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Phosphatidylinositol 3-Kinase Is Required for the Regulation of Hepatitis B Surface Antigen Production and Mitogen-Activated Protein Kinase Activation by Insulin but Not by TPA

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Insulin suppresses hepatitis B surface antigen (HBsAg) gene expression and stimulates cell proliferation in human hepatoma Hep3B cells. 12-*O*-tetradecanoyl phorbol-13-acetate, TPA, has been demonstrated to mimic insulin actions in these cells. We examined the role of phosphatidylinositol 3-kinase (PI 3-kinase) in the signaling pathways of insulin and TPA towards these two biological phenomena in Hep3B cells. The pre-treatment of 5 μM of wortmannin diminished insulin suppressed HBsAg production and completely abolished insulin stimulated cell proliferation. However, wortmannin had no effect on TPA actions in both HBsAg suppression and cell growth stimulation. We further investigated the effect of wortmannin in mitogen-activated protein kinases (MAPKs) activation induced by insulin or TPA. After the pretreatment of wortmannin, insulin activated MAPKs was completely blocked, but TPA was still capable to activate MAPKs. These results suggest that PI 3-kinase is involved in insulin actions but not in TPA effects, and allow us to dissociate the signaling pathways of insulin and TPA in human hepatoma Hep3B cells.

Insulin induces a wide variety of physiological responses, including modulation of glucose, amino acid transport, specific gene expression, cellular growth and differentiation (1). These cellular events are elicited by the binding of insulin to a specific cell surface receptor which activates the protein tyrosine kinase intrinsic to the β-subunit of the receptor (2,3). Two immediate substrates of the insulin receptor kinase, the insulin receptor substrate-1 and 2 (IRS-1 and IRS-2), have been identified (4–6). The IRS-1 protein can be phosphorylated by the activated insulin receptor kinase and then acts as a docking protein for the binding of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-K) and growth factor receptor binding protein-2 (GRB2) through their Src homology-2 (SH2) domain (7–9), while IRS-2 mediates insulin signaling through a different kinetic in the interaction with PI 3-kinase and GRB2 (10,11). Interactions between activated insulin receptor and downstream targets trigger the phosphorylation cascade of cellular signaling components, leading to the induction of different cellular events (12–14).

PI 3-K consists of two subunits, a p110 catalytic subunit and a p85 regulatory subunit which contains two SH2 domains. In the insulin signaling pathways, the activity of p110 catalytic subunit is stimulated by the interaction of SH2 domains of p85 subunit with tyrosine-phosphorylated IRS-1 and IRS-2. Microinjection of the SH2 domain of p85 subunit inhibits insulin induced DNA synthesis and c-fos expression (15). This kinase was also demonstrated to be involved in NFAT activation in T cells (16). The importance of PI 3-K in receptor tyrosine kinase and cytokine receptors signalings have been intensively studied (17, 18).

Human hepatoma Hep3B cell line contains an integrated hepatitis B virus (HBV) genome, which constantly expresses and secretes hepatitis B surface antigen (HBsAg) into culture media. The HBsAg suppression appears to be one of the few cases in insulin regulated gene expression comparing to its activation effects (19, 20). We have demonstrated that both insulin and TPA stimulate Hep3B cell proliferation through a PKC-α dependent pathway, while insulin suppresses HBsAg production through a PKC independent pathway (21). Herein, we used wortmannin to further clarify the role of PI 3-K relative to PKC in insulin signaling towards these two phenomena and demonstrated that PI 3-K is so far the only signaling component identified in the regulation of HBsAg production by insulin.

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MATERIALS AND METHODS

Cell culture. Human hepatoma Hep3B cells were kindly given to us by Dr. B. Knowles (Wista Institute, PA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% fetal calf serum (GIBCO) in an atmosphere of 5% CO_2, 95% air at 37°C.

Assay for secreted HBsAg. Culture fluids were collected and the presence of HBsAg was measured using an enzyme immunoassay kit (Abbott Austria II).

Western blot analysis of mitogen-activated protein kinase. Hep3B cells were seeded and serum starved for 48 hr. These cells were then pretreated with 5 μM of wortmannin or 0.5% DMSO as a control for 10 min. After the pretreatment, the cells were exposed to 10^{-8} M of insulin or 50 ng/ml of TPA for 10 min or 5 min, respectively. The treated cells were then washed in PBS [pH7.4] and lysed using lysis buffer containing 50 mM Hepes, [pH7.8]; 4 mM EDTA; 2 mM EGTA; 5 mg/ml leupeptin; 2 mM PMSF; 5 mg/ml aprotinin and 1% Triton X-100. Cell lysates were centrifuged at 100,000 $\times$g for 30 min. at 4°C. The supernatant (100 μg of protein) was resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose in transfer solution containing 25 mM Tris; 192 mM glycine; 20% methanol. The blot was incubated overnight in buffer containing 25 mM Tris, [pH8.0]; 125 mM NaCl; 0.05% Tween 20; 0.1% sodium azide and 3% skim milk. The nitrocellulose paper was then probed with anti-MAPKs polyclonal antibody for 3 hr at room temperature. The filter was washed three times with Tris-buffered saline (TBS, 25 mM Tris, [pH8.0]; 125 mM NaCl), and the mobility shift of MAPKs was detected using the enhanced chemiluminescence (ECL) detection system (Amersham).

Materials. Recombinant human insulin was a gift from the Eli Lilly Co. Fetal calf serum and culture medium were from GIBCO. Other chemicals were from Sigma.

RESULTS AND DISCUSSION

Wortmannin has been shown to be a PI 3-K activity inhibitor and blocks many functional responses in different cell systems (22, 23). To study the involvement of PI 3-K in insulin and TPA actions, we treated the cells with wortmannin, and observed its effects on cell proliferation and HBsAg production. We pretreated Hep3B cells with 5 μM of wortmannin for 10 min and then exposed the cells to various concentrations of insulin or TPA in the presence of wortmannin, for further 48 hr. As shown in Fig. 1A, insulin stimulated cell proliferation in a dose-dependent manner in the control cells, but the insulin effect was blocked in wortmannin-pretreated cells. However, TPA was able to stimulate cell proliferation in both control and wortmannin-pretreated cells (Fig. 1B). From our result, we found that PI 3-K might be involved in insulin stimulated cell proliferation but not in TPA effect. The consistence of tyrosine phosphorylation patterns after insulin stimulation in both treated and non-treated cells suggested that the wortmannin pretreatment does not diminish the tyrosine phosphorylation of insulin receptor and IRS-1/IRS-2 (data not shown).

We also investigated the effect of wortmannin in the HBsAg suppression. After wortmannin treatment, the cells were then challenged with different concentrations of insulin or TPA for further 48 hr, in the presence of wortmannin. As seen in Fig. 2A, the effect upon HBsAg suppression was reduced at the concentrations of 10^{-10} and 10^{-9} M of insulin in wortmannin-pretreated cells. However, wortmannin had no effect in blocking the insulin suppressed HBsAg at high concentrations of insulin. We cannot exclude the possibility that the residual PI 3-K activity after wortmannin treatment still had effect in transducing the insulin

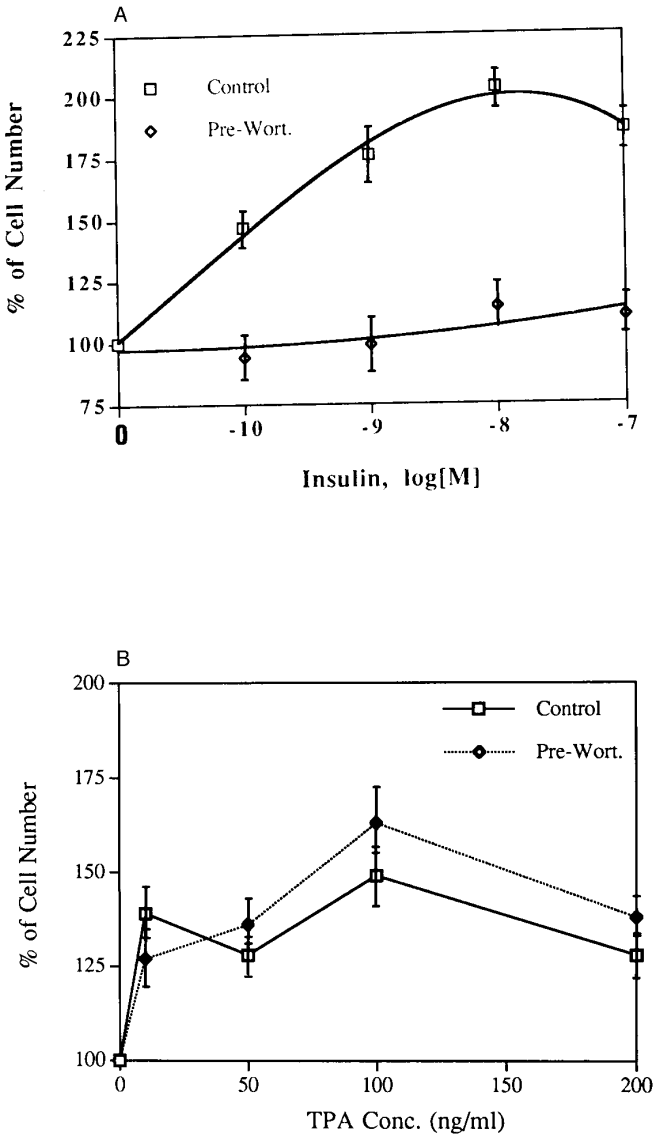

FIG. 1. Insulin or TPA stimulated cell proliferation in both control and wortmannin-treated Hep3B cells. Hep3B cells were seeded in 24-well plates. After 48 hr serum starvation, they were treated with serum-free DMEM containing 0.5% DMSO as a control or 0.5 μM wortmannin for 10 min. They were then exposed to various concentrations of (A) insulin or (B) TPA in the presence of wortmannin or DMSO for another 48 hr. Cell numbers were counted using a hemocytometer. (Control, control cells; Pre-Wort., wortmannin-pretreated cells).

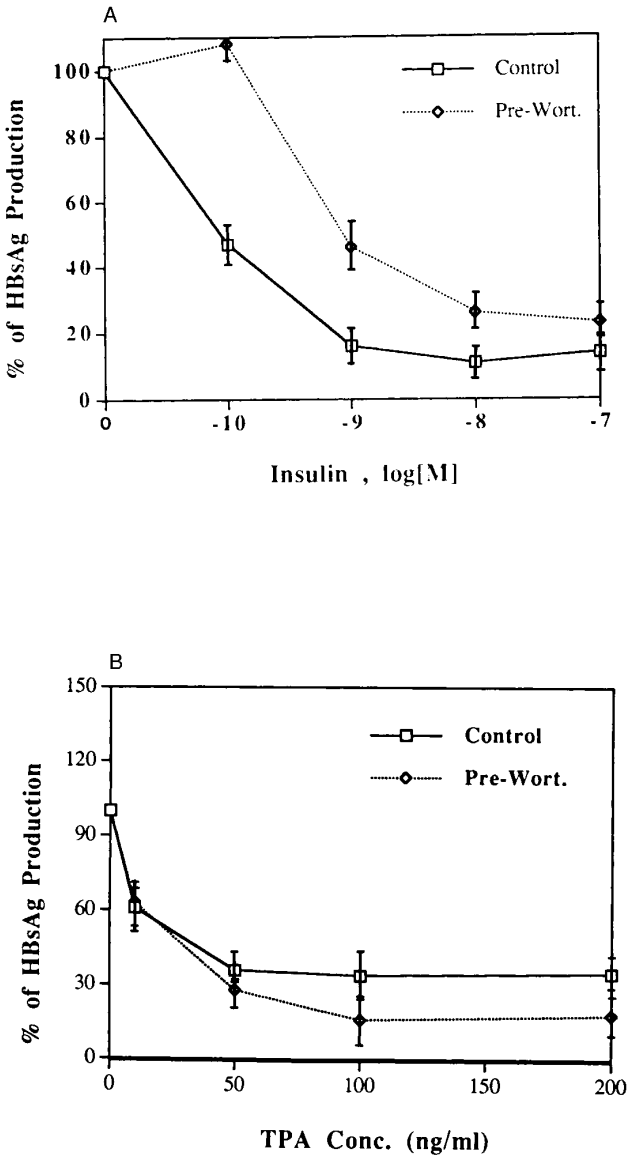

FIG. 2. Insulin or TPA suppressed HBsAg production in both control and wortmannin-treated Hep3B cells. Hep3B cells were seeded and serum starved for 4 hr in 24-well plate. They were then treated as mentioned in the legend of Fig. 1. The amounts of HBsAg in culture media were determined using ELISA method. (Control, control cells; Pre-Wort., wortmannin-pretreated cells).

signals or the existence of a PI 3-K-independent pathway could transduce the signal induced by high concentrations of insulin, in the suppression of HBsAg. However, as seen in Fig. 2B, the pretreatment of wortmannin had no effect on the suppression of HBsAg by TPA. TPA suppressed HBsAg production in a dose-dependent manner in both control and wortmannin-treated cells. In our previous studies, we have shown that PKC-α is involved in both insulin and TPA stimulated cell growth, but it is only involved in TPA but not insulin suppressed HBsAg expression (21). Taken together, PI 3-K but no PKC is required for the regulation of HBsAg production by insulin. The proximity of PI 3-K to insulin receptor relative to that of PKC in insulin signaling pathway might explain the two suppression effects of wortmannin.

Mitogen-activated protein kinases, MAPKs (Erk-1 and 2), are serine/threonine protein kinases which rapidly become phosphorylated on threonine and tyrosine residues (24, 25) in quiescent cells stimulated with various agonists, resulting in enzymatic activation. We pretreated Hep3B cells with wortmannin and characterized the MAPKs activation induced by insulin or TPA. As shown in Fig. 3, both insulin and TPA induced the mobility shifts of both p44 and p42 MAPKs in the control cells. In wortmannin-pretreated cells, TPA still induced the activation of MAPKs, but the insulin effect was completely blocked.

The production of HBsAg is considered as a diagnostic marker for hepatitis B virus infection. The studies of HBsAg gene regulation provides an opportunity to understand the virus life cycle and the infection mechanism which play an important role on the carcinogenesis of human liver cells (26, 27). Moreover, the suppression of HBsAg production by insulin also serves as a good model for studying insulin signal transduction. We attempted to identify the signaling pathways of insulin and TPA towards the suppression of HBsAg expression and stimulation of cell proliferation using wortmannin. Sutherland et al. have shown that wort-

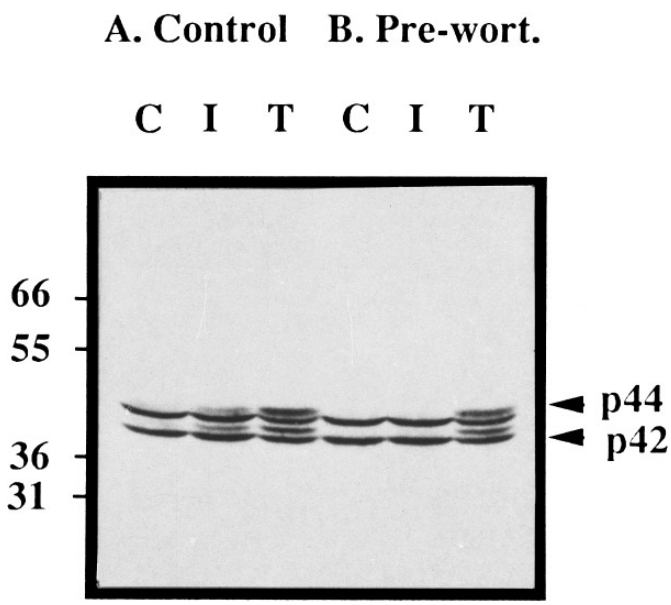

FIG. 3. Wortmannin effect on the mobility shift of MAPKs induced by insulin or TPA in an acrylamide gel. Hep3B cells were treated with wortmannin or 0.5% DMSO for 10 min. They were then treated with 10^{-8} M insulin or 50 ng/ml TPA for 10 or 5 min, respectively. The mobility shift was determined by Western blotting using anti-MAPKs antibody as described in "MATERIALS AND METHODS". (C, control cells; I, insulin-treated cells; T, TPA-treated cells).

mannin inhibited the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin but not by TPA (28). Our data proposed a similar mechanism upon the suppression of HBsAg expression by insulin. Moreover, our results showed that PI 3-K is required for insulin stimulated but not TPA induced MAPK activation and cell proliferation, which have been demonstrated to mediate through the same PKC-α dependent pathway.

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