Activation of Protein Kinase B/Akt Is Sufficient to Repress the Glucocorticoid and cAMP Induction of Phosphoenolpyruvate Carboxykinase Gene

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A rat hepatoma cell line, H4IIE, was stably transfected with a tamoxifen regulatable Akt-1 construct. Treatment of these cells with tamoxifen caused a rapid stimulation of Akt enzymatic activity that was comparable with the activity observed with the endogenous Akt after insulin stimulation. Prior studies have extensively documented that insulin can repress the glucocorticoid and cAMP-stimulated increase in phosphoenolpyruvate carboxykinase (PEPCK) gene transcription. Activation of this regulatable Akt with tamoxifen was found to mimic the dominant inhibitory effect of insulin on PEPCK gene transcription. Dose response curves to insulin and tamoxifen demonstrated that this response was very sensitive to Akt activation although the maximal response observed with tamoxifen activation was slightly less than that observed with insulin, indicating that the response to insulin may also involve other signaling cascades. The regulation of PEPCK transcription via Akt was, like that previously described for insulin, not dependent upon 70 kDa S6 kinase activity in that it was not inhibited by rapamycin. Finally, the expression of a kinase dead Akt was able to partially inhibit the ability of insulin to stimulate this response. In summary, the present results indicate that activation of Akt alone is sufficient to repress the glucocorticoid and cAMP-stimulated increase in PEPCK gene transcription.

Insulin regulates the expression of more than 100 genes (1). One of the most extensively studied transcription processes regulated by insulin is the ability of insulin to repress the induction of phosphoenolpyruvate carboxykinase (PEPCK) mRNA by glucocorticoids and cAMP (1, 2). This response to insulin is important in regulating hepatic glucose output because PEPCK is the rate-limiting step in hepatic gluconeogenesis, and PEPCK activity is primarily regulated at the transcriptional level (1, 2).

Insulin inhibition of the glucocorticoid and cAMP induction of PEPCK mRNA has been shown to be mediated via the activation of the lipid kinase, phosphatidylinositol 3-kinase (3). Recent studies have documented that insulin activates this enzyme by stimulating the tyrosine phosphorylation of a number of endogenous substrates (including insulin receptor substrates 1, 2, and 3), and these tyrosine-phosphorylated endogenous substrates bind and activate the PI 3-kinase, resulting in the generation of the phospholipids phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate (4, 5). In addition to playing a role in the regulation of PEPCK mRNA, the activation of PI 3-kinase has been found to play a role in a number of other insulin-stimulated biological responses including stimulation of glucose uptake and GLUT4 translocation, activation of the 70-kDa S6 kinase, stimulation of glycogen synthesis and in the inhibition of apoptosis (4–7).

In contrast, activation of the 70-kDa S6 kinase and the estrogen-activated protein kinase cascade were found not to be required for the repression of PEPCK gene transcription by insulin (3, 8, 9).

Recent studies have identified a number of downstream targets of the phospholipid products of the PI 3-kinase. These include a serine/threonine kinase named Akt or PKB as well as several isoforms of the protein kinase C family of serine/threonine kinases (6, 10, 11). In addition, the PI 3-lipids also bind and activate other classes of molecules including an ADP-ribosylation exchange factor named GRP1 (12). To determine which biological responses can be mediated via the activation of the Akt serine/threonine kinase, we have designed a regulatable Akt with tamoxifen was found to mimic the dominant inhibitory effect of insulin on PEPCK gene transcription. Dose response curves to insulin and tamoxifen demonstrated that this response was very sensitive to Akt activation although the maximal response observed with tamoxifen activation was slightly less than that observed with insulin, indicating that the response to insulin may also involve other signaling cascades. The regulation of PEPCK transcription via Akt was, like that previously described for insulin, not dependent upon 70 kDa S6 kinase activity in that it was not inhibited by rapamycin. Finally, the expression of a kinase dead Akt was able to partially inhibit the ability of insulin to stimulate this response. In summary, the present results indicate that activation of Akt alone is sufficient to repress the glucocorticoid and cAMP-stimulated increase in PEPCK gene transcription.

Insulin inhibition of the glucocorticoid and cAMP induction of PEPCK mRNA has been shown to be mediated via the activation of the lipid kinase, phosphatidylinositol 3-kinase (3).

EXPERIMENTAL PROCEDURES

Materials—The following were purchased: [γ-32P]ATP from NEN Life Science Products; insulin from Boehringer Mannheim; 8-bromoadenosine 3′,5′-cyclic monophosphate and dexamethasone from Sigma; hydroxytamoxifen from Research Biochemicals; LY294002 from Alexis Biochemicals; rapamycin and basicidin S from Calbiochem; polyclonal antibodies to p70/p85 ribosomal S6 protein kinase from Upstate Biotechnology; monoclonal antibodies to Akt from Transduction Laboratories; Anti-HA monoclonal antibody 12CA5 from Boehringer Mannheim; and G418 from Life Technologies, Inc. The Phoenix retroviral packaging cell line and the retroviral vectors were gifts of Dr. Garry Nolan. Plasmid Constructions—The pWZL-neo retroviral vector encoding MER-Akt was as described (13). An inactive Akt was made by site-directed mutagenesis of the important regulatory phosphorylation sites threonine 308 and serine 473 to alanine in the plasmid encoding the
Akt/Protein Kinase B and PEPCK Gene Expression

RESULTS

H4IIE hepatoma cells stably expressing a tamoxifen-regulatable form of Akt-1 were developed by infecting these cells with retroviruses encoding MER-Akt (13) and selecting those cells that became resistant to the drug-selectable marker encoded by the retrovirus. The amount of tamoxifen-regulatable Akt enzymatic activity expressed in these cells was compared with the amount of insulin-stimulated endogenous Akt present in these cells. To this end, cells were treated with different concentrations of either insulin or tamoxifen, lysed, and either the endogenous or expressed Akt chimeras were precipitated with specific antibodies to each molecule. The amount of enzymatic activity in the precipitates was then determined via the use of a synthetic peptide-based assay specific for the expressed Akt. The expressed MER-Akt and endogenous Akt.

Because PEPCK mRNA transcription is low in the absence of any stimulus, we studied the ability of insulin and Akt to repress the induction of PEPCK mRNA stimulated by a combination of a nonhydrolyzable cAMP analog and the synthetic glucocorticoid dexamethasone. The levels of endogenous PEPCK mRNA were monitored by primer extension analysis. As previously reported (1), insulin completely inhibited the induction of PEPCK mRNA by the dexamethasone/cAMP treatment (Fig. 2). Activation of the regulatable Akt by tamoxifen was also capable of largely inhibiting the increase in mRNA caused by the dexamethasone/cAMP treatment in the cells expressing the construct encoding the chimeric MER-Akt (Fig. 2). In contrast, tamoxifen had no effect on the induction of the PEPCK mRNA in control cells not expressing the regulatable full-length Akt (14). This plasmid was then subcloned into the retroviral vector pBMN-Z-I-Blasto (a gift from Garry P. Nolan).

Retroviral Infections—H4IIE cells were infected with pWZLneo/ MER-Akt or pBMN-Z-I-Blasto/Akt T308A, S473A as described previously (14). The former were selected by using 1 mg/ml of G418, whereas the latter were selected by using 10 μg/ml of blasticidin S.

Cell Culture and Hormone Treatments—Cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose, 2.5% fetal calf serum and 2.5% newborn calf serum in an atmosphere of 5% CO₂, 95% air. Before treatment, cells were deprived of serum overnight in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin. Cells were then incubated with hormones and inhibitors as indicated in the figure legends.

Akt Kinase Assays and Immunoblotting—The expressed MER-Akt was specifically immunoprecipitated by using a monoclonal antibody to the HA-tag present on this enzyme. The endogenous Akt was specifically precipitated by using a polyclonal antibody to the Akt-1 pleckstrin homology domain (15), because the MER-Akt does not contain the pleckstrin homology domain (15). Western blots were performed with either the monoclonal antibody to HA (for the expressed MER-Akt) or with a monoclonal antibody to Akt-1 (for the endogenous Akt). Akt kinase assays were performed with the GSK-3 peptide (sequence GRPRTSFAEG) (16), and the phosphorylation of the peptide was monitored by analyzing the samples on a 6% urea, 40% polyacrylamide gel as described previously (13).

RNA Isolation and Primer Extension Analysis—Total cellular RNA was isolated by using the Qiagen RNeasy Mini Kit as detailed in their manual. Primer extension analyses for the PEPCK and β-actin mRNAs were performed essesntially as described (17). In brief, oligonucleotides PC28 (5'-GGAGAGAGGGAGACTCTGACCGACC-3') and ACT25 (5'-GGGATGCGGACCAGGAGGATGCT-3') (complementary to positions +102 to +129 and positions +42 to +67, relative to the transcription start sites in PEPCK and β-actin, respectively) (18, 19) were used to amplify the 5'-end RNA. These fragments were then labeled with [γ-32P]ATP and then incubated with 10 μg of total cellular RNA. After the primer extension reaction, the products were analyzed by electrophoresis on a 7.5% urea, 6% polyacrylamide gel, visualized by autoradiography, and quantified by PhosphorImager analysis.

Akt (Fig. 2), demonstrating that this effect of tamoxifen was mediated via the expressed MER-Akt.

To better compare the repression of PEPCK mRNA induction with tamoxifen and insulin, a dose curve was performed with different concentrations of insulin and tamoxifen (Fig. 3). As previously reported for insulin (17), the inhibition of PEPCK mRNA transcription was exquisitely sensitive to insulin, with 0.1 nM insulin causing almost a maximal response. The response to tamoxifen was also quite sensitive with 1 nM tamoxifen also causing an almost maximal response. The maximal inhibition observed with insulin was greater than that observed with tamoxifen (Fig. 3).

Because previous studies have demonstrated that Akt can also activate the 70-kDa S6 kinase (20), we sought to determine whether this enzyme was playing a role in the Akt regulation of PEPCK mRNA. Cells were therefore stimulated with tamoxifen in the presence or absence of rapamycin, an inhibitor of the activation of the 70-kDa S6 kinase (21, 22). Rapamycin was found to have only a slight effect on the ability of Akt to regulate PEPCK mRNA, similar to that observed with insulin (Fig. 4). Controls verified that rapamycin completely prevented the activation of the 70-kDa S6 kinase by insulin and MER-Akt as monitored by a shift in the migration of the 70-kDa S6 kinase (Fig. 5). As expected, rapamycin had no effect on the activation of the enzymatic activity of either the endogenous Akt or the chimeric MER-Akt (Fig. 5). In contrast, a specific
inhibitor of the PI 3-kinase (23), LY294002, completely inhibited the ability of both insulin and Akt to repress PEPCK mRNA transcription (Fig. 4). This inhibition correlated with the ability of this compound to block the activation of the enzymatic activity of both the endogenous and the expressed MER-Akt by insulin and tamoxifen, respectively (Fig. 5).

Finally, to test the role of endogenous Akt in mediating the insulin-stimulated repression of PEPCK mRNA, we expressed a kinase dead Akt in the H4IIE cells. This mutant Akt has had its two regulatory phosphorylation sites (24) (threonine 308 and serine 473) changed to alanine and thus is enzymatically inactive (24). The expression of this kinase dead Akt partially inhibited the ability of insulin to repress the dexamethasone/cAMP induction of PEPCK mRNA (Fig. 6).

**DISCUSSION**

Recent studies have documented that insulin activates PI 3-kinase, and this enzymatic activity appears to be required for insulin to elicit several of its biological responses including the regulation of the PEPCK mRNA (3–8). However, the step after activation of PI 3-kinase, which is responsible for the regulation of PEPCK mRNA, is not known. A number of serine/threonine kinases have been shown to be activated by the PI 3-kinase pathway. These include ERK1/ERK2 (at least in some cell types) (25), the 70-kDa S6 kinase (26), the family of Akt kinases (10) and at least several isoforms of protein kinase including PKCδ and λ (6, 11). Studies with inhibitors of the ERK1/ERK2 and the 70-kDa S6 kinase cascades have shown that it is unlikely that these enzymes play a role in mediating the ability of insulin to regulate PEPCK gene expression (3, 8, 9).

In the present work, we have examined the ability of the serine/threonine kinase Akt to regulate PEPCK mRNA. To do
this, a line of H4IIE hepatoma cells was constructed which expressed a tamoxifen-regulatable Akt-1. These cells exhibited a rapid and large increase in Akt enzymatic activity after tamoxifen stimulation. The amount of expressed activity after tamoxifen stimulation as assessed in vitro with an exogenous substrate appears to be comparable with that of the endogenous Akt-1 stimulated with insulin. In addition, these cells also appear to have an insulin-stimulated Akt-3 at approximately one-third the levels of the Akt-1 stimulated with insulin. In addition, these cells also appear to have an insulin-stimulated Akt-3 at approximately one-third the levels of the endogenous Akt (lanes 1–4), or a monoclonal antibody to the HA-tag on the expressed Akt chimeras (lanes 5–8). The immunoprecipitates were assayed for Akt enzymatic activity by using the GSK-3 peptide and then analyzed for Akt by Western blotting. Panel B shows a representative Western blot of the precipitates and panel C shows a representative GSK-3 assay. The means ± S.E. of three different experiments are shown in panel D.

The activation of the regulatable Akt with tamoxifen treatment was found to be sufficient to repress the dexamethasone/cAMP-stimulated increase in PEPCK mRNA. This response was observed with even a submaximal stimulatory dose of tamoxifen, in agreement with the data with the insulin stimulation. Also like the insulin regulation of PEPCK mRNA, the response to tamoxifen was not inhibited by rapamycin, an inhibitor of the activation of the 70-kDa S6 kinase (21, 22). The response to tamoxifen required the presence of the MER-Akt, because cells not expressing this construct showed no response to the tamoxifen. The maximal response observed with tamoxifen was less than the maximal response observed with insulin, possibly suggesting that the response to insulin may in part be mediated through a combination of signaling pathways. Alternatively, it is possible that this chimeric Akt molecule does not signal as efficiently as the endogenous Akt. The finding that overexpression of a kinase inactive Akt at least partly inhibits the ability of insulin to regulate PEPCK mRNA is consistent with a role for the endogenous Akt in mediating the normal insulin response.

(ii) Akt and the PEPCK gene.

(a) Akt overexpression. The activation of various insulin-stimulated biological responses. It is also consistent with a recent report that transient overexpression of a constitutively active Akt can negatively regulate the basal expression of a reporter construct containing a conserved insulin response sequence from the promoter of the insulin-like growth factor binding protein-1 gene (30). These authors also showed that this constitutively active Akt regulated the expression of a conserved sequence from the promoter of the PEPCK gene. The present work extends this previous

Fig. 5. Effects of the PI 3-kinase inhibitor LY294002 and the S6 kinase inhibitor rapamycin on Akt enzymatic activity and the 70-kDa S6 kinase shift. H4IIE/MER-Akt cells were treated with either buffer, 50 μM LY294002 (LT) or 200 nM rapamycin (Rapa) for 1 h before the addition of either buffer, insulin, or tamoxifen (HT). After an additional 30 min of incubation, the cells were lysed, and a fraction of the total lysate was used to monitor the shift in p70 S6 kinase migration by Western blotting (top panel). The remainder of the cell lysates were immunoprecipitated with either an antibody to the endogenous Akt (lanes 1–4), or a monoclonal antibody to the HA-tag on the expressed Akt chimeras (lanes 5–8). The immunoprecipitates were assayed for Akt enzymatic activity by using the GSK-3 peptide and then analyzed for Akt by Western blotting. Panel B shows a representative Western blot of the precipitates and panel C shows a representative GSK-3 assay. The means ± S.E. of three different experiments are shown in panel D.

Fig. 6. Expression of an inactive Akt-1 partially inhibits the ability of insulin to regulate PEPCK mRNA. H4IIE cells were infected with a retroviral vector encoding an inactive Akt (pBMNZIB-Akt1T308A, S473A), drug selected, and the cells that were resistant were grown up. The levels of the expressed inactive Akt are shown in panel A by Western blotting of total cell lysates with an anti-HA epitope antibody. H4IIE/mer-Akt cells or H4IIE/Akt T308A, S473A cells were treated with either buffer, dexamethasone, and cAMP (Dex/cAMP) and either buffer or insulin for 3 h. Total RNA was isolated and primer extension analyses were performed. The amount of PEPCK mRNA present was expressed as a percentage of the nontreated control cells, and the results shown in panel B are means ± S.E. of three different experiments. Where not shown, the error bars were below the amount detectable.

K. Nakatani, J. Liao, and R.A. Roth, unpublished studies.
study because it demonstrates for the first time that transcription of the endogenous PEPCK gene can also be regulated via Akt. In addition, we show that Akt can repress the induction of PEPCK transcription by dexamethasone and cAMP. And finally, the present studies demonstrate that these effects of Akt occur with Akt enzymatic activities comparable with those induced by insulin. During the course of the review of this manuscript, Agati et al. (31) also reported the results of studies examining the ability of transiently expressed Akt to regulate a luciferase reporter construct containing the promoter region \((-600/+69)\) of the PEPCK gene. In these studies the authors found that a constitutively active Akt did inhibit the cAMP kinase-stimulated increase in the expression of the reporter construct by approximately 50%. The remaining expression of this construct was further inhibited by insulin treatment. In addition, these authors did not observe any inhibition of the insulin effect on this reporter construct after the transient expression of three different defective Akt constructs. These results were interpreted to indicate that Akt was not involved in the insulin inhibition of PKA-induced PEPCK gene expression. Because these studies only utilized transient transfections, it was impossible to determine how much Akt enzymatic activity was expressed with the constitutively active form of the enzyme relative to what was stimulated by insulin. In addition, because only a few percent of the cells expressed these constructs, the authors could not determine whether the defective Akt constructs actually inhibited the insulin-stimulated activation of the endogenous Akt. Recent studies have shown that inhibition of endogenous Akt by these defective Akt constructs is problematic (32). In addition, as demonstrated in the present work, only a very low level of Akt enzymatic activity is sufficient to repress the PEPCK gene transcription. The blockage of the insulin response would therefore require that the expression of the defective Akt almost completely inhibit the activation of the endogenous Akt. Thus, the role of Akt in mediating a particular biological response is difficult to rule out via the use of transient transfections of defective Akt molecules.

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