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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].

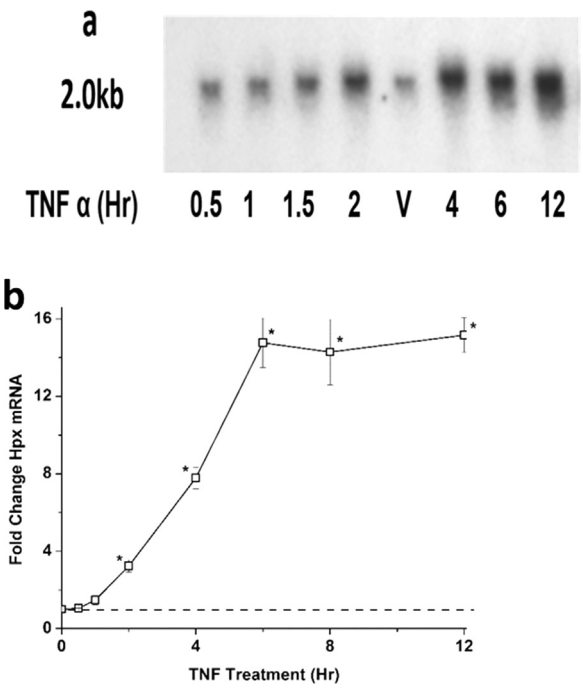

Fig. 1. Time Course of TNF-α stimulation of hemopexin mRNA in H4 cells. Serum-deprived rat H4IIE (H4) hepatoma cells were treated with recombinant rat TNF-α (5 nM) for the indicated times. Total RNA was purified and subjected to Northern Blotting as described in the methods. A representative autoradiograph 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;