Original Article
Insulin Dependant Gene Expression of Heat Shock Protein 60 in H4IIE Hepatoma Cells

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Abstract: Insulin regulates metabolism and growth in cells of hepatic origin by specifically binding to and activating the tyrosine kinase insulin receptor. Insulin-induced intracellular signaling is conducted via multiple pathways, including the MAP kinase (MEK/ERK) and the phosphatidylinositol 3-kinase (PI3K) pathways, which in turn activate multiple downstream signaling molecules. Heat shock protein 60 (HSP60; chaperonin 60kD) was selected by screening to be regulated by insulin in rat hepatoma cells. Heat shock proteins are a family of molecular chaperones whose main cellular function is to mediate the proper folding of newly synthesized proteins. The cellular response to stress is characterized by an overall decrease in protein synthesis, and upregulation of the heat shock protein family, including HSP60. A role for HSP60 has been implied in many diseases and in the responses to hypoxia. The present study was designed to ask whether insulin stimulated HSP60 gene expression. The rate of HSP60 transcription and mRNA accumulation were measured in rat H4IIE hepatoma cells and insulin-induced expression of HSP60 was predominantly via the MEK/ERK pathway. Inhibition of the p38 and PI3K pathways suggest complex feedback interactions of other insulin-, cell stressor- and cytokine-regulated pathways on the primary role of the MEK/ERK signaling in the regulation of HSP60 gene expression by insulin.

Key Words: Insulin, heat shock proteins, HSP60, transcription, MEK/ERK

Introduction

Insulin is a key regulator of metabolism and growth in cells of hepatic origin. After first specifically binding to and activating the tyrosine kinase insulin receptor, insulin-induced intracellular signaling is conducted via multiple pathways, including the MAP kinase (MEK/ERK) and the phosphatidylinositol 3-OH-kinase (PI3K) pathways, which in turn activates multiple downstream signaling molecules [1]. Activation of these two pathways is usually considered separately, and activation of the MEK/ERK pathway has been shown to be independent of the PI3-K pathway [2,3]. However, MEK/ERK activation by insulin has been reported to require activation of the PI3-K pathway in adipocytes [4,5], muscle cells [6,7], Chinese hamster ovary cells expressing the insulin receptor [8,9], and in rat H4IIE hepatoma cells [10].

Heat Shock Proteins (HSP) are a family of molecular chaperones whose main cellular function is to mediate the proper folding of newly synthesized proteins. Additionally, it has been shown that they aid intracellular transport and possibly modulate degradation by ubiquitination [11]. Multiple heat shock proteins have been identified, but we have focused on HSP60 (chaperonin 60kD), as this was selected by screening to be regulated by insulin in rat hepatoma cells.

HSP60 and its closely related HSP10 have been well characterized and are now known to form a complex which, in the presence of ATP, assists in protein folding. This complex has been found to be expressed throughout the cell, but is predominantly located in the mitochondria where it is involved in the assembly of the ATP synthase complex.
Cytosolic HSP60 is limited to about 10% of the total cellular content. However, localization is an important regulatory process during the stress response. The cellular response to stress is characterized by an overall decrease in protein synthesis, and upregulation of the heat shock protein family, including HSP60 [12]. In H35 hepatoma cells, the progenitor cell line of the H4IIE cell line used in the present work, HSP60 was induced in response to increasing heat exposure or exposure to arsenite [13]. A role for HSP60, as yet not clearly defined, has been implied in many diseases, including autoimmune diabetes and atherosclerosis and in the responses to hypoxia, and streptozocin induced insulinitis [14-16]. Additionally, IGF-1 receptor (IGF1R) signaling may play a contributory role in the induction of HSP60 in at least some of these conditions [11]. Due to the close sequence identity and physical structure of the insulin and IGF1 receptors, and the shared usage of signaling pathways by these two similar receptors, a role of insulin in HSP60 expression would not be surprising.

We previously identified insulin-responsive genes through differential production of an insulin/anisomycin treated cDNA library isolated from rat H4IIE hepatoma cells and screening insulin-regulated genes [17]. Following this screening protocol [17], we selected a single clone that by DNA sequence analysis was identified as HSP60. The present study was designed to test whether insulin directly stimulated HSP60 gene expression, and, if so, to ask by what insulin-regulated intracellular signaling pathway(s). By measuring the rate of HSP60 transcription and accumulation of mRNA in rat H4IIE hepatoma cells, and use of specific inhibitors of intracellular signaling pathways, the MEK/ERK pathway was found to be of foremost importance in the insulin-induced stimulation of HSP60 gene expression. Use of inhibitors of the p38 and PI3K pathways indicated that there are multiple potential positive and negative feedback inputs by other insulin-, cell stressor- and cytokine-regulated pathways that may play a role in insulin regulation of HSP60 gene expression.

Materials 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 Swims S-77 (U.S. Biological; Swampsccott, 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 [18]. This hepatoma cell line is highly sensitive to insulin and has been found to respond in a similar fashion as the liver to insulin.

Plates were treated with 10 nM porcine insulin (Sigma; St. Louis, MO) for the indicated times 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 used, the concentrations used, and references justifying the concentrations used were: PD98059 (50 μM; Cell Signaling Technology, Inc.), to block activation of MEK by insulin (please see [10,19-23]); LY294002 (50 μM; BIOMOL International L.P.; Plymouth Meeting, PA; [10,21-23]) and wortmannin (100 nM; Sigma; [21,22,24]) are PI3-K inhibitors; and SB202190, an inhibitor of p38 was obtained from Calbiochem (10 μM; San Diego, CA; [20,10]). 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 [17]. 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 X 10⁻⁸ M) and anisomycin (100 M) for 2 h. Colonies which exhibited expression changes were selected and subsequently sequenced [17]. A single clone was selected and identified as HSP60 using the NCBI GenBank database.

RNA extraction

Total RNA was isolated using Ultraspec RNA...
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isolation reagent (Biocex; Houston, TX) following the manufacturer's protocol. Briefly, for a 100 mm plate, 800 μL of the Chaosolv based denaturing reagent was used to isolate and homogenize the cells, followed by isolation of the aqueous phase, which was then precipitated by isopropanol and sodium acetate/ethanol [25]. The concentration and purity was determined by spectrophotometric analysis.

Northern analysis

Total RNA samples (10 μg) were electrophoresed using 2.2M formaldehyde, 1.2% agarose denaturing gels [26]. Proper loading was confirmed by staining the 28S/18S ribosomal RNA bands with acridine orange and a broad range RNA ladder (Invitrogen; Carlsbad, CA). RNA was transferred to a nylon membrane (Ambion; Brightstar-Plus; Austin, TX), membranes were incubated with an [32P]dCTP-labeled (Stratagene; LaJolla, CA) HSP60 cDNA, and were autoradiographed and analyzed using scanning densitometry.

Nuclear run-on analysis

Transcription rates were assayed as previously described [21]. Transcriptionally active nuclei were isolated, then incubated in the presence of [32P]-UTP in order to extend nascent labeled mRNA. Transcripts were purified and hybridized against a nitrocellulose bound cDNA array. Membranes were autoradiographed and analyzed using scanning densitometry.

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 ± standard error (SEM).

Statistical analysis

All data was analyzed by analysis of variance (ANOVA) and the Dunnett ANOVA post test for comparison of all experimental groups to the control of each experiment or the Student's 2-tailed t-test using Instat (Graphpad v3.0; San Diego, CA) software. Significance was established when P<0.05, with all comparisons indicated.

Results

Time course of insulin-induced HSP60 transcription

Serum-deprived H4 hepatoma cells were treated with porcine insulin over a 6 hour time course. At each time point studied, cellular nuclei were isolated and transcription rates were measured using the nuclear run-on assay. Insulin rapidly induced HSP60 transcription, a significant increase was
measured as early as 7.5 minutes, and the increase was maximal, at approximately 4-fold, at 15 and 30 minutes (Figure 1). The transcription of the HSP60 gene returned to control levels by 6 hours even in the continued presence of insulin.

**Insulin signaling inhibition and HSP60 transcription**

Further experiments explored the effects of signaling pathway inhibitors on the peak rate of insulin-induced transcription measured at 30 minutes. Specific inhibition of p38 was obtained with SB202190, and the presence of the inhibitor alone did not alter HSP60 expression when compared to vehicle alone (Figure 2A). Following pretreatment with this p38 inhibitor prior to addition of insulin, stimulation of HSP60 transcription was not observed to be reduced, but in fact increased; insulin alone resulted in a 4-fold increase, whereas pretreatment with SB202190 followed by addition of insulin resulted in a 7.4-fold induction of HSP60 transcription.

A potent, specific inhibitor of MEK1/MKK1 (PD98059) was then used and as presented in Figure 2B, inhibition of the MEK/ERK pathway did not alter basal HSP60 transcription rates. However, pretreatment with PD98059 prior to addition of insulin completely inhibited the
insulin-dependant stimulation of HSP60 transcription. Inclusion of the p38 inhibitor, SB202190, in combination with PD98059, resulted in a complete inhibition of the larger synergistic effect of insulin in the presence of SB202190 (compare the last bar of Figure 2A with the last bar of Figure 2B). These results are reminiscent of several previous studies suggesting that inhibition of the p38 pathway results in a hyper-induction of ERK activation and a hyper-induction of ERK dependent genes [20, 21]. Thus, insulin induction of HSP60 transcription occurs via regulation of the MEK/ERK pathway. The p38 pathway may normally inhibit this pathway, and when the p38 pathway is inhibited, there is a greater insulin stimulation of the MEK/ERK pathway and there is an enhanced induction of insulin and MEK/ERK-dependent genes, such as HSP60.

Next, the specific phosphoinositol-3 kinase (PI3K) inhibitor, LY294002, was used prior to insulin addition to determine if PI3K blockade would interfere with insulin induced HSP60 transcription. Cells cultured in the presence of the inhibitor alone exhibited no change relative to vehicle controls. Pretreatment of the H4 cells with LY294002 prior to addition of insulin seemed to partially inhibit the insulin stimulation of HSP60 transcription, but this inhibition did not reach statistical significance (Figure 2C). Therefore, the PI3K signaling pathway, that is highly stimulated by insulin in this cell line, does not seem to play a major role in the induction regulation of HSP60 gene expression at concentrations that fully inhibit insulin-stimulated PI3K activity [10, 21].

However, when a second PI3K inhibitor, wortmannin, is used a slightly different result is obtained. Cells cultured in the presence of the wortmannin alone exhibited no change relative to vehicle controls, but unlike with LY294002, pretreatment of H4 cells with wortmannin prior to addition of insulin completely inhibited the insulin stimulation of HSP60 transcription (Figure 2C). As described at length in previous publications, insulin activates ERK1/2 in two temporally distinct phases, a rapidly induced peak phase of ERK1/2, followed by a plateau of ERK1/2 activation [10,21]. The peak of insulin induced ERK1/2 activation was significantly blocked by wortmannin, but not LY294002 [10]. Regulation of HSP60 by insulin was dependent upon activation of the MEK/ERK pathway (Fig. 2B), not affected by LY294002 (Figure 2C), but blocked by wortmannin (Figure 2D), suggesting that HSP60 is temporally regulated by the rapid peak of ERK1/2 activation by insulin.

**Time course of insulin stimulation on HSP60 mRNA**

Following addition of insulin to H4 cells, total cellular RNA was isolated and Northern analysis was employed using a HSP60 specific cDNA in order to assay the relative concentration of HSP60 mRNA. Compared to the basal value of HSP60 mRNA, that in H4...
cells treated with the vehicle control, insulin rapidly increased HSP60 mRNA, which reached statistical significance by 60 minutes following the addition of insulin (Figure 3). Unlike the transient induction of transcription, HSP mRNA continued to rise up to 6 hours, and then increased even further and remained elevated at 20 and 24 hours, the longest time tested in the present experiments, with a maximum induction of 9.2-fold.

**Inhibitor of MEK1 blocks insulin induced HSP60 mRNA**

As presented above, when PD98059 was used to specifically inhibit MEK1, the insulin stimulation of HSP60 gene transcription was inhibited. Therefore, we investigated whether the insulin-induced increase of HSP60 mRNA accumulation was also sensitive to MEK1 inhibition. Cells were pretreated with PD98059 for 60 minutes prior to the addition of insulin for a further 60 minutes. Cells cultured in the presence of the inhibitor alone exhibited no change in the basal expression of HSP60 mRNA when compared vehicle-treated (control) cells (Figure 4). However, following pretreatment of cells with the MEK1 inhibitor, the induction by insulin of HSP60 mRNA accumulation was completely blocked, to approximately the levels measured in vehicle treated controls. These results parallel the findings on gene transcription rates and confirm transcriptional control of HSP60 by insulin is predominantly via the MEK/ERK pathway.

**Discussion**

Heat shock proteins have many important functional roles in metabolically active tissues, including those responsive to insulin. HSP60 is normally found to be highly expressed in liver, with preferential expression in mitochondria of hepatocytes and Kupffer cells. There is increased HSP60 expression in Kupffer cells following heat shock and in hepatocytes following liver inflammation [27]. In mitochondria, HSP60 is associated with and involved in the proper assembly of the ATP synthase complex and may be important for the proper assembly of other enzyme complexes [28,29]. Cytosolic HSP60 associates with intracellular p21Ras, and this association increases upon serum stimulation of fibroblasts [30]. HSP60 can bind and activate specific integrins and may be involved in the IGF-1 activation of "3$1 integrin [31]. These studies suggest a positive role of cytosolic HSP60 in intracellular signaling. However, excessive HSP60 accumulation in the cytosol may serve a pro-apoptotic function, and movement from the cytosol to the plasma membrane, with a concomitant release of the pro-apoptotic protein BAX following hypoxia, may be one of the mechanisms by which HSP60 is involved in apoptosis [32,33].

While there is evidence that HSP60 may play an important role in normal beta cell physiology, and perturbation of HSP60 expression and/or location is coincident with beta cell destruction [14,34,35], most relevant
to the present work are several studies indicating an association of HSP60 expression and insulin or insulin-like signaling. As yet there is not data for a direct effect of HSP60 on the insulin receptor or insulin signaling. However, in the myocardium HSP60 inhibits ubiquitination and degradation of the insulin-like growth factor 1 receptor (IGF-1R) with no change in IGF-1R mRNA. Overexpression of HSP60 results in increased IGF-1R levels and an amplification of intracellular signaling in of the response to IGF-1 [11]. Expression of HSP60 is decreased in the myocardium and in adipocytes of animal models of diabetes, such as Zucker Fatty rats, and in streptozotocin-induced insulin deficiency [11,15,36]. This decrease in HSP60 correlates with a loss of IGF-1R signaling. Both HSP60 expression and IGF-1 signaling is restored following treatment with insulin. Thus, there is now interest in the contribution of decreased HSP60 expression, and the subsequent decline of IGF-1 signaling, to diabetic cardiomyopathy.

There is little published data on the specific regulation of HSP60 gene expression, except the well-known inducers of heat shock proteins, such as heat stress, cellular poisons and heavy metals [13]. We selected the HSP60 cDNA out of a library to screen for insulin-induced hepatic genes, and therefore became interested in how insulin influenced HSP60 gene expression. Using specific pharmacological inhibitors, the MEK/ERK pathway was found to play a dominant role in the insulin induction of both HSP60 transcription and mRNA levels in this rat hepatoma cell line. And using the PI3K inhibitor, LY294002, which effectively blocks insulin activation of AKT in H4IIE cells [21], we found little evidence of a direct contribution of the PI3K-AKT signaling pathway to the insulin stimulation of HSP60 transcription.

Previous studies indicate at least two, distinct, temporal patterns of insulin effects on the MEK/ERK pathway which may regulate gene expression of multiple genes. For instance, the immediate early genes ATF3 and Pip92 appear to be regulated by the rapid peak of ERK that occurs within the first five minutes after adding insulin, whereas the induction of Insig-1 requires not the peak, but the slightly delayed plateau phase of insulin-induced MEK/ERK [10]. In H4IIE cells, the PI3K pathway may play a feedback role on insulin regulation of the MEK/ERK pathway. While the PI3K inhibitor LY294002 has only a minimal effect on the rapid peak phase of insulin induced ERK1/2, wortmannin, another inhibitor of PI3K was found to significantly inhibit the peak activation of ERK1/2 (please see [10]). The present study indicates that HSP60 gene transcription is also temporally induced by insulin, with a strong induction at early time points, coincident with the early phase of MEK/ERK activation. In addition, due to the blockade of insulin-induced HSP60 transcription by wortmannin, which greatly reduces the rapid peak of insulin-induced ERK1/2 [10], it indicates that the HSP60 gene is similar to insulin regulation of the immediate early genes ATF3 and Pip92. Thus, the rapid peak of MEK/ERK activation by insulin (which is blunted by wortmannin) is necessary for insulin induction of HSP60 gene expression.

When a specific inhibitor of the p38 pathway was administered prior to insulin treatment there was no reduction of HSP60 gene expression, but an enhancement of the insulin effect. This has some similarities to previous studies which suggest that p38 may negatively regulate the MEK/ERK [21,37]. Clearly, inhibition of MEK1 completely blocked the insulin-stimulated transcription of HSP60, as well as the synergistic effect of p38 blockade plus insulin on HSP60 gene expression, indicating the necessary involvement of insulin-regulated MEK/ERK. This feedback by the p38 pathway and by the PI3K pathway (see previous paragraph) suggest that there are complex negative feedback pathways involved in the insulin induction of HSP60 transcription, such that other insulin signaling pathways (PI3K) and other insulin and stress-regulated pathways (such as p38) can alter the insulin regulation of this gene. Also, there is a significant post-transcriptional effect on HSP60, beyond the effects on transcription, since HSP60 mRNA continues to rise and remains elevated after the induction of transcription has waned. However, since this later increase in HSP60 mRNA, most likely due to increased mRNA stability, is coincident with increased DNA synthesis and cell division in response to insulin [38-40], it is difficult to tell whether this is a direct effect of insulin, or dependent on the effect insulin to induce DNA synthesis in this cell line.

In summary, we report here that HSP60 gene expression was induced in H4IIE hepatoma...
cells by insulin. A specific inhibitor of MEK1, was able to block the induction of HSP60 gene expression and the early phase of HSP60 mRNA accumulation. Thus, insulin regulation is via a MEK/ERK-dependant pathway. Further, there are multiple potential feedback inputs by other insulin-, cell stressor- and cytokine-regulated pathways that may play a role in insulin regulation of HSP60 gene expression. Due to the cellular roles of HSP60 as a chaperone, especially for mitochondrial proteins and the IGF-1R, the study of its regulation may be important in the potential anti-inflammatory actions of insulin.

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