Published in final edited form as:

Biochem Biophys Res Commun. 2016 June 03; 474(3): 594–598. doi:10.1016/j.bbrc.2016.04.067.

Prenylation Differentially Inhibits Insulin-Dependent Immediate Early Gene mRNA Expression

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Abstract

Increased activity of prenyl transferases is observed in pathological states of insulin resistance, diabetes, and obesity. Thus, functional inhibitors of farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) may be promising therapeutic treatments. We previously identified insulin responsive genes from a rat H4IIE hepatoma cell cDNA library, including β -actin, EGR1, Pip92, c-fos, and Hsp60. In the present study, we investigated whether acute treatment with FTase and GGTase inhibitors would alter insulin responsive gene initiation and/or elongation rates. We observed differential regulation of insulin responsive gene expression, suggesting a differential sensitivity of these genes to one or both of the specific protein prenylation inhibitors.

Keywords

insulin; gene expression; transcription; immediate early genes; prenylation

Introduction

Insulin is a key regulator of metabolism and growth in cells of hepatic origin. Insulin dependent gene expression has been a focus of our research using rat liver derived H4IIE hepatoma cells as a model system [1; 2]. This cell line expresses both insulin and growth hormone receptors, and responds to physiologic concentrations of both [1–8]. Therefore, this line has been utilized in the study of intracellular signaling molecules and subsequent gene expression as we have previously identified cytoskeletal, cell division, and cellular stress-regulated genes that are transcriptionally regulated by insulin [9–18]. Many of these genes

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are described as immediate early genes due to their robust and rapid activation in response to various growth factors or other environmental stimuli [19; 20].

Insulin initiates intracellular signaling by first binding to, and activating, the tyrosine kinase insulin receptor, and then, via multiple pathways including the MAP kinase (MEK/ERK) and the phosphatidylinositol 3-OH-kinase (PI3K) pathways, activates multiple downstream signaling molecules [21]. We have previously demonstrated that the pattern of insulin induced ERK1/2 activation is important for the temporal expression patterns of insulin-induced genes [18]. Our findings also indicate that a complex coordination of signaling pathways activated by insulin is required for the expression of the EGR1 and 2 genes [22].

Post-translational modification of proteins is a central concept of intracellular signaling. Prenylation of GTP-binding proteins, nuclear lamins, and many protein kinases increase membrane and/or protein-protein interaction [reviewed elsewhere [23; 24]]. Two classes of related lipid transfer enzymes are the CAAX-motif recognition farnesyl transferase (FTase) / geranylgeranyl transferase-I (GGTase-I), and the Rab-GGTase (GGTase-II). The FTase and GGTase-I enzymes are structurally similar, but achieve specificity dictated by the type of Ras protein acceptor [24–26]. GGTase-II is specific to Rab proteins involved in membrane trafficking and receptor endocytosis [24].

Insulin activates FTase and GGTase-I via Shc [27], and subsequently increases the amount of farnesylated p21-Ras [28] and geranylgeranylated Rho-A [29]. Increased activity of prenyl transferases have been observed in pathological states of insulin resistance, diabetes, and obesity [28; 30]. This led to an effort to functionally inhibit FTase and GGTase toward potential therapeutic treatments [31; 32]. Insulin has also been observed to activate GGTase-II and increase geranylgeranylated Rab-4 in a MEK/ERK dependent manner, but the physiological significance of this is still unclear [33]. Therefore, we have focused on the CAAX-type prenyl transferases as possible mediators of insulin regulated gene expression. In the present experiments, we investigated whether acute treatment with these inhibitors would alter mRNA initiation and/or elongation rates of multiple insulin responsive genes.

We previously identified insulin responsive genes from a cDNA library isolated from rat H4IIE hepatoma cells [34–36] and selected multiple clones that by DNA sequence analysis were identified as immediate early genes. The present study was designed to ask whether inhibition of prenylation would alter acute insulin-regulated gene initiation and/or elongation and we observed differential regulation by use of specific protein prenylation inhibitors.

Material and Methods

Cell Culture

Rat H4IIE (H4) hepatoma cells (ATCC; CRL-1548; Rockville, MD) were grown at 37°C in 5% CO₂, 95% humidity in Swins S-77 (U.S. Biological; Swampscott, MA) supplemented with 2% fetal bovine serum (Hyclone; Logan, UT), 3% calf serum, and 5% horse serum (Gibco; Carlsbad, CA). Prior to experimental treatments, cells were washed and transferred into serum-free medium for 20–24 hours. All experiments were performed on 70–80% confluent plates following previously established protocols [6].

Plates were treated with 10 nM porcine insulin (Sigma; St. Louis, MO) for 15 or 30 minutes and media was aspirated and cells were isolated by scraping. When inhibitors were used they were added 30 min prior to the addition of insulin to allow for blockade of the specific pathway. The inhibitors and concentrations used were: the farnesyl transferase inhibitor, [1 μ M in ethanol] α -hydroxyfarnesylphosphonic acid; abbreviated HFPA; (Biomol; Plymouth Meeting, PA) and the geranylgeranyl transferase inhibitor, [3 μ M in DMSO] GGTI-286; abbreviated GGTI; (Calbiochem/EMD; Gibbstown, NJ). Unless noted, all other reagents were purchased from Fisher Scientific (Waltham, MA).

Differential screening of cDNA libraries

Libraries were constructed from H4IIE cells treated with insulin and anisomycin for 2 hours as described previously [34]. Library colonies were plated, transferred to nylon membranes in duplicate, and denatured. Membranes were probed with radiolabeled cDNA produced from the mRNA of H4IIE cells treated with either vehicle or a postprandial concentration of insulin (1×10^{-8} M) and anisomycin (100 μ M) for 2 h. Colonies which exhibited expression changes were selected and subsequently sequenced [34]. For the purpose of this manuscript, the following genes were identified and used as an indicator of insulin action: β -actin (ACTB), Hsp60 (HSPD1), EGR1 (NGF1A), c-fos (FOS), and Pip92 (IER2).

Nuclear Run-On Analysis

Messenger RNA (mRNA) initiation/elongation rates were assayed as previously described [22]. Transcriptionally active nuclei were isolated, then incubated in the presence of 32 P-UTP in order to extend and label nascent mRNA. Transcripts were purified and hybridized against a nitrocellulose bound cDNA array. Membranes were exposed to autoradiograph film and analyzed using scanning densitometry. It is important to note that the current experiments are performed at time points following insulin too early to measure steady-state mRNA levels, and much too soon to be able to measure protein levels expressed from the mature mRNA. Thus, this assay is designed to study the elongation of mRNA previously initiated in response to the addition of insulin to H4IIE cells.

Densitometry

Each autoradiogram was scanned and then analyzed using Scanalytics ZeroD scan (v1.1; Fairfax, VA). Unity was assigned to an experimental control and change from that control is presented as the fold-difference. Unless noted, at least three independent experiments were averaged and presented as mean $\pm$ standard error (SEM).

Statistical Analysis

All data was analyzed by analysis of variance (ANOVA) using the Tukey-Kramer post-test for comparison of all experimental groups to the control of each experiment or the Student's *t*-test using Instat (Graphpad v3.0; San Diego, CA) software. Significance was established when $P < 0.05$, with all comparisons indicated.

Results

Selecting Genes of Interest

The H4IIE hepatoma cell line was used as a model for the hepatic response to insulin [9–18]. Differential screening allowed us to identify genes highly responsive to insulin. Each was subcloned and the sequence was identified using the NCBI database. These target genes were used in nuclear run-on assays to verify the insulin responsive increase in transcription. The genes are listed in Table 1 and include targets previously identified by our lab as immediate early genes [11; 12; 14; 34; 35].

Differential Gene Expression with Inhibitors of Prenylation

In the present studies H4IIE cells were pre-treated with inhibitors of FTase or GGTase-I for 30 minutes prior to insulin challenge. For all targets, there was no effect of the inhibitors alone as compared to vehicle treated control cells.

Our earlier studies indicate that cytoskeletal genes are regulated by insulin and this includes β -actin, which was rapidly induced following addition of insulin to H4IIE cells [11]. The insulin regulation of β -actin required a proximal promoter CCArGG motif, similar to that in other mitogen-induced genes [37]. In the present study, β -actin gene expression was induced by insulin alone, and was not significantly altered upon use of either inhibitor (Fig. 1a).

EGR1 (NGF1A) is a member of a family of zinc-finger transcription factors known to be expressed in liver and other tissues and are regulated by mitogenic stimuli that induce cellular proliferation [38; 39]. We reported that EGR1 gene expression is rapidly induced by insulin, and regulated by feedback between the p38 and MEK/ERK pathways [22]. Similar, to β -actin, there was a rapid and large induction of EGR1 gene expression by insulin alone, which was not significantly changed upon use of either inhibitor (Fig. 1b).

c-fos (FOS) is a mitogen and cellular activation responsive proto-oncogene [40–42], and the *c-fos* protein associates with *c-jun* creating the AP-1 DNA binding complex which regulates many other genes [43; 44]. We have previously reported that *c-fos* gene transcription is insulin responsive and was sensitive to reduction in PKC activity [9; 12]. The current experiments indicate that inhibition of GGTase-I resulted in an increase in insulin sensitivity unlike HFPA which did not alter the insulin effect (Fig. 2a).

Pip92 (IER2) was originally identified and cloned as a proline-rich serum/growth factor-induced protein whose gene is regulated by growth factors via MAPK-dependent pathways [45–48]. We have previously reported that Pip92 gene expression was acutely activated by insulin and regulated predominantly via the peak levels of ERK1/2 activation [34]. As with the *c-fos* gene, Pip92 was more insulin sensitive upon treatment with GGTI, but not with HFPA (Fig. 2b).

Lastly, we identified Hsp60 as an insulin responsive immediate early gene whose regulation is via the MEK/ERK pathway [35]. In the current study Hsp60 transcription was induced by insulin alone (Fig. 3) and inhibition of FTase resulted in increased insulin sensitivity which was specific, as GGTase inhibition did not alter the insulin response.

Discussion

The cellular response to insulin includes the regulation of numerous genes, including transcription factors which can promote a metabolic or mitogenic response of the cell. The present studies have explored the contribution of protein prenyltransferases to insulin-regulated gene expression. We have found that inhibition of GGTase-I or FTase have non-overlapping and specific effects on insulin-responsive genes resulting in increased sensitivity to an acute administration of insulin in a subset of the genes studied.

Inhibition of GGTase-I or FTase had no effect on the insulin-induced initiation and/or elongation of the β -actin and EGR1 genes. Insulin is known to have a biphasic action on FTase activity, with two peaks at 5 and 60 minutes [49], via a Shc/MAPK dependent mechanism [50; 51]. This correlates well with the insulin induction of ERK1/2 activity previously reported by our laboratory in H4IIE cells [22]. Since the insulin induction of the β -actin and EGR1 genes is solely dependent upon insulin activation of MEK/ERK activity, and do not change in response to inhibition of GGTase-I or FTase, it likely suggests that the GGTase and FTase inhibitors do not alter the ability of insulin to maximally induce MEK/ERK.

However, initiation and/or elongation of c-fos and Pip92 mRNAs were found to be more insulin sensitive upon pre-treatment with GGTI. This alteration in sensitivity to insulin is similar to our previous observation using SB202190, an inhibitor of p38 activity [22; 52]. Regulation of MEK1 via p38 is an inhibitory feedback mechanism to ERK dependent gene expression, and when blocked, increased insulin sensitivity is observed. It is possible that GGTI is specifically acting via this pathway resulting in these genes becoming more sensitive to insulin upon inhibition of GGTase-I activity.

We have recently found that insulin induced HSP60 gene expression is ERK dependent and more sensitive to insulin upon inhibition of p38 [35], but unlike c-Fos and Pip92, HSP60 gene initiation and/or elongation is not altered in the presence of GGTI. However, HSPA is shown to specifically increase the insulin-sensitive induction of HSP60. We speculate that this may be due to differential effects on the temporal activation of ERK. Previously an inhibitor of insulin signaling, Wortmannin, was shown to block insulin dependent HSP60 transcription via abrogation of rapid ERK activity and is also known to inhibit the mTOR kinase and the action of rictor-mTOR to activate AKT/PKB [35; 53; 54]. This suggests multiple signaling pathways regulate the insulin induced expression of HSP60, and upon FTase inhibition that a regulatory feedback mechanism may be lost.

Our previous data indicate that insulin-induced and ERK-dependent genes are expressed at different time points following insulin activation of ERK, and that many are under the control of additional pathways, including the p38 pathway [18; 22; 52]. Thus, both the time period of insulin-induced ERK activity, and the activity of other MAPK pathways help regulate these ERK-dependent genes. Our current data suggest that some insulin induced, ERK-dependent gene expression can be further modified by GGTases and FTases. The insulin-induced mRNA initiation and/or elongation rates of the β -actin and EGR1 genes were not affected by inhibition of either GGTase or FTase and, thus, are not under the

control of these pathways. The insulin and ERK pathway dependent induction of the c-fos and Pip92 genes is under tonic inhibition by GGTase, since inhibition of GGTase activity increased the insulin effect. Conversely, the insulin and ERK pathway induction the Hsp60 gene is under tonic inhibition by FTase, since inhibition of FTase activity increased the insulin effect. Thus, even though insulin, via activating the MEK-ERK signaling pathway can regulate a number of genes, multiple other signaling pathways can directly or indirectly modulate insulin-induced ERK gene expression, resulting in a specific gene expression profile in response to insulin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Derwei Venable, Dr. Adam Keeton, Dr. William Bennett and current members of the laboratory for insightful discussions and suggestions during this manuscript's preparation. This work is supported by grants from the National Institutes of Health (DK62071), and the Veterans Administration Merit Review (2IO1BX000611-05) to J.L.M.

**Abbreviations

FTase	farnesyl transferase
GGTase	geranylgeranyl transferase
HFPA	α -hydroxyfarnesylphosphonic acid
GGTI	geranylgeranyl transferase inhibitor GGTI-286

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Highlights

We determined that:

- Inhibition of prenylation, specifically inhibition of GGTase-I or FTase, have non-overlapping and specific effects on insulin-responsive genes, resulting in increased sensitivity to an acute administration of insulin.
- Inhibition of GGTase-I or FTase had no effect on the insulin-induced initiation and/or elongation of the β -actin and EGR1 genes.
- Initiation and/or elongation of c-fos and Pip92 mRNAs were found to be more insulin sensitive upon pre-treatment only with the GGTase inhibitor, GGTI.
- HSP60 gene initiation and/or elongation was not altered in the presence of GGTI. However, an FTase inhibitor, HFPa, was shown to specifically increase the insulin-sensitive induction of HSP60.
- Both the time period of insulin-induced ERK activity, and the activity of other MAPK pathways help regulate these ERK-dependent genes, which may be further modified by inhibition of protein prenylation.

Fig. 1a

Fig. 1b

Figure 1. Insulin induced β -actin and EGR1 mRNA initiation and/or elongation is unchanged upon prenylation inhibition

Serum-deprived H4IIE cells were treated with insulin (10 nM) alone or after pre-treatment with prenylation inhibitors, GGTI (3 μ M) or HFPA (1 μ M), as indicated. Nuclei were purified and subjected to the Nuclear Run-On assay as described in methods. The vehicle treated control level was set to unity and the fold-change of incorporated dCTP is plotted. The symbols indicate mean $\pm$ SEM for at least three experiments at each time point. * indicates a significance of $p < 0.05$ versus the vehicle control. \$ indicates a significance of $p < 0.05$ versus the inhibitor alone.

Fig. 2a

Fig. 2b

Figure 2. Insulin sensitivity of *c-fos*, and Pip92 mRNA initiation and/or elongation is increased upon GGTI treatment

Serum-deprived H4IIE cells were treated with insulin (10 nM) alone or after pre-treatment with prenylation inhibitors, GGTI (3 μ M) or HFPA (1 μ M), as indicated and the Nuclear Run-On assay was performed and analyzed as described in Fig. 1. The symbols indicate mean $\pm$ SEM for at least three experiments at each time point. * indicates a significance of $p < 0.05$ versus the vehicle control. \$ indicates a significance of $p < 0.05$ versus the inhibitor alone. # indicates a significance of $p < 0.05$ versus insulin alone.

Figure 3. HSP60 mRNA initiation and/or elongation is more insulin sensitive upon HFPA treatment

Serum-deprived H4IIE cells were treated with insulin (10 nM) alone or after pre-treatment with prenylation inhibitors, GGTI (3 μ M) or HFPA (1 μ M), as indicated and the Nuclear Run-On assay was performed and analyzed as described in Fig. 1. The symbols indicate mean $\pm$ SEM for at least three experiments at each time point. * indicates a significance of $p < 0.05$ versus the vehicle control. \$ indicates a significance of $p < 0.05$ versus the inhibitor alone. # indicates a significance of $p < 0.05$ versus insulin alone.

Table 1
Immediate Early Genes Identified by Differential Screening

Differential screening of H4IIE cells treated with either vehicle or a postprandial concentration of insulin (1×10^{-8} M) and anisomycin (100 μ M) for 2 h. Colonies which exhibited expression changes were selected and subsequently sequenced as described in the methods [34]. The targets are listed with a description of known cellular functions.

Gene ID	Accession#	Function
β -actin (ACTB)	AK130157	Cell migration
EGR1	BC043224	Zinc-Finger transcription factor
Pip92 (IER2)	AA235294	Proline-rich transcription factor
c-fos (FOS)	AF111167	AP1 complex cell differentiation, transcription factor
Hsp60 (HSPD1)	AU098504	Protein assembly, molecular chaperone