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Blockade of Rapid Versus Prolonged Extracellularly Regulated Kinase 1/2 Activation Has Differential Effects on Insulin-Induced Gene Expression

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In the present work, insulin's regulation of expression of activating transcription factor 3 (ATF-3), the putative transcription factor proline-rich induced protein (Pip)92, and insulin-inducible gene-1 (Insig-1) (an ER resident protein involved in regulation of sterol-responsive element-binding protein 1 activation) have been examined in a liver-derived cell line (rat H4IIE hepatoma cells). We report that: 1) insulin-induced transcription of ATF-3, Pip92, and Insig-1 required MEK-ERK activation; 2) insulin-induced transcription of ATF-3 and Pip92 reached maximum levels within 15 min and was blocked by wortmannin but not LY294002; 3) in contrast, the maximum level of insulin-induced transcription of Insig-1 was delayed and was not blocked by either wortmannin or LY294002; 4) insulin activated ERK1/2 in two distinct phases, a rapid peak and a later plateau; 5) the delayed plateau phase of insulin-induced ERK1/2 activation was partially phosphatidylinositol 3-OH-kinase dependent; and 6) however, the rapid, insulin-induced peak of ERK1/2 activation was blocked by wortmannin but not LY294002. (*Endocrinology* 146: 2716–2725, 2005)

INSULIN IS A KEY regulator of metabolism and growth in cells of hepatic origin. It affects these parameters through specific binding to, and activation of, the tyrosine kinase insulin receptor heterotetramer. The intracellular signaling of insulin is conducted via multiple pathways, including the Ras/Raf/MEK/ERK (MEK-ERK) kinase cascade and the phosphatidylinositol 3-OH-kinase (PI3-K), which in turn can activate multiple downstream signaling molecules (1). Often, activation of these two pathways is considered separately. Activation of the MEK-ERK pathway can be independent of the PI3-K pathway (2, 3). However, it has been reported that ERK activation by insulin requires activation of the PI3-K pathway in adipocytes (4, 5), muscle cells (6, 7), and Chinese hamster ovary cells expressing the insulin receptor (8, 9).

Activating transcription factor 3 (ATF-3, also known as LRF-1, LRG-21, CRG-5, and TI-241) is a transcription factor of the basic region leucine zipper family of proteins (10). It was originally cloned as a phorbol ester-responsive gene in HeLa cells and found to be a transcriptional repressor as a heterodimeric complex, but it may also function as a transactivator when heterodimerized with other basic region leucine zipper proteins such as JunD. It is rapidly induced by a variety of cellular stresses such as ischemia-reperfusion, oxidative stress, or hepatotoxic insult (reviewed in Refs. 10 and 11). ATF-3 was also found to be inducible by protein synthesis inhibitors in combination with insulin or serum and by hepatocyte growth factor or epidermal growth factor (EGF) in primary rat hepatocytes (11). Some target genes of ATF-3 that have been identified include gadd153, E-selectin, and cyclinD1, as well as hepatic gluconeogenic enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase. Constitutive overexpression of exogenous ATF-3 in the liver alone, or in both liver and pancreas, results in hepatic and adipose tissue dysfunction as well as disturbances in glucose metabolism, suggesting that ATF-3 may play a role in maintenance of hepatic homeostasis and metabolism (10, 12, 13).

As previously described, proline-rich induced protein (Pip92) and insulin-inducible gene-1 (Insig-1) were identified through differential screening of insulin-induced genes (14). Pip92 is also known as CHX-1, ETR101, and IER2. It was cloned as an immediate early gene induced by serum, cycloheximide, or phorbol esters in fibroblasts, T-cells, and promyelocytic cells, respectively (15, 16). It is also induced by specific receptor agonists (fibroblast, platelet derived, and nerve growth factors as well as carbachol and insulin) (14, 17–20) and by activation of the MEK-ERK signal transduction pathway (21, 22). Cellular stresses such as anisomycin, bacterial infection, UV light, clotting factors, and partial hepatectomy can also induce Pip92 (23–27). Pip92 encodes a proline-rich protein that may either be predominantly cytoplasmic or nuclear in distribution, depending on the experimental system (17, 23). Although its sequence provides no obvious clues to its function, it has been noted that short regions of Pip92 are highly homologous to the transcription factors JunB, JunD, and c-Jun, leading some to speculate that it may function as a transcription factor (15, 16). Depending on the stimulus, Pip92 induction requires the protein kinase C, MEK-ERK, p38, or c-Jun N-terminal kinase pathways,

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Abbreviations: ATF-3, Activating transcription factor 3; EGF, epidermal growth factor; Insig-1, insulin-inducible gene-1; MEK, MAPK/ERK kinase; P-Akt, phospho-Akt; P-ERK, phospho-ERK1/2; PI3-K, phosphatidylinositol 3-OH-kinase; Pip92, proline-rich induced protein; SDS, sodium dodecyl sulfate; SREBP, sterol-responsive element-binding protein; T-Akt, total Akt; T-ERK, total ERK1/2.

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usually by activating transcription via a serum-responsive element in its promoter region (17, 21–23, 28).

Insig-1 (also known as CL-6) was originally cloned from a library of cDNAs induced in regenerating liver, where it is highly expressed. In addition to liver, it is expressed at lower levels in kidney and heart (29, 30). Insig-1 is also induced by insulin or serum treatment of hepatoma cells and fibroblasts and decreased by oxysterols (14, 31). Recent work has described a role for Insig-1 in sterol metabolism. Insig-1 is a component of a ternary complex along with sterol-responsive element-binding protein (SREBP) cleavage activator protein and the transcription factor, SREBP1. This complex retains SREBP1 in an inactive state in the endoplasmic reticulum in the presence of elevated sterol levels (32). It appears that Insig-1 along with a newly identified family member, Insig-2, tightly regulates the activity of SREBP1-dependent processes via interaction with the sterol-sensing domain of SREBP cleavage activator protein (30, 33, 34). Insig-1 also plays a similar role in regulating the proteasome-mediated degradation of another sterol sensing protein, β-hydroxy-β-methylglutaryl-CoA reductase (34). In adipocytes, Insig-1 exerts a limiting role in lipogenesis and is up-regulated in differentiating adipocytes (35).

In the present work, we studied the regulation of ATF-3, Pip92, and Insig-1 in response to insulin stimulation of a liver-derived cell line (H4IIE rat hepatoma cells). All three genes are induced in a MEK-ERK-dependent fashion but may be dependent upon different durations of MEK-ERK induction. This property of time dependence was illustrated by our finding that blunting insulin's rapid peak of ERK1/2 activation blocked subsequent transcription of ATF-3 and Pip92 but not Insig-1. In contrast, reduction in later time points of insulin-induced ERK1/2 activation or PI3-K activation had no effect on insulin-induced gene transcription.

Materials and Methods

Cell culture

H4IIE cells were maintained in Swim's 77 medium (Sigma, St. Louis, MO) supplemented with 2% fetal bovine serum, 3% calf serum, and 5% horse serum. 3T3-L1 fibroblasts were maintained in subconfluent cultures in complete medium [DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin supplement (Cellgro; Mediatech, Inc., Herndon, VA)]. For adipocyte differentiation, cells were grown to confluence, then transferred to differentiation medium containing 500 nM 1-methyl-3-isobutylxanthine, 250 nM dexamethasone, and 170 nM insulin for 2 d. Cells were removed from differentiation medium and maintained for 2 more days in complete medium plus insulin, followed by 6 d in complete medium (36). Insulin, dexamethasone, and 1-methyl-3-isobutylxanthine were obtained from Sigma. Polyclonal phospho-(active)-ERK1/2 (P-ERK), total ERK1/2 (T-ERK), phospho-Ser-473-Akt (P-Akt), total Akt (T-Akt), and secondary antirabbit antisera were obtained from Cell Signaling Technology, Inc. (Beverly, MA).

Treatments

Serum was withdrawn from subconfluent cultures of H4IIE cells or confluent monolayers of differentiated adipocytes 24–48 h before experimental treatments. PD98059 (Cell Signaling Technology, Inc.) was added to prevent activation of MEK by insulin (37, 38). LY294002 (BIOMOL International L.P., Plymouth Meeting, PA) is a competitive PI3-K inhibitor (39). The distinct PI3-K inhibitor, wortmannin (Sigma), binds covalently to the ATP binding region (40). ML-7 competitively inhibits myosin light chain kinase II protein kinase (41). Propranolol (Calbiochem, San Diego, CA) was added to inhibit phospholipase-D-derived diacylglycerol by inhibition of phosphatidate phosphohydrolase (42, 43). As suggested by previous reports, these enzyme inhibitors are fully effective within 10–30 min, thus all were added 30 min before insulin addition (37–39, 41–47).

Transcription

Rates of transcription were assayed by the nuclear run-on method as previously described (14, 20). Transcriptionally active nuclei were labeled with ^{32}P-UTP and incubated to allow extension of nascent mRNA. Transcripts were isolated and hybridized with cDNAs immobilized on nitrocellulose, followed by autoradiography. Densitometric data were analyzed using ZeroD Scan from Scanalytics (Fairfax, VA), and values from experimental treatments compared with vehicle controls were expressed as fold change.

Western blot analysis

Sodium dodecyl sulfate (SDS) whole-cell lysates (1% SDS; 10 mM Tris, pH 7.5; 7.5 μg/ml aprotonin; 5 mM benzamidine; 5 mM phenylmethylsulfonylfluoride; 50 mM NaF; and 1.25 mM $NaVaO_4$) were assayed for protein content using the DC assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins (25 μg) were resolved by SDS-PAGE in a Bio-Rad Mini-Protean II electrophoresis cell, transferred to Protran BA85 membranes (Schleicher & Schuell, Inc., Keene, NH), developed with ECL Plus (Amersham Biosciences, Buckinghamshire, UK), and visualized by autoradiography or by direct digital imaging of chemiluminescent blots using the Fluorchem FC imager system [Alpha Innotech, San Leandro, CA (20)]. Relative band intensity quantitation and background subtraction were carried out using the onboard software of the Fluorchem FC digital imager (20, 48).

Statistical analysis

Student's *t* test and ANOVA were performed using the Instat version 3.0 software (GraphPad Software, Inc., San Diego, CA). Fold increases in transcription were compared with those in vehicle-treated control cultures. In Western blot experiments, values obtained at 5 min insulin treatment were assigned the maximum value of 100% for comparison to other treatments.

Results

ATF-3, Pip92, and Insig-1 were identified from a cDNA library prepared from H4IIE cells treated with insulin and the translational inhibitor, anisomycin, as previously described (14). Library colonies were denatured and transferred to duplicate membranes and were then probed with radiolabeled cDNAs prepared either from cells treated with insulin and anisomycin for 120 min or from untreated cells. Differentially expressed mRNAs were selected for further screening and sequence analysis. Among the genes identified in this screen were ATF-3, Pip92, and Insig-1. Further studies of insulin's effect on these genes in the absence of anisomycin or other translational inhibitors are presented below.

In Fig. 1, the time course of the induction of transcription of ATF-3, Pip92, and Insig-1 by treatment of H4IIE cells with insulin alone is presented. Both ATF-3 and Pip92 were rapidly induced, with peak level of transcription (3- and 14-fold, respectively) occurring by 15 min, followed by a return to basal level of transcription by 120 min. In contrast, transcription of Insig-1 was delayed; it was not significantly increased from basal levels 15 min after insulin addition, and only reached its peak level of an 8-fold increase by 30 min, then declined to 6-fold by 60 min and returned to the basal level by 120 min.

To understand the signaling mechanisms used by insulin to regulate expression of these genes, specific inhibitors were

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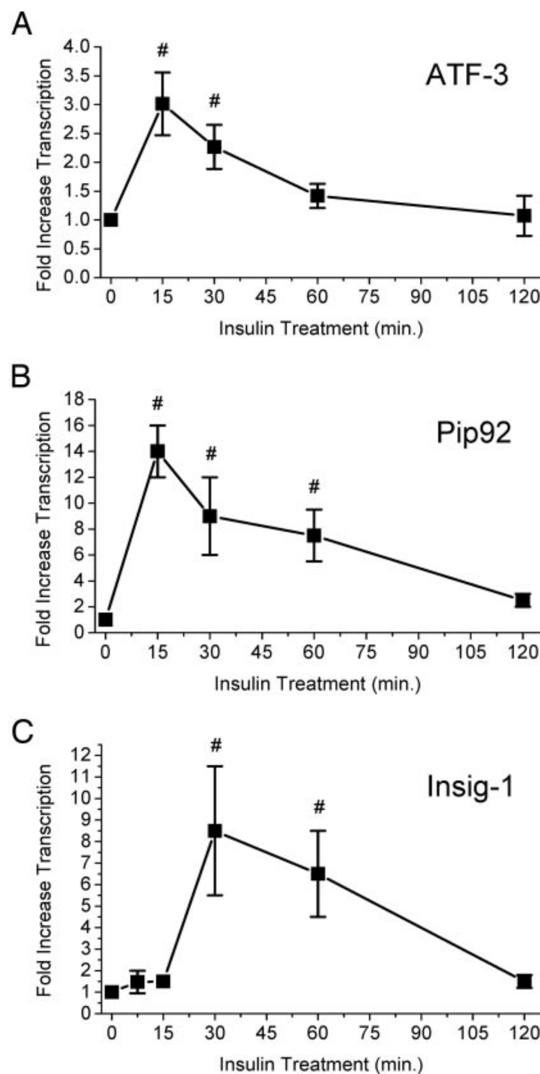

FIG. 1. Kinetics of insulin-induced transcription of ATF-3, Pip92, and Insig-1. Serum-deprived H4IIE cells were treated with 10 nM insulin for the indicated times, and transcription was measured by nuclear run-on assay, as described in *Materials and Methods*. Mean data collected from three or more separate experiments is presented with SEM. Statistical significance of differences from control: #, $P < 0.001$ *vs.* vehicle control.

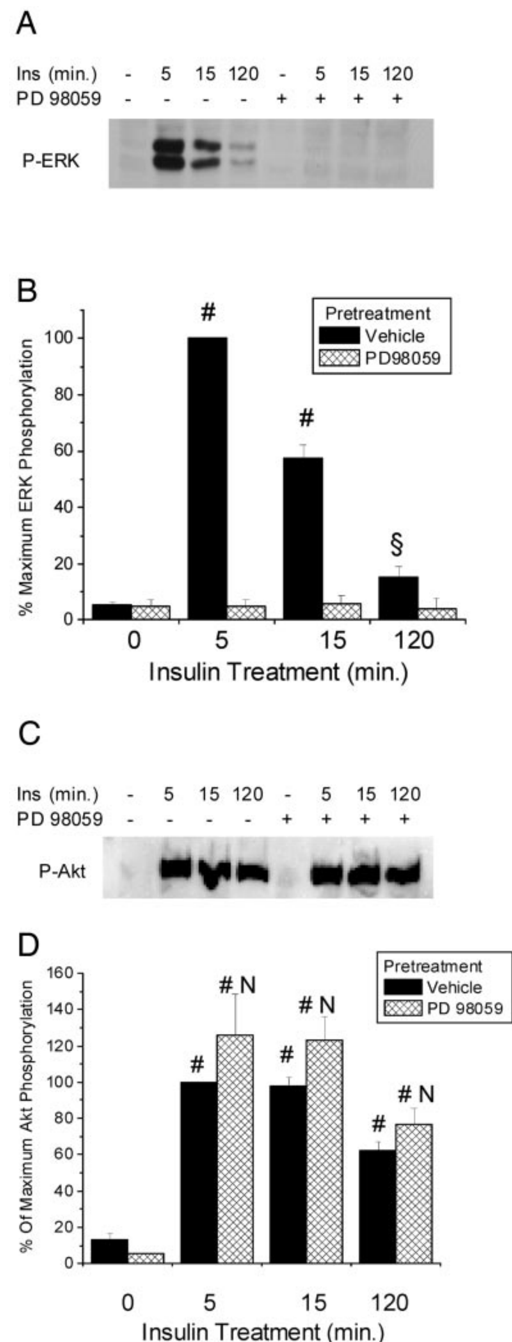

FIG. 2. Rapid activation of ERK1/2 and PI3-kinase pathways by insulin in H4IIE cells. Serum-deprived H4IIE cells were treated with 10 nM insulin alone or in the presence of the MEK inhibitor, PD98059 (50 μM). Levels of ERK1/2 phosphorylation (P-ERK; A and B) and Akt phosphorylation (P-Akt) (C and D) were determined by Western blot of whole-cell lysates with phospho-specific antisera. Representative autoradiograms are shown (A and C) along with mean data collected from three or more separate experiments at each time point (B and D), in which the maximum effect by insulin was arbitrarily set to 100%. Ins, Insulin; §, $P < 0.05$ *vs.* vehicle control; #, $P < 0.001$ *vs.* vehicle control; N, not significant *vs.* vehicle plus insulin.

used in combination with activation-state-specific antisera in Western blot experiments. Insulin treatment of serum-starved H4IIE cells resulted in the rapid phosphorylation of the ERK1/2 MAPKs, which reached a maximal level (arbitrarily assigned a value of 100%) within 5 min, as previously reported (20, 47). ERK1/2 phosphorylation decreased to 58% at 15 min, and further to 15% of maximum activation by 120 min of insulin treatment. Pretreatment of cells with the MEK inhibitor PD98059 completely blocked the activation of ERK1/2 to levels that were not significantly different from those of vehicle controls, indicating that all of the ERK1/2 phosphorylation induced by insulin is dependent on MEK (Fig. 2, A and B). Another highly activated insulin signaling pathway is the PI3-K pathway. We examined phosphorylation of the downstream serine kinase, Akt (Ser-473), as a proxy for PI3-K activation. It was induced by insulin within 5 min and remained within 40% of maximum for at least 120 min. PD98059 had no significant effect on Akt phosphorylation, indicating that MEK is not required for insulin's activation of this pathway in H4IIE cells (Fig. 2, C and D).

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Transcription of ATF-3, Pip92, and Insig-1 were assayed after pretreatment with the MEK inhibitor. The insulin effect on all of these genes was blocked after PD98059 treatment (Fig. 3). The level of insulin-induced transcription of all genes was not significantly different from that of either vehicle control or cells treated with PD98059 alone, indicating that insulin's effect on these genes requires activation of the MEK-ERK pathway. The inhibitor itself had no significant effect on basal transcription rates (Fig. 3, A–C).

It has been alternately reported that insulin activation of the ERK1/2 and PI3-K pathways proceeds independently, or that insulin may activate the ERK1/2 pathway via a PI3-kinase-dependent mechanism (2–9). To investigate which of these possible mechanisms was involved in the regulation of these MEK-ERK-dependent genes, we used two different PI3-K inhibitors. Wortmannin and LY294002 are unrelated compounds that inhibit PI3-K by different mechanisms. As expected, both wortmannin and LY294002 were able to reduce insulin-induced Akt phosphorylation to levels not significantly different from those in unstimulated cells, with maximum inhibitory effects observed at 100 nM and 50 μM, respectively (Fig. 4A). Although wortmannin treatment caused a marginally greater inhibition, levels were not significantly different from either vehicle alone or LY294002 pretreated values at all times tested, indicating that there were no differences in effectiveness or kinetics of these two inhibitors, with respect to PI3-K inhibition (Fig. 4B). However, the effects of the two inhibitors on ERK1/2 phosphorylation differed significantly. Wortmannin caused a dramatic blunting of the rapid activation of ERK1/2 (60% inhibition), whereas LY294002 caused no significant alteration in this early peak of insulin-induced ERK1/2 phosphorylation (Fig. 4, C and D). At later times of insulin treatment, there was a similar reduction of ERK1/2 activation by both wortmannin and LY294002. At 15 min, insulin-induced P-ERK remained at 51% of maximum, and this effect was reduced to 26 and 20% by wortmannin and LY294002 pretreatment, respectively. Similarly, after 60 min of insulin treatment alone, P-ERK declined to 42% of maximum, and this effect was further reduced to 14 or 12% by wortmannin or LY294002 pretreatment, respectively. Although neither inhibitor altered the level of T-ERK protein, the effect of wortmannin on rapid ERK1/2 activation was pronounced at a concentration of 100 nM, whereas even a concentration of 100 μM LY294002 (twice the concentration required for maximum inhibition of Akt phosphorylation) had no significant effect on ERK1/2 activation (Fig. 4C).

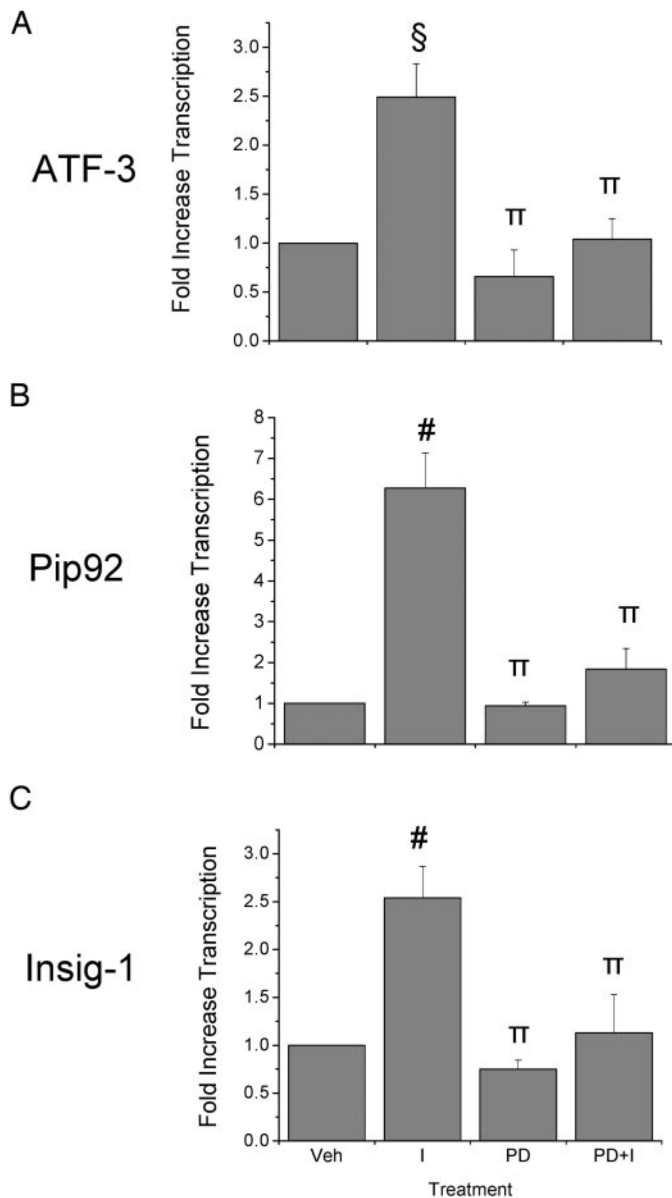

FIG. 3. Insulin-induced transcription of ATF-3, Pip92, and Insig-1 is MEK-ERK dependent. Serum-deprived H4IIE cells were pretreated for 30 min with 50 μM PD98059 or vehicle, then stimulated with 10 nM insulin for 30 min. Transcription was measured by nuclear run-on assay, compared with the effect of vehicle alone, and expressed as fold effect. Veh, Vehicle control; I, insulin treated; PD, PD98059 pretreated; §, $P < 0.05$ *vs.* vehicle control; #, $P < 0.001$ *vs.* vehicle control; Π, not significant *vs.* vehicle control.

Because these results were unexpected, it was asked whether comparable results could be obtained in another well-characterized insulin-responsive cell line. Previous studies in murine 3T3-L1 adipocytes have indicated that insulin activates ERK1/2 via the PI3-K pathway (5). As in H4IIE cells, insulin caused a rapid increase of activated ERK1/2 within 5 min and returned to levels not significantly different from control by 15 min (Fig. 5, A and C). The activation of ERK1/2 was also different from that in H4IIE cells, in that ERK2 (p42) was more strongly activated than ERK1 (p44), as has previously been reported (49). In 3T3-L1 adipocytes, we observed that pretreatment with either wortmannin or LY294002 was equally effective at blocking activation of Akt phosphorylation in response to insulin treatment (Fig. 5, A and B). However, as in H4IIE cells, wortmannin was more effective than LY294002 at blocking insulin's induction of ERK1/2 phosphorylation. In these cells, wortmannin pretreatment completely abolished insulin-mediated activation of ERK1/2, whereas LY294002 pretreatment only partially inhibited the peak of insulin-activated ERK1/2. At 15 min of insulin treatment, there was no significant difference between the effects of the two inhibitors, because P-ERK1/2 levels were no different from vehicle-treated controls with either treatment (Fig. 5, A and C).

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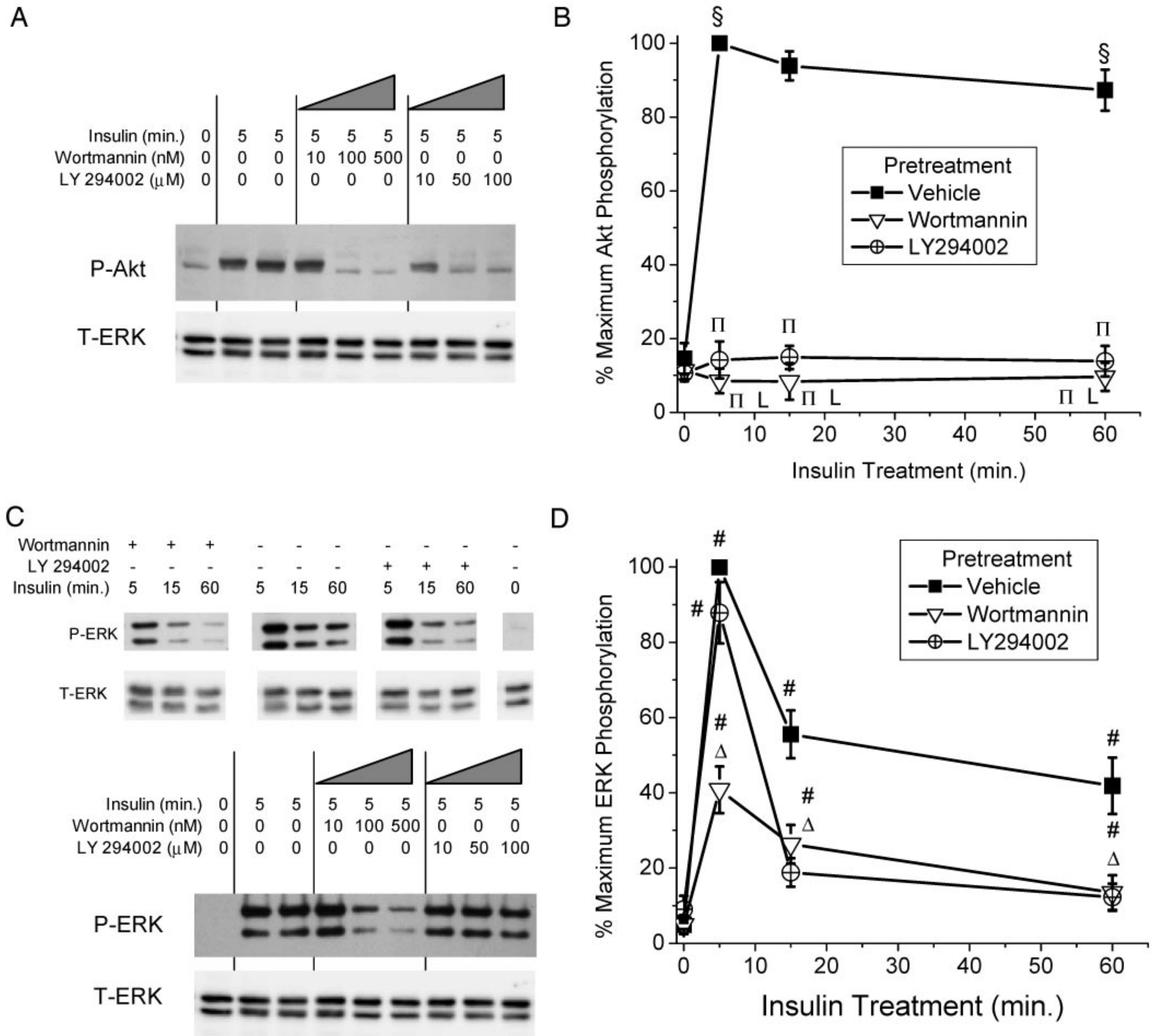

FIG. 4. Wortmannin and LY294002 differentially inhibit insulin-induced phosphorylation of ERK1/2 but not Akt in H4IIE cells. Serum-deprived H4IIE cells were treated with 10 nM insulin alone or after 30 min pretreatment with the indicated concentrations of inhibitor (A and C), or 100 nM wortmannin or 50 μM LY294002 (B and D). Levels of Akt Ser-473 phosphorylation (A and B), ERK1/2 phosphorylation (C and D), and total ERK (T-ERK) (C) were determined by Western blot of whole-cell lysates. Representative autoradiograms are shown (A and C) along with mean data collected from three or more separate experiments (B and D) in which the maximum effect by insulin was arbitrarily set to 100%. §, $P < 0.05$ *vs.* vehicle control; #, $P < 0.001$ *vs.* vehicle control; Π, not significant *vs.* vehicle control; L, not significant *vs.* LY294002 plus insulin; Δ, $P < 0.05$ *vs.* vehicle plus insulin.

The data from 3T3-L1 cells are consistent with our findings in H4IIE cells in which wortmannin inhibits insulin-induced ERK1/2 activation more effectively than LY294002, but the two compounds have similar inhibitory effects on the PI3-K pathway while not effecting the total level of either protein (Fig. 5A). This indicates the PI3-K inhibitor, wortmannin, can have effects on insulin-induced signaling separate from its inhibition of PI3-K activity, and data using wortmannin should be analyzed with caution in such experiments.

Previous reports indicate that wortmannin may interfere with activation of myosin light chain kinase II or phospholipase D (44, 50). To determine whether either of these pathways was involved in the observed effects of wortmannin, we examined effects of inhibitors of these signaling molecules on insulin-induced ERK1/2 phosphorylation. Inhibition of myosin light chain kinase II with ML-7 pretreatment did not alter insulin's effect on ERK1/2 activation, the level of ERK1/2 phosphorylation in unstimulated cells, or the level of T-ERK proteins (Fig. 6). In contrast, treatment with propranolol to inhibit production of phospholipase D-derived diacylglycerol (42, 51) caused phosphorylation of ERK1/2 to a level similar to that of insulin treatment, and this

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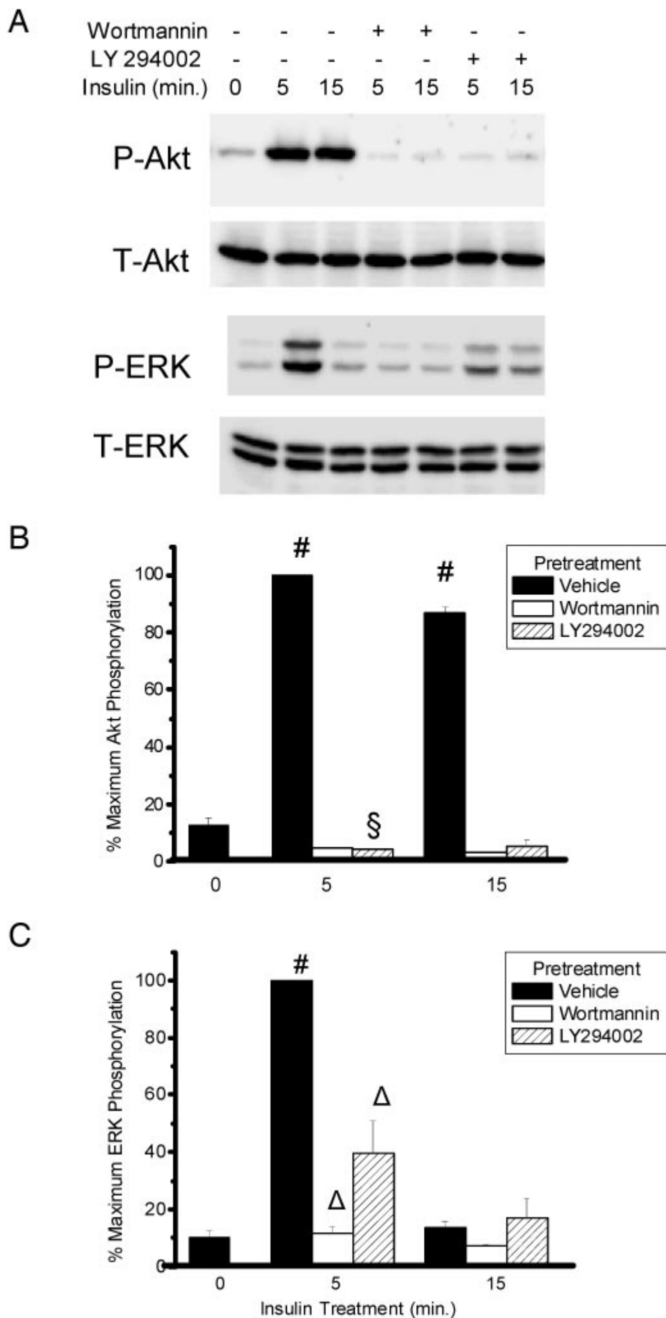

FIG. 5. 3T3-L1 adipocytes display similar disparity of effects of wortmannin and LY294002 on insulin-induced phosphorylation of ERK1/2 and Akt. Serum-deprived adipocyte-differentiated 3T3-L1 cells were treated with 10 nM insulin alone or after 30 min pretreatment with 100 nM wortmannin or 50 μM LY294002. Levels of T-ERK (A) and P-ERK (A and C) and total- (A) and phospho-Akt (A and B) were determined by Western blot of whole-cell lysates. Representative autoradiograms are shown (A) along with mean data collected from separate experiments (B and C) in which the maximum effect by insulin was arbitrarily set to 100%. §, $P < 0.05$ *vs.* vehicle control; #, $P < 0.001$ *vs.* vehicle control; Δ, $P < 0.05$ *vs.* vehicle plus insulin.

effect was further increased by insulin treatment, whereas the total level of ERK1/2 proteins were unaffected (Fig. 6). This effect of propranolol to induce ERK1/2 phosphorylation indicated that it was acting in a manner opposite from that

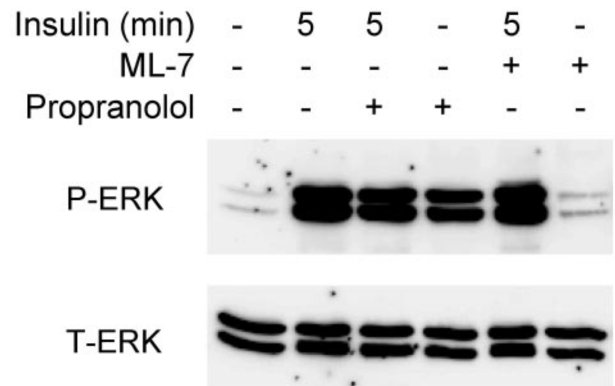

FIG. 6. Effects of phospholipase D and myosin light chain kinase II inhibitors on ERK1/2 phosphorylation. Serum-deprived H4IIE cells were treated with 10 nM insulin alone or after pretreatment with 1 μM ML-7 or 333 μM propranolol. Levels of T-ERK and P-ERK were determined by Western blot of whole-cell lysates. Representative autoradiograms are shown from one of three or more separate experiments with each inhibitor.

of wortmannin, and thus suggested that wortmannin was not affecting the ERK1/2 pathway by limiting phospholipase D-derived signals. Propranolol was not used further because of these unexpected effects. Neither of these inhibitors recapitulates the effect of wortmannin on insulin-induced ERK1/2 activation.

After our findings of a difference in wortmannin *vs.* LY294002 on insulin-induced MEK/ERK signaling, we set out to investigate the role of PI3-K in insulin's regulation of MEK/ERK-dependent genes. Transcription of all three genes was assayed in the presence or absence of each of the PI3-K inhibitors. LY294002 alone did not significantly affect transcription of any of the genes studied, nor did it significantly affect insulin's induction of ATF-3, Pip92, or Insig-1 (Fig. 7). Like LY294002, wortmannin did not by itself affect transcription of any of the genes studied. However, wortmannin pretreatment blocked the ability of insulin to induce transcription of both ATF-3 and Pip92. The inhibitory effect of wortmannin and nonsignificant effect of LY294002 on insulin-induced transcription of ATF-3 and Pip92 corresponded with the effects of the two inhibitors on the rapid peak of ERK1/2 phosphorylation, with wortmannin being inhibitory and LY294002 not having an effect. In contrast to ATF-3 and Pip92, insulin-induced Insig-1 transcription was not significantly affected by wortmannin.

Discussion

The present studies extended findings by our lab and others regarding the mechanism of insulin's regulation of the Insig-1 gene (14). Another gene, Pip92, which may serve as a transcription factor or a signaling intermediate [as inferred from sequence homology (15–17)], was also found to be induced by insulin (20). To our knowledge, this is the first report of a direct regulation of the third gene, ATF-3, by insulin alone. A previous report indicated that the combination of the protein synthesis inhibitor, cycloheximide, and insulin induced ATF-3 mRNA (11), but we have previously reported the transcriptional or posttranscriptional induction of several immediate early genes by protein synthesis inhib-

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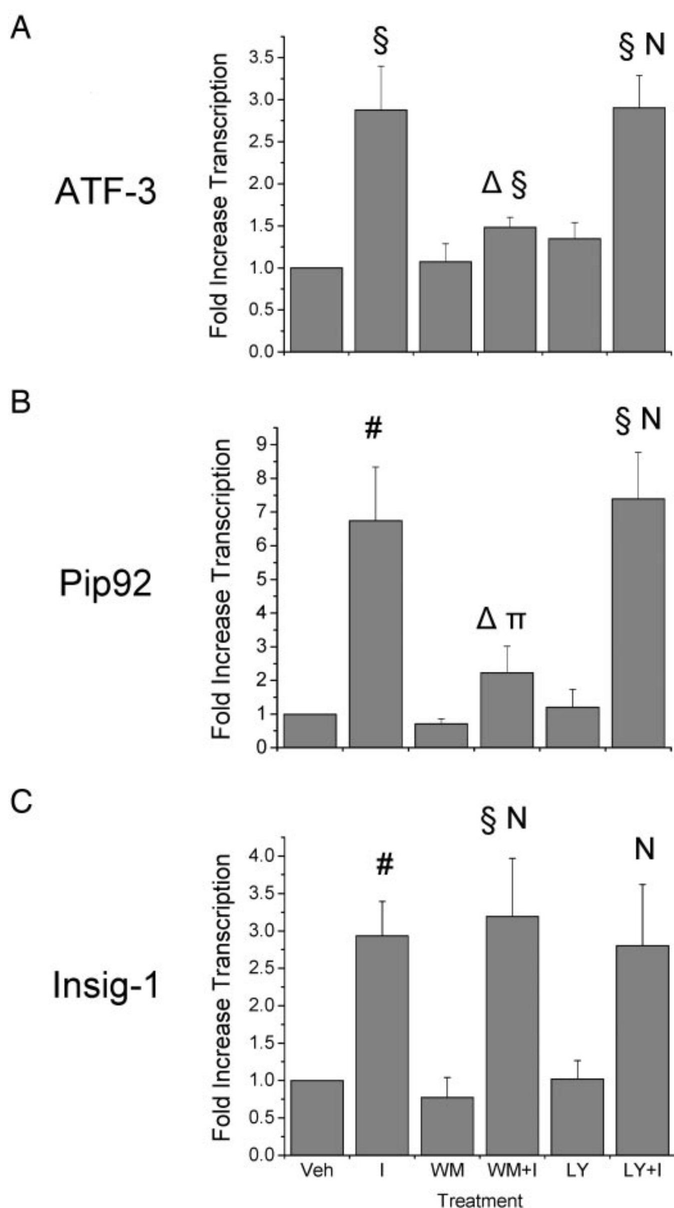

FIG. 7. Insulin-induced transcription of ATF-3 and Pip92, but not Insig-1, are differentially inhibited by wortmannin and LY294002. Serum-deprived H4IIE cells were pretreated for 30 min with 100 nM wortmannin, 50 μM LY294002, or vehicle, then stimulated with 10 nM insulin for 30 min. Transcription was measured by nuclear run-on assay, compared with the effect of vehicle alone, and expressed as fold effect. Veh, Vehicle control; I, insulin-treated; LY, LY294002 pretreated; WM, wortmannin pretreated; §, $P < 0.05$ *vs.* vehicle control; #, $P < 0.001$ *vs.* vehicle control; Π, not significant *vs.* vehicle control; Δ, $P < 0.05$ *vs.* vehicle plus insulin; N, not significant *vs.* vehicle plus insulin.

itors alone in the absence of any other agent (14, 52, 53). The present studies indicate a direct effect of insulin on inducing ATF-3 transcription.

In H4IIE cells, insulin treatment induced activation of both the PI3-K and ERK1/2 pathways, but with different temporal patterns of induction. Phosphorylation of Akt reached a maximum level within 5 min, which was sustained throughout the 120-min time course of insulin treatment. In contrast, insulin appeared to activate ERK1/2 with a very different pattern, consisting of two phases. Insulin caused a rapid rise and fall (*peak phase*, 0–15 min) of ERK1/2 phosphorylation, followed by a return to a lower (*plateau phase*) level of phosphorylation, which gradually decreased toward basal values after 15 min of insulin treatment but were still significantly elevated at 120 min of insulin.

Specific pharmacological inhibitors of cellular signaling pathways are powerful tools for understanding the mechanism of action of insulin's effect on gene expression. Inhibition of the activation of ERK1/2 was affected using the compound PD98059, which specifically blocks activation of the upstream kinase MEK (37). Two inhibitors have been widely used to determine the involvement of PI3-K in various processes. The fungal metabolite, wortmannin, is a direct inhibitor of PI3-K (lipid and serine kinase activity) both *in vitro* and in the context of intact cells. It has no direct effect on enzymes important to the ERK1/2 cascade, such as MEK, ERK2, or protein kinase C (38, 40, 54, 55). A chemically distinct compound, LY294002, is a competitive inhibitor of PI3-K (39). These compounds have been used to identify required signaling pathways in a variety of systems.

Our results indicated that all time points of insulin-induced Akt phosphorylation are blocked by either inhibitor of the PI3-K pathway in H4IIE cells. Similarly, the later phase plateau of ERK1/2 activation (15 min and later) was reduced by treatment with either PI3-K inhibitor. This suggests that there is a partial requirement for PI3-K in the insulin-induced plateau of ERK1/2 activity in H4IIE cells. This is consistent with the substantial dependence on PI3-K activity for ERK1/2 activation by insulin in 3T3-L1 adipocytes reported here and in earlier reports (4, 5). Previous works have suggested that cross-talk between the PI3-K and ERK1/2 pathways may involve activation of Raf and that this process requires insulin activation of both phosphoinositide-dependent kinase-1 and protein kinase C (5, 45).

Inhibition of PI3-K with LY294002 had no significant effect on the peak of ERK1/2 activation. However, wortmannin dramatically blunted the rapid peak of insulin-induced ERK1/2 activation. This suggests that wortmannin, but not LY294002, inhibits insulin-induced ERK1/2 activation separately from its effect on PI3-K, because both wortmannin and LY249002 equally blocked Akt phosphorylation without effecting the total level of either protein. Similar to the present work, a previous study in rat hepatocytes indicated that LY294002 does not inhibit insulin-mediated activation of ERK1/2, whereas 500 nM wortmannin does (56).

Previous studies have indicated that insulin can use different mechanisms to activate ERK1/2 in different cell types or even in the same cell type after differentiation (8, 49, 57). Likewise, insulin's activation of ERK1/2 in different cell types also varies with respect to the requirement of PI3-K activation. In 3T3-L1 adipocytes and Rat-1 fibroblasts, inhibition of PI3-K signaling blocks insulin-induced ERK1/2 activation, indicating that PI3-K is necessary for ERK1/2 activation by insulin (45, 58). In contrast, insulin-induced ERK1/2 activation occurs independently of PI3-K activation in Chinese hampster ovary cells overexpressing the human insulin receptor, 3T3-L1 fibroblasts, and isolated human muscle strips (2, 45, 59). In another rat hepatoma cell line,

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KR-7, a high concentration of wortmannin (1 μM) inhibits insulin-induced Src homology 2 domain containing tyrosine phosphorylation, resulting in inhibition of ERK1/2 phosphorylation, and this is likely independent of the insulin receptor substrate (IRS) 1-PI3-K pathway (60). Thus, it is clear from previously published reports that there are multiple mechanisms for insulin-induced activation of ERK1/2. In the present work, however, it appears that the time point one chooses to examine the involvement of PI3-K in ERK1/2 activation by insulin (as well as which inhibitor one uses) may also influence the answer to this question.

Previous studies in Cos-1 cells have revealed that another growth factor, EGF, can lead to activation of Ras and ERK in two, temporally distinct, phases. Rapid activation of Ras occurs at the plasma membrane that is transient due to the activity of the RasGAP, CAPRI. In contrast, a delayed and sustained activation of Ras occurs in the Golgi apparatus that is induced via RasGRP1 (61). Activation of Golgi-restricted Ras by EGF or by UV radiation requires phospholipase Cγ (62). This activation of Ras requires activation of protein kinase C, similar to insulin-induced activation of ERK1/2 in adipocytes (63). Thus, differences of the location of Ras within the cell may explain the two temporally distinct phases of ERK1/2 activation by insulin in H4IIE cells observed in the present work. Future studies will seek to determine whether H4IIE cells have a significant pool of Ras located in the Golgi apparatus and whether it is activated by insulin. If so, the time course of activation of Golgi-restricted Ras *vs.* plasma membrane-associated Ras, the mechanism of activation of each pool of Ras, and, finally, how activation of distinct pools of Ras might lead to activation of the two categories of genes identified here represent intriguing future directions for the present studies.

The differences between the effects of LY294002 and wortmannin on transcription correlate with the effects of these inhibitors on the peak of ERK1/2 activation. Insulin-mediated activation of the peak of ERK1/2 was implicated in the induction of both ATF-3 and Pip92 transcription by several lines of evidence. Insulin-induced transcription was blocked by treatment with the MEK inhibitor, PD98059, indicating the requirement of the MEK-ERK pathway for insulin's effect. The effect of insulin on both ATF-3 and Pip92 transcription is extremely rapid, reaching maximum levels within 15 min, before the postpeak plateau of ERK1/2 activity is reached. This stimulation of ATF-3 and Pip92 transcription by insulin was inhibited by wortmannin, which blocked the rapid peak phase of ERK1/2 activation. However, blockade of PI3-K signaling without a significant effect on the rapid peak of ERK1/2 activation (LY294002 pretreatment) did not block insulin-induced transcription of ATF-3 or Pip92. Together, these results suggest that insulin's effect on these two genes may be achieved during the rapid peak of ERK1/2 activation, before the later phase plateau of ERK1/2 activation.

Induction of Insig-1 transcription is also dependent on ERK1/2 activation, as indicated by the ability of PD98059 to block this effect. However, unlike ATF-3 and Pip92, wortmannin did not block insulin-induced Insig-1 transcription. This may indicate that Insig-1 has a lower threshold of peak ERK1/2 activation required for its transcriptional regulation by insulin, given that we have previously reported apparent differences in the thresholds of ERK1/2 activation between MEK-dependent genes (20). Alternatively, this may suggest that only a physically distinct pool of ERK1/2 (that is unaffected by wortmannin) is required to fully induce transcription of Insig-1. In transcriptional studies, we have found rapid induction of genes by insulin, including ATF-3 and Pip92. In genes that are induced rapidly, by 7.5–10 min there is a large induction of transcription, approaching or equal to transcription rate levels induced after 15 min of insulin treatment. However, in support of our contention that insulin-induced transcription of Insig-1 is delayed, there is no induction of transcription by 7.5–10 min (see Fig. 1). This is consistent with our observation that, unlike ATF-3 and Pip92, Insig-1 has a peak of transcription that is temporally separated from the peak of ERK1/2 activation and is consistent with the plateau of ERK1/2 activation.

As suggested by Marshall, the eventual outcome of signaling through tyrosine kinase receptors will depend not only upon the extent of ERK activation but also the duration of ERK activation and the particular ERK-responsive transcription factors that are present in a given cell (64). For example, in PC-12 cells, EGF transiently activates ERK, resulting in stimulation of proliferation, whereas NGF causes a sustained activation of ERK and induces differentiation (64). Other studies indicate that for fibroblasts to become competent to proliferate, sustained growth factor exposure is necessary, which results in stabilization of the immediate early gene product, c-*fos* (65). More recent work indicates that stabilization of c-*fos*, as well as other immediate early gene products, many of which are transcription factors, is achieved by phosphorylation of the immediate early protein product by sustained ERK activation (66, 67). The present studies complement the previous work describing the importance of sustained ERK activation (induction of ERK activity for many minutes to hours) with the finding that rapid changes (a few minutes) in ERK activity stimulated by insulin treatment had significant effects on specific immediate early gene expression, including that of a transcription factor not previously known to be regulated by insulin alone (ATF-3).

In summary, we have presented data indicating that in H4IIE cells, insulin activated ERK1/2 in two temporally distinct phases, a rapidly induced peak phase of ERK1/2, followed by a plateau of ERK1/2 activation. The peak of ERK1/2 activation was only significantly blocked by wortmannin, but the plateau of ERK1/2 activation was equally sensitive to either PI3-K inhibitor (wortmannin or LY294002). Regulation of ATF-3, Pip92, and Insig-1 by insulin was dependent upon activation of the MEK-ERK pathway. However, unlike ATF-3 and Pip92, insulin-induced transcription of Insig-1 was insensitive to wortmannin. This suggests that there may be two groups of insulin-induced, MEK-ERK-dependent genes. ATF-3 and Pip92 represent one group of genes that is temporally related to the rapid peak of ERK1/2 activation by insulin and is blocked by wortmannin. In contrast, Insig-1 is distinct from the other group of genes in that it is temporally separated from this rapid peak and is insensitive to wortmannin.

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