EXPERIMENTAL STUDY

Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells

Shinhae Kang¹, Jihoon Song¹, Heekyoung Kang, Sejae Kim¹, Youngki Lee and Deokbae Park

Department of Medicine, College of Medicine, Institute of Basic Science and ¹Department of Biology, College of Natural Science, Cheju National University, Ara-1, Cheju, 690-756, South Korea

(Correspondence should be addressed to D Park, Department of Medicine, College of Medicine, Cheju National University, Ara-1, Cheju, 690-756, South Korea; Email: parkdb@cheju.ac.kr)

Abstract

Objective: 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 in different cell lines; however, little is known about the nature of its anti-apoptotic activity. In many clinical disorders, including type 2 diabetes mellitus, oxidative stress and the production of reactive oxygen species (ROS) is increased. With these facts as a background, we examined here whether insulin protects HepG2 cells from apoptosis by decreasing oxidative stress and, if so, which signaling steps are involved in this process.

Methods: Intracellular DNA content, the degree of nuclear condensation or poly(ADP-ribose) polymerase hydrolysis was measured to verify the occurrence of apoptotic events. Caspase-3 activity and ROS accumulation within cells were also measured. Western blot analysis was performed to identify signaling molecules activated in response to insulin.

Results: Serum starvation resulted in a marked accumulation of ROS, activation of caspase-3, and subsequent apoptotic cell death which were, in turn, markedly blocked by the addition of insulin. The anti-apoptotic activity of insulin was sensitive to blockade of two different signaling steps, activations of phosphatidylinositol 3-kinase (PI3 kinase) and extracellular signal-regulated protein kinase (ERK).

Conclusion: Insulin exerts an anti-apoptotic activity by suppressing the excessive accumulation of ROS within cells through signaling pathways including stimulation of PI3 kinase and ERK in HepG2 cells.

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Introduction

Insulin has a variety of functions such as glucose uptake, glycogen biosynthesis, inhibition of lipolysis and stimulation of cellular proliferation. It can also protect cells from apoptosis mediated by growth factor removal in different cell lines (1–3). We have recently investigated the anti-apoptotic function of insulin and its related signaling pathways in Chinese hamster ovary cells expressing wild-type human insulin receptors (CHO-IR) (4, 5). 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.

Reactive oxygen species (ROS) have been implicated as important pathologic mediators in many clinical

disorders (6), including type 2 diabetes mellitus (7, 8). In addition, oxidative stress disrupts insulin-induced signaling events such as insulin receptor tyrosine phosphorylation, insulin receptor substrate-1 (IRS-1) phosphorylation and activation of phosphatidylinositol 3-kinase (PI3 kinase) (9) as well as redistribution of IRS-1 and PI3 kinase in adipocytes (10). However, conflicting results on the effect of H₂O₂ on insulin signaling are also available. H₂O₂ mimics the stimulatory effects of insulin on glucose transport and lipid synthesis in adipocytes (11, 12) and insulin transiently generates H₂O₂ via NADPH oxidase, which is integral to activation of the insulin signaling cascades in adipocytes (13). Insulin-induced generation of H₂O₂ reversibly inhibits protein-tyrosine phosphatase 1B, thereby enhancing the early insulin action cascades (14). So

far there has been no general agreement on which oxidative stress affects insulin signaling and its physiological functions, or on which insulin modulates ROS generation.

Following insulin binding, the insulin receptors undergo activation of their intrinsic tyrosine kinase function and subsequent stimulation of signaling molecules (15). We have reported that insulin delayed apoptosis induced by serum starvation (4), by a mechanism that is dependent on short-lived farnesylated proteins in CHO-IR cells (5). However, there is little evidence as to whether insulin really acts as a survival factor in physiological cell systems expressing intrinsic insulin receptors. With these facts as a background, we examined whether insulin exerts an anti-apoptotic activity and, if so, which signaling steps are important for completing this process in HepG2 cells expressing intrinsic insulin receptor molecules.

Materials and methods

Materials

Human recombinant insulin, PD98059, SB202190, the fluorogenic caspase-3 substrate, Ac-DEVD-AMC, the caspase inhibitor, z-DEVD-fmk and propidium iodide, 2',7'-dichlorofluorescein diacetate (H₂DCFDA) were obtained from Calbiochem (La Jolla, CA, USA). Wortmannin, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's-phosphate-buffered saline (D-PBS) and trypsin-EDTA solution were obtained from Sigma Chemical Corp. (St Louis, MO, USA), and fetal bovine serum (FBS) from Life Technologies Inc. (Rockville, MD, USA). Monoclonal antibodies against phospho-ERK1/2 (E-4), phospho-C-Jun NH₂ terminal kinase (phospho-JNK) (G-7), phospho-p38Map kinase (D-8), ERK2 (D-2) and polyclonal antibodies against phospho-Akt1 (ser473) and poly(ADP-ribose) polymerase (PARP) (H-250) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrophoresis reagents, such as gels, Tris-glycine SDS running buffer, and poly(vinylidene difluoride) (PVDF) membrane were from Novex Corp. (San Diego, CA, USA).

Cell culture

HepG2, a hepatoblastoma cell line used in this study, was obtained from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM containing 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS, and maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. Two days after plating in 35 mm tissue culture dishes, HepG2 cells were serum starved for 24 h and then treated with different reagents. Cells were quickly frozen in liquid nitrogen and stored at -70 °C until analysis.

SDS-PAGE and immunoblotting

Unless otherwise indicated, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µM aprotinin, 1 µM leupeptin and 1 µM pepstatin A). The same amount of proteins was separated by SDS-PAGE on 4–20% polyacrylamide gels and electrotransferred onto PVDF membrane. The membrane was incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20) for 1 h at room temperature and then probed with different primary antibodies (1:1000–1:5000). After a series of washes, the membrane was further incubated with different horseradish peroxidase-conjugated secondary antibodies (1:2000–1:10 000). The signal was detected with an enhanced chemiluminescence detection system (Intron, Seongnam, Korea).

Determination of caspase-3 activity

After treatment with reagents, cells were collected, lysed in ice-cold 0.5 ml caspase assay buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% sodium deoxycholate and 1 mmol/l EGTA) for 15 min. After centrifugation at 12 000 *g* for 15 min at 4 °C, aliquots of supernatant were incubated with 10 µmol/l Ac-DEVD-AMC for 3 h at 37 °C. The fluorescence from the cleaved product was detected with a Spectrafluor multiwell fluorescence reader (Tecan, Grödig, Austria) at 360 nm and 465 nm wavelengths 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 & Steinkamp (16). The samples were analyzed with a Coulter Epics™ cytometer (Beckman, Fullerton, CA, USA). Ten thousand 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, Meyer, Houston, TX, USA) to examine the degree of nuclear condensation.

Measurement of ROS

H₂DCFDA, 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 and further incubated in D-PBS containing 10 mmol/l H_2DCFDA for 10 min at 37°C. The fluorescence intensity was measured with a Spectrafluor multiwell fluorescence reader 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.

Statistical analysis

Statistical analysis was performed using an analysis program, StatView (Abacus Concepts, Berkeley, CA, USA). The Student's *t*-test was used to analyze the difference between control and experimental groups. $P < 0.05$ was considered to be significant.

Results

Insulin delays apoptosis induced by serum starvation or oxidative stress

Our previous studies (4, 5) have shown that insulin delayed apoptosis induced by serum starvation in CHO-IR cells. However, it is not clear whether insulin can really play a role in inhibiting apoptosis in certain cells having intrinsic insulin receptors such as adipocytes, muscle cells or liver cells. HepG2 is a hepatoblastonema cell line that expresses intrinsic insulin receptors. HepG2 cells were preincubated in serum-free medium for 24 h and then washed with D-PBS twice, then treated with 100 nmol/l insulin for an additional 48 h. 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. 1A). The percentages of apoptotic cells were $23.2 \pm 3.5\%$ (control, serum-free) and $4.1 \pm 0.7\%$ (100 nmol/l insulin) respectively. The

Figure 1 Insulin blocks apoptosis induced by serum starvation or H_2O_2 addition. (A) Cells were serum starved (24 h), preincubated with 10 μ mol/l α -DEVD-fmk (30 min), and further incubated with 100 nmol/l insulin and 1 mmol/l H_2O_2 for an additional 48 h. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis and the photographs show cells with highly condensed nuclei stained with H33342. (B) Proteolytic cleavage of PARP induced by H_2O_2 and (C) its suppression by insulin after treatment of cells for 48 h. Equal amounts of cell lysates were subjected to electrophoresis and analyzed by Western blot for PARP, and ERK as an internal standard.

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 ($35.3 \pm 4.2\%$) compared with control groups ($P < 0.05$). Insulin treatment also significantly decreased ($8.9 \pm 2.6\%$, $P < 0.01$) the degree of apoptosis compared with H_2O_2 alone. 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. 1A).

A 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 cell 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 the results of flow cytometric analysis and H33342 staining. The degree of proteolytic cleavage of PARP, a target molecule of caspases, was parallel

A **B**

Figure 2 Insulin suppresses caspase-3 stimulation and ROS production induced by serum starvation. (A) HepG2 cells were serum starved for 24 h and preincubated with inhibitors for 30 min before insulin addition as described in Results. Caspase-3 activity was measured at 24 h after insulin treatment and is represented as relative units. Results are the means $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ vs serum free; # $P < 0.05$ vs insulin; *ND, non-detected. (B) Dichloro-fluorescein (DCF) fluorescence from cells imaged with a CoolSNAP-Pro digital camera attached to the inverted fluorescent microscope. Cells were serum starved for 24 h and further treated with insulin and/or H_2O_2 for an additional 3 h.

with the dose of H_2O_2 (Fig. 1B). 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. 1C). These results suggested that insulin can protect HepG2 cells from apoptosis induced by serum starvation or oxidative stress.

Insulin suppresses caspase-3 activity and ROS production

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 (17, 18). The function

Figure 3 The anti-apoptotic activity of insulin is sensitive to ERK inhibition and PI3 kinase inhibition. Cells were serum starved (24 h), preincubated with inhibitors for 30 min and further incubated with insulin for an additional 48 h. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis and the photographs show cells with highly condensed nuclei stained with H333342.

of caspase-3 activity has been described (19) as being involved in the execution of apoptosis in a tissue-, cell type- or death stimulus-specific manner. From the results shown in Fig. 1A, serum starvation or H_2O_2 treatment may induce apoptosis via mechanisms including caspase-3 activation. We further investigated the effect of insulin on the caspase-3 activity and intracellular ROS accumulation. Moreover, inhibitors against signaling molecules activated in response to insulin were administered to cultured cells to examine which signaling steps play significant roles in protecting cells from apoptosis. First, caspase-3 activity was measured 24 h after insulin (100 nmol/l) treatment in the absence or presence of inhibitors as indicated (Fig. 2A). Serum starvation sharply elevated caspase-3 activity when compared with the presence of 10% FBS in the medium. Insulin markedly suppressed caspase-3 activity induced by serum starvation. Caspase-3 activity was undetectable where z-DEVD-fmk (10 μ mol/l) was added, demonstrating the specific nature of this assay. Inhibition of ERK with PD98059 (50 μ mol/l) and inhibition of PI3 kinase with wortman-

nin (100 nmol/l) markedly blocked insulin's function in suppressing caspase-3 activity, whereas inhibition of p38Map kinase with SB202190 (10 μ mol/l) showed no effect. We also examined whether insulin affects intracellular ROS accumulation (Fig. 2B). H_2O_2 (1 mmol/l) 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 observations, the intensity of fluorescence from dichlorofluorescein (DCF) 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 mmol/l). Insulin lowered both of the intensities of fluorescence elicited by serum starvation and H_2O_2 addition.

Inhibition of PI3 kinase and ERK block the anti-apoptotic activity of insulin

Activation of the insulin receptor increases PI3 kinase activity, whose function has been associated with the anti-apoptotic signaling in various cell types (20, 21).

Figure 4 ERK activation is necessary for the anti-apoptotic protection of cells by insulin. Cells were serum starved for 24 h, preincubated with inhibitors for 30 min and further incubated with insulin for (A) 5 min or (B) 36 h. Equal amounts of cell lysates were subjected to electrophoresis and analyzed by Western blot for phospho(p)-Akt, p-ERK, p-p38Map kinase (p38MapK), p-JNK, PARP, and ERK as an internal standard.

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 insulin's anti-apoptotic protection was not altered by wortmannin or LY294002 (4), two chemically unrelated inhibitors of PI3 kinase. In the present study, however, HepG2 cells pretreated with 100 nmol/l wortmannin were apoptotic to a large extent even in the presence of insulin (Fig. 3). A specific inhibitor of MAP kinase kinase 1 (MEK1), PD98059, was also used to assess the role of ERK activity in insulin-mediated protection against apoptosis. The protective effect of insulin was markedly blocked by 50 μ mol/l PD98059 (Fig. 3) and the insulin stimulation of ERK activity was also completely blocked (Fig. 4A). Unexpectedly, insulin stimulation of ERK activity was clearly suppressed by wortmannin whereas Akt stimulation by insulin was unaffected by PD98059 (Fig. 4A). Neither active JNK nor p38Map kinase, other members of Map kinase families, were changed in the presence of insulin (Fig. 4A). Separate experiments were performed to determine the effects of those inhibitors on the cleavage of PARP (Fig. 4B). Treatment of cells with PD98059 or wortmannin accelerated PARP cleavage, which was suppressed by insulin. These results suggest that the anti-apoptotic function of insulin is mediated by PI3 kinase as well as ERK, but not by Akt.

Discussion

In the present study, we examined the ability of insulin to exert an anti-apoptotic function in HepG2 cells and its related signaling pathways. Insulin is a potent survival factor in different cell systems (1–3). Our present study has shown that insulin inhibits apoptosis induced by serum starvation in HepG2 cells that express insulin receptors. This finding is in agreement with our previous results using CHO-IR cells (4, 5) but has different kinetics. HepG2 cells were apoptotic at least 36 h later in serum-free conditions whereas most CHO-IR cells were readily apoptotic within 24 h (5) from the flow cytometric analysis. The degree of intracellular ROS accumulation and caspase-3 activity was paralleled by the progress of apoptosis. Information regarding the biological significance of ROS has increased considerably in recent years, revealing diverse functions (22, 23). Exposure of cells to ROS in a variety of experimental systems leads to apoptosis and to cell damage (23). 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 (24). Other studies have also reported that oxidative stress reduces glucose uptake in response to insulin through the changes of the level of glucose transporter (GLUT)1 and GLUT4 transcription (25, 26). However, at low concentrations, ROS may function as physiological

mediators of cellular responses (27). For example, several studies have shown that H₂O₂ had insulinomimetic effects in different cell systems (12, 28, 29). A very recent study has suggested that the generation of H₂O₂ in response to insulin is integral to the activation of the distal insulin signaling cascade, stimulating PI3 kinase, Akt and then glucose uptake in adipocytes (13). Growth factors such as platelet-derived growth factor and basic fibroblast growth factor are also capable of producing ROS (30, 31). From these results it appears that the transient generation of ROS and the prolonged accumulation of ROS might have distinctive significance with regard to 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 the proapoptotic activities of ROS. The 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 H₂O₂, blocked caspase-3 activation and protected cells from apoptosis induced by serum starvation in HepG2 cells in a separate experiment (data not shown). However, in CHO-IR cells, z-DEVD-fmk failed to rescue cells from apoptosis whereas it inhibited caspase-3 activity (5). These results suggest that the apoptotic steps caused by serum starvation are different between CHO-IR and HepG2 cells with respect to the significance of caspase-3. The present study has shown a strong relevance of insulin's anti-apoptotic activity to the suppression of ROS accumulation and the subsequent inhibition of caspase-3 activation induced by serum starvation in HepG2 cells. However, we did not examine the fluctuation in ROS at the early phases after insulin treatment; thus, the significance of the transient changes in intracellular ROS content remains to be clarified.

The signal transduction pathways stimulated by insulin to confer anti-apoptotic protection were also investigated. Various inhibitors of key steps in signaling cascades activated by growth factors have been widely used and have provided important insights into the understanding of the role of signaling molecules (32–34). In the present study, insulin led to the activation of Akt, which was fully inhibited by 100 nmol/l wortmannin. The anti-apoptotic protection of insulin 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 kainite in rat striatal slices (35) and by acetylcholine in colonic

smooth muscle (36). However, the mechanism by which the activation of PI3 kinase plays a role in stimulating ERK activity is not yet understood. Vanadium salts, which have insulinomimetic effects, activate Ras, Raf-1 and MEK and their activation can be blocked by wortmannin (37). Moreover, a member (PI3K γ) of the PI3 kinase family activates ERK as well as Akt (38). 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 (39). On the contrary, Akt inhibits the Raf-MEK-ERK pathway in the course of myotube differentiation (40). Thus, the PI3 kinase-Akt axis might affect the Raf-MEK-ERK pathway positively or negatively in different cell systems or under different physiological conditions. Our 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 the anti-apoptotic activity of insulin is not yet clear, because the activity of Akt was still considerably high, even in cells which were clearly made apoptotic by the addition of PD98059 together with insulin. Blockade of insulin-induced ERK activation by PD98059 or wortmannin was paralleled by the stimulation of caspase-3 activity and the induction of apoptosis. We also tested whether insulin can affect the activities of JNK and p38Map kinase, other members of the MAP kinase families. Neither the activities of JNK nor p38Map kinase were changed by the addition of insulin. From these results, it is suggested the only ERK among the members of the MAP kinase families plays a role in protecting HepG2 cells from apoptosis in response to insulin.

Taken together, we have demonstrated that the anti-apoptotic action of insulin is paralleled by the reduction of ROS generation and suppression of caspase-3 stimulation induced by serum starvation or H₂O₂ addition in HepG2 cells. Moreover, insulin's survival function is dependent on the activation of PI3 kinase and ERK.

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