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Insulin attenuates TNFα-induced hemopexin mRNA: An anti-inflammatory action of insulin in rat H4IIE hepatoma cells

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ABSTRACT

Proinflammatory cytokines, including TNF-α and IL-6, can contribute to insulin resistance. Conversely, insulin has some actions that can be considered anti-inflammatory. Hemopexin is a Class 2 acute phase reactant and control of its transcription is predominantly regulated by IL-6, with TNF-α and IL-1β also inducing hemopexin gene expression. Thus, we asked whether insulin could inhibit the ability of TNF-α to stimulate hemopexin mRNA expression. In cultured rat hepatoma (H4IIE) cells, TNF-α significantly increased hemopexin mRNA accumulation. The TNF-α-induced increase of hemopexin mRNA was dramatically attenuated by insulin, even though TNF-α reduced peak insulin activation of ERK. Thus, even though TNF-α can contribute to insulin resistance, the residual insulin response was still able to counteract TNF-α actions.

1. Introduction

Hemopexin (Hx) is a serum glycoprotein produced in the liver, which can bind free heme and promotes the scavenging of heme by the liver. Upon internalization by the liver, heme is catabolized to bilirubin resulting in conservation of cellular iron [1]. Due to the high affinity of hemopexin for heme, additional benefits have been observed including suppression of bacterial replication by removal of excess iron [2], and prevention of heme-catalyzed oxygen radical formation and oxidative cellular damage [3]. Since hemopexin is also a major mammalian hyaluronidase, it is important for the immune response and angiogenesis at the site of wound repair [4].

The regulation of hemopexin is complex and poorly understood [5], but is primarily controlled at the transcriptional level. Hemopexin is a class 2 acute phase reactant and control of its transcription is best characterized during the acute phase response [6]. The predominant regulator of hemopexin gene expression is IL-6, but TNF-α and IL-1β can also induce hemopexin mRNA expression [7–9]. Recently, we identified hemopexin as a growth hormone regulated gene [10].

Hemopexin has numerous anti-inflammatory actions, for instance by limiting the macrophage response to LPS [11–13]. Knockout of the Hx gene accelerated disease progression via regulation of IL-17 secreting Th cells (Th17) in a mouse model of multiple sclerosis [14]. In a model of heme overload there was increased infiltration of CD18+ macrophages in liver and increased oxidative stress in Hx-null mice. And both hepatic overexpression and exogenous administration of Hx in murine sickle cell disease models improved markers of inflammation, likely via regulation of the Nrf2/HO-1 antioxidant defense axis [15–17].

The proinflammatory cytokines, in particular TNF-α, but also IL-6 and IL-1β, can cause insulin resistance *in vivo* and in cultured cells [18–20]. Conversely, insulin is sometimes referred to as an anti-inflammatory hormone [21–23]. Insulin can inhibit IL-6 induced gene expression [24], the dominant stimulator of hemopexin and other acute phase gene expression, via inhibiting IL-6 stimulation of STAT3 activation [25,26]. Increased TNF-α, partially via activation of the JNK signaling pathway, is known to contribute to chronic states of insulin resistance [27–29]. One isoform of Gadd45, Gadd45-β, can antagonize the cytotoxicity of TNF-α by suppressing TNFα-induced c-Jun N-terminal kinase (JNK) activation by forming an inhibitory complex with MKK7, the upstream regulator of JNK [30,31]. We recently found that insulin can increase transcription of Gadd45-β, and by this increase, insulin is able to decrease JNK activity, decreasing the inflammatory response and insulin resistance [23]. Therefore, we hypothesized an anti-inflammatory action of insulin could be to inhibit TNF-α-induced gene expression. We found that in cultured hepatoma cells, TNF-α significantly increased hemopexin mRNA accumulation. This increase of hemopexin mRNA by TNF-α was dramatically attenuated by insulin, an anti-inflammatory action, even though TNF-α treatment caused insulin resistance.

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2. Material and methods

2.1. Cell culture

Rat H4-II-E (H4) hepatoma cells (ATCC; CRL-1548; Rockville, MD) were grown in Swims 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) in 5% CO2, 95% humidity, and 37 °C. Cells were washed into serum-free medium for 20–24 h before each experiment and all experiments were performed at 70–80% confluence following previously described protocols [32–36].

Added were recombinant rat TNFα (Biosource; Camarillo, CA) at a final concentration of 5 nM (~80 ng/mL) in 0.1% BSA in 1× PBS and/or insulin (porcine, Sigma; St. Louis, MO) 10 nM for the times indicated. Unless noted, all reagents were supplied by Fisher Scientific (Waltham, MA).

2.2. RNA extraction

Total RNA was isolated using Ultraspec RNA isolation reagent (Biotecx; Houston, TX) following the manufacturer's protocol. Briefly, for a 100 mm plate, 800 µl of the denaturing reagent was used, the cells were homogenized, and the RNA isolated from the aqueous phase by sequential isopropanol and sodium acetate/ethanol precipitations [37]. The concentration and purity was determined by spectrophotometric analysis.

2.3. Northern analysis

Total RNA (10 µg) was electrophoresed using 2.2 M formaldehyde, 1.2% agarose denaturing gels [37]. Equal loading was confirmed by staining the 28 S/18 S ribosomal RNA bands with acridine orange and sizes estimated by including a broad range RNA ladder (Invitrogen; Carlsbad, CA). RNA was transferred to a positively-charged nylon membrane (Brightstar-Plus; Ambion; Austin, TX), which were then incubated with an [α32P] dCTP-labeled (Stratagene; LaJolla, CA) full-length rat hemopexin cDNA [8] a gift from Drs. H. Bauman and U. Muller-Eberhard (Roswell Park Cancer Institute; Buffalo, NY). Membranes were autoradiographed and analyzed using scanning densitometry.

2.4. Western analysis

Sodium dodecyl sulfate (SDS} whole-cell lysates [1% SDS; 10 mM Tris; 7.5 µg/mL aprotinin; 5 mM bezamidine; 5 mM phenylmethylsulfonyl fluoride (PMSF); 50 mM sodium fluoride (NaF); 1.25 mM sodium vanadate (NaVO4)] were isolated by gentle scraping, homogenized, and assayed for protein content using the DC method (Bio-Rad; Hercules, CA) as previously described [19,32]. Proteins (40 µg) were resolved by 5–9% gradient SDS-PAGE and transferred to Protran BA85 nitrocellulose membrane (Whatman; Florham Park, NJ). Unless noted, antiserums were purchased from Cell Signaling (Danvers, MA) and used according to manufacturer's recommendation. Blots were developed using HRP-conjugated goat anti-rabbit IgG and visualized using ECL reagent (Amersham Biosciences; Piscataway, NJ).

All blots measuring phosphorylated proteins were re-probed using the corresponding total protein to ensure equal loading of samples and have not been included in the figures for brevity. At least three independent experiments were averaged and presented as mean ± standard error (SEM) as a time course of activation.

2.5. Densitometry

Each autoradiogram was scanned and then analyzed using Scanalytics ZeroD scan (v1.1; Fairfax, VA). Unity was assigned to the experimental control and change from that control is presented as fold difference [32].

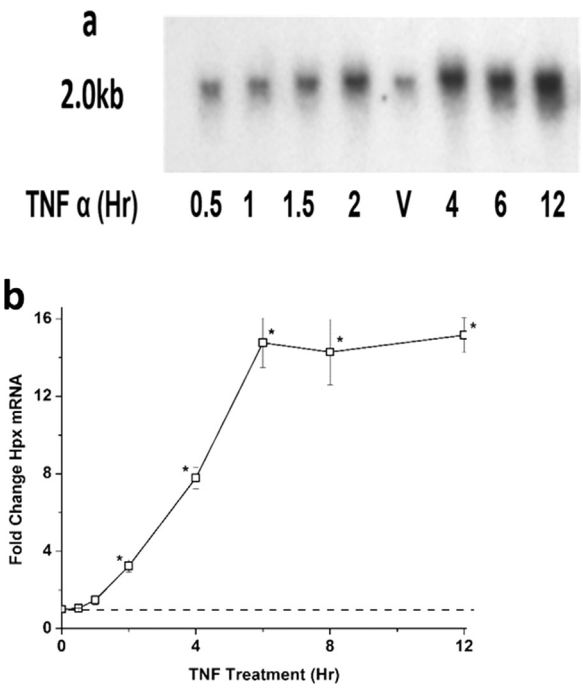

Fig. 1. Time Course of TNF-α stimulation of hemopexin mRNA in H4 cells. Serum-deprived rat H4IIE (H4) hepatoma cells were treated with recombinant rat TNF-α (5 nM) for the indicated times. Total RNA was purified and subjected to Northern Blotting as described in the methods. A representative autoradiograph is shown (a) and the fold-change of hemopexin mRNA (2.0 kb) versus the vehicle control (V) measured in response to the duration of TNF-α treatment is plotted (b). The symbols indicate mean ± SEM for at least three experiments at each time point. The vehicle (V)-treated (control) level was set to unity, indicated by a dashed line. * indicates a significance of $p < 0.05$ versus the vehicle-treated control.

2.6. Statistical analysis

All data was analyzed by analysis of variance (ANOVA) or Student's 2-tailed *t*-test using Instat (Graphpad v3.0; San Diego, CA) software. Significance was established when $p \leq 0.05$ with all comparisons indicated.

3. Results

3.1. Time course of TNF-α stimulation of hemopexin mRNA

Serum-deprived H4 hepatoma cells were treated with recombinant rat TNF-α (5 nM) for up to 24 h. Total RNA was purified and subjected to Northern analysis. A predominate transcript of approximately 2.0 kb was observed and correlated to the known size of hemopexin mRNA (Fig. 1a) [8]. Setting the vehicle-treated control level as unity, the fold-increase of hemopexin mRNA was approximately 4-fold by 2 h, with a maximum 15-fold by 6 h in response to TNF-α administration which was maintained for an additional 6 h (Fig. 1b). Between 12 and 20 h hemopexin mRNA levels decreased steadily, and then stabilized at approximately 3-fold above basal between 20 and 24 h in the continual presence of TNF-α (data not shown).

3.2. Time course of insulin on of hemopexin mRNA

Serum-deprived H4 hepatoma cells were treated with insulin (10 nM) over a 16-h period. Compared with the vehicle treated control, insulin, surprisingly, had a biphasic effect on hemopexin mRNA, with a much smaller but steady induction between 0.5 and 4 h (~1.6-fold, 4 h;

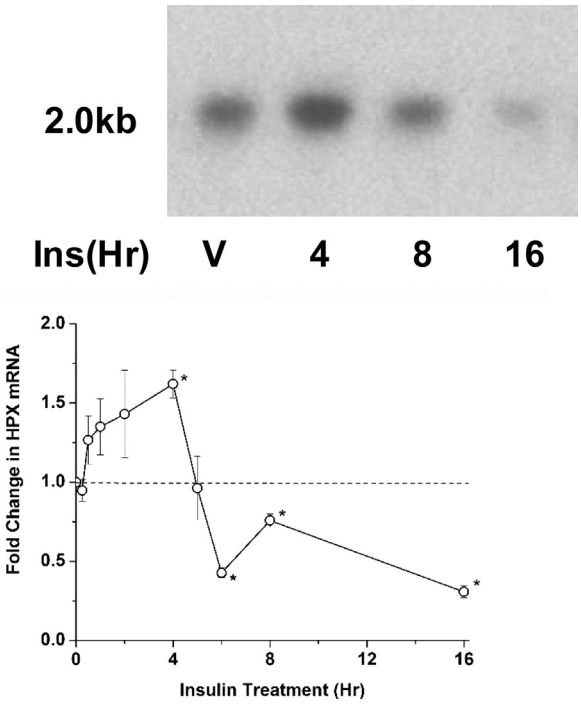

Fig. 2. Time Course of insulin stimulation of hemopexin mRNA in H4 hepatoma cells. Serum-deprived H4 cells were treated with porcine insulin (10 nM) as indicated. Total RNA was purified and subjected to Northern Blotting as described in the methods. A representative autoradiograph is shown and the fold-change of hemopexin mRNA (2.0 kb) versus the vehicle control (V) measured in response to the duration of Insulin treatment is plotted. The symbols indicate mean ± SEM for at least four experiments at each time point. The vehicle (V)-treated (control) level was set to unity, indicated by a dashed line. * indicates a significance of $p < 0.05$ versus the vehicle-treated control.

Fig. 2). However, hemopexin mRNA then decreased to one half of starting (basal) levels by 6 h in the continued presence of insulin which was maintained throughout the duration of the experiment, consistent with previous observations [38].

3.3. Insulin counteracts TNF-α stimulation of hemopexin mRNA in H4 hepatoma cells

To determine if insulin was additive or could counteract TNF-α, H4 hepatoma cells were treated with TNF-α followed 30 min later by the addition of insulin (arrow, Fig. 3a). The addition of insulin did not increase, but significantly reduced the TNF-α-induced increase of hemopexin mRNA at the 4, 6 and 8 h time points. Insulin was not able to suppress gene expression below ~3-fold, the level of induction by TNF-α alone at 2 h. And it took over 1 ½ h for insulin to have an effect after its addition since there was no effect of insulin at the 2 h time point of TNF-α, 1 ½ h after the addition of insulin.

To determine whether insulin could still have an effect longer after TNF-α was added, cells were treated with TNF-α for 4 h before the addition of insulin. The effects of TNF-α were not yet maximal at 4 h, and the addition of insulin (Fig. 3b, arrow) significantly blocked any further increase of hemopexin mRNA so there was no increase by the 8-h time point, the first time point measured. Hemopexin mRNA further decreased to ~2–3-fold by 12 h in the continuous presence of both TNF-α and insulin. Thus, insulin addition, whether shortly after (30 min, Fig. 3a) or many hours after TNF-α addition (4 h, Fig. 3b) greatly blunted the effects of TNF-α.

3.4. TNF-α alters insulin induced phosphorylation/activation of ERK

We then studied the ability of TNF-α to modulate insulin's activation of its two main signaling pathways in H4 hepatoma cells.

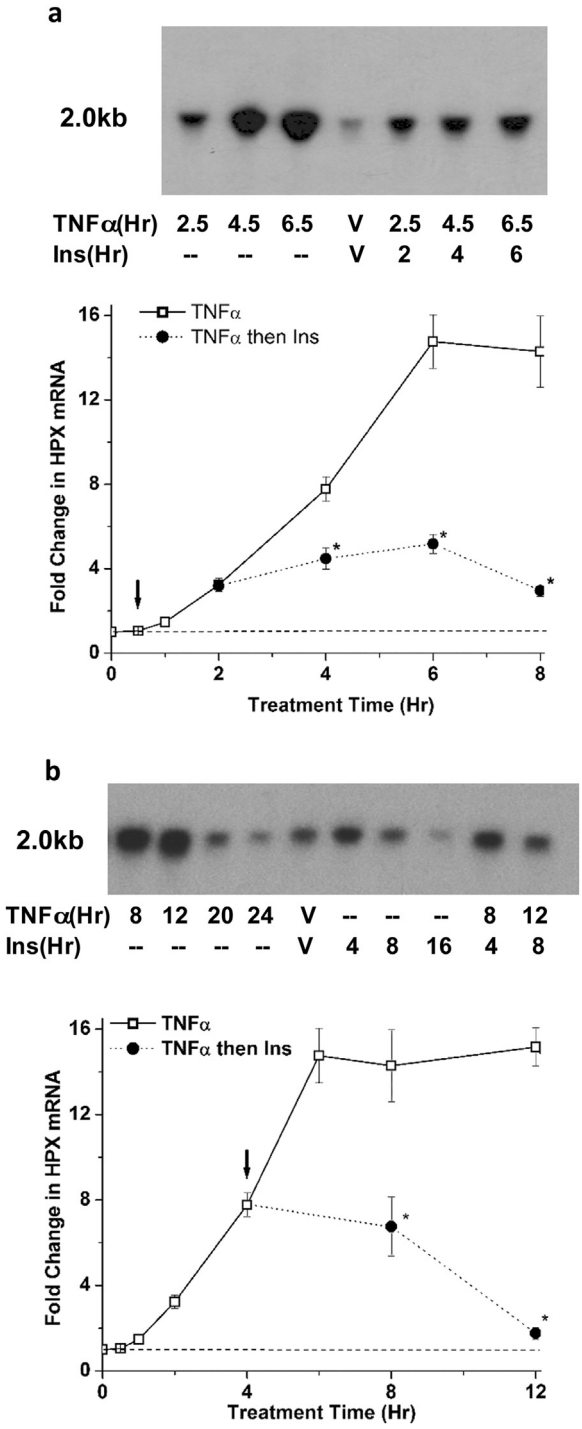

Fig. 3. Insulin counteracts TNF-α stimulation of hemopexin mRNA in H4 hepatoma cells. Serum-deprived H4 cells were treated with recombinant rat TNF-α (5 nM) alone, solid line, or treated with TNF-α for the first 30 min followed by the addition of insulin (10 nM; arrow; dotted line) and the incubation was continued for the times indicated (a). Serum-deprived H4 cells were treated with recombinant rat TNF-α (5 nM) alone, solid line, or treated with TNF-α for 4 h followed by the addition of insulin (10 nM; arrow; dotted line) and the incubation continued for the times indicated (b). Total RNA was purified and subjected to Northern Blotting as described in the methods. A representative autoradiograph is shown and the fold-change of hemopexin mRNA (2.0 kb) versus the vehicle control (V) measured in response to the duration of each treatment is plotted. The symbols indicate mean ± SEM for at least three experiments at each time point. The arrow indicates when insulin was added to the medium. The vehicle (V)-treated (control) level was set to unity, indicated by a dashed line. * indicates a significance of $p < 0.05$ versus the TNF-α alone treatment groups at the same time point.

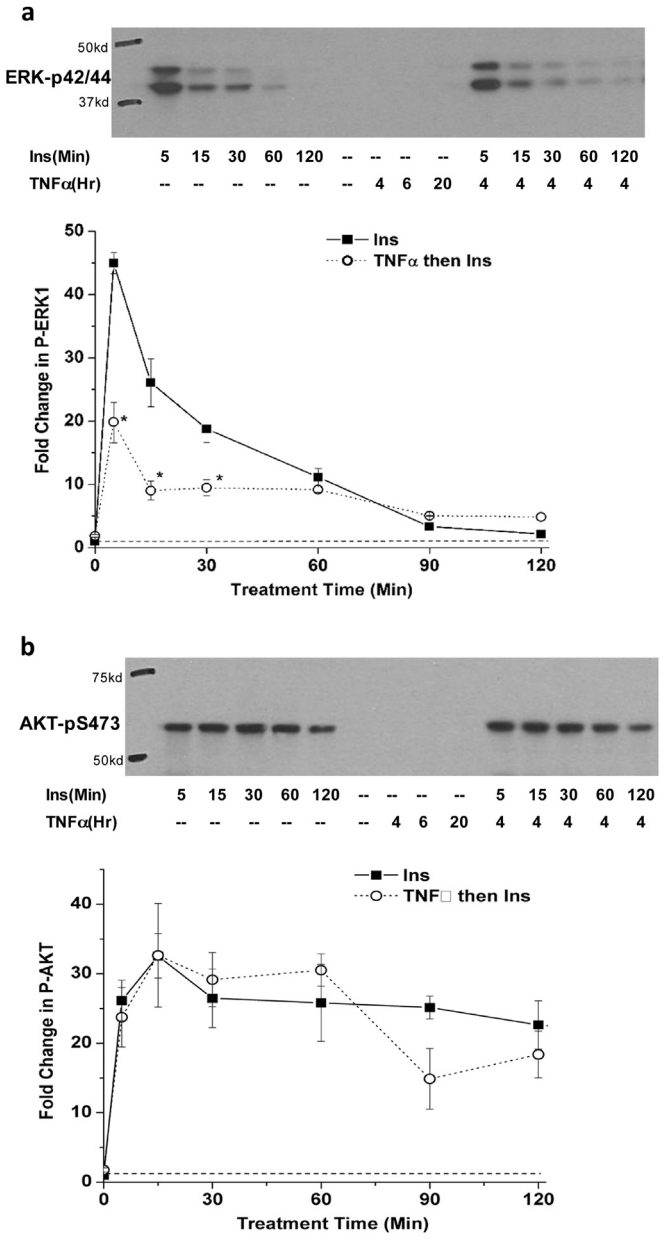

Fig. 4. TNFα effects insulin phosphorylation/activation of ERK1 and AKT in H4 hepatoma cells. Serum-deprived H4 cells were treated with insulin (10 nM), solid line, or treated for 4 h with TNFα (5 nM), dotted line, before the addition of insulin (10 nM). In this figure, the zero time point is the time of addition of insulin, so TNFα, when added, was added at minus 4 h. Total cellular protein lysates were isolated and subjected to Western analysis as described in methods. A representative autoradiograph is shown and the fold-change of phospho-ERK or phosphor-AKT versus the vehicle control (–) measured in response to the duration of each treatment is plotted. The symbols indicate mean ± SEM for at least three experiments at each time point. Insulin induction of ERK phosphorylation/activation was compared to basal (vehicle treated cells) which was set to unity, indicated by a dashed line (a). Insulin induction of AKT phosphorylation/activation was compared to basal (vehicle treated cells) which was set to unity, indicated by a dashed line (b). * indicates a significance of $p < 0.05$ versus the insulin alone treatment groups at the same time point.

Following the addition of insulin, total cellular protein lysates were isolated and subjected to Western analysis using a phospho-specific antiserum to ERK (MAPK) p42/44. As we have previously demonstrated in H4IIE cells, insulin rapidly induced ERK activation by 5 min [32–34]. This was transient, and was reduced to 50% of maximum by 15 min. By 120 min, the activation approximates the control [Fig. 4a; and see [33,34]].

When rat hepatoma cells were pre-treated with rat TNF-α for 4 h before the addition of insulin, the peak insulin induction of ERK was attenuated by over 50%, at the three earliest time points, thus reducing the peak of ERK activation (Fig. 4a). However, by 60 min, the insulin activation of P-ERK was indistinguishable between untreated and TNF-α pretreated cells.

Insulin also rapidly induced AKT (S473) phosphorylation/activation by 5–15 min after addition to H4 cells, which was maintained much longer than the transient induction of P-ERK in the continuous presence of insulin (Fig. 4b). Unlike P-ERK, following pretreatment with TNF-α for 4 h, the peak induction of P-AKT activation by insulin was not attenuated. Thus, the induced level of P-AKT remains near maximal even at 90–120 min following addition of insulin (Fig. 4b).

4. Discussion

Hyperglycemia and insulin resistance often occur following injury and/or critical illness [39,40]. However, following injury or infection, the ability of insulin to help regulate the inflammatory response [21,22,41] may be equally or more important than its control of intermediary metabolism. Among the anti-inflammatory actions of insulin is the reduction of proinflammatory mediators such as TNF-α, IL6, JE, and KC in animal models of endotoxemia [42]. However, if or how insulin acts to inhibit TNF-α action is not well understood. *In vivo* experiments are problematic in dissecting the role of an individual cytokine to the insulin resistant state, and the ability of insulin to counteract that cytokine. To obviate many of these problems, the present study uses cultured rat H4IIE hepatoma cells to explore TNF-α mediated insulin resistance and the ability of insulin to inhibit TNF-α induction of hemopexin mRNA.

H4IIE hepatoma cells are responsive to both insulin and growth hormone [36;43–45] and we have identified hemopexin as a growth hormone regulated gene both *in vivo* and in H4 cells [10,38]. In the present study, we found that hemopexin mRNA was regulated by both TNF-α and insulin, and that H4IIE hepatoma cells develop at least partial insulin resistance following chronic administration of TNF-α. Therefore, this cell line is an ideal model for investigating the interplay between TNF-α and insulin. We found that insulin can inhibit the TNF-α-induced accumulation of hemopexin mRNA. Thus, even though H4 cells become less sensitive to the actions of insulin following exposure to TNF-α [present work and [18,46]], insulin is still able to inhibit the accumulation of hemopexin mRNA by TNF-α.

Another important regulator of hemopexin mRNA is the proinflammatory cytokine, IL-6, which signals primarily via STAT3. We and others have found that a potential anti-inflammatory action of insulin is to decrease inducible STAT3 activity [24–26]. We have recently published an additional potential mechanism for insulin's anti-inflammatory actions on TNFα: insulin can modulate JNK activity by inducing Gadd45-β expression, an antagonist of TNFα-induced JNK activity [23]. In an example of cross-regulation, the development of insulin resistance by IL-6 and TNFα may dampen, but not abolish, the anti-inflammatory effects of insulin.

We have used hemopexin mRNA as a marker for the anti-TNFα effects of insulin, and we did not examine whether hemopexin mRNA regulation was due to modulation of transcription or altered post-transcriptional mRNA processing. Although hemopexin mRNA is predominately transcriptionally regulated, there is some evidence that hemopexin mRNA stability can be modulated [9,38,47]. In addition, we have found that insulin can regulate the c-myc gene via modulation of intragenic pausing [48], allowing for multiple possible regulatory steps in the transcription and processing of hemopexin mRNA by insulin. Future work is planned to elucidate the exact cellular mechanisms by which insulin can regulate TNFα-induced hemopexin mRNA levels.

TNF-α contributes to obesity-induced insulin resistance, by decreasing insulin signaling via the insulin receptor, decreasing tyrosine phosphorylation of IRS-1, and increasing the inhibitory phosphoryla-

tion of IRS-1 at serine 307 [49–53]. The complete mechanism(s) used by TNF-α in the development of chronic insulin resistance are still being explored, but include activation of MAP4K4, JNK, p38, ERK1/2, PI3K, and several PKC isoforms [20,54–57]. Our previous studies also indicate that p38 activation may play an important role in the regulation of the ERK pathway [33]. Another stress signaling pathway, the c-Jun N-terminal kinase (JNK) pathway, plays a role in obesity and injury-induced hepatic insulin resistance [19,40,58]. Since TNF-α can induce both the p38 and JNK pathways, there are multiple mechanisms by which TNF-α can modulate insulin actions and are being explored by many groups including ours.

In H4 cells, insulin activates ERK1/2 in two distinct phases, an initial, rapid peak, followed by a plateau of MEK/ERK activation [34]. The two phases of insulin-induced MEK/ERK activation results in the regulation of different sets of genes [34]. In the present work, TNFα treatment for only 4 h inhibits the early peak of insulin-induced ERK phosphorylation/activation, while having minimal effect on the later plateau phase. This predicts that only the rapidly induced insulin- and ERK-dependent genes would be altered by TNF-α. The lack of action of 4 h pretreatment with TNF-α to alter AKT activation was unexpected. Possibly a longer time of TNF-α is necessary for it to have an effect on this pathway. Interestingly, the majority of genes regulated by insulin are primarily through its induction of the ERK pathway [32,33,59], whereas the majority of insulin's actions on metabolism are via the AKT pathway. Thus, our data suggests that the effects of TNF-α treatment may function differently on different aspects of the insulin signaling, and these effects may be dependent upon the length of time of TNF-α exposure or other factors, such as additional proinflammatory cytokines. From the presented data, it is clear that the converse is also true; insulin can alter the response to TNF-α, explaining at least in part, insulin's anti-inflammatory actions.

Acknowledgments

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.013.

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