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Insulin Regulates Cleavage of Procaspase-9 via Binding of X Chromosome-Linked Inhibitor of Apoptosis Protein in HT-29 Cells

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ABSTRACT

Insulin significantly reduced tumor necrosis factor (TNF)-α–induced cleavage of procaspase-8, -9, and -3 and poly(ADP-ribose) polymerase when observed for up to 24 hours in a dose-dependent manner. Signaling pathways responsible for the inhibitory effects of insulin were investigated by using protein kinase inhibitors. Both phosphatidylinositol 3′-kinase (PI3K) and mitogen-activated protein kinase kinase pathways mediate the ability of insulin to decrease the TNF-α–induced cleavage of procaspase-8. In contrast, only the PI3K inhibitor reversed the effect of insulin on the TNF-α–induced cleavage of procaspase-9. Moreover, insulin decreased the apoptotic level induced by TNF-α, whereas the PI3K inhibitor enhanced it. The protein level of Apaf-1, an activator of procaspase-9, remained constant with the application of agents affecting the cleavage of procaspase-9. In examining another regulator of cleaved caspase-9, X chromosome-linked inhibitor of apoptosis protein (XIAP), we observed that TNF-α treatment induced fragmentation of XIAP, which was also enhanced by the PI3K inhibitor. In addition, XIAP was coimmunoprecipitated with procaspase-9. The treatment with TNF-α reduced the level of XIAP precipitated with procaspase-9, whereas insulin reversed this effect. Moreover, PI3K and Akt inhibitors, but not mammalian target of rapamycin inhibitor, inhibited the effect of insulin on the coprecipitation of procaspase-9 and XIAP. Our data suggest that insulin decreases the TNF-α–induced cleavage of procaspase-9 and subsequent apoptosis by regulating XIAP via the PI3K/Akt pathway.

INTRODUCTION

Apoptosis plays an essential physiologic role in development and the immune defense system by removing unnecessary or harmful cells (1). Cysteine-containing aspartate-specific proteases (caspases), including initiator (*e.g.,* caspase-8 and -9) and executioner (*e.g.,* caspase-3, -6, and -7) caspases (2), are key mediators of apoptosis. Triggered by apoptotic signals such as death receptor ligands or cell-damaging agents (3, 4), procaspases, which are inactive zymogens under normal conditions, become cleaved into their active forms (2). Binding of a death receptor ligand, tumor necrosis factor (TNF)-α, to its receptor results in the association of the receptor with an adaptor protein, TNF receptor-associated death domain protein, via a death domain, which subsequently binds to Fas-associated death domain protein (5). This association forms a death-inducing signaling complex (6), in which procaspase-8 binds to the Fas-associated death domain protein via a death effector domain (7) and becomes activated through homolytic cleavage (8). In type I cells, active caspase-8 directly cleaves downstream procaspase-3 (9), whereas a small amount of caspase-8 activated in type II cells truncates the BH3 domain-containing proapoptotic Bcl-2 family protein (BID). Truncated BID may be myristoylated and consequently translocated into mitochondrial membrane (10). The translocation of truncated BID induces the release of cytochrome *c* into cytosol (11, 12), which also occurs through cell-damaging agents (3, 4). In the presence of cytochrome *c* and dATP (13, 14), Apaf-1 binds to procaspase-9 via a caspase activation recruitment domain (15), forming an apoptosome (16, 17), in which procaspase-9 becomes activated. Cleaved caspase-9 processes other downstream procaspases such as procaspase-3 (18), which further cleaves downstream substrates such as poly(ADP-ribose) polymerase (PARP; ref. 19), leading to apoptotic changes (20, 21). Regulating the cleavage of various caspases by survival factors is therefore essential for the cellular balance between survival and death.

Survival factors such as insulin and growth factors rescue cells from apoptosis induced by death receptor ligands (22) or DNA-damaging agents (23). Phosphatidylinositol 3′-kinase (PI3K)/Akt (22, 24, 25), mitogen-activated protein kinase (MAPK; ref. 26), and nuclear factor κB (27–29) become activated to inhibit apoptosis. Also, antiapoptotic molecules regulated by these kinases and thereby regulating proapoptotic proteins have been studied to elucidate protective mechanisms by survival factors. For instance, FLICE-like inhibitory protein (FLIP), a dominant negative form of procaspase-8, has been suggested to inhibit the activation of procaspase-8 by playing the role of its competitor (30, 31). Also, Akt was reported to phosphorylate the proform and large domain of recombinant caspase-9 in an *in vitro* kinase experiment (32). X chromosome-linked inhibitor of apoptosis protein (XIAP), a multifunctional protein involved in cell cycle regulation, protein ubiquitination, and receptor-mediated signaling (33), is known as the most potent endogenous inhibitor of cleaved caspase-3, -7, and -9 (34). In cell-free experiments, recombinant XIAP bound to and inactivated the cleaved forms of caspase-3, -7, and -9 (35–37). Likewise, it has been suggested that survival stimuli tightly control the activation of caspases via posttranslational modifications or protein–protein interactions.

Assuming that survival factors protect cells from apoptosis by inactivating key apoptotic mediators, caspases, via antiapoptotic proteins, we examined the effects of a survival factor, insulin, on the cleavage of major procaspases and a substrate, PARP. Also, we defined an insulin kinase pathway(s) responsible for the inhibitory effects of insulin on the cleavage of procaspases. Focusing on caspase-9, we observed that XIAP was coimmunoprecipitated with procaspase-9 and also cleaved after TNF-α treatment. The action of XIAP was therefore investigated at the level of interaction with procaspase-9. Our results suggest that insulin decreases the TNF-α–induced cleavage of procaspase-9 and apoptosis by regulating binding of procaspase-9 and XIAP via PI3K/Akt pathway.

MATERIALS AND METHODS

Cell Culture and Chemical Treatment. Human colon epithelial adenocarcinoma cell line HT-29 (generously provided by the Peter Sorger laboratory in the Massachusetts Institute of Technology biology department) was seeded with a density of 5×10^4 cells per cm in McCoy's 5A medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal bovine serum and L-glutamine (Life Technologies, Inc.) at 37°C in 5% CO_2 incubator. Interferon (IFN)-γ (200 units/mL; Roche Applied Science, Indianapolis, IN) was applied for 24 hours to sensitize the cells to apoptotic agents (38). Then, 50 ng/mL TNF-α (Peprotech, Rocky Hill, NJ), with or without insulin (Cal-

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biochem, San Diego, CA), was applied to the cells for the specified hours. In some experiments, cells were pretreated with protein kinase inhibitors, LY294002, PD98059, rapamycin, and Akt inhibitors (Calbiochem) for 1 hour.

Western Blotting. Cell lysates were prepared by three freeze/thaw cycles in lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 0.5% Igepal, 2.5 mmol/L sodium PP_i, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 μmol/L okadaic acid, 1 μg/mL leupeptin, 1 μg/mL bestatin, and 1 mmol/L phenymethylsulfonyl fluoride) followed by centrifugation at 14,000 $\times$ *g* for 30 minutes. For cytochrome *c* detection, the cytosolic fraction was prepared by incubating cells in a lysis buffer with 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 2.5 mmol/L Na PP_i, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 μmol/L okadaic acid, 1 μg/mL leupeptin, 1 μg/mL bestatin, and 1 mmol/L phenymethylsulfonyl fluoride at 4°C for 20 minutes, followed by 15 passages through a 26-gauge needle. Then, lysates were centrifuged at 14,000 $\times$ *g* for 30 minutes, followed by centrifugation of the supernatant at 100,000 $\times$ *g* for 30 minutes. Fifty or 100 μg of total protein measured by the bicinchoninic acid protein assay (Pierce, Rockford, IL) were separated in 15% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) by SDS-PAGE (with the exception of PAPR, for which a 10% gel was used) and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline (TBS) with 0.1% Tween 20 for 1 hour and then incubated with the indicated primary antibody in the same blocking buffer at 4°C overnight. Mouse anti–caspase-8 antibody, rabbit anti–cleaved caspase-9 antibody, rabbit anti–cleaved caspase-3 antibody, mouse anti-BID antibody, rabbit anti-XIAP antibody (Cell Signaling Technology, Beverly, MA), mouse anti-cytochrome *c* antibody, mouse anti-PARP antibody, mouse anti–caspase-9 antibody, mouse anti–Apaf-1 antibody, mouse anti-XIAP antibody (BD Bioscience, San Jose, CA), and mouse anti–caspase-9 antibody (Upstate Biotechnology, Waltham, MA) were used as primary antibodies (1:1,000 dilution). After washing with TBS and 0.1% Tween 20, the membrane was incubated with secondary anti-IgG antibody conjugated with horseradish peroxidase (1:100,000 dilution; Pierce) for 1 hour. Then, blots were developed with supersignal West Femto substrate (Pierce). If necessary, the membrane was reprobed with goat antiactin antibody (Calbiochem) for normalization.

Apoptosis Measurement. Apoptosis levels were measured with cell death detection enzyme-linked immunosorbent assay plus (Roche Applied Science) according to the manufacturer's instructions. Briefly, cell lysates equivalent to 10^3 cells were reacted with both antihistone antibody labeled with biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates for 2 hours. Microplate wells were washed and incubated with substrates for colorimetric measurement at a wavelength of 405 nm with reference at 490 nm.

Immunoprecipitation. Cell lysates containing 2 mg of protein were incubated with rabbit anti–procaspase-9 antibody (BD Bioscience) immobilized onto Aminolink plus coupling gel (Pierce) at 4°C overnight. Beads were washed with TBS containing 0.1% Igepal and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma). Then, bound proteins were eluted with 0.1 mol/L glycine (pH 2.9) for 15 minutes and immediately neutralized with ammonium hydroxide. For immunoprecipitation of XIAP, rabbit anti-XIAP antibody (Cell Signaling Technology) was incubated with 2 mg of total proteins at 4°C overnight. Protein A-agarose beads were added, incubated for 2 hours, and washed. Beads were boiled in SDS sample buffer, and supernatants were separated by SDS electrophoresis followed by Western blotting.

RESULTS

Insulin Decreased the TNF-α–Induced Cleavage of Procaspases and Their Substrates. We investigated whether a survival signal, insulin, affected the cleavage of procaspases and other related proteins in HT-29 cells by using Western blotting. We observed the earliest TNF-α–induced cleavage products of caspase-8 at 4 hours, the earliest TNF-α–induced cleavage products of caspase-9 at 8 hours, the earliest TNF-α–induced cleavage products of caspase-3 at 12 hours, and the earliest TNF-α–induced cleavage products of PARP at 8 hours (Fig. 1). Due to differences in affinities of each antibody, absolute comparison between proteins at each time point is not possible. However, an overall trend is that upstream procaspases become cleaved before downstream substrates in response to TNF-α treatment. On the other hand, insulin delayed the TNF-α–induced cleavage of all four proteins (Fig. 1). Also, insulin decreased the TNF-α–induced cleavage of procaspase-8 and -9 and their downstream substrates, BID, procaspase-3, and PARP, in a dose-dependent manner (Fig. 2). The cleavage of the downstream substrates represents the activity of upstream enzymes (*e.g.,* activated caspase-9 cleaves procaspase-3). We also detected release of cytochrome *c* into the cytosol, a downstream event of caspase-8 activation. Treatment with insulin caused little decrease in the level of cytosolic cytochrome *c* but reduced the cleavage of procaspase-9 significantly (Fig. 2). This result implies that insulin inhibits the cleavage of procaspase-9 via a mech-

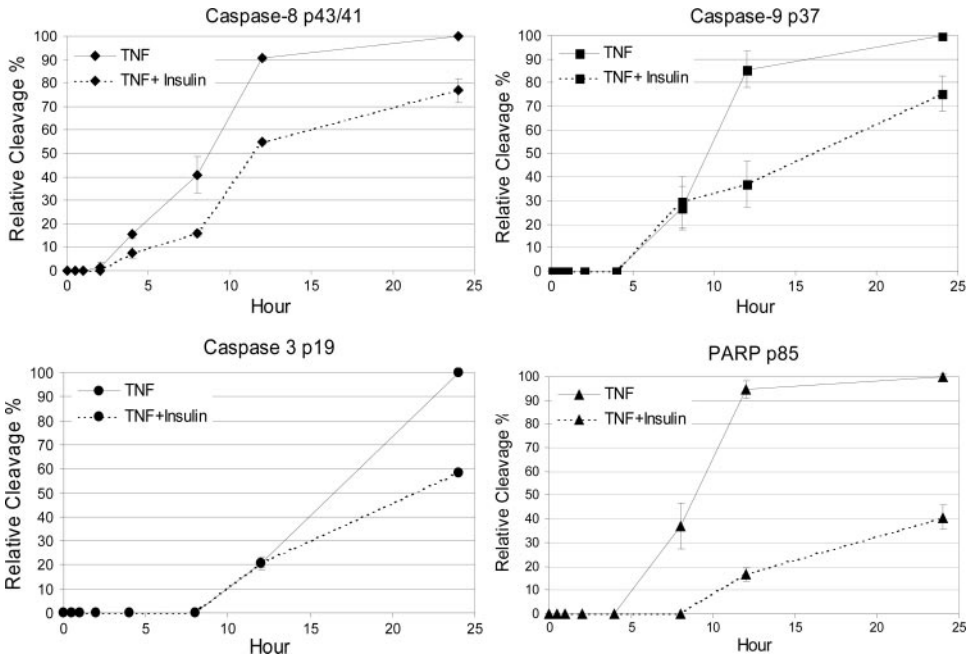

Fig. 1. Time course of cleaved caspases and PARP induced by TNF-α with or without insulin. The cleaved forms of caspase-8, caspase-9, caspase-3, and PARP were detected by Western blotting from cells treated with 50 ng/mL TNF-α with or without 200 nmol/L insulin for the indicated times (in hours) after incubation with 200 units/mL IFN-γ for 24 hours. P41/43 of caspase-8, p37 of caspase-9, p17 of caspse-3, and p85 of PARP are their cleaved forms. The density of each band was measured by using Scion Image software and plotted with the TNF-α–induced cleavage of each protein at the 24 hour point as 100%.

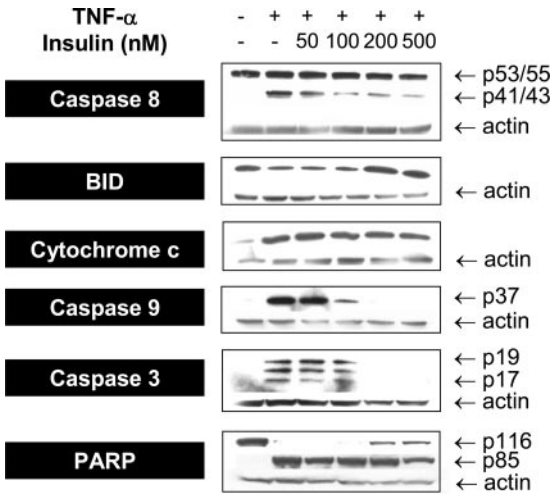

Fig. 2. The effects of different doses of insulin on TNF-α–induced cleavage of apoptosis-related proteins. The proform and/or cleaved form of each protein were detected by Western blotting from cells treated with 50 ng/mL TNF-α with or without the indicated concentrations of insulin for 8 hours after incubation with 200 units/mL IFN-γ for 24 hours. P53/55 of caspase-8 and p116 of PARP are their intact forms. P41/43 of caspase-8, p37 of caspase-9, p17/19 of caspse-3, and p85 of PARP represent their partial/complete cleaved forms. Bands of BID and cytosolic cytochrome *c* represent their intact forms. Actin was blotted as a loading control. The figures represent three similar experimental results.

anism separate from the one that affects the cleavage of procaspase-8, an early event of the TNF-α signaling pathway.

PI3K and MAPK Pathways Mediate the Inhibitory Effects of Insulin. Next, we examined insulin signaling pathways that inhibit the cleavage of procaspases by using protein kinase inhibitors. LY294002 and PD98059 are inhibitors of PI3K and MAPK kinase (MAPKK), respectively, which are kinases activated by insulin. Both LY294002 and PD98059 not only reversed the inhibitory effects of insulin but also enhanced the TNF-α–induced cleavage of procaspase-8. In contrast, only LY294002 reversed the ability of insulin to inhibit the TNF-α–induced cleavage of procaspase-9 (Fig. 3*A*). Based on these results, both MAPKK/extracellular signal-regulated kinase (ERK) and PI3K/Akt pathways activated by insulin reduce the cleavage of procaspase-8, whereas PI3K/Akt pathway seems to play a major inhibitory role in regulating the cleavage of procaspase-9. Western blotting of their substrates, caspase-3 and PARP, showed corresponding results to those of their upstream proteins. Because both caspase-8 and -9 can cleave procaspase-3, we could not determine whether insulin affects procaspase-3 and PARP directly or via alterations of one or both upstream caspases. In addition, protein kinase inhibitors alone could induce the cleavage of procaspase-8, -9, and -3 and PARP, suggesting that an endogenous protective mechanism(s) exists in this cell line.

The Effects of Insulin and a PI3K Inhibitor on TNF-α–Induced Apoptosis and Regulatory Proteins of Procaspase-9. To confirm the antiapoptotic role of insulin via the PI3K/Akt pathway, levels of apoptosis were measured in response to insulin and/or LY294002 in combination with TNF-α. Insulin rescued cells from TNF-α–induced apoptosis, whereas LY294002 enhanced it (Fig. 3*B*). This result, combined with the Western blotting data of caspase-9, suggests that the PI3K/Akt pathway mediates one of the antiapoptotic mechanisms activated by insulin via inhibition of procaspase-9 cleavage. Accordingly, we investigated regulatory proteins of caspase-9. Although we could observe that TNF-α cleaved procaspase-9 (Figs. 2 and 3*A*), treatment with TNF-α for up to 12 hours did not change the protein level of Apaf-1, which is necessary for the activation of procaspase-9 (data not shown). Also, insulin with or without LY294002 in combination with TNF-α did not alter the level of Apaf-1 (data not shown). Therefore, we examined another regulatory molecule, XIAP, a potent endogenous inhibitor of cleaved caspase-3 and -9. We observed that the treatment with TNF-α produced an approximately 30-kDa fragment of XIAP (Fig. 4*A*). To obtain complementary data, we used two separate XIAP antibodies that are sensitive to either intact or fragmented XIAP. The application of LY294002 enhanced the fragmentation of XIAP by TNF-α, whereas insulin reversed this effect (Fig. 4*B*, *Ab1*). Also, TNF-α treatment decreased the level of intact XIAP at 57 kDa, which was reversed by insulin and enhanced by LY294002 (Fig. 4*B*, *Ab2*). In addition, insulin restored the level of intact XIAP decreased by TNF-α in a dose-dependent manner (Fig. 4*C*).

The Effects of TNF-α and Insulin on the Coprecipitation of XIAP and Procaspase-9. Because the agents affecting the cleavage of procaspase-9 changed the level of intact XIAP, we examined the interaction of XIAP with caspase-9. Contrary to previous literature showing that XIAP binds only to the cleaved form of caspase-9 (37, 39), we observed that XIAP was coimmunoprecipitated with procaspase-9 in control HT-29 cells (Fig. 5*A*). The cleaved form of caspase-9 was not precipitated with the antibody used for the immunoprecipitation (Fig. 5*B*). Based on these results, we investigated the effects of TNF-α with or without insulin on the binding of XIAP to

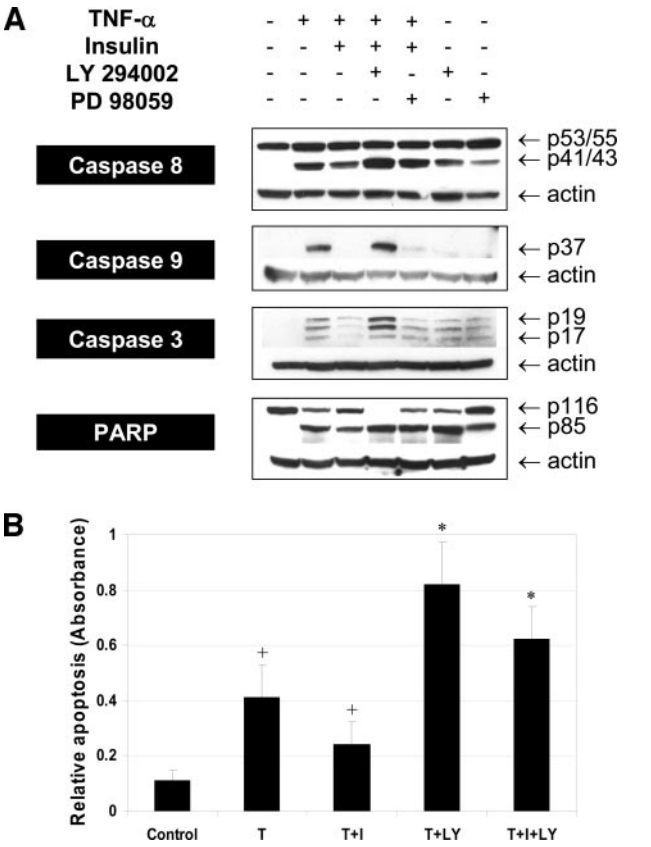

Fig. 3. The effects of protein kinases on the level of proteins and apoptosis. *A*. The effects of protein kinase inhibitors on apoptosis-related proteins regulated by TNF-α and insulin. The proform and/or cleaved form of each protein were detected by Western blotting from the cells treated with 50 ng/mL TNF-α with or without 500 nmol/L insulin for 8 hours, in some cases after pretreatment with either 20 μmol/L LY294002 or PD98059 for 1 hour. P53/55 of caspase-8 and p116 of PARP are their intact forms. P41/43 of caspase-8, p37 of caspase-9, p17/19 of caspse-3, and p85 of PARP represent their partial/complete cleaved forms. Actin was blotted as a loading control. The figures represent three similar experimental results. *B*. The effects of insulin and a PI3K inhibitor on TNF-α–induced apoptosis. Apoptotic levels were measured by enzyme-linked immunosorbent assay for cytosolic histone-associated DNA fragment. Cells were treated with 50 ng/mL TNF-α (*T*) with or without 500 nmol/L insulin (*I*) for 24 hours, in some cases with pretreatment with 20 μmol/L LY294002 (*LY*) for 1 hour. *, statistically significant, $P < 0.01$. +, statistically significant, $P < 0.05$. Paired Student's *t* test; $n = 8$.

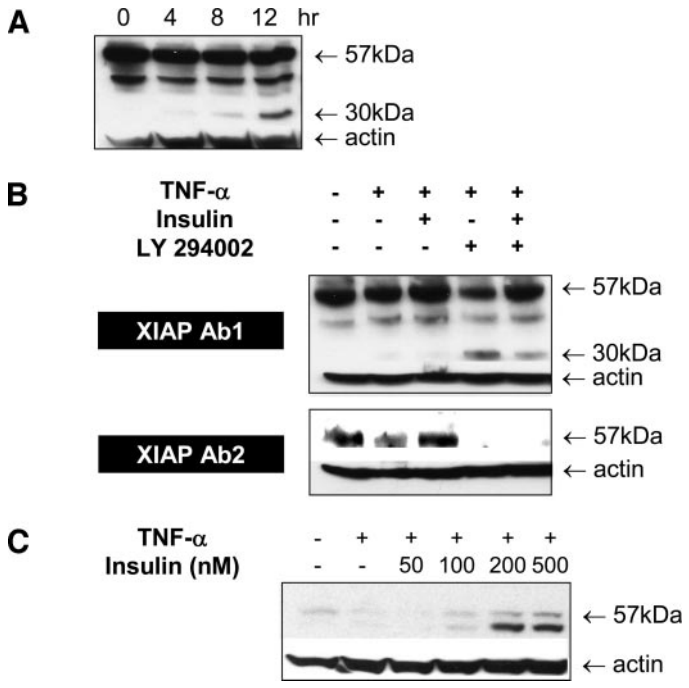

Fig. 4. The effects of the chemicals affecting the cleavage of caspase-9 on XIAP. *A*. The protein level of XIAP was detected by Western blotting from cells incubated with 50 ng/mL TNF-α for the indicated time periods (in hours). *B*. The intact form and cleaved form of XIAP were detected by two different antibodies 8 hours after application of 50 ng/mL TNF-α with or without 500 nmol/L insulin, in some cases, after pretreatment with 20 μmol/L LY294002 for 1 hour. Antibody 1 is antimouse XIAP antibody (BD Bioscience), and antibody 2 is antirabbit XIAP antibody (Cell Signaling Technology). *C*. The intact form of XIAP was detected by using Western blotting from cells incubated with 50 ng/mL TNF-α with or without the indicated concentrations of insulin for 8 hours after treatment with 200 units/mL IFN-γ. Actin was blotted as a loading control. The figures represent three similar experimental results.

procaspase-9. Fig. 5*C* shows that TNF-α treatment decreased the level of XIAP precipitated with procaspase-9, which was reversed by insulin. Also, only procaspase-9 was coprecipitated with XIAP (Fig. 5*C*, *bottom panel*), but not with the cleaved form of caspase-9 while we could still detect the cleaved form from the same treatment (data not shown). The level of procaspase-9 precipitated with XIAP was decreased by TNF-α, which was restored by insulin.

Protein Kinase(s) Responsible for the Coprecipitation of Procaspase-9 and XIAP. Because LY294002 is an inhibitor of PI3K, which is an upstream kinase of the insulin signaling pathway, effects of inhibitors for downstream kinases were examined. Among three Akt inhibitors and the mammalian target of rapamycin inhibitor, rapamycin, Akt inhibitor II showed the strongest inhibition of the insulin effect to decrease TNF-α–induced cleavage of procaspase-9 (Fig. 6*A*). All three Akt inhibitors are synthetic phosphatidylinositol analogs. Akt inhibitor II and III inhibited the effect of insulin more strongly than inhibitor I, which seems to result from improved cell permeability, judging from their structures. Based on this result, precipitation levels of XIAP with procaspase-9 were examined. LY294002 and Akt inhibitor II demolished the inhibitory effect of insulin on the TNF-α–induced decrease of XIAP precipitation with procaspase-9, whereas rapamycin did not show any effect (Fig. 6*B*). These results confirm that insulin decreases the TNF-α–induced activation of procaspase-9 by regulating XIAP via the PI3K/Akt pathway.

DISCUSSION

Insulin has been reported to rescue diverse cell types from death. Two survival pathways activated by insulin are the MAPKK/ERK and PI3K/Akt pathways. Their overexpression or activation by other survival factors also prevents cells from undergoing apoptosis. Important questions are which antiapoptotic proteins are affected by these kinases, and which proapoptotic proteins are their targets. Caspases, key apoptosis mediators, accordingly, have been studied as the most likely proapoptotic proteins regulated by antiapoptotic effectors, yet we still do not know the complete mechanisms for survival signals to inhibit caspases. FLIP, which is up-regulated by survival factors (40, 41), has been suggested to inhibit the activation of procaspase-8 by interfering with its binding to a death effector domain of Fas-associated death domain proteins (30, 31). The literature reported conflicting data on which survival kinase regulates FLIP, probably due to different experimental conditions, including cell types. Based on our results with pharmacological inhibitors, both MAPK/ERK and PI3K/Akt signaling pathways are involved in inhibiting the cleavage of procaspase-8. It might be the case that both kinases regulate FLIP in HT-29 cells at

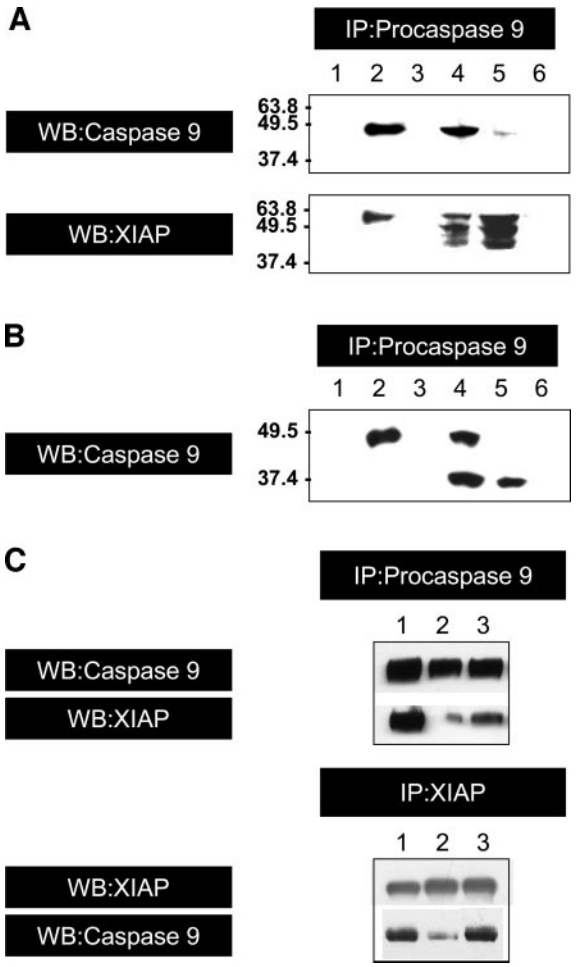

Fig. 5. The effects of TNF-α and insulin on the coprecipitation of procaspase-9 and XIAP. *A*. Procaspase-9 and XIAP were detected from the control cell lysates by Western blotting after immunoprecipitation with procaspase-9 antibody. *Lane 1*, cell lysates precipitated with control beads; *Lane 2*, cell lysates precipitated with antibody-immobilized beads; *Lane 3*, no cell lysates precipitated with antibody-immobilized beads; *Lane 4*, supernatant of *Lane 1*; *Lane 5*, supernatant of *Lane 2*; *Lane 6*, supernatant of *Lane 3*. *B*. Caspase-9 was detected from the control cell lysates with an antibody that recognizes both the proform and cleaved form after immunoprecipitation with procaspase-9 antibody. *Lane 1*, cell lysates precipitated with control beads; *Lane 2*, cell lysates precipitated with antibody-immobilized beads; *Lane 3*, no cell lysates precipitated with antibody-immobilized beads; *Lane 4*, supernatant of *Lane 1*; *Lane 5*, supernatant of *Lane 2*; *Lane 6*, supernatant of *Lane 3*. *C*. Caspase-9 and XIAP were detected by Western blotting after immunoprecipitation of procaspase-9 or XIAP from the HT-29 cells. *Lane 1*, control cells treated with 200 units/mL IFN-γ; *Lane 2*, cells incubated with 50 ng/mL TNF-α after 200 units/mL IFN-γ; *Lane 3*, cells incubated with 50 ng/mL TNF-α and 500 nmol/L insulin for 12 hours after 200 units/mL of IFN-γ.

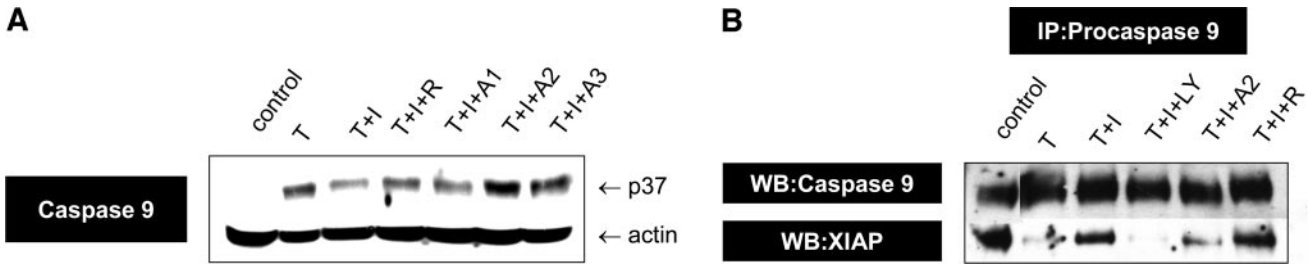

Fig. 6. The effects of kinase inhibitors on the precipitation of procaspase-9 and XIAP. *A*. The protein level of cleaved caspase-9 was detected by Western blotting from cells incubated with 50 ng/mL TNF-α for 8 hours with or without insulin after pretreatment with kinase inhibitors for 1 hour. *T*, 50 ng/mL TNF-α; *I*, 500 nmol/L insulin; *R*, 200 nmol/L rapamycin; *A1*, 20 μmol/L Akt inhibitor I; *A2*, 20 μmol/L Akt inhibitor II; *A3*, 20 μmol/L Akt inhibitor III. *B*. Caspase-9 and XIAP were detected by Western blotting after immunoprecipitation of procaspase-9 from the HT-29 cells incubated with 50 ng/mL TNF-α with or without 500 nmol/L insulin for 12 hours after pretreatment of kinase inhibitors for 1 hour. *T*, 50 ng/mL TNF-α; *I*, 500 nmol/L insulin; *LY*, 20 μmol/L LY294002; *R*, 200 nmol/L rapamycin; *A2*, 20 μmol/L Akt inhibitor II.

different levels or different degrees. Alternatively, one kinase might decrease the cleavage of procaspase-8 via FLIP, whereas the other operates via a different mechanism. We observed that insulin treatment slightly increased the protein level of FLIP (data not shown). Phosphorylation induced by survival signals has also been suggested to inhibit the activity of caspase-9. Phosphorylation of recombinant procaspase-9 at a serine residue by Akt was shown to inhibit its activity (32), whereas phosphorylation of endogenous procaspase-9 at a threonine residue via the MAPKK/ERK pathway was reported in a HeLa cell line (42). Based on these reports, different kinases may phosphorylate various sites of procaspase-9.

We observed that insulin decreased the rate of apoptosis and the cleavage of procaspase-8, -9, and -3 induced by TNF-α. Also, our experiments with kinase inhibitors demonstrated that the most likely pathway activated by insulin to inhibit the activation of procaspase-9 is the PI3K/Akt pathway. Janes *et al.* (43) emphasized that in HT-29 cells treated with TNF-α, Akt was the only kinase significantly activated over a long time period by insulin, compared with other kinases (ERK, Iκ-B kinase, c-Jun NH_2-terminal kinase 1, MAPKK, and MAPK-associated protein kinase 2). In addition, the activation of Akt by insulin showed a biphasic trend, which consists of early time activation and sustained activation from 4 to 24 hours (43). By applying a PI3K inhibitor, Janes *et al.* (43) confirmed that late-phase activation of Akt is important in decreasing apoptosis. Sustained activity of Akt in this report agrees with our observation that insulin decreased TNF-α–induced apoptosis by reducing the cleavage of procaspase-9, a late apoptotic event, via the PI3K/Akt pathway.

These results led us to investigate the mechanisms by which insulin reduces the cleavage of procaspase-9, one of which might be that alterations in the interaction of procaspase-9 with other proteins regulated by the PI3K/Akt pathway may affect its cleavage. Investigating potential molecules capable of mediating the survival effect of insulin, we found that TNF-α induced cleavage of XIAP, which was enhanced by a PI3K inhibitor. Considering that XIAP is an important survival molecule to inhibit caspases, particularly those bound to procaspase-9 as discussed below, its fragmentation probably accelerates the apoptotic process. Similarly, autoubiquitination and degradation of XIAP occur in response to dexamethasone and etoposide (44), and a mitochondrial serine protease, Omi/HtrA2, was suggested to degrade inhibitor of apoptosis proteins including XIAP (45, 46). Also, a member of the inhibitor of apoptosis protein family, c-IAP1, was cleaved by caspases, producing a proapoptotic fragment (47). Activation of Akt by overexpression of vascular endothelial growth factor (48) and insulin-like growth factor I (49) increased the gene expression or protein level of XIAP. These reports correspond to our results showing that an inhibitor of PI3K, an upstream kinase of Akt, decreased the level of intact XIAP and induced its fragmentation.

We observed that XIAP was coimmunoprecipitated with procaspase-9. Our observation of XIAP binding to the proform of caspase-9 can explain why insulin decreased both the cleavage of caspase-9 and its downstream substrates. If XIAP bound to only the cleaved form of caspase-9, the level of TNF-α–induced caspase-9 cleavage should not be decreased by insulin treatment, whereas the level of downstream substrate cleavage is decreased. The possibility of procaspase-9 binding to XIAP was mentioned in earlier literature in the field (36), but later reports emphasized that XIAP binds only to the cleaved form of caspase-9 in either a cell-free system with mutated procaspase-9 or in human U-937 myeloid leukemia cells overexpressing XIAP (37, 39). On the other hand, we investigated wild-type endogenous procaspase-9 and XIAP from HT-29 cells. These differences in experimental protocols could lead to different results. Although we cannot exclude the possibility that XIAP binds to both the proform and the cleaved form of caspase-9 in other systems, our results with HT-29 cells demonstrated that only the proform of caspase-9, and not the cleaved form, was precipitated with XIAP. Moreover, Western blotting of XIAP after immunoprecipitation of procaspase-9 demonstrated that some amount of XIAP was not precipitated with procaspase-9 (Fig. 5*A*), which implies that XIAP may interact with other proteins, possibly including cleaved caspase-9, for its multiple functions. Also, this antibody recognized several bands, which might be multiple forms of XIAP. Various forms of XIAP might interact with either the proform or cleaved form of caspase-9.

We suggest that an interaction between XIAP and procaspase-9 is one of the regulatory systems by which insulin decreases the TNF-α–induced cleavage of procaspase-9 and subsequent apoptosis. Our results, along with those of Janes *et al.* (43), strongly support an antiapoptotic mechanism in which insulin acts through the PI3K/Akt pathway. Furthermore, a phosphorylation event(s) on an Akt substrate(s) may prevent release of XIAP from procaspase-9. Procaspase-9 is a potential substrate for Akt (32), and phosphorylation of XIAP by Akt was also reported recently (50). Therefore, it is feasible that phosphorylation of procaspase-9 and/or XIAP by Akt plays a role in their interaction (and possibly other inhibitory proteins) and thus inhibits subsequent XIAP cleavage. The results shown in this study, in addition to our previous report that demonstrated regulation of procaspase-9 via S-nitrosation in HT-29 cells (51), suggest that there are multiple inhibitory factors regulating a single component in the caspase cascade leading to apoptosis in cultured cancer cells.

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