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DIVERGENT EFFECTS OF PHORBOL ESTERS AND INSULIN ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP-1) PRODUCTION AND mRNA IN RAT H4IIE HEPATOMA CELLS

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Summary: ^{125}I-IGF-I binding assay, western ligand and immunoblotting, and northern analysis of total RNA reveal that phorbol ester agonists of protein kinase C rapidly enhance IGFBP-1 production and increase the abundance of IGFBP-1 mRNA in rat H4IIE hepatoma cells. In combination with insulin, a potent inhibitor of IGFBP-1 gene transcription, this early effect of phorbol esters is dominant. These results demonstrate divergent regulation of IGFBP-1 by phorbol esters and insulin and indicate that protein kinase C may play a critical role in the regulation of IGFBP-1 and modulation IGF bioactivity in metabolic disease.

Insulin-like growth factors (IGFs) circulate in association with specific binding proteins (IGFBPs) which are thought to modulate the availability and biological effects of IGFs on target tissues (1). IGF binding protein-1 (IGFBP-1) is a ~30 kDa protein that is expressed in the liver and reproductive tissues in man (1, 2). Hepatic expression and circulating levels of IGFBP-1 are rapidly regulated by insulin and nutrition (3,4), suggesting that hepatic production of IGFBP-1 may modulate the bioactivity of IGFs in response to changes in metabolic state.

Recently, we reported that rat H4IIE hepatoma cells produce IGFBP-1 (5) and provide a useful model for studying multihormonal regulation of hepatocellular expression of IGFBP-1 (4). We observed that insulin rapidly inhibits the production of IGFBP-1 by H4IIE cells, and that this effect of insulin super-

ABBREVIATIONS: BCS, bovine calf serum; kDa, kilodalton; IGF-I, insulin-like growth factor I; IGFBP-1, insulin-like growth factor binding protein-1; INS, insulin; PDB, phorbol-12, 13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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cedes the stimulation of IGFBP-1 expression by cyclic AMP and glucocorticoids (4). Since phorbol esters exert insulin-like effects on the expression of a number of genes (6-9) and are agonists of protein kinase C (10), we asked whether phorbol esters, and protein kinase C, also might regulate the expression of IGFBP-1 in H4IIE cells.

MATERIALS AND METHODS

Materials: Materials for cell culture were purchased from Gibco (Grand Island, NY). Purified porcine insulin was a gift of Eli Lilly Co. (Indianapolis, IN). Recombinant human IGF-I was bought from BACHEM (Torrance, CA) and [^{125}I]Na, and [α-^{32}P]dATP were purchased from Amersham (Arlington Heights, IL). Phorbol-12,13-dibutyrate (PDB), and phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO). Polyclonal antiserum against purified rat IGFBP-1 (5) was raised in white New Zealand rabbits and does not cross-react with rat IGFBP-2, -3, or -4 in adult or fetal rat serum or conditioned medium by western blot. cDNA clones for rat IGFBP-1 (11), PEPCK (12) and human ß-actin were kindly provided by Drs. L. Murphy, D. Granner, and E. Fuchs.

Preparation of Cells and Conditioned Medium: Rat H4IIE hepatoma cells (from D. Granner) were grown to near confluency with Dulbecco's Modified Eagle's Medium (DMEM) plus 10% calf serum, Stabilized overnight in serum free DMEM, and then refed with fresh DMEM plus 10 µM PDB or PMA and/or 100 nM insulin, sufficient to exert maximal effects on IGFBP-1 expression (4). 1 ml aliquots of medium were centrifuged and stored at -20°C, or RNA harvested at appropriate time points (below).

IGF Binding Assay: IGF-I was iodinated by lactoperoxidase as before (13). IGF binding activity in medium was estimated by 1 hr, 22°C incubation of tracer and precipitation with polyethylene glycol, as per Clemmons et al (14). Non-specific binding was subtracted from all values (14). Samples were diluted so that binding in control medium was 20-65% of maximum in this assay.

Western Ligand and Immunoblot Analysis: IGFBPs in conditioned medium were separated by 13% non-reduced SDS/PAGE, then transferred to nitrocellulose and probed with [^{125}I]IGF-I (15) or with 1:2000 dilution of anti-IGFBP-1 anti-serum. Ligand blots were imaged by autoradiography with enhancing screens at -80°C and quantified by scanning densitometry (Joyce Loeble Co., Gateshead, England). Immune complexes were identified with goat anti-IgG tagged with horseradish peroxidase.

Northern Blot Analysis of RNA: Total cellular RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform (16), and 10 µg loaded for agarose electrophoresis and transfer. Ribosomal RNA was visualized with ethidium bromide, then membranes probed with cDNA clones (above) labeled with ^{32}P by random priming. Following autoradiography and densitometry, hybridization intensity was expressed relative to control.

Statistical Analysis: Results are expressed as the mean $\pm$ SEM and differences between groups examined by unpaired two-tailed *t* test. Statistical significance is assigned for $P<0.05$.

RESULTS

Initial studies revealed that [^{125}I]IGF-I binding activity in H4IIE conditioned medium is increased above control after 4 and 8 hr treatment with 10 μM PDB (Fig 1). In contrast, incubation with 100 nM insulin lowered binding activity in conditioned medium, as previously reported (4). Of note, PDB prevented this effect of insulin (Fig 1), suggesting that PDB may exert a dominant effect on the production of IGFBPs by H4IIE cells.

Western analysis revealed that levels of 32-34 kDa IGFBPs are increased in conditioned medium after 4 and 8 hr treatment with PDB compared to control (Fig 2, Panel A), reflecting changes in levels of immunoreactive IGFBP-1 (Panel B). Further, PDB prevents the negative effect of insulin on the production of IGFBP-1 in this cell line (Panels A and B) Densitometric analysis of ligand blots demonstrated that the early positive effect of PDB are dominant in the presence of insulin (Fig 2, Panel C). Of note, a 25 kDa IGFBP also is produced by H4IIE cells (Fig 2, Panel A); this IGFBP is not recognized by anti-IGFBP-1 antiserum and levels are not altered by insulin or PDB.

Northern blot analysis revealed that 3 hr treatment with PDB increases the abundance of IGFBP-1 mRNA relative to control (Figure 3, Panel A) and that PMA exerts similar effects (not shown). In contrast, insulin rapidly lowers the level of IGFBP-1 mRNA in H4IIE cells (lanes 10-12), while the positive effect of PDB on IGFBP-1 mRNA is dominant, even in the presence of insulin (lanes 7-9). This divergence in the effects of insulin and

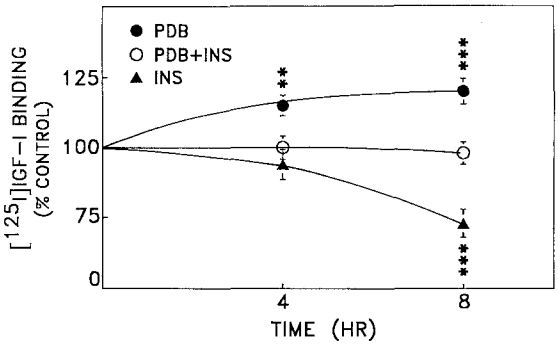

Fig. 1. [^{125}I]IGF-I binding activity in H4IIE conditioned medium. H4IIE cells were incubated with 10 μM PDB and/or 100 nM insulin (INS), then aliquots taken for measurement of IGF binding activity, as in Methods. Results are expressed relative to medium from control incubations. ** P<0.005 vs. control.

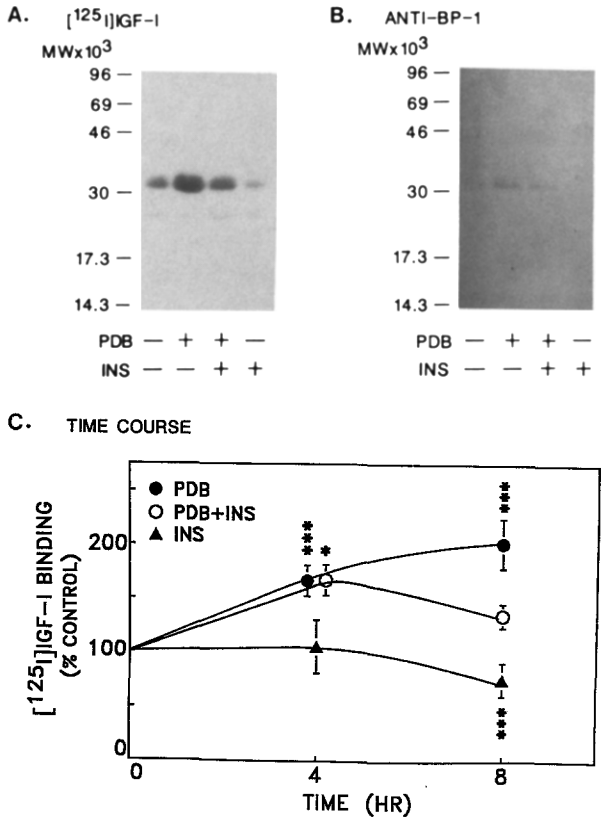

Fig. 2. Western analysis of IGFBPs. Panel A. Ligand blot. Medium collected after 8 hr incubation with PDB and/or INS was prepared for non-reduced 13% SDS/PAGE and transfer to nitrocellulose. Membranes were probed with [^{125}I]IGF-I prior to autoradiography. Panel B. Immunoblot. Membranes were prepared as above, then probed with anti-IGFBP-1 antiserum, as in Methods. C. Time course. Ligand blots were quantified by scanning densitometry, and results for IGFBP-1 expressed relative to control.
* P<0.01 vs. control; *** P<0.001 vs. control.

phorbol ester on the abundance of IGFBP-1 mRNA is specific, since PDB and insulin both decrease the abundance of PEPCK mRNA (Figure 3, Panel B) and increase ß-actin mRNA (Figure 3, Panel C).

Time course studies revealed that PDB rapidly increases the abundance of IGFBP-1 mRNA in H4IIE cells and that levels remain high for at least 9 hr (P<0.001 vs. control at t = 1.5, 3, 6, and 9 hr) (Figure 4). In combination with insulin, the abundance of IGFBP-1 mRNA subsequently falls to control levels, but remains above the level observed with insulin alone (Fig 4). This decline appears to reflect an effect of insulin; it is not present when cells are treated with PDB alone, but it is reproduced when insulin is added to cells continuously exposed to PDB (Fig 4).

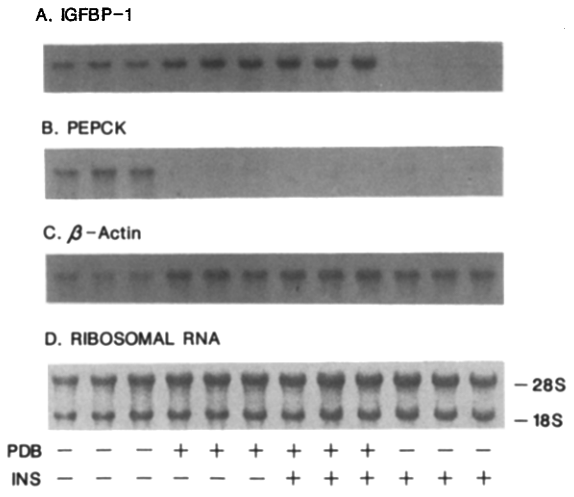

Fig. 3. Northern blot analysis of RNA. Total RNA from H4IIE cells after 3 hr incubation with PDB and/or INS was loaded for northern blotting with cDNA clones for IGFBP-1 (A), PEPCK (B) or ß-actin (C). Ribosomal RNA was visualized with ethidium bromide (D).

DISCUSSION

Clinical and laboratory studies suggest that the liver is a major source of circulating IGFBP-1 (2,11), and that hepatic production of IGFBP-1 is regulated by insulin (3,17) and counter-regulatory hormones (4,18,19). We utilized well differentiated rat hepatoma cells to examine the effects of phorbol esters on hepatocellular expression IGFBP-1. IGF binding assays, and ligand and immunobloting demonstrated that PDB stimulates production of IGFBP-1, and northern analysis revealed that this

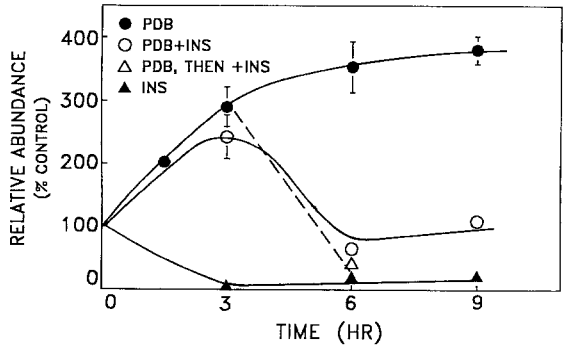

Fig. 4. Time course for IGFBP-1 mRNA. RNA was extracted after 1.5-9 hr treatment with PDB and/or INS, then analyzed by northern blotting with an IGFBP-1 cDNA probe. Following autoradiography, hybridization intensity was quantified by scanning densitometry, and expressed relative to control.

effect of phorbol esters is accounted for by an increase in the abundance of IGFBP-1 mRNA.

Of note, the positive effects of phorbol esters are rapid, and initially are dominant in combination with insulin. Since phorbol esters activate protein kinase C (PKC) (9), it may well be that these initial effects on the expression of IGFBP-1 are mediated through PKC. In this regard, recent studies indicate that PKC is activated in the liver of fasted rats (20), where the expression of IGFBP-1 is increased even after the administration of insulin (21). Pittner and Fain have reported that glucagon and other calcium-mobilizing hormones activate PKC in rat hepatocytes (22). Taken together, these observations suggest that PKC may mediate some hepatic effects of counter-regulatory hormones and play an important role in the regulation of IGFBP-1.

The observation that phorbol esters and insulin exert opposite effects on the abundance of IGFBP-1 also is of interest. Insulin rapidly and profoundly inhibits transcription of the IGFBP-1 gene (22,23), similar to its effects on the gene for PEPCK (24). Granner and co-workers have identified a 10-base cis-acting DNA sequence which mediates inhibitory effects of insulin on the PEPCK promoter (25) and they have demonstrated that this same DNA sequence mediates negative effects of phorbol esters on PEPCK transcription in H4IIE cells (26). Further, a closely related DNA sequence is present in the 5′ promoter region of the human IGFBP-1 gene (27). While this putative negative response element may account for the ability of insulin to suppress IGFBP-1 transcription in H4IIE cells, it does not explain the divergent and dominant effect that phorbol esters exert on the expression of IGFBP-1 in the same cell line.

Further studies are required to better understand specific mechanisms by which insulin and protein kinase C may interact to regulate the hepatic expression of IGFBP-1, and thereby modulate the biological effects of IGFs in metabolic disease.

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