

Rapid Activation of Protein Kinase B/Akt Has a Key Role in Antiapoptotic Signaling during Liver Regeneration

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Liver regeneration is controlled by multiple signaling pathways induced by a variety of growth factors, hormones, and cytokines. Here we report that protein kinase B (PKB)/Akt, part of a key cell survival signaling pathway, is markedly activated after partial hepatectomy (PHX). The antiapoptotic protein Bad, a downstream target of PKB/Akt, is also phosphorylated. This cascade can be activated by various factors in primary hepatocytes, with the strongest activation by insulin and the α_1 -adrenergic agonist phenylephrine (PE), followed by IL-6, epidermal growth factor (EGF), and hepatocyte growth factor (HGF). Pretreatment of cells with the specific PI3 kinase inhibitor LY294002 abolished insulin- or PE-activation of PKB/Akt, suggesting that activation of PKB/Akt is mediated by a PI3 kinase-dependent mechanism. *In vivo* administration of PE, insulin, IL-6, HGF, or EGF to mice markedly stimulated PKB/Akt in the liver, with the strongest stimulation induced by insulin and PE. Moreover, HGF and insulin were able to attenuate transforming growth factor β -induced apoptosis in hepatic cells, and these effects were antagonized by LY294002. Taken together, these findings suggest that rapid activation of PKB/Akt is a key antiapoptotic signaling pathway involved in liver regeneration. © 2000

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Key Words: PKB/Akt; PI3 kinase; Bad; liver regeneration; apoptosis; growth factors.

Although the process of liver regeneration has intrigued scientists for over a century, the underlying molecular mechanisms are still not fully understood.

Abbreviations used: PI3 kinase, phosphatidylinositol 3 kinase; PKB/Akt, protein kinase B/Akt; PHX, partial hepatectomy; α_1 AR, α_1 adrenergic receptor; IL-6, interleukin-6; HGF, hepatocyte growth factor; EGF, epidermal growth factor; TGF β , transforming growth factor- β ; MAPK, mitogen-activated protein kinase.

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Two-thirds partial hepatectomy (PHX) is the most commonly used experimental model to study liver regeneration. After PHX, the concentrations of many growth factors (such as HGF and EGF), cytokines (such as TNF α and IL-6), and hormones (such as norepinephrine) are dramatically elevated in the plasma or within the liver, followed by activation of multiple signaling pathways that initiate and stimulate liver regeneration (reviewed in 1–3). These signaling pathways/proteins include nuclear factor κ B (NF κ B) (4, 5), signal transducer and activator of transcription 3 (STAT3) (6), and mitogen-activated protein kinases (MAPK) (7–10). Observations in knockout mice suggest that the following signaling sequence is critical for initiating liver regeneration: TNF α \rightarrow TNF α receptor \rightarrow NF κ B \rightarrow IL-6 \rightarrow IL-6 receptor \rightarrow Stat3 \rightarrow induction of critical target genes \rightarrow liver regeneration (5, 6). It has also been shown that various growth factors and hormones, as well as partial hepatectomy can activate p42/44 mitogen-activated protein kinase (p42/44 MAPK, also termed Erk1/2), p38 MAPK (also termed p38-RK or p38 stress-activated protein kinase) and c-Jun NH₂-terminal kinase (JNK, also termed p46/54 stress-activated protein kinase) in the liver (7–10). These kinases have been shown to play a pivotal role in cellular growth, transformation, differentiation, and apoptosis in a variety of cell types including hepatocytes (8, 10, 11).

Recently, the phosphatidylinositol 3 kinase (PI3 kinase)/protein kinase B (PKB, also known as Akt) cascade was identified as a key component of survival signaling in a wide variety of cell types (reviewed in 12–14). It has been reported that PI3 kinase can be activated by a wide variety of growth factors, cytokines, and hormones, and activated PI3 kinase can stimulate a number of cellular intermediates, including tyrosine kinases, GTPase activating proteins for small G-proteins, and a number of serine/threonine protein kinases, such as the atypical PKC isoforms, SGK, pp70^{S6K}, and PKB/Akt (15). Among these, PKB/

Akt has been identified as the most important mediator of the PI3 kinase survival signal (16). The mechanism by which PKB/Akt prevents apoptosis is not fully understood, although it is generally believed that PKB/Akt protects cells from apoptosis by phosphorylating BAD on serine residues. Phosphorylated BAD dissociates from Bcl-XL and interacts with the 14-3-3 protein instead. Bcl-XL is then free to exert its activity to prevent apoptosis (reviewed in 13). Although the role of PKB/Akt in suppressing apoptosis has been extensively investigated in a wide variety of cell types, including human hepatocellular carcinoma Hep3B cells (17, 18), little is known about the role of PKB/Akt in liver regeneration. Here we examined the activation of PKB/Akt in the regenerating liver, using a mouse partial hepatectomy model. Our data show that PKB/Akt is rapidly activated in the residual liver tissue, and this activation is a critical cell survival signal during the regenerative process.

MATERIALS AND METHODS

Materials. Anti-PKB/Akt, anti-phospho-PKB/Akt (Ser⁴⁷³), anti-STAT3, anti-phospho-STAT3 (Tyr⁷⁰⁵), anti-Bad, anti-phospho-Bad (Ser¹¹²), anti-GSK3, anti-GSK3 (Ser²¹⁹), anti-p42/44MAPK, and anti-phospho-p42/44MAPK (Tyr²⁰⁴) antibodies, and the PI3 kinase inhibitor LY294002 were obtained from Cell Signaling (Beverly, MA). The following reagents were purchased from Sigma Chemicals (St. Louis, MO): IL-6, HGF, insulin, EGF, and PE. Radiolabeled (γ -³²P)ATP was purchased from DuPont NEN (Boston, MA). HepG2 cells were obtained from ATCC (Rockville, MD).

Partial hepatectomy. Mice were anesthetized with sodium pentobarbital, 50 mg/kg i.p., and the median and left lateral lobes of the liver were ligated at their stem and excised under aseptic conditions. Control mice were subjected to sham operation, which consisted of laparotomy and a brief manipulation of the intestines but not the liver with a cotton swab prior to wound closure. The animals were allowed to recover and were sacrificed by decapitation at the indicated times following surgery.

Western blot analysis. Liver tissue was homogenized in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1% Nonidet P-40, 10% glycerol) and then centrifuged for 10 minutes at 4°C. Protein concentration of the supernatant (protein fraction) was calculated using the Bio-Rad protein assay. An aliquot of 40 μ g of protein was mixed with an equivalent volume of 2 \times protein loading buffer containing β -mercaptoethanol and boiled for 5 min before loading onto an SDS/8% polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies. Membranes were washed with TPBS (0.05% [vol/vol] Tween 20 in phosphate-buffered saline [pH 7.4]) and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

PKB/Akt activity assay. Liver extracts were immunoprecipitated with anti-PKB/Akt antibodies. The resulting immunoprecipitate was then incubated with GSK-3 fusion protein (CGPKGPGRRRRTSS-FAEG) (Cell Signaling, Beverly, MA) in the presence of ATP and kinase buffer (25 mM Tris [pH 7.5], 5 mM β -glycerol phosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂), which allowed the immunoprecipitated Akt to phosphorylate GSK-3. Phosphorylation of

GSK-3 was quantified by Western blotting using a phospho-GSK-3 α/β (Ser21/9) antibody.

Isolation of hepatocytes. Male Sprague-Dawley rats weighing 150–200 g were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the portal vein was cannulated under aseptic conditions. Liver cells were isolated by a collagenase-perfusion protocol as described previously (19). The isolated cells were washed twice and resuspended with Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, NaHCO₃ and 10 mM glucose) containing 1.5% gelatin, and further treated with drugs, cytokines, or growth factors.

Apoptosis assay. Cell apoptosis was detected by 3'-end labeling DNA with terminal deoxynucleotidyl transferase (Life Technologies, NY) as described previously (20). Briefly, genomic DNA was isolated and subjected to labeling reaction, which was performed in a final volume of 50 μ l, containing 31 μ l of DNA, 10 μ l of 5 \times reaction buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6), 5 μ l CoCl₂, 5 μ l [α -³²P]-ddATP (17 pmol; 50 μ Ci), and 1 μ l (25 U) terminal transferase enzyme. The reaction was allowed to proceed for 60 min at 37°C, and terminated by addition of 5 μ l of 0.25 M EDTA (pH 8.0). Labeled DNA was separated from unincorporated radionucleotide by using Chroma spin column (Clontech, Palo Alto, CA). The labeled sample is loaded onto a 2% agarose gel and separated by electrophoresis. The gel is then dried and exposed to X-ray film. The amount of radiolabeled ddATP incorporated into the high (>20 kb) and low (<20 kb) molecular weight DNA fractions is quantified by cutting the respective fraction of DNA from the dried gel and counting for 1 min in a β -counter.

RESULTS

Rapid Activation of PKB/Akt during Liver Regeneration Induced by Partial Hepatectomy

To test whether PKB/Akt is activated during liver regeneration, mice were subjected to sham operation or partial hepatectomy (PHX). Extracts of the remnant liver were assayed for PKB/Akt phosphorylation by using Western blot analysis with an anti-phospho-PKB/Akt antibody. As shown in Fig. 1A, sham operation caused a slight increase in PKB/Akt phosphorylation (2- to 3-fold, quantified in Fig. 1D), whereas PHX induced PKB/Akt phosphorylation 7- to 8-fold (Fig. 1B, quantified in Fig. 1D). This was already evident at 30 min and maximum stimulation was observed between 2 and 24 hours after PHX. The levels of PKB/Akt protein expression were not significantly changed after PHX (3rd panel in Fig. 1B). PKB/Akt activity was also determined by using GSK as a substrate. As shown in Fig. 1C, PKB/Akt activities were markedly elevated after PHX, which is consistent with the rapid PKB/Akt phosphorylation after PHX as demonstrated in Fig. 1B. During the course of this study, we noticed that stress also slightly stimulated PKB/Akt activation in the liver (data not shown). To minimize the effect of stress, mice were kept in individual cages and remained in the laboratory overnight before surgery. Interestingly, both PHX and sham operation markedly induced STAT3 tyrosine phosphorylation (see Figs. 1A and B), which is consistent with previous reports (21, 22).

Activation of Bad, which is a downstream target of PKB/Akt, was also examined by Western blotting us-

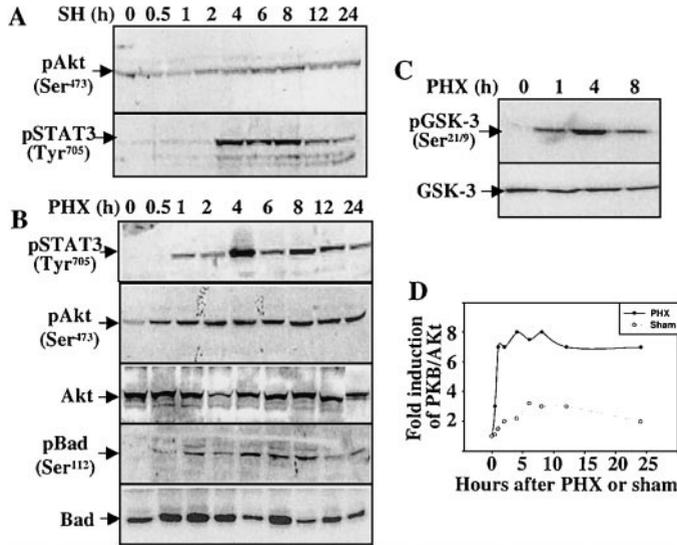


FIG. 1. Partial hepatectomy activates PKB/Akt and Bad. Adult ICR mice (25–30 g) were subjected to two-thirds partial hepatectomy (PHX) or sham operation (SH) as described under Materials and Methods. After various time periods as indicated, total protein extracts from the remnant livers were subjected to Western blotting analysis using antibodies as indicated in A and B, or kinase assay for PKB/Akt activity in panel (C) as described under Materials and Methods. (D). The densities on blots in A and B were quantitated from two independent experiments and plotted. Autoradiograms shown are representative of two independent experiments.

ing an anti-phospho-Bad antibody. As shown in Fig. 1B, PHX resulted in rapid and marked phosphorylation of Bad at residue 112 (4th panel in Fig. 1B). Interestingly, there was a transient increase in the levels of Bad protein between 0.5 and 8 h following PHX (5th panel in Fig. 1B), which is consistent with a previous report (23).

Activation of PKB/Akt in Hepatocytes *In Vitro* and *In Vivo*

Many growth factors, cytokines, and hormones are markedly elevated after PHX (reviewed in 1–3). To test which factors may be involved in the activation of PKB/Akt during liver regeneration, primary hepatocytes were stimulated with various agonist for 10 min. As shown in Fig. 2A, a number of agents markedly induced PKB/Akt activation, with the strongest stimulation exhibited by insulin and the α 1-adrenergic agonist phenylephrine (PE). Both EGF and HGF slightly activated PKB/Akt while they markedly activated p42/44 MAP kinase. IL-6 also slightly stimulated PKB/Akt phosphorylation. The kinetics of PKB/Akt activation induced by PE and insulin was further examined in Fig. 2B. PE treatment rapidly induced PKB/Akt activation, with the peak effect occurring between 10–60 min and returning to control levels after 120 min. Insulin also rapidly stimulated PKB/Akt activation with a peak effect observed at 20 min. To examine

whether PKB/Akt activation is mediated by PI3 kinase, the specific PI3 kinase inhibitor LY294002 was used. As shown in Fig. 2C, inhibition of PI3 kinase significantly attenuated PE- or insulin-activation of PKB/Akt. These findings suggest that activation of PKB/Akt is mediated through a PI3 kinase-dependent mechanism.

To determine whether these agonists activate PKB/Akt *in vivo*, mice were injected intravenously with PE, IL-6, HGF, EGF, or insulin at various intervals before sacrificing the animals. Liver extracts were then prepared and examined for PKB/Akt phosphorylation. As shown in Fig. 2D, all of the agonists tested induced PKB/Akt activation in the liver with the strongest stimulation again observed with PE and insulin, which is consistent with the above *in vitro* data.

Activation of PKB/Akt Is a Critical Cell Survival Signal in Hepatocytes

To determine the role of PKB/Akt in liver regeneration, we examined the effects of LY294002, a specific PI3 kinase inhibitor, which has been shown to inhibit PKB/Akt activation (Fig. 2C), on hepatocyte apoptosis *in vitro*. As shown in Fig. 3, when HepG2 cells were treated with TGF- β , significant apoptosis was observed, whereas pretreatment with insulin or HGF markedly prevented TGF β -induced apoptosis. Blocking PKB/Akt activation by LY294002 markedly antag-

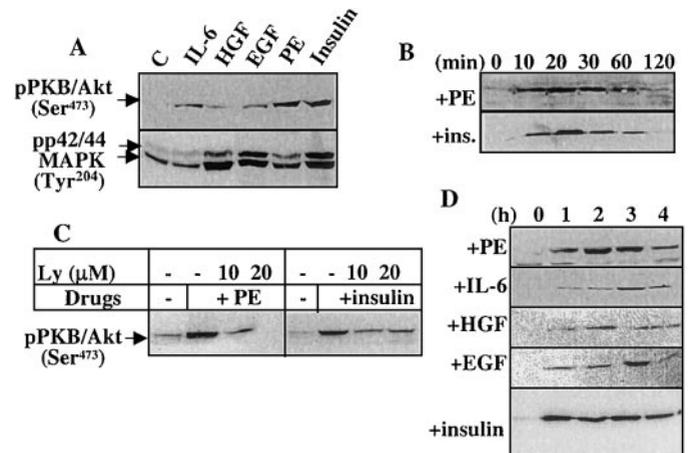


FIG. 2. Various factors activate PKB/Akt in primary hepatocytes *in vitro* and in the liver *in vivo*. (A) Freshly isolated adult rat hepatocytes were stimulated with IL-6 (10 ng/ml), HGF (10 ng/ml), EGF (10 ng/ml), phenylephrine (10^{-6} M), or insulin (5 μ g/ml) for 10 min (A), or stimulated with PE or insulin for various time periods (B). (C) Hepatocytes were pretreated with various concentrations of LY 294002 for 30 min, followed by a 20-min stimulation with PE or insulin. (D) Mice were injected intravenously with PE (100 ng/g body weight [bwt]), IL-6 (80 ng/g bwt), HGF (40 ng/g bwt), EGF (200 ng/g bwt), or insulin (200 ng/g bwt) for various time periods. Total cell or liver extracts in A–D were prepared and subjected to Western analysis by using an anti-phospho-Akt (Ser⁴⁷³) antibody. Autoradiograms shown are representative of two or three independent experiments.

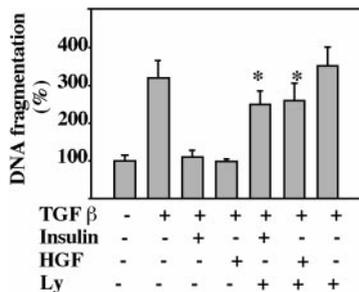


FIG. 3. Evidence for the involvement of PI3 kinase/PKB/Akt in antiapoptosis in hepatic cells. HepG2 cells were incubated with or without LY294002 (25 μ M), then stimulated with insulin or HGF for 30 min, followed by a 24-h treatment with TGF- β (5 ng/ml). DNA was isolated and subjected to DNA fragmentation assay as described under Materials and Methods. Values shown are means \pm SE from three independent experiments, expressed % radioactivity relative to the control. * ($P < 0.01$), significant difference from the corresponding insulin plus TGF β or HGF plus TGF β groups.

onized the protective role of insulin or HGF on TGF β -induced apoptosis. These findings suggest that activation of PKB/Akt is a critical survival signal for hepatocytes.

DISCUSSION

It has been shown that TGF β can induce hepatocyte apoptosis *in vitro* (24, 25) and *in vivo* (26, 27). Although the expression of TGF β and its receptors is markedly increased during liver regeneration (28–30), no significant apoptosis was observed during the regenerative process. Recently, Desbarats and Newell (31) demonstrated that regenerating hepatocytes were resistant to Fas-induced apoptosis. Taken together, these findings suggest that the apoptotic pathways are suppressed during liver regeneration, although the underlying mechanism remains unknown. Rapid activation of NF κ B and STAT3 has been implicated in protecting hepatocytes from apoptosis during liver regeneration (17, 18, 32, 33). Here we provide the first evidence that PKB/Akt is rapidly activated during liver regeneration and activation of PKB/Akt protects hepatocytes from TGF- β -induced apoptosis *in vitro* (Fig. 3, and Refs. 17, 18, 34–36). This suggests that rapid activation of PKB/Akt is another critical cell survival signal that prevents hepatocyte apoptosis during liver regeneration.

Activation of PKB/Akt has been shown to protect a wide variety of cells from apoptosis, but the underlying mechanism is not clear. Several downstream targets of PKB/Akt (such as E2F, GSK3 β , PFK2, c-Myc, caspase-9, IKK α , Raf, eNOS, Bad, etc.) have been implicated in cell cycle progression and anti-apoptosis (13). Among these, the phosphorylation of BAD and its consequent dissociation from Bcl-Xl is the most important signaling event linked to the antiapoptotic effect of PKB/Akt (16). This pathway is probably also impli-

cated in the observed effect of PKB/Akt in the regenerating liver, because BAD was rapidly phosphorylated after PHX as shown in Fig. 1. It has been shown that Bcl-X mRNA as well as protein levels are markedly elevated after PHX (37, 38). Therefore, the antiapoptotic effects of Bcl-X after PHX may be due not only to its dissociation from phosphorylated BAD, but also to an increase in its gene expression. Further studies are required to clarify whether other downstream targets of PKB/Akt are also involved in protecting hepatocytes from apoptosis.

The mechanism by which PHX activates PKB/Akt was also explored in the present study. We demonstrated that several biological mediators including HGF, insulin, EGF, IL-6 and phenylephrine activate PKB/Akt in hepatocytes *in vitro* and *in vivo*. Since the levels of many of these factors (including norepinephrine, the endogenous counterpart of phenylephrine) are dramatically elevated in the plasma or within the liver after PHX (1, 2), they may contribute to the activation of PKB/Akt during liver regeneration. Among the compounds tested, insulin and phenylephrine induced the strongest activation of PKB/Akt both *in vitro* and *in vivo*. Whereas plasma levels of norepinephrine are markedly elevated, plasma insulin levels are slightly decreased after PHX (39, 40). This suggests that norepinephrine, but not insulin, may be one of the major factors involved in rapid activation PKB/Akt during liver regeneration.

In summary, our data described here demonstrate for the first time that PKB/Akt is rapidly activated and plays an important role in antiapoptosis during liver regeneration, and multiple factors including norepinephrine, HGF, EGF, and IL-6 may contribute to this activation *in vivo*. PKB/Akt has also been implicated in the control of a variety of metabolic processes in the liver (12–14, 41), and its rapid activation may thus play an important, albeit yet undefined, role in maintaining metabolic homeostasis during liver regeneration.

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