (Fig. 27). Neither c-Jun NH_2-terminal kinase1/2 (active-JNK) nor p38Map kinase, other members of Map kinase families, were not changed in the presence of insulin (Fig. 27). Separate experiments were performed to determine the effects of those inhibitors on the cleavage of PARP (Fig. 29). Treatment of cells with PD98059 or wortmannin accelerated PARP cleavage, which was suppressed by insulin. These results suggest that

insulin's antiapoptotic function is mediated by PI3 kinase as well as ERK, but not by Akt in HepG2 cells.

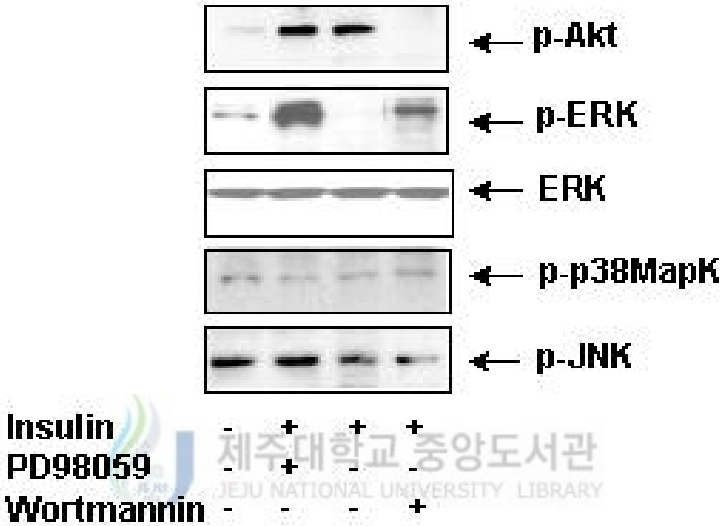


Figure 27. Modulation of insulin receptor-specific signaling by insulin and specific inhibitors in HepG2. HepG2 cells were serum-starved for 24 h and then pretreated with wortmannin (100 nM, PI3 kinase inhibitor) or PD98059 (50 μM, MEK1/2 inhibitor) for 1 h before insulin or FBS treatment (10 min). Cells were lysed and immunoblotting was carried out using different antibodies against phosphorylated active signaling molecules. Immunoblot of ERK protein is an internal standard of each group.

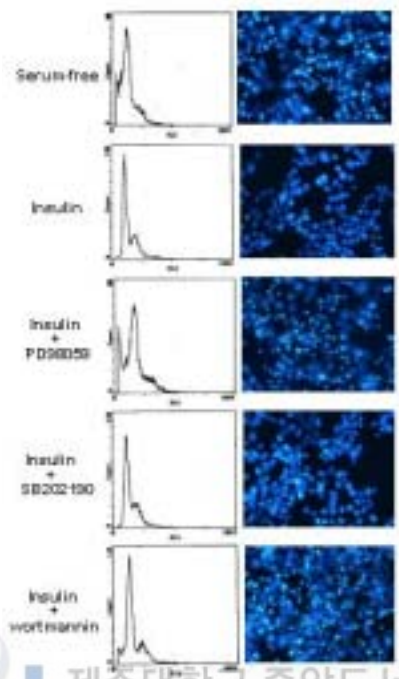


Figure 28. Suppressive effect of inhibition of PI3 kinase as well as MEK1/2 on insulin's antiapoptotic activity in CHO-IR cells. HepG2 cells were serum-starved for 24 h and then pretreated with wortmannin (100 nM, PI3 kinase inhibitor), PD98059 (50 μM, MEK1/2 inhibitor) or SB202190 (20 μM, p38Map kinase inhibitor) for 1 h before insulin (100 nM) treatment (48 h). After treatment, the membrane-permeable, DNA-specific fluorescent dye, H33342 (10 μ g/ml medium at final) was directly added to cultured cells. The fluorescent image of nuclei of cells were observed and photographed under inverted fluorescent microscope equipped with a CoolSNAP-Pro digital camera. Condensed, fragmented nuclei are shown in apoptotic cells. Otherwise, the degree of apoptosis is represented as the DNA content measured by flow cytometric analysis as described in "Material and Methods". The first peak of each panel indicates the peak of population of apoptotic cells having sub G1 (< 2N) DNA content per cell.

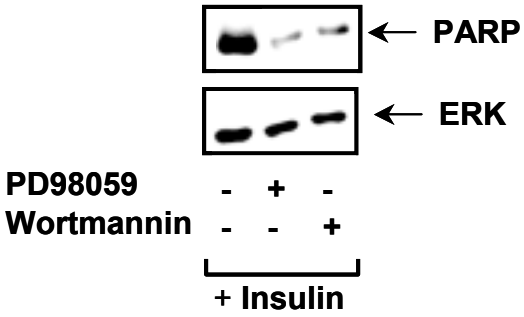


Figure 29. Inhibition of PI3 kinase or MEK1/2 leading cleavage of PARP in HepG2 cells. HepG2 cells were serum-starved for 24 h and then pretreated with wortmannin (100 nM, PI3 kinase inhibitor) or PD98059 (50 μM, MEK1/2 inhibitor) for 1 h before insulin (100 nM) treatment (48 h). Cells were lysed and immunoblotting was carried out using an antibody against PARP. Immunoblot of ERK protein is an internal standard of each group.

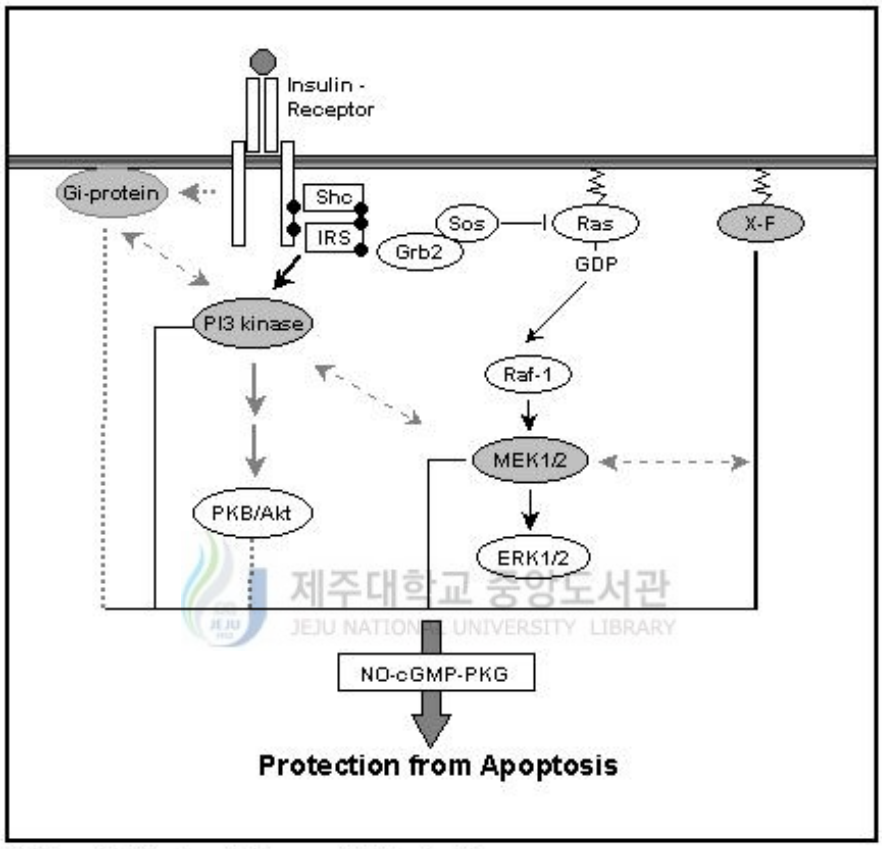


Figure 30. Summary, an illustration of hypothetical action mechanism of insulin's antiapoptotic function. PI3 kinase, MEK1/2 not ERK1/2, and a farnesylated protein excluding p21Ras are thought to play important roles in mediating insulin's antiapoptotic function. The involvement of Gi protein seems to be inconsistent among different cell types.

Discussion

The present study examined the ability of insulin to exert antiapoptotic function in CHO-IR and HepG2 cells and its related signaling pathways. Insulin is a potent survival factor in different cell systems (Diaz *et al.*, 1999; Bertrand *et a.l*, 1998; Rampalli & Zelenka, 1995). The present results showed that insulin inhibited apoptosis induced by serum starvation in CHO-IR and HepG2 cells that express insulin receptors. This result and others support the notion that the use of CHO-IR cells which express human insulin receptors are valid experimental models to investigate the antiapoptotic function by the insulin receptor-derived biochemical events. This finding is in agreement with previous results using CHO-IR cells (Lee-Kwon *et al.*, 1998; Park *et al.*, 2000) but has different kinetics. HepG2 cells were apoptotic at least 36 h later in serum-free conditions whereas most of CHO-IR cells were readily apoptotic within 24 h (Park *et al.*, 2000) from the data of flow cytometric analysis. Moreover, insulin reduced the degree of DNA fragmentation that was induced in response to serum withdrawal from culture medium. These results suggest that insulin can protect CHO-IR cells from apoptosis induced by serum starvation. However, z-DEVD-fmk, a caspase-3 inhibitor, failed to rescue cells from apoptosis whereas it inhibited caspase-3 activity in CHO-IR cells (Park *et al.*, 2000). On the contrary, in HepG2 cells, z-DEVD-fmk *per se* effectively protected cells from apoptosis in the present study. These results suggested that the apoptotic steps by serum starvation are different between CHO-IR

and HepG2 cells with respects to the significance of caspase-3.

The signal transduction pathways stimulated by insulin to confer antipoptotic protection were also investigated. Various inhibitors of key steps in signaling cascades activated by growth factors have been widely used and have provided important insight into the understanding of the role of signaling molecules (Farrelly *et al.*, 1999; Kulik & Webber *et al.*, 1998; Erin *et al.*, 1999). Figure 5 showed the diagram of insulin signaling cascades and specific inhibitors. Various functions of insulin are mediated by the insulin receptor, a member of a large family of receptor tyrosine kinases (RTK). Signal transduction by th insulin receptor follows a paradigm for RTK signalling. Many intracellular signalling molecules contain multiple modular domains that mediate protein-protein interactions and participate in the formation of signalling complexes, phosphorylation cascades are also a prominent features of RTK signalling. After insulin binding to α-subunits, insulin receptors are phosphorylated at a number of tyrosine residues (autophosphorylaton) with intrinsic receptor tyroine kinase (RTK) activity of β-subunits. Although PI3-kinase-Akt cascade and p21Ras-Raf-MEK-ERK cascade are well-known signaling cascades derived by insulin-receptor binding, a number of different signaling molecules have known to be activated by insulin. Activation of insulin receptor tyrosine kinase is essential for many of the biological actions of insulin. The liganded receptor initiates intracellular signals by stimulation tyrosine phosphorylation of endogenous substrates (Insulin receptor substrate; IRS). Several lines of evidence suggest that insulin receptor tyrosine kinase

activity may be involved in most of the physiological functions of insulin. To assess the requirement for kinase function of the antiapoptotic protection, CHO/neo cells lacking insulin receptor expression and CHO-IR cells were used. Insulin stimulated activities of Akt as well as ERK in CHO-IR cells, however, neither were stimulated in CHO/neo cells. This result showed an insulin receptor-specific signaling nature of CHO-IR cells.

In addition, the Ras pathway appears to connect signaling events that begin at the plasma membrane with nuclear events. Insulin is one of the major stimulants of the Ras signaling pathway. The biological importance of the Ras pathway has been studied extensively with the use of inhibitors of Ras-farnesylation and dominant negative mutants of Ras (Wiese *et al*., 1995; Sasaoka *et al*., 1994; Jhun *et al*., 1994; De Meyts *et al*., 1996). Farnesylation is a regulated posttranslational modification that allows attachment of a number of proteins, including p21Ras. By inducing the activity of the enzyme farnesyl protein transferase, insulin increases the pool of membrane-associated p21Ras and promotes GTP loading on Ras (Goalstone *et al*., 1996; Goalstone *et al*., 1997). It has been documented that manumycin, a selective protein farnesylation inhibitor (FTI), blocks the antiapoptotic protection exerted by insulin in CHO-IR cells in the absence of growth factors (Lee-Kwon *et al*., 1998). The present study showed that manumycin inhibit insulin's antiapoptotic function. α-HFTA and FTI-277 (another specific farnesyltransferase inhibitor) also inhibited insulin's antiapoptitic activity like as manumycin. These results suggest that p21Ras activity seems to be important for the insulin's antiapoptotic function in

CHO-IR cells. Thus, the importance of p21Ras activation in the survival function of insulin was examined further by using CHO-IR cells transfected with ΔSOS, a transdominant negative mutant of the Ras-specific exchange factor, mSOS1. mSOS1 is the mammalian homologue of the Drosophila gene son-of-sevenless, which becomes bound to the plasma membrane and thereby localized close to membrane-bound p21ras (Christian H., 1995). For example, Grb-2 is normally pre-bound to the guanine nucleotide exchange facor SOS (two SH_3 domains of Grb-2 bind proline rich regions of SOS). When phosphotyrosine motifs on IRS-1 or Shc bind to the SH_2 domain of Grb-2, the pre-bound SOS catalyzes the exchange of GTP form GDP on Ras leading to its activation. CHO-IR/Δ*SOS* cell is lacking intrinsic p21Ras activity because they express a deletion mutant, mSOS1 protein that lacks the guanine nucleotide exchange domain of p21 Ras, and p21Ras is not activated in CHO-IR/Δ*SOS* cells (Fig. 8). Although manumycin inhibited insulin's antiapoptotic function in CHO/ΔSOS cells, insulin *per se* still could protect CHO/ΔSOS cells from apoptosis. These results suggest the significance of protein farnesylation process of other proteins rather than p21Ras.

In the present study, insulin led to the activation of Akt, which was fully inhibited by 100 nM wortmannin. The insulin's antiapoptotic protection was also sharply blocked by the addition of wortmannin, suggesting the role of PI3 kinase in protecting cells from apoptosis. Insulin stimulated the ERK activity, which was completely blocked by PD98059, an inhibitor of ERK activation. Interestingly, the stimulation of ERK by insulin was clearly

suppressed by wortmannin. Blockade of ERK activation by PI3 kinase inhibitors has been reported in a few cell systems. For example, wortmannin blocks the activation of ERK by kainate in rat striatal slices (Fuller *et al*., 2001) and by acetylcholine in colonic smooth muscle (Yamboliev *et al.*, 2000). However, the mechanism by which the activation of PI3 kinase plays a role in stimulating ERK activity has not been understood. Vanadium salts which have insulinomimetic effects, activate Ras, C-raf-1 and MEK and their activations can be blocked by wortmannin (Pandey *et al.*, 1999). Moreover, a member of PI3 kinase families activates ERK as well as Akt (Bondeva *et al.*, 1998). Tyrosine phosphorylation of Gab1, a Grb-2-associated binder, also plays a pivotal role in PI3 kinase-dependent ERK activation in response to endothelin-1 which activates G-protein-coupled receptor signaling cascades (Bisotto and Fixman, 2000). On the contrary, Akt inhibits Raf-MEK-ERK pathway in the course of myotube differentiation (Rommel *et al.*, 1999). Thus, PI3 kinase-Akt axis might affect Raf-MEK-ERK pathway positively or negatively in different cell systems or under different physiological conditions. These data showed that the activations of PI3 kinase and ERK in response to insulin play roles in protecting cells from apoptosis. However, the significance of Akt in exerting insulin's antiapoptotic activity is not clear yet, because the activity of Akt was still considerably high, even in cells which were clearly apoptotic by the addition of PD98059 together with insulin.

The degree of intracellular ROS accumulation and caspase-3 activity was paralleled with the progress of apoptosis. The information regarding the

biological significance of ROS has been increased considerably in recent years, revealing its diverse functions (Palmer & Paulson, 1997; Papa & Skulachev, 1997). Exposing cells to ROS in various experimental systems leads to apoptosis and to cell damage (Papa & Skulachev, 1997). Moreover, targeted disruption of inducible nitric oxide synthase (iNOS) protects against insulin resistance in muscle, indicating the involvement of iNOS in the development of muscle insulin resistance (Perreault & marette, 2001). Other studies also reported that oxidative stress reduced glucose uptake in response to insulin through the changes of the level of GLUT1 and GLUT4 transcription (Rudich *et al.*, 1997; Rudich *et al.*, 1998). However, at low concentrations, ROS may function as physiological mediators of cellular responses (Schreck *et al.*, 1991). For example, several studies showed that H_2O_2 had insulinomimetic effects in different cell systems (May & de Haen, 1979; Heffetz *et al.*, 1990; Wilden & Broadway, 1995). A very recent study suggested that the generation of H_2O_2 in response to insulin is integral to activation of distal insulin signaling cascade, stimulating PI3 kinase, Akt and then glucose uptake in adipocytes (Mahadev *et al.*, 2001). Growth factors like as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are also capable of producing ROS (Lo & Cruz, 1995; Sundaresan *et al.*, 1995). From these results, the transient generation of ROS and the prolonged accumulation of ROS might have distinctive significances regarding their signaling pathways and physiological roles. In the present study, serum starvation induced ROS accumulation, which was suppressed by the addition

of insulin. Caspase-3 activity was also increased by serum starvation, indicating an aspect of proapoptotic activities of ROS. Involvement of caspase-3 stimulation in ROS-induced apoptotic process is supported by the protection of cells from apoptosis by the addition of z-DEVD-fmk, an inhibitor of caspase-3. Moreover, n-acetylcysteine, a scavenger of H_2O_2, blocked caspase-3 activation and protected cells from apoptisis whereas it inhibited caspase-3 activity (Park et al., 2000).

Taken together, we demonstrated that the antiapoptotic action of insulin is paralleled with the reduction of ROS generation and suppression of caspase-3 stimulation induced by serum starvation or H_2O_2 addition in HepG2 cells. Among diverse signaling cascades stimulated by insulin, insulin's survival function is dependent on the activations of PI3 kinase and ERK whereas p21Ras does not play a significant role in exerting insulin's antiapoptitic function. Although protein farnesylation was shown to be an important step in insulin's antiapoptotic function, it was remained to be elucidated which protein rather than p21Ras is a target protein to be farnesylated in response to insulin to exert antiapoptotic function.

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초록

인슐린은 주로 에너지 대사를 조절하는 호르몬으로 알려져 있는데, 그밖에도 세포증식 및 물질의 생합성을 조절함으로서 생물체의 항상성을 유지하는데 중요한 역할을 하는 것으로 알려져 있다. 최근 인슐린이 일단의 세포모델에서 세포사멸 (예정세포사)을 억제할 수 있다는 연구 결과들이 보고되었으나, 인슐린의 세포사멸 억제 현상에 대한 기전은 알려져 있지 않다. 이러한 사실로부터 본 연구는 사람의 인슐린 수용체를 과발현 하도록 조작된 세포주인 Chinese hamster ovary 세포주 (CHO-IR)와 사람의 인슐린 수용체를 발현하는 간암세포주의 일종인 HepG2 세포주를 재료로 하여 인슐린이 세포사멸 과정을 억제하고 세포 생존을 유도할 수 있는 지를 조사하였다. 세포사멸 현상을 규명하기위한 실험 방법으로 세포 내의 DNA량, 핵 응축의 정도, PARP 가수분해 정도를 측정하였으며 아울러 caspase-3 활성과 ROS(활성 산소종)의 양도 조사하였다.

CHO-IR 세포주는 배양액에서 혈청을 제거(영양 결핍)하면 세포사멸 현상이 야기되었다. 그러나 배양액에 인슐린을 처리하면 영양 결핍에 의해 야기된 세포사멸 작용이 억제되었다. 인슐린의 처리는 세포사멸 유발 경로중의 하나인 caspase-3 활성도 증가시켰다. 한편 활성 산소종 (ROS)은 영양 결핍 환경에서 그 양이 증가하였고 인슐린은 이러한 증가를 억제시켰다. 배양액에 NO donor를 처리하였을 때 세포사멸이 유도되지 않았으나 cGMP analogue를 처리하거나 cGMP-specific phosphodiesterase를 억제할 경우 인슐린의 세포사멸 억제 활성이 저해되었다.

아울러 본 연구는 인슐린이 세포사멸을 억제하는 과정에 어떤 신호 전달 경로가 관여하는 지를 조사하였다. 이러한 실험 목적에 따라 성장 인자들에 의해 활성화되는 여러 가지 신호 전달 경로 단계에 따른 다양한 저해제들이 인슐린의 세포사멸 억제 효과에 영향을 미치는 지를 조사하였다. 그 결과

ERK, PI3-kinase, 그리고 Gi protein의 활성이 억제될 경우 인슐린의 세포사멸 억제 기능이 저해되었으나, ERK를 활성화시키는 상위 단계중의 하나인 p21Ras 활성의 억제는 인슐린의 세포사멸 억제작용과 관계가 없는 것으로 보였다.

CHO-IR세포에서 보여진 인슐린의 세포사멸 억제효과가 과연 사람의 세포주 모델에서도 재현되는 가를 밝히기 위하여 간암 세포주의 일종인 HepG2세포에서 인슐린의 세포사멸 억제 작용을 조사하였다. CHO-IR 세포와 같이 HepG2 세포에서도 인슐린에 의해 세포사멸이 억제되었다. 이 과정에서 ERK 와 PI3-kinase가 중요한 작용을 하였으며 인슐린은 활성산소종의 생산과 caspase-3 활성을 억제하였다.

이상의 결과들로부터, 인슐린은 세포사멸 유발과정에 수반되는 caspase-3 활성의 증가를 억제시키고 산화 스트레스 또한 억제함으로서 궁극적으로 세포사멸을 억제하는 기능을 갖고 있다는 점을 시사하였다. 이러한 과정에서 ERK, PI3-kinase와 Gi protein같은 신호전달 물질들이 인슐린의 세포사멸 억제에 중요한 작용을 할 것으로 사료되었다.

주요어 : 인슐린, 세포사멸, 세포사멸 억제 활성, CHO-IR, HepG2, 활성 산소종, caspase-3, PI3-kinase, ERK

감사의 글

본 논문을 마무리하면서 많은 얼굴들이 제 머리를 스쳐갑니다. 그분들께 깊은 감사를 드립니다. 먼저 본 논문에 대한 연구의 수행과 논문의 완성이 있기까지 항상 격려와 애정을 아끼지 않으시고 지도해주신 김세재 교수님께 진심으로 감사드립니다. 그리고 바쁘신 가운데에도 격려와 지도로 부족함이 많은 논문을 세심하게 심사하여 주신 오문유 교수님, 김원택 교수님, 의과대학의 강희경교수님, 유은숙 교수님께도 감사드립니다. 또한 대학원 과정동안 늘 관심과 조언으로 이끌어주신 김문홍 교수님, 이용필 교수님, 오덕철교수님, 이화자 교수님, 고석찬 교수님께도 깊은 감사를 드립니다.

또한 본 연구를 수행할 수 있도록 그 터전을 마련해주신 의과대학 조직학교실의 이영기교수님께도 이 지면을 빌어 깊은 감사를 드립니다. 또한 늘 관심을 가지고 지켜봐 주신 의과대학 교수님들께도 뒤늦은 감사를 드립니다. 아울러 생물학과 대학원 선배님과 후배들에게도 감사의 말씀을 드리며, 특히 여러 가지로 많은 조언과 도움을 주었던 분자생물학 실험실의 박지권 선생님과 정형복, 윤지현, 진영준, 최진영, 현은아 후배와 의과대학 조직학 교실의 송지훈 후배, 오유성 선생님, 장재영에게도 진한 고마움을 전합니다. 또한 박사과정 처음부터 끝까지 함께 과정을 밟으면서 많은 도움을 준 박수영 선생님, 한상현 선생님께도 감사의 마음을 전합니다.

한편, 오늘의 제가 있기까지 늘 끊임없는 사랑과 기도를 보내주신 부모님과 동생들에게도 감사의 마음을 전합니다. 무엇보다도 오랜 시간동안 제대로 아내, 엄마 노릇을 못했음에도 이해하고 참아준 사랑하는 아들 석진, 석범과 남편에게 미안한 마음과 아울러 고마움을 전합니다.

마지막으로 만학의 길을 걸을 수 있도록 이끌어주시고 용기를 주신 하나님께도 이제서야 감히 감사를 드립니다.