Akt protects mouse hepatocytes from TNF-α- and Fas-mediated apoptosis through NK-κB activation

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Akt protects mouse hepatocytes from TNF-α- and Fas-mediated apoptosis through NK-κB activation. Am J Physiol Gastrointest Liver Physiol 282: G1357–G1368, 2002.—To determine the role of phosphatidylinositol 3-kinase (PI3K)Akt and nuclear factor-κB (NF-κB) in protecting hepatocytes from tumor necrosis factor-α (TNF-α)- and Fas-mediated apoptosis, we pretreated primary cultures of mouse hepatocytes with pharmacological and adenovirus-mediated inhibitors of the PI3K/Akt and NF-κB pathways followed by treatment with TNF-α or Jo2, an anti-Fas antibody. Jo2 and, to a lesser extent, TNF-α phosphorylate Akt. The PI3K inhibitor LY-294002 blocks TNF-α- and Fas-mediated Akt phosphorylation. LY-294002 pretreatment reduces NF-κB binding activity and transcriptional activity and NF-κB-responsive gene expression by TNF-α or Jo2. LY-294002 promotes apoptosis after TNF-α or Jo2. The expression of dominant-negative Akt blocks NF-κB activation and sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis. The expression of constitutively active Akt rescues LY-294002-pretreated cells from TNF-α- and Fas-mediated apoptosis. Active Akt induces NF-κB transcriptional activity but not NF-κB binding activity or IκB degradation. Furthermore, LY-294002 pretreatment blocks TNF-α- and Jo2-induced Bcl-xl levels in hepatocytes, with no effect on the phosphorylation levels of Bad. Bcl-xl overexpression protects hepatocytes from Fas- but not TNF-α-induced apoptosis after sensitization by actinomycin D or the IκB superrepressor. Together, the PI3K/Akt pathway has a protective role in Fas-mediated apoptosis, which requires NF-κB activation, partially through the subsequent induction of Bcl-xl.

Bcl-xl; phosphatidylinositol 3-kinase; IκB kinase; transcription; signaling

DEATH FACTORS SUCH AS tumor necrosis factor-α (TNF-α) and Fas ligand (FasL) induce apoptosis, or programmed cell death (33, 34). Overexpression of the TNF/TNF receptor (TNFR) or FasL/Fas system causes hepatic damage in animal models and is associated with diverse diseases in patients. TNF-α injection with pretreatment of protein synthesis inhibitor or RNA transcriptional inhibitor induces massive apoptosis in the mouse liver (27). Intrapitoneal injection of anti-Fas agonistic antibody (Jo2) in mice results in fulminating hepatitis and death within hours (35). On the other hand, TNF-α is a comitogen for hepatocytes (17). Furthermore, a recent study (12) showed that Fas engagement of hepatocytes in regeneration promotes cell growth. This indicates that TNF-α and Fas may mediate either liver damage or stimulate protective regenerative responses.

Nuclear factor-κB (NF-κB) has been strongly implicated in the regulation of apoptosis induced by death factors, such as TNF-α (49, 50). The NF-κB superfamily consists of transcriptional activators such as p65 and p50 that form homo- or heterodimers as well as inhibitory subunits such as IκBα that function to retain the transcription factor in the cytoplasm (25). Various stimuli, such as TNF-α, interleukin-1 (IL-1), viral infection, and lipopolysaccharide, activate the IκB kinase complex (IKK), which results in the phosphorylation, ubiquination, and degradation of IκBα, followed by the translocation of NF-κB and activation of NF-κB-responsive genes. Additional mechanisms activate NF-κB transcriptional activity, such as the phosphorylation of the NF-κB transactivation domains, without affecting its DNA binding (31). NF-κB activation suppresses TNF-α-induced apoptosis through NF-κB-responsive protective genes (49). TNFR-associated factor (TRAF) 1, TRAF2, and the inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 are gene targets of NF-κB transcriptional activity (51). We (19) have demonstrated that NF-κB activation and nitric oxide (NO), synthesized by inducible NO synthase (iNOS), protect primary mouse hepatocytes from TNF-α- and Fas-mediated apoptosis. Therefore, iNOS is an NF-κB-inducible gene that mediates resistance to TNF-α- and Fas-induced hepatotoxicity. However, there must be additional NF-κB-responsive genes whose expression in hepatocytes is required for protection from TNF-α- and Fas-mediated apoptosis, because iNOS is not sufficient for complete protection.

Akt/protein kinase B is a serine/threonine protein kinase that mediates cell-survival signals from growth factors and cytokines (8). Several targets of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway have been identified to promote survival or inhibit apoptosis. These substrates include Bad, caspase-9,
transcription factors of the forkhead family, and IKK. TNF-α activates PI3K and its downstream target Akt in 293 cells (36). Both Akt and NF-κB-inducing kinase (NIK) are necessary for TNF-α-induced activation of NF-κB, and Akt mediates IKKα phosphorylation in 293 cells (36). On the other hand, in MCF7 breast carcinoma cells, whereas both TNFR1 and NIK are partially involved in Akt-induced NF-κB stimulation, IkB superrepressor completely blocked Akt-NF-κB cross-talk (4). Also NF-κB activation by platelet-derived growth factor (PDGF) is a target of the antiapoptotic Ras/PI3K/Akt pathway (42). However, PDGF-induced Akt phosphorylation does not activate NF-κB in human vascular smooth muscle cells and fibroblasts (38). These results indicate NF-κB activation by Akt might be a cell-specific event. Growth factors such as epidermal growth factor (EGF) and interleukin (IL)-6 inhibit transforming growth factor-β (TGF-β)-mediated apoptosis through PI3K/Akt signaling pathway in rat hepatocytes and human hepatoma cell lines, respectively (7, 41). PI3K/Akt mediates the antiapoptotic effects of EGF and intestinal trefoil factor 18, 48). However, the role of Akt in death receptor-mediated apoptosis in hepatocytes remains unclear.

We hypothesized that TNF-α/TNFR and FasL/Fas stimulate survival signals to protect hepatocytes from apoptosis, because TNF-α and anti-Fas agonistic antibody alone induce minimal cytotoxicity in cultured hepatocytes. In this study, we aimed to determine whether 1) TNF-α or Jo2 activates PI3K/Akt pathway in mouse hepatocytes, 2) active Akt induces NF-κB activation, and 3) active Akt protects hepatocytes from TNF-α- or Fas-mediated apoptosis.

MATERIAL AND METHODS

Primary hepatocyte cultures. C57Bl6 male mice (~8 wk old) were anesthetized with ketamine-acepromazine maleate administered by intraperitoneal injection. For some experiments, we used Bcl-xL transgenic mice for hepatocyte cultures (a kind gift from Dr. A. Mignon), in which expression of human Bcl-xL cDNA is directed by the regulatory sequences of the rat L-type pyruvate kinase gene (10). Transgenic mice were identified by Southern blot analysis using the coding 0.9-kb Bcl-xL sequence as a probe (10). Hepatocytes were then isolated by a retrograde, nonrecirculating in situ collagenase perfusion of livers cannulating through the inferior vena cava by a procedure modified from Moldeus et al. (32). Livers were first perfused in situ with oxygenated 0.5 mM EGTA containing calcium-free salt solution (10 ml/min at 37°C for 5 min), followed by perfusion with solution containing 0.04% collagenase type I (Worthington Biochemical, Lakewood, NJ) for 10 min. The liver was then gently minced on a Petri dish and filtered with polyamide mesh (I 003 Y Nitex 3-60/45, Tetko). Hepatocytes were washed two times and centrifuged at 50 g for 2 min. Cell viability was consistently >90% as determined by trypan blue exclusion. Hepatocyte cultures contained <1% Kupffer cells and hepatic stellate cells as determined by FITC-labeled latex beads (1 μm, Polysciences, Warrington, PA) and autofluorescence, respectively. Cells (4 × 10⁵) were plated on six-well plates coated with mouse collagen type I in Waymouth’s medium containing 10% fetal bovine serum, 0.1 μM insulin, and 0.1 μM dexamethasone. In preliminary experiments, 0.1 μM insulin did not affect Akt phosphorylation. Next, 1.5 × 10⁶ or 4 × 10⁶ cells were plated on 60- or 100-mm dishes, respectively. After 1.5–2 h, the culture was washed with PBS and changed to hormonally defined medium (HDM) containing 0.1 μM insulin, 2 mM l-glutamine, 5 μg/ml transferrin, 3 μM selenium, and 10 nM free fatty acids in RPMI basal medium. Cells were pretreated with the PI3K inhibitor LY-294002 (Calbiochem-Novabiochem, San Diego, CA) or wortmannin (Sigma, St. Louis, MO) for 1 h before the exposure of recombinant murine TNF-α (R&D Systems, Minneapolis, MN) or Jo-2 (Pharmingen, San Diego, CA). In some experiments, cells were infected with recombinant adenoviruses in HDM containing 30 plaque-forming units/cell for 3 h at 37°C and then changed to HDM containing TNF-α or Jo2. All animals received humane care in compliance with the guidelines of the University of North Carolina.

Western blot analysis for Akt, Ikβ α, Bad, and Bcl-xL. Whole cell extracts were prepared by lysing the cells in lysis buffer (10 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5% Nonidet P-40, and 25% glycerol containing protease inhibitors: 5 μM apatatin, 0.5 mM Pefabloc, 700 ng/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin; all from Roche, Indianapolis, IN). To detect the phosphorylation status of the protein, we used cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin) plus 1 mM phenylmethylsulfonyl fluoride according to the manufacturer’s instructions. The protein concentration of the extracts was determined by the Bradford method. Lysates containing 500 μg of protein were separated by electrophoresis on 10% acrylamide SDS gels. The proteins were transferred into nitocellulose membranes (Schleicher & Schuell, Keene, NH). Equal loading was confirmed by Ponceau S staining. Phosphorylated Akt was detected using rabbit polyclonal phospho-Akt (serine-473) antibody (New England Biolabs, Beverly, MA) and secondary anti-rabbit horseradish peroxidase-conjugated antibody (New England Biolabs). Anti-total Akt antibody (New England Biolabs) was used for internal control. Antibody was used at 1:1,000 dilution. Proteins were detected with enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, IL). The protein levels of phosphorylated Akt were quantified using AlphaImager 2000 (Alpha Innotech, San Leandro, CA). For Ikβ α, Bcl-xL, and Bad Western blot analysis, lysates containing 50 μg protein were separated by 12% acrylamide SDS gels. Ikβ α expression was detected using rabbit polyclonal Ikβ α antibody (C-21, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution. Phosphorylated Bad was detected using rabbit polyclonal phospho-Bad (serine-136) antibody and phospho-Bad (serine-112) (New England Biolabs) at 1:500 dilution. Anti-total Bad antibody (New England Biolabs) was used for internal control. Bcl-xL was detected using rabbit polyclonal Bcl-xL antibody (S-18, Santa Cruz Biotechnology) at 1:1,000 dilution.

Measurements of apoptosis. For quantitation of cell viability (presented as means ± SE), cells were infected and treated as described above. After 20 h of TNF-α or Jo2 treatment, cell viability was determined by exclusion of trypan blue. Viable cells were counted in three different ×200 power fields, and the percentage of treated viable cells to untreated viable cells was expressed as a percentage of viability. For propidium iodide nuclear staining, cells were fixed in methanol-acetic acid (3:1), stained with 10 μg/ml propidium iodide, and viewed with an Olympus fluorescence microscope using a rhodamine filter set. Hepatocyte cell death was confirmed as apoptosis by terminal deoxynucleo-
tidyl transferase-mediated dUTP nick-end labeling (TUNEL) (Boehringer Mannheim, Mannheim, Germany). TUNEL staining was performed according to the manufacturer’s suggested protocol. Positive (apoptotic) cells were counted in three different \( \times 200 \) power fields. As described previously (19), we used the FluorAce kit (Bio-Rad Laboratories, Hercules, CA) to perform 7-aminomethyl-coumarin (AFC) release assays for caspase-3.

**Nuclear extraction and electrophoretic mobility shift assay.** Four million cells were cultured overnight after min on ice, using 5\( \times \)22). Protein-DNA binding reactions were carried out for 20 min after Jo2 treatment. Nuclear protein extracts were prepared from primary mouse hepatocytes as previously described (2, 22). Protein-DNA binding reactions were carried out for 20 min on ice, using 5 \( \mu \)g of nuclear extract and \( ^{32} \)P-labeled DNA probes for the NF-\( \kappa \)B consensus binding site (3). Complexes were separated by electrophoresis on nondenaturing 5% acrylamide gels and assayed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). For supershift assays, 8 \( \mu \)g of antibody against the p65 or p50 subunit of the NF-\( \kappa \)B complex was added to the reaction mixture, and the incubation time was extended for an additional 30 min.

**Transfection and luciferase reporter assays.** Primary mouse hepatocytes plated on six-well plates were transiently transfected using the Targefect F-1 (Targeting Systems, San Diego, CA) in a final volume of 25 \( \mu \)l of F-1 reagent. Complexes were allowed to form for 20 min at 37°C. Hepatocytes were washed twice with Opti-MEM, and then F-1-DNA complexes were added to the cells. Twelve hours after transfection, cells were washed with PBS and pretreated with LY-294002 for 1 h. Five hours after treatment of TNF-\( \alpha \) or Jo2, cells were lysed in cell lysis buffer, and luciferase assays were performed with enhanced luciferase assay systems (Analytical Luminescence Laboratory, San Diego, CA) according to the manufacturer’s instructions. For some experiments, cells were infected with adenovirus vector 12 h after transfection and then lysed at 22 h after infection.

**RT-PCR for iNOS and intercellular adhesion molecule-1.** Total RNA was extracted with RNaseasy mini kit (Qiagen, Chatsworth, CA) according to the manufacturer’s protocol. First-strand cDNA was synthesized using 1 \( \mu \)g of total RNA, 10 mM dNTPs (Pharmacia, Piscataway, NJ), and 200 U of Moloney murine leukemia virus RT (GIBCO, Grand Island, NY) in a final volume of 25 \( \mu \)l. The reaction was carried out for 60 min at 42°C. The synthesized cDNA was amplified using specific sets of primers for iNOS and \( \beta \)-actin. The iNOS sense primer sequence was 5'-TGGAGAACACAG-GAACCTACCA-3', and the antisense primer was 5'-ACAGGGGTGATGCTCATGACA-3'. The primers for \( \beta \)-actin were as described previously (5). The PCR reactions were cycled as follows: after initial denaturation for 4 min at 99.9°C, 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; final extension was carried out at 72°C for 5 min. The intercellular adhesion molecule-1 (ICAM-1) sense primer sequence was 5'-TGGAACTGACGTCTGTAT-3', and the antisense primer was 5'-GACGAAAATCTGTCTACCA-3'. The PCR reactions were cycled as follows: after initial denaturation for 4 min at 99.9°C, 30 cycles at 95°C for 60 s, 59°C for 90 s, and 72°C for 10 s; final extension was carried out at 72°C for 5 min. The PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed.

**Adenoviruses.** The adenovirus 5 variants Ad5IkB and Ad5GFP, expressing hemagglutinin (HA)-IkB\( \alpha \) (S32A, S36A) and green fluorescent protein, respectively, were as described previously (2, 22). Dominant-negative (dn) Ad5IKK\( \beta \) expresses a catalytic mutant with lysine changed to methionine (19). AddeltaNIK expressing dnNIK was as described previously (20). An adenovirus expressing constitutively active Akt encoding an amino-terminal myristylation signal (AdmyrAkt) was a kind gift from Dr. J. A. Romashkova (42). An adenovirus expressing dnAkt (AddnAkt) was a kind gift from Dr. W. Ogawa (26). Lysates were prepared from 4 \( \times \)10\(^5\) hepatocytes at 24 h after adenoviral infection. The HA-tagged myrAkt and dnAkt were detected using mouse anti-HA antibody (Boehringer Mannheim).

**RESULTS**

**TNF-\( \alpha \) and anti-Fas antibody phosphorylate Akt through PI-3 K in primary mouse hepatocytes.** To determine whether TNF-\( \alpha \) or Jo2 phosphorylates Akt in hepatocytes, we performed Western blot analysis using cell lysates from primary mouse hepatocytes. Hepatocytes were incubated for 12 h without serum before treatment to reduce the basal level of phosphorylated Akt. Phospho-Akt antibody detects Akt only when phosphorylated at serine-473. Cell lysate from hepatocytes treated with 10 ng/ml of EGF was used as a positive control (41). TNF-\( \alpha \) phosphorylated Akt at 5 min after treatment, and Jo2 phosphorylated Akt 15 min after treatment (Fig. 1A). The results from densitometry showed that Jo2 is a more potent inducer for Akt phosphorylation than TNF-\( \alpha \) (Fig. 1B). Furthermore, to determine whether PI3K is involved in TNF-\( \alpha \)- and Jo2-induced Akt phosphorylation, we pretreated cells with the PI3K-specific inhibitor LY-294002 for 1 h at 25 \( \mu \)M, as performed previously (41). Immunoblot analysis demonstrated that the PI3K inhibitor LY-294002 blocks TNF-\( \alpha \)- and Fas-mediated Akt phosphorylation, indicating that PI3K is upstream in TNF-\( \alpha \)- and Jo2-induced Akt phosphorylation.

**PI3K inhibitor sensitizes mouse hepatocytes to TNF-\( \alpha \)- and Fas-mediated apoptosis.** To determine the role of PI3K/Akt pathway on survival for TNF-\( \alpha \)- and Fas-mediated apoptosis, primary mouse hepatocytes were pretreated with LY-294002 for 1 h and treated with TNF-\( \alpha \) or Jo2. Percent viability was determined after 20 h by the trypan blue exclusion test. TNF-\( \alpha \), Jo2, or LY-294002 alone induced minimal cytotoxicity. However, pretreatment with LY-294002 promotes cell death after TNF-\( \alpha \) or Jo2 (35% and 70%, respectively, Fig. 2B). The morphological changes demonstrated by the phase-contrast microscopy included cellular rounding, loss of attachment, and increased reactivity (Fig. 2A). The hepatocytes treated with LY-294002 plus TNF-\( \alpha \) or LY-294002 plus Jo2 displayed nuclear condensation and fragmentation by propidium iodide staining, characteristics of apoptosis (Fig. 2C, middle and right), whereas cells treated with LY-294002 displayed normal nuclear morphology (Fig. 2C, left). To confirm death by apoptosis, a TUNEL assay was performed. Although TUNEL-positive cells were minimal after TNF-\( \alpha \) or Jo2 treatment in hepatocytes (2 \( \pm \) 1, 11.7 \( \pm \) 1.5, means \( \pm \) SE cells/100, respectively), positive hepatocytes were observed after TNF-\( \alpha \) or Jo2 treatment in hepatocytes pretreated with LY-294002.
NF-κB activation by the IκB superrepressor, a proteasome inhibitor, dnNIK, or dnIκKβ sensitizes mouse hepatocytes to TNF-α- and Fas-mediated apoptosis (19, 20, 45). To explore the mechanism by which LY-294002 sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis, we examined NF-κB DNA-binding activity by electrophoretic mobility shift assay (EMSA) (Fig. 3A), NF-κB transcriptional activity by reporter gene assay (Fig. 3B), and NF-κB-responsive gene expression by RT-PCR (Fig. 3C). EMSA showed that TNF-α or Jo2 induced NF-κB binding activities (Fig. 3A, middle band in lanes 2 and 4), which is composed of p65/p50 heterodimers, as demonstrated by the supershift analysis (data not shown). Pretreatment with LY-294002 reduced NF-κB binding activities by TNF-α...
These results indicate that inhibition of PI3K with LY-294002 attenuated TNF-α-induced gene expression of iNOS and ICAM-1 in hepatocytes. The pretreatment with LY-294002 reduced TNF-α- and Jo2-induced NF-κB binding activity, whereas GFP expression had no effect on NF-κB binding activity (Fig. 4B). TNF-α induced IkB degradation in hepatocytes from 15 to 30 min after treatment (Fig. 4C, top), whereas IkB degradation was not observed in or Jo2 (59% or 33% reduction, respectively, demonstrated with PhosphorImager analysis). Pretreatment with LY-294002 also reduced NF-κB transcriptional activities by TNF-α or Jo2, as demonstrated by the (κB)3Luc luciferase activity assessed by TNF-α or Jo2, as demonstrated by the (κB)3Luc luciferase activity assays.

NF-κB activation induces the expression of several NF-κB-responsive genes, including iNOS and ICAM-1. Total RNA was extracted from the hepatocytes 4 h after treatment with TNF-α or Jo2. mRNA induction of iNOS and ICAM-1 was observed in TNF-α- and Jo2-treated hepatocytes and, to lesser content, in Jo2-treated hepatocytes. The pretreatment with LY-294002 reduced TNF-α-induced gene expression of iNOS and ICAM-1 and completely inhibited Fas-induced gene expression. These results indicate that inhibition of PI3K with LY-294002 attenuated TNF-α-induced NF-κB binding activity and transcriptional activity and NF-κB-responsive gene expression and inhibited Jo2-induced NF-κB activation to the level of untreated cells.

dnAkt blocks NF-κB binding activity and IkB degradation and sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis. To extend the results obtained with the pharmacological PI3K inhibitor LY-294002, we used AddnAkt to block activation of Akt. HA-tagged mutant Akt (dnAkt) has a lysine-179 in the kinase domain replacing Asp, as previously described (26). The adenovirus-mediated expression of dnAkt was confirmed by detecting HA (Fig. 4A). dnAkt abolished TNF-α- and Jo2-induced NF-κB binding activity, whereas GFP expression had no effect on NF-κB binding activity (Fig. 4B). TNF-α induced IkB degradation in hepatocytes from 15 to 30 min after treatment (Fig. 4C, top), whereas IkB degradation was not observed in

Fig. 3. Inhibition of PI3K attenuates nuclear factor-κB (NF-κB) binding activity and transcriptional activity and NF-κB-mediated gene expression by TNF-α or anti-Fas antibody. A: NF-κB DNA binding activity was assessed by an electrophoretic mobility shift assay (EMSA) using an NF-κB binding site as the probe with nuclear extracts prepared after a 30-min incubation with TNF-α or Jo2 with or without LY-294002 pretreatment for 1 h. Data are shown as average %viability ± SE of 3 different experiments. B: reporter gene assays were performed using 3×κB luciferase [(κB)3-Luc] plasmid. NF-κB activation was induced by a 5-h incubation with TNF-α (30 ng/ml) or Jo2 (0.5 μg/ml). Some cultures were pretreated with LY-294002. Results from 1 representative experiment performed in triplicate are shown. C: total RNA was extracted at 4 h after treatment with TNF-α (30 ng/ml) or Jo2 (0.5 μg/ml). Some cultures were pretreated with LY-294002. Inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and β-actin mRNAs were measured using RT-PCR analysis.

Fig. 4. A dominant-negative (dn) form of Akt (dnAkt) sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis. A: lysates were prepared from 4 × 10⁵ hepatocytes at 24 h after adenoviral infection. The hemagglutinin (HA)-tagged dnAkt was detected using mouse anti-HA antibody. B: NF-κB DNA binding activity was assessed by an EMSA using an NF-κB binding site as the probe with nuclear extracts prepared from hepatocytes expressing green fluorescent protein (GFP) or dnAkt after a 30-min incubation with TNF-α or Jo2. Experiments were repeated 3 times with similar results. C: IkBα expression was detected by Western blotting using anti-IkBα antibody after AddnAkt infection. The dnAkt inhibited IkBα expression was detected by Western blotting using anti-IkBα antibody after Ad5dnAkt infection. The dnAkt inhibited IkB degradation by TNF-α. D: hepatocytes were infected with AdGFP or AddnAkt and then treated with TNF-α (30 ng/ml) or Jo2 (0.5 μg/ml). Cell viability was assessed at 19 h after TNF-α or Jo2 treatment by a trypan blue exclusion test. Data are shown as average %viability ± SE of 3 different experiments. E: propidium iodide-stained images were obtained at 22 h after TNF-α (middle) or Jo2 (right) treatment (original magnification, ×400). MOI, multiplicities of infection.
Jo2-treated cells (data not shown). Consistent with this result, the Fas-interacting proteins FADD, Casper, and caspase-8 also activate NF-κB by an alternative pathway in other cell types (21). dnAkt expression (Fig. 4C, bottom) blocks TNF-α-induced IκB degradation (Fig. 4C, middle). To confirm the effect of Akt on survival after treatment with TNF-α or Jo2, hepatocytes were infected with AddnAkt or control adenovirus AdGFP and treated with TNF-α or Jo2. Measurements of cell viability demonstrate that dnAkt sensitizes hepatocytes to killing by TNF-α or Fas (Fig. 4D). The hepatocytes expressing dnAkt treated with TNF-α or Jo2 displayed nuclear condensation and fragmentation by propidium iodide staining, characteristics of apoptosis (Fig. 4E, middle and right), whereas cells expressing dnAkt without the treatment displayed normal nuclear morphology (Fig. 4E, left). These results demonstrate that either dnAkt or LY-294002 sensitize hepatocytes to TNF-α- and Fas-mediated apoptosis through inhibition of NF-κB activation.

Constitutively active Akt rescues hepatocytes from sensitization by LY-294002 to TNF-α- and Fas-mediated apoptosis. To confirm that Akt is involved in the antiapoptotic pathway in TNF-α- and Fas-mediated apoptosis, we used AdmyrAkt (42). After infection with AdmyrAkt, cells were pretreated with LY-294002, followed by treatment with TNF-α or Jo2. Myristylated Akt expression was confirmed by HA Western blotting (Fig. 5A). Constitutively active Akt rescues LY-294002-pretreated cells from TNF-α- and Fas-mediated apoptosis (Fig. 5B). Thus Akt is the downstream effector of PI3K that mediates protection from TNF-α- or Jo2-induced apoptosis.

In addition to nuclear translocation, signal-induced phosphorylation of the RelA subunit of NF-κB is critical for the induction of NF-κB-dependent transcription (1, 31, 52). Activated Akt is reported (30, 31) to stimulate NF-κB-dependent transcription rather than inducing NF-κB nuclear translocation via IκB degradation. To determine the mechanism of the survival effect of Akt on TNF-α- and Fas-mediated apoptosis, we focused on NF-κB activation by Akt. Adenovirus-mediated expression of active Akt did not induce significant NF-κB binding activity (Fig. 6A, lanes 1 and 2). Furthermore, active Akt has no synergistic effect on NF-κB binding activity by TNF-α or Jo2 (Fig. 6A, lanes 3 and 4 and lanes 6 and 7). Adenovirus-mediated IκBsr expression blocked NF-κB activation by TNF-α or Jo2 in hepatocytes expressing active Akt (Fig. 6A, lanes 5 and 8), indicating that Akt is upstream of NF-κB and IκB. Furthermore, AdmyrAkt infection did not induce IκB degradation (Fig. 6B) or IKK activity (data not shown). However, myrAkt markedly induced NF-κB transcriptional activity, whereas control adenovirus AdGFP had no effect, as assayed by the NF-κB luciferase assay (Fig. 6C). These results indicate active Akt induces NF-κB transcriptional activity, but not NF-κB binding activity or IκB degradation, consistent with previous findings (30, 31).

NF-κB is a downstream target of Akt. To explore the signaling pathways of Akt/NF-κB in primary hepatocytes, we tested whether dnNIK, dnIKKα, dnIKKβ, or IκBsr would influence TNF-α-induced NF-κB activation in hepatocytes expressing active myrAkt, using EMSA and IκBs Western blot analysis. Similar experiments could not be performed with Jo2, since Jo2 appears to activate NF-κB independent of IκB. As shown in Figs. 3A and 4C, TNF-α-induced NF-κB binding activity and IκB degradation (Fig. 7, A, lane 2, and B, top left). Active myrAkt alone did not induce NF-κB binding activity but accelerated IκB degradation by TNF-α (Fig. 7, A, lanes 3 and 4, and B, middle left). As expected, IκBsr completely blocked NF-κB activation and IκB degradation by TNF-α in hepatocytes expressing active myrAkt (Fig. 7, A, lane 5, and B, bottom left). Furthermore, expression of either dnIKKβ or dnNIK, but not dnIKKα, blocked NF-κB activation and IκB degradation by TNF-α in hepatocytes expressing active myrAkt (Fig. 7). These results are similar to those obtained from hepatocytes without adenovirus-mediated active myrAkt (19, 20).

Previous studies (19, 20) showed that expression of either deltaNIK or dnIKKβ, but not dnIKKα, sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis. Therefore, we tested the survival effect of active myrAkt on TNF-α- and Fas-mediated cell killing. However, active myrAkt fails to rescue hepatocytes expressing myrAkt by a trypan blue exclusion test. Data are shown as average %viability ± SE of 3 different experiments.
pressing the IκB superrepressor, the truncated form of NIK, or dnIKKβ from TNF-α- and Fas-mediated apoptosis (Fig. 8). These results are consistent with the results shown above, in which expression of either dnIKKβ or deltaNIK blocked NF-κB activation and IκB degradation by TNF-α in hepatocytes expressing active myrAkt. Thus blocking the classic NF-κB translocation pathway eliminates the antiapoptotic function of active Akt.

TNF-α and Jo2 induce antiapoptotic Bcl-xL. Several putative antiapoptotic targets of PI3K/Akt pathway have been proposed, including Bad, caspase-9, and the Forkhead transcriptional factors (8). To determine the involvement of Bad in sensitization by LY-294002 to TNF-α- and Fas-mediated apoptosis, we performed Western blot analysis with phospho-Bad (serine-136), phospho-Bad (serine-112), and total Bad antibodies. Phosphorylated Bad was not detected (data not shown), and total Bad expression was unchanged during TNF-α- and Fas-mediated apoptosis (Fig. 9A). Further pressing the IκB superrepressor, the truncated form of NIK, or dnIKKβ from TNF-α- and Fas-mediated apoptosis (Fig. 8). These results are consistent with the results shown above, in which expression of either dnIKKβ or deltaNIK blocked NF-κB activation and IκB degradation by TNF-α in hepatocytes expressing active myrAkt. Thus blocking the classic NF-κB translocation pathway eliminates the antiapoptotic function of active Akt.

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thermore, active myrAkt had no influence on Bad levels. In addition, the long form of Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein (FLIP) is downregulated in T cells and endothelial cells when they become susceptible to apoptosis (23, 40). Partial hepatectomy exerts its protective effect by maintaining levels of FLIP sufficient to inhibit Fas-induced apoptosis (12). However, pretreatment with LY-294002 or the expression of IκBsr did not affect intracellular FLIP, as demonstrated by Western blotting (data not shown).

TNF-α and Jo2 induce Bcl-xL, an antiapoptotic member of the Bcl2 family. However, in hepatocytes sensitized by LY-294002, the expression of Bcl-xL was reduced after the treatment with TNF-α or Jo2. Furthermore, active myrAkt partially restored the level of Bcl-xL expression especially after Jo2. Importantly, adenoviral-mediated IκBsr expression inhibited Bcl-xL expression, indicating that Bcl-xL is one of the NF-κB-responsive genes (Fig. 9B).

To confirm the protective effect of Bcl-xL in Fas-mediated apoptosis, we isolated hepatocytes from Bcl-xL transgenic mice or wild-type littermates (10) and treated these hepatocytes with Jo2 or TNF-α after sensitization with actinomycin D or AdIκBsr. Overexpression of Bcl-xL in hepatocytes was confirmed with Western blotting (data not shown). Overexpression of Bel-xL protects hepatocytes from Fas- but not TNF-α-mediated apoptosis, compared with hepatocytes from wild-type mice (Fig. 10). Together, these results indicate that Bcl-xL might be a key antiapoptotic NF-κB-responsive gene in Fas-mediated apoptosis in hepatocytes.

DISCUSSION

In this study, we demonstrate that 1) TNF-α and anti-Fas agonistic antibody Jo2 phosphorylate Akt through PI3K, 2) pretreatment with a PI3K inhibitor or by adenovirus-mediated expression of dnAkt reduces NF-κB activation and sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis, 3) the expression of constitutively active Akt rescues PI3K inhibitor-pretreated cells from TNF-α- and Fas-mediated apoptosis, 4) active Akt induces NF-κB transcriptional activity, but not NF-κB binding activity or IκB degradation, 5) active Akt had no effect on dnNIK, dnIKKα, or dnIKKβ, or the IκBsr in TNF-α-induced NF-κB binding activity, IκB degradation, or apoptosis in hepatocytes, and 6) TNF-α and Jo2 induce Bcl-xL through NF-κB activation, which is blocked by PI3K inhibition. These results indicate that the PI3K/Akt pathway has a protective role in death receptor-induced apoptosis mediated in part by NF-κB via Bcl-xL (Fig. 11).

Ozes et al. (36) suggested that Akt is involved in the TNF-α-mediated activation of NF-κB in HeLa cells, implying that the antiapoptotic activity of Akt may be mediated in part through NF-κB. Furthermore, in MCF7 breast carcinoma cells, PI3K and Akt suppressed a dose-dependent induction of apoptosis by TNF-α and stimulated NF-κB activation in a dose-dependent manner, suggesting a common link between these two pathways (4). However, Delhase et al. (11) failed to detect any involvement of Akt in the signaling pathway through which TNF-α leads to NF-κB activation in HeLa cells. In human endothelial cells, TNF-α and IL-1 activate the PI3K/Akt antiapoptotic pathway, but the antiapoptotic effects of Akt are independent of NF-κB (29). Together, the role of Akt and the extent to which it is activated by TNF-α must be cell-type specific.
In our study, Jo2 and, to a lesser extent, TNF-α phosphorylated Akt (Fig. 1A). The PI3K inhibitor LY-294002 blocks TNF-α- and Fas-mediated Akt phosphorylation (Fig. 1C). Pretreatment with LY-294002 blocks TNF-α phosphorylated Akt (Fig. 1A). NF-κB binding activity, NF-κB-mediated apoptosis. IκB, a zinc finger protein, phosphorylated Akt. Akt phosphorylates IκB, leading to its degradation and release of NF-κB into the cytoplasm, where it can bind to DNA and activate transcription of target genes. However, the role of Akt in regulating NF-κB activity is complex and may vary depending on the cell type and context.

Fig. 11. Protective pathways in TNF-α- and Fas-mediated apoptosis in cultured hepatocytes. First, TNF-α and Jo2 activate IκK, which induces IκB phosphorylation and degradation, resulting in NF-κB nuclear translocation. Second, TNF-α and Jo2 phosphorylate Akt through PI3K. Activated Akt induces NF-κB transcriptional activity. Third, TNF-α and Jo2 activate p38 mitogen-activated protein kinase (MAPK), which may induce the cAMP-response element binding protein binding protein (CBP) coactivator to further increase NF-κB transcription. The NF-κB-responsive (NF-κB RE) genes, including Bcl-xL and iNOS, protect hepatocytes from TNF-α- and Fas-mediated apoptosis. Death R, death receptor; p, phosphorylation; MAPKKK, MAPK kinase kinase.

In our study, Jo2 and, to a lesser extent, TNF-α phosphorylated Akt (Fig. 1A). The PI3K inhibitor LY-294002 blocks TNF-α- and Fas-mediated Akt phosphorylation (Fig. 1C). Pretreatment with LY-294002 blocks TNF-α phosphorylated Akt (Fig. 1A). This observation might result from the more potent phosphorylation activity of Akt by Jo2. These results are consistent with the previous reports (41) suggesting TNF-α-mediated PI3K/Akt activation in isolated rat hepatocytes treated with TGF-β is minimal compared with EGF-mediated PI3K/Akt activation. Furthermore, our study revealed the novel finding that PI3K/Akt protects cultured hepatocytes from Fas-mediated apoptosis. Although the mechanism by which Fas stimulates the PI3K/Akt pathway is not clear, there are several studies supporting PI3K/Akt activation as a protective mechanism in Fas-mediated apoptosis. In a rat hepatoma cell line, bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas (47) and some hydrophobic bile acids activate PI3K-dependent survival pathways, which prevents their toxicity (44). The protective effect of hepatocyte growth factor in bile acid-induced apoptosis requires Akt activation (53). PTEN, a phosphatidylinositol phosphatase, acts as a tumor suppressor, at least in part, by antagonizing PI3K/Akt signaling (5). Fas-mediated apoptosis was impaired in PTEN +/- mice, and T lymphocytes from these mice show reduced activation-induced cell death (13). PI3K inhibitors restored Fas responsiveness in PTEN +/- cells (13). These results indicate that PTEN is an essential mediator of the Fas response and a repressor of autoimmunity and thus implicate the PI3K/Akt pathway in Fas-mediated apoptosis.

Pharmacological inhibition of PI3K attenuates NF-κB binding activity, NF-κB transcriptional activity, and NF-κB-mediated gene expression by TNF-α or anti-Fas antibody (Fig. 3). Adenovirus-mediated expression of dnAkt reduces NF-κB activation and sensitizes hepatocytes to TNF-α- and Fas-mediated apoptosis (Fig. 4). The expression of constitutively active Akt rescues PI3K inhibitor-pretreated cells from TNF-α- and Fas-mediated apoptosis (Fig. 5B) and active Akt induces NF-κB transcriptional activity, but not NF-κB binding activity and IκB degradation (Fig. 6). Taken together, active Akt is necessary but not sufficient for optimal death receptor-mediated NF-κB activation.

In HeLa cells, IκB is a target for Akt (36). However, other studies (30, 31, 39, 46) have found no evidence for the involvement of Akt in IκB degradation but rather propose an IκB-independent mechanism in which Akt affects the transcriptional activity of NF-κB. IL-1 stimulates the PI3K-dependent phosphorylation and transactivation of NF-κB, a distinct process from the liberation of NF-κB from its cytoplasmic inhibitor IκB (30, 46). Akt stimulates NF-κB transcriptional activity by phosphorylating the transactivation domain of NF-κB p65 (30, 31). These recent studies (30, 31) are consistent with our observation that active Akt induces NF-κB transcriptional activity but not NF-κB binding activity and IκB degradation in primary hepatocytes.

NF-κB reporter gene activity in HeLa cells is induced by constitutively active Akt, and this is inhibited by dnIκKα (55). Similarly, in our study, expression of either dnIκKβ or dnNIK, but not dnIκKα, blocked NF-κB activation and IκB degradation by TNF-α in hepatocytes expressing active myrAkt. Active myrAkt fails to rescue hepatocytes expressing the IκB superrepressor, dnNIK, and dnIκKβ from TNF-α- and Fas-mediated apoptosis. Together, these observations show that an intact mitogen-activated protein kinase kinase kinase (MAPKKK/IκKβ/NF-κB) is required for the protective effect of activated Akt in cultured hepatocytes. What is the relationship between the classic IκK signaling pathway with nuclear translocation of NF-κB and the increased NF-κB transcriptional activity mediated by Akt? Based on our present study and the recent literature (30, 31, 45), we propose a working
model in Fig. 11. Here the death receptors activate an upstream MAPKKK such as NIK (56) and then the IKK complex, resulting in IκB phosphorylation and degradation, with subsequent release and nuclear translocation of NF-κB. Through a distinct second pathway, Akt is activated by the same death receptors and in turn activates NF-κB transcriptional activity, probably through the phosphorylation of the NF-κB transactivation domain (30, 31, 45). p65 is not a direct substrate for Akt, so unknown intermediate steps are required (30, 31). Furthermore, Akt potentiates the activation of IKK, demonstrating cross talk between these two pathways. Finally, the death receptors activate p38 MAPK in primary hepatocytes, which is required for optimal induction of NF-κB (Hatano, unpublished observations). Recently (30), p38 was shown to increase the transcription of NF-κB-responsive genes by interacting with the coactivator cAMP-response element binding protein binding protein. This model explains why a low level of the NF-κB translocation pathway is required to reveal the Akt-NF-κB transcriptional activation, since NF-κB is required to act as a substrate for activation by the Akt pathway.

Several targets of the PI3K/Akt signaling pathway have been recently identified (8) that may underlie the ability of this regulatory cascade to promote survival. These substrates include components of the intrinsic cell death machinery (Bad, caspase-9, and c-FLIP), transcription factors of the forkhead family, and a kinase (IKK) that regulates the NF-κB transcription factor. Growth factor activation of the PI3K/Akt signaling pathway culminates in the phosphorylation of the Bcl-2 family member Bad, thereby suppressing apoptosis and promoting cell survival (9). Akt phosphorylates Bad in vitro and in vivo and blocks the Bad-induced death of primary neurons in a site-specific manner (9). However, we could not detect phosphorylation of Bad in mouse hepatocytes stimulated by TNF-α, Jo2, or active Akt. Similarly, phosphorylated Bad was not detected in EGF-mediated protection from TNF-α-mediated apoptosis, especially by Fas, including the activation of NF-κB and induction of Bcl-xL. Activation of PI3K/Akt might be a therapeutic target for several types of death factor-related liver diseases.

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