2012

Effects of Stress on Growth Hormone Receptor Signaling

Ryan Marshall Corrick
University of Alabama at Birmingham

EFFECTS OF STRESS ON GROWTH HORMONE RECEPTOR SIGNALING

by

RYAN M. CORRICK

JOSEPH L. MESSINA, COMMITTEE CHAIR
MARCAS M. BAMMAN
STUART J. FRANK
JEFFREY D. KERBY
RAKESH P. PATEL

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2012

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RYAN M. CORRICK

PATHOLOGY GRADUATE PROGRAM

ABSTRACT

Growth hormone (GH) regulates body composition via stimulation of protein synthesis and catabolism of adipose tissue, generally promoting maintenance of lean body mass. Following severe injury, GH resistance contributes to muscle protein wasting, adversely impacting morbidity and mortality. In this dissertation research, we sought to determine the mechanisms of GH resistance following injury. To accomplish this, we evaluated GH signaling in a mouse model of severe injury. In the first section of this thesis, we demonstrate severe impairments in hepatic GH signaling occurring in association with an apparent, hemorrhage-dependent cleavage of the GH receptor (GHR). In the second section, we demonstrate the rapid development of cardiac GH resistance following injury. Finally, we detail the effects of injury on GH signaling in skeletal muscle and adipose tissue. Throughout these studies, the effects of fasting (alone or in combination with hemorrhage) on GH signaling were also investigated. The results of our studies suggest that 1) hepatic GH signaling is influenced by rapid alterations in the availability of functional GHR during stress, 2) fasting prevents cardiac GH resistance following injury, and 3) GH resistance is a common response to stress in multiple tissues.

DEDICATION

I dedicate this dissertation to God, who has given me so much to be grateful for. I also dedicate this dissertation to my family, both near and far. To my beautiful wife (and best friend) Katie, and our son Taylor, who have inspired me to more mature thinking about the reasons for working hard, and who have shown me the things that are most important in life. And to my parents and my uncle, who have supported me and believed in me unwaveringly.

ACKNOWLEDGMENTS

To my mentor, Dr. Joe Messina, thank you for your guidance and mentorship, and for allowing me to participate in your research. To the members of my committee, Drs. Marcas Bamman, Stuart Frank, Jeff Kerby, and Rakesh Patel, thank you all for your support and encouragement. To Dr. Robin Lorenz, Paula Wiley, Mindy Robbins, and Randy Seay, thank you all so much for everything you do for us. You are what make our MSTP great. To Adrianne Raymond, and Drs Sandra Frazier, Waid Shelton, and Hughes Evans, thank you for being true friends. To my family, thank you for everything.

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CONCLUSIONS AND FUTURE DIRECTIONS

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular-Signal Regulated Kinase
FFA	Free Fatty Acids
F/N	Combination of PNGase F and Neuraminidase
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
GHR	Growth Hormone Receptor
GHRKO	Growth Hormone Receptor Knockout Mouse
IGF-I	Insulin-Like Growth Factor-I
IL	Interleukin
JAK	Janus Kinase
MAP	Mean Arterial Pressure
MEK	Mitogen Activated Protein Kinase Kinase
mRNA	Messenger Ribonucleic Acid
SH2	Src-Homology 2
STAT	Signal Transducer and Activator of Transcription

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOCS	Suppressors of Cytokine Signaling
TNF	Tumor Necrosis Factor

INTRODUCTION

Growth hormone (GH) is a 22 kDa peptide hormone that is secreted by the anterior pituitary gland (1;2). Named for its role in development, GH is necessary for mammals to achieve full adult size. However, in addition to longitudinal growth, GH is an important regulator of body composition via its effects on intermediary metabolism. In general, GH favors the accumulation of lean body mass, promoting the use of adipose tissue as metabolic fuel while preserving skeletal and muscle mass (3). Other identified roles for GH include immune modulation, wound healing, and normal organ function, in addition to its many purported anti-aging and performance-enhancing effects (4-7). Despite a name that suggests a singular role, it is now clear that GH is responsible for regulating a wide range of physiological functions and is important throughout all stages of development.

Growth Hormone Physiology

GH is produced in the somatotroph cells of the anterior pituitary and secreted into the circulation before traveling to target tissues. Secretion occurs in a pulsatile fashion, resulting in fluctuations of plasma GH concentrations. In rodents, GH periodicity is approximately 3 hours, resulting in about 8 peaks per day (8). This rhythm approximates the rodent sleep-wakefulness cycle (as measured by electroencephalography); in addition, sleep deprivation effectively prevents GH pulses (9;10). Similarly, the major daily GH

pulse in humans coincides with the onset of slow-wave sleep during the first sleep cycle of the night, and can also be inhibited by sleep deprivation (11).

The primary modulators of pituitary GH secretion are growth hormone-releasing hormone (GHRH) and somatostatin, which have stimulatory and inhibitory effects, respectively (12;13). The relative concentrations of GHRH and somatostatin in the hypophyseal-portal circulation determine the GH secretion rate. However, the balance between circulating GHRH and somatostatin is regulated by a number of factors, including sleep (9-11;14), blood sugar (15;16), and physiological stress (17;18). In addition, the GH secretagogue ghrelin also acts to directly stimulate GH release from the pituitary, but via a receptor distinct from the GHRH receptor (19;20). These factors, combined with the relatively short half-life of GH in plasma (approximately 15 min) (21-24), allow rapid modulation of plasma GH concentrations. This is important, as plasma GH peak frequency and amplitude are essential determinants of GH-induced gene expression. For example, sex-specific patterns of GH-induced gene expression are dependent on GH pulse frequency and amplitude (25;26).

The two principal effects of GH are regulation of longitudinal growth and accumulation/preservation of lean body mass. Longitudinal growth is regulated by GH primarily via its effects on bone and cartilage, occurring throughout childhood until the end of puberty (27). Following closure of the epiphyseal plates, GH continues to play an important role in skeletal health by regulating bone mass. GH acts to preserve muscle mass via two distinct actions: 1) stimulation of protein synthesis and reduction of protein degradation, and 2) stimulation of lipolysis and free fatty acid oxidation (3). During periods of energy surplus, the net effect of these two actions is to increase muscle size

and strength. Conversely, when energy stores are depleted, the net effect of GH is to reduce muscle breakdown while simultaneously liberating free fatty acids from adipose tissue. The resulting increase in plasma free fatty acids provides a metabolic alternative to the amino acid stores retained by skeletal muscle. The protein sparing effects of GH in the fasted state are at least partially dependent on appearance of free fatty acids in the blood, since protein degradation resumes when GH-induced lipolysis is inhibited (28). Considering the relationship between strength, mobility, and overall health, the importance of GH in skeletal and muscle maintenance cannot be overstated.

Although the long-term effects are complementary to insulin, the acute effects of GH oppose those of insulin (3). For example, insulin promotes storage while GH promotes liberation of free fatty acids. Overall, GH acutely reduces insulin sensitivity of insulin target tissues, due in part to the increase in plasma free fatty acids. However, since insulin resistance following GH stimulation can be detected before the rise in plasma free fatty acids, it is likely that GH antagonism of insulin action occurs by multiple mechanisms (29). Conditions of GH deficiency and excess confirm the important role of GH in glucose homeostasis. For example, one of the long-term consequences of acromegaly is the development of diabetes, and pituitary dwarfism is usually accompanied by heightened sensitivity to the effects of insulin, contributing to frequent episodes of fasting hypoglycemia (30;31). Conversely, insulin stimulation inhibits GH signaling, which may be why individuals with chronic hyperinsulinemia exhibit central adiposity and reduced skeletal muscle mass (32). Adding to this complex interplay, insulin enhances certain aspects of GH signaling, and periodic insulin stimulation of tissues may be necessary to maintain GH sensitivity (33;34). This

multifaceted relationship between GH and insulin signaling may be the reason for the alternating predominance of insulin during waking hours (in association with eating) and GH during sleep, enabling the full complement of actions of both hormones.

In addition to its important roles in overall size and strength, as well as intermediary metabolism, GH is also necessary for normal growth and development of most tissues, including the heart, lungs, kidneys, pancreas, intestines, and liver. Normal growth and development are regulated by GH primarily via stimulation of cell proliferation and differentiation, as well as tissue hypertrophy (35). In addition, GH continues to regulate organ structure and function throughout adulthood, including effects on gut function, immune function, and skin structure and health (36). Representing another example of the importance of GH throughout life, maintenance of cardiovascular health is partially dependent on GH action, as illustrated by the cardiac structural abnormalities that accumulate over time in individuals with GH deficiency or excess (37). In summary, GH exerts important physiological effects on virtually every organ system from infancy through adulthood.

Growth Hormone Signal Transduction

After binding GH, growth hormone receptor (GHR) monomers undergo relative rotation, bringing receptor-associated JAK2 kinases into close proximity, resulting in JAK2 transphosphorylation (38-40). Once phosphorylated, JAK2 becomes active and phosphorylates tyrosine residues on the GHR (Fig. 1). This is followed by recruitment of various signaling proteins which are then phosphorylated by JAK2, resulting in the downstream activation of several pathways. JAK2 directly phosphorylates signal

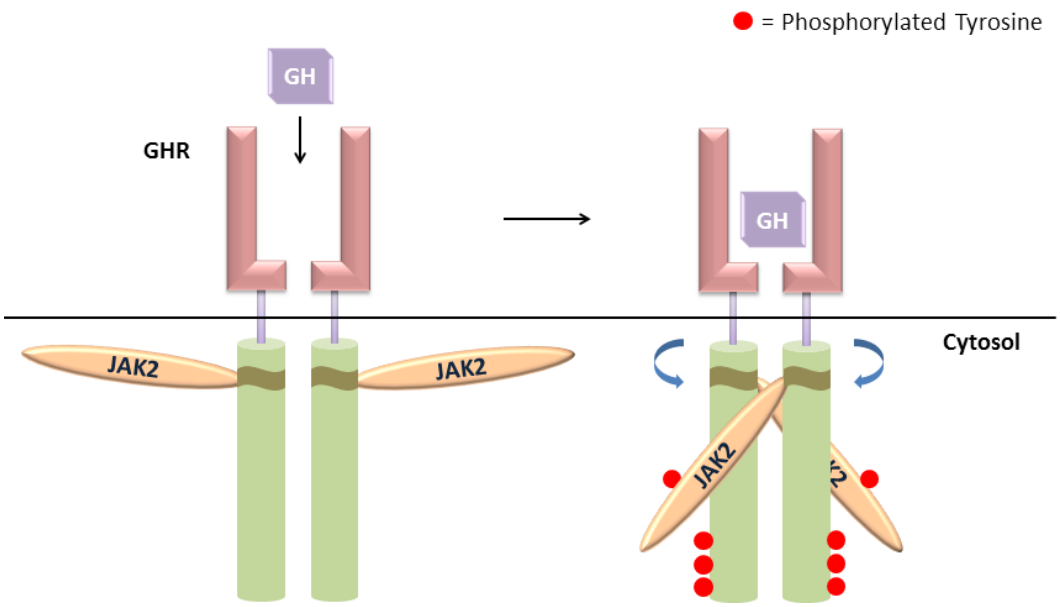

Figure 1. Schematic of GH-induced JAK2 activation.

transducers and activators of transcription 1 and 3 (STAT1 and STAT3), resulting in STAT dimerization, nuclear translocation, and subsequent transcriptional regulation (41-43). GH-activated JAK2 also activates the PI3K/Akt pathway and the MEK/ERK pathway via various adapter proteins (44). However, the specific downstream effects of GH-induced STAT1, STAT3, PI3K/Akt, or MEK/ERK activation are not well understood at this time.

The best-studied GH-activated pathway is the JAK2/STAT5 pathway. In contrast to STAT1 and STAT3, STAT5 must associate with the cytoplasmic domain of GHR to be tyrosine phosphorylated by JAK2 (41;42;45). In order to accomplish this, activated JAK2 phosphorylates tyrosine residues within the cytoplasmic domain of GHR. These phospho-tyrosines serve as docking sites for the SH2 (src-homology 2) domain of STAT5 (42;45). Once docked, STAT5 is tyrosine-phosphorylated by JAK2, enabling STAT5

dimerization and nuclear translocation (Fig. 2) (41;46;47). Nuclear STAT5 regulates transcription of GH-responsive genes. In particular, GH-activated STAT5 stimulates transcription of insulin-like growth factor-I (IGF-I) (48).

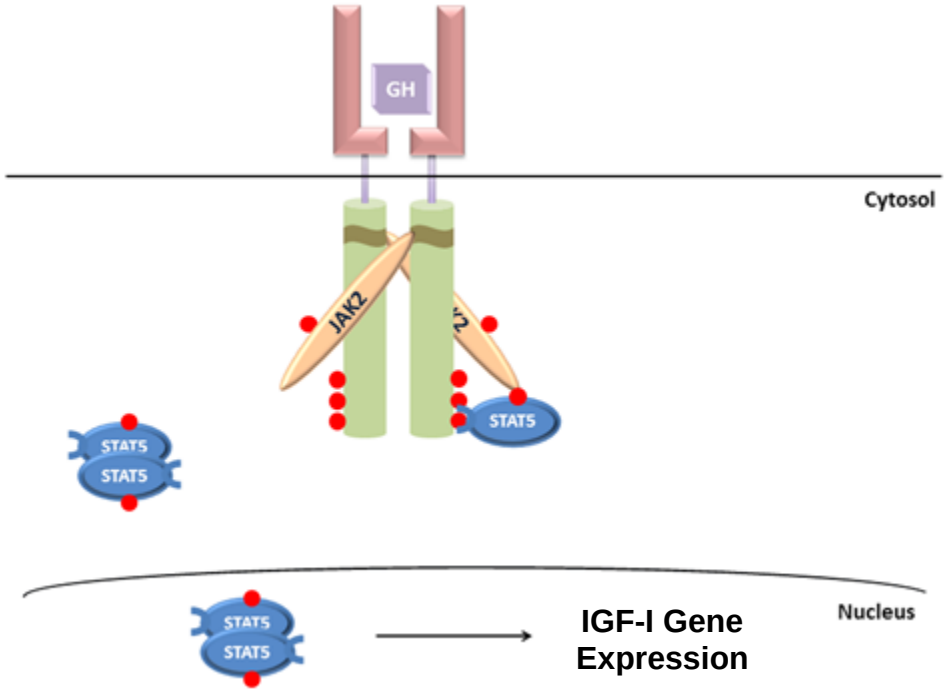

Figure 2. Schematic of GH-induced STAT5 activation.

Direct and Indirect Effects of Growth Hormone

Many, but not all of the effects of GH are mediated indirectly by IGF-I. GH stimulates production of IGF-I in the liver, which is secreted into the circulation. Hepatic IGF-I accounts for the bulk of circulating IGF-I, as evidenced by a 75% reduction of circulating IGF-I in mice with a liver-specific deletion of IGF-I (49). GH also stimulates IGF-I expression in several other tissues (50;51), which likely contribute the remaining 25% of circulating IGF-I. It has been demonstrated that IGF-I produced by these tissues

also functions locally in a paracrine or autocrine fashion. However, the paucity of IGF receptors in the rodent liver suggests that GH affects the liver primarily via the direct route (52-54). Therefore, GH stimulation of extra-hepatic tissues occurs by three different routes: 1) directly via GHR, 2) indirectly via circulating IGF-I, and 3) indirectly via locally-produced IGF-I.

The finding that growth retardation was more severe in GHR and IGF-I double knockout mice when compared with IGF-I knockout mice (55) demonstrated that there are significant direct effects of GH on somatic growth. Recent studies have suggested that myostatin and the androgen receptor are regulated directly by GH (56;57), which may partly explain the direct anabolic effects of GH. However, the major anabolic effects of GH are mediated indirectly by IGF-I (58). IGF-I stimulates amino acid uptake and protein synthesis, and decreases muscle protein degradation (59;60). Local IGF-I appears to be more important than circulating IGF-I in regulating skeletal muscle mass (4;61), and circulating IGF-I is insufficient to regulate longitudinal bone growth and bone mass in the absence of local IGF-I (62). Therefore, the ability of bone and muscle to produce local IGF-I in response to GH is important for the growth and maintenance of both tissues.

GH also plays an important role in normal structure and function of visceral organs, primarily via IGF-I. The renal and cardiovascular systems provide two important examples of this. The kidneys express both GH and IGF-I receptors (63-66), and both GH and IGF-I influence kidney size, renal blood flow, glomerular filtration rate, and sodium excretion (62;67-69). The heart and vasculature also express GH and IGF-I receptors (70-73). In addition to regulating cardiac size and structure, GH and IGF-I also

influence vascular tone and cardiac contractility (74-76). In both systems (renal and cardiovascular), local IGF-I produced in response to GH and circulating IGF-I have distinct effects. Therefore, the ability of each system to respond to circulating GH appears to be important for proper maintenance and function.

In addition to influencing somatic growth and organ function, hepatic (circulating) IGF-I also regulates plasma GH concentrations. Circulating IGF-I feeds back to the hypothalamus and pituitary gland to inhibit further GH secretion (49;77). By regulating GH secretion, the indirect (IGF-I-mediated) effects of GH are also important for maintaining the balance between the direct and indirect effects of GH on all other tissues.

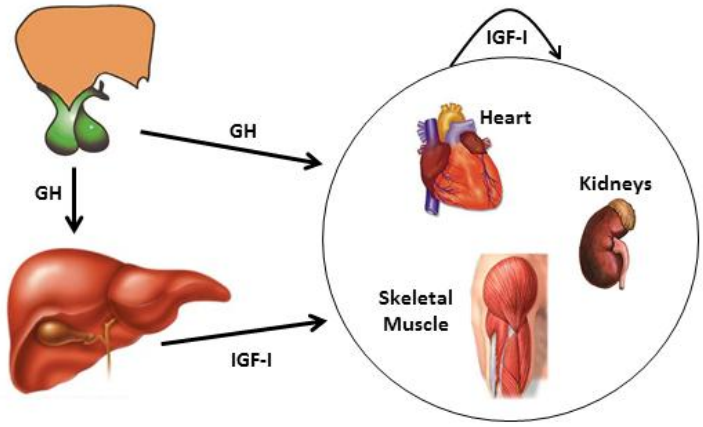

Figure 3. Schematic of direct and indirect effects of GH on target tissues.

Thus, GH communicates with tissues by three distinct routes: 1) directly via GHR, 2) indirectly, via IGF-I produced by the liver and secreted into the circulation, and 3) indirectly via paracrine/autocrine IGF-I produced locally in response to GH (Fig. 3). Most tissues (with the possible exception of the liver) are affected by all three routes.

The hepatic response to GH is important for maintenance and function of most tissues, but each tissue also has a separate, liver-independent response to GH that is also important for its own maintenance and function.

The Metabolic Response to Injury

Trauma, burns, and major surgery are associated with severe metabolic disturbances that generally develop 24-72 hours after the initial insult (78). These disturbances are often not clinically apparent during the resuscitative phase following injury, but have severe clinical consequences, even contributing to multiple organ failure and death. Two of the prominent clinical features of metabolic dysfunction following severe injury are insulin resistance and protein wasting, both of which adversely impact morbidity and mortality. These are attributed, at least in part, to a severe derangement of the GH-IGF axis frequently referred to as GH resistance. GH resistance is common following major injury and other forms of severe physiological stress (79-81), and is characterized by an absent or blunted response of tissues to GH stimulation. This can result from changes in GHR function, altered GH signaling, and/or changes in GH-induced gene expression. Hepatic GH resistance is frequently encountered following severe injury, and is easily detected in humans as an increase in circulating GH combined with decreased circulating IGF-I (82-84). However, evaluation of GH signaling and action in extra-hepatic tissues is more complex, and it is therefore not currently known whether other tissues become GH-resistant in humans following severe injury. As discussed previously, GH has important roles in many tissues, and the potential implications of GH resistance are distinct for each.

GH resistance following severe injury is multifactorial, but inflammation is thought to play a major role. Pro-inflammatory cytokines are rapidly increased following severe injury (85-89). IL-6 can be detected within 30 min of a surgical incision, and the rise in plasma IL-6 appears to be proportional to injury severity (90;91). Work by our group and others suggests that, while soft-tissue injury alone is sufficient to increase circulating IL-6, hemorrhage is required to increase circulating TNF-α (92-94). However, TNF-α by itself is capable of increasing circulating IL-6, and hemorrhage-induced elevations in TNF-α result in IL-6 increases above that achieved with soft-tissue trauma alone (88;92;95). Our previous work has demonstrated important roles for TNF-α in the development of hepatic insulin resistance (92;93), and work by others has suggested roles for both TNF-α and IL-6 in the development of GH resistance (96;97).

Pro-inflammatory cytokines are released by immune cells, fibroblasts, and damaged epithelial cells in response to injury (98). Circulating cytokines in turn activate the systemic acute phase response, which functions to limit further tissue damage, activate repair processes, and restore homeostasis. As a result, normal tissue functions are suspended, and new functions are prioritized in order to respond to the insult. Constitutive protein synthesis is reduced in the liver, and hepatic acute phase protein synthesis is simultaneously increased to support coagulation, wound healing, and immune function (99-101). Injured tissues undergo repair processes including cell proliferation, angiogenesis, and re-epithelialization, followed by extracellular matrix production, deposition, and tissue remodeling (102). The acute phase response also stimulates the hypothalamus to increase core temperature, which is accomplished partly via the profound increase in futile substrate cycling observed following injury (103). In addition,

hematopoiesis and appropriate immune responses are stimulated by the acute phase response (98). These simultaneous responses to injury result in a massive increase in metabolic requirements.

The concomitant rise in oxygen consumption and carbon dioxide production, respiratory rate, heart rate, and cardiac output, reflects the increased energy demand and expenditure following injury (104). To satisfy these demands, there is a dramatic increase in protein catabolism. Due to its anabolic properties, tissue responsiveness to GH would interfere with liberation and use of energy stores, particularly from skeletal muscle protein, during the response to injury. Therefore, the GH resistance observed following injury may represent a physiological mechanism for diverting resources away from anabolic processes toward cells and tissues actively engaged in repair or defense processes. An important question then is whether or not GH resistance and the associated muscle protein catabolism are essential for recovery following injury.

Protein catabolism in skeletal muscle provides amino acids for gluconeogenesis as well as new protein synthesis (105;106). However, this also leads to skeletal muscle wasting, weakness, and impaired mobility, as muscle protein catabolism primarily reflects proteosomal degradation of the myofibrillar proteins actin and myosin (107-111). Weakness, particularly of respiratory muscles, prolongs the need for mechanical ventilation. In addition, protein catabolism is also associated with delayed wound healing and impaired structure and function of organs including the liver and kidneys (79;98;112;113), increasing the risk for multiple organ failure. It should be noted that multiple organ failure accounts for more thanhalf of all deaths in critical illness (114;115). Taken together, the clinical consequences of protein catabolism following

severe injury are frequently so severe that they jeopardize survival. Therefore, since GH resistance is necessary for protein catabolism following injury, restoration of the GH-IGF-I axis represents an appropriate therapeutic target.

Clinical Efforts to Reverse Protein Catabolism

Early attempts to reduce protein catabolism following injury involved providing aggressive nutritional support to meet the enhanced metabolic requirements. However, hypercaloric feeding resulted in impaired immunity and liver function, and increased mortality (116;117). Additional studies were undertaken to determine more precisely the actual caloric requirements following injury (118;119). However, when the resulting values were used to determine caloric delivery in a subsequent study, preservation of lean mass was not achieved despite an increase in total body fat (120). The failure of these initial attempts to preserve lean mass indicated a need for a more targeted approach to the problem of protein catabolism following injury.

These earlier studies were followed by numerous attempts to alleviate protein catabolism by administration of exogenous GH. Many of these achieved some degree of success in maintaining lean mass following injury (121-124). In addition, other beneficial effects of GH administration were discovered, including increased IGF-I (125;126), improved wound healing (127;128), attenuation of inflammation and acute phase responses (129-131), and improved immunity (132;133). However, clinical administration of GH during critical illness was abruptly halted when the first large-scale study demonstrated increased mortality in critically ill patients receiving GH compared with placebo (134). The increase in mortality may have been due to the increased

incidence of sepsis and multiple organ failure in the GH group. It remains to be discovered how GH might contribute to an increase in sepsis and multiple organ failure. However, hepatic GH resistance during critical illness results in increased GH secretion due to decreased IGF-I production. Therefore, exogenous GH administration may have resulted in excessive stimulation of the direct effects of GH, including insulin resistance and hyperglycemia, which are known to contribute to increased incidence of multiple organ failure and death during critical illness (135;136).

Attempting to restore the GH-IGF-I axis may be a safer approach to combating protein catabolism and its associated deleterious effects. Restoration of GH signaling and action in liver as well as extra-hepatic tissues would allow endogenous GH to function properly, without increasing insulin resistance. It is possible that reversing GH resistance may impair recovery processes by reducing the availability of plasma amino acids. However, these could easily be replaced via clinical nutrition without the complications associated with hypercaloric feeding. The reason for this is that when GH resistance is reversed, maintenance of lean body mass will likely require a smaller caloric load. However, the mechanisms of GH resistance following injury remain ill-defined, and studies are necessary to determine the optimal safe approach for restoring GH action.

Mechanisms of Growth Hormone Resistance

GH resistance occurs in response to a variety of physical stresses. In addition to injury and critical illness, fasting also results in GH resistance. The mechanisms of GH resistance have been studied in a variety of *in vivo* models of critical illness, including models of sepsis and renal failure (137;138), as well as fasting (139-141). Impaired GH-

induced STAT5 phosphorylation is the most common finding in studies of GH signaling in models of inflammation/sepsis, renal failure, and fasting, and is associated with decreased IGF-I gene expression. The dependence of GH-induced IGF-1 gene expression on STAT5 phosphorylation has been confirmed in both liver and skeletal muscle in studies employing STAT5 knockout mice (48;56). Taken together, these studies suggest that decreased GH-induced STAT5 phosphorylation is an important part of the mechanism of GH resistance, acting as a reliable predictor of diminished IGF-I expression.

It has been suggested that GH resistance following injury results from reduced GHR expression/abundance (142;143). This could explain decreased GH-induced STAT5 phosphorylation, since STAT5 must associate with GHR in order to be phosphorylated (41;42;45). However, some studies of GHR in models of GH resistance have reported decreased GHR gene expression but no change in protein abundance (96;97;144). Other studies have reported reduced GHR protein expression following elective surgery (145), and metalloprotease cleavage of GHR in response to endotoxin injection (146). It remains unclear whether the discrepancy in GHR protein abundance between these studies is due to differences in the experimental models, or to varying sensitivity of the GHR detection methods. However, the GHR represents an important starting point in determining the mechanism for reduced STAT5 phosphorylation during GH resistance, and should not be ignored.

HEPATIC GROWTH HORMONE RESISTANCE FOLLOWING ACUTE INJURY

by

RYAN M. CORRICK, LI LI, STUART J. FRANK, AND JOSEPH L. MESSINA

Submitted to *American Journal of Physiology – Endocrinology and Metabolism*

Format adapted for dissertation

Abstract

Severe injury and infection are often followed by accelerated protein catabolism and acute insulin resistance. This results in several effects that complicate and prolong recovery, including weakness, immobility, impaired wound healing, and organ dysfunction. Recent studies have demonstrated the development of GH resistance during severe inflammation, providing a potential mechanism for the protein loss that follows injury and infection. In order to understand this GH resistance, we recently developed a murine model of acute injury. Mice were subjected to soft-tissue injury, alone or combined with hemorrhage, and injected intravenously with GH 30, 60, or 90 min later. Hepatic GH signaling was measured via Western analysis. GH-induced STAT5 phosphorylation was decreased immediately following completion of the trauma procedure, and at the 30 and 60 min time points, but further decreased by 90 min after trauma. Combined trauma and hemorrhage resulted in severely decreased GH-induced STAT5 phosphorylation compared to trauma alone, and this was true at all time points studied. Western analysis revealed an apparent decrease in the molecular weight of the hepatic growth hormone receptor (GHR) following trauma and hemorrhage, but not trauma alone. Additional studies determined that the hemorrhage-induced decrease in receptor size was not due to changes in GHR N-linked glycosylation. These results suggest that growth hormone sensitivity is rapidly impaired following acute injury, and that trauma combined with hemorrhage results in a more severe form of growth hormone resistance resulting from alteration or inactivation of hepatic GHR.

Introduction

Recovery from trauma, burns, and sepsis is frequently complicated by hyperglycemia, insulin resistance and metabolic dysfunction. Also often observed is accelerated muscle catabolism, resulting from decreased skeletal muscle protein synthesis and increased protein degradation (1-3). Levels of circulating free amino acids are consequently increased, providing substrate for the acute phase response as well as gluconeogenesis (4;5). These metabolic changes may shift substrate away from costly anabolic processes toward those necessary for survival (6). However, prolonged insulin resistance/hyperglycemia and acute phase responses frequently associated with critical illness adversely impact morbidity and mortality. As urinary nitrogen excretion increases (7;8), the associated cachexia contributes to weakness, reduced mobility, prolonged ventilator support, and increased risk of thrombus formation (9-11). Other consequences of protein wasting include impaired wound healing and organ dysfunction (12;13). The catabolic state is not easily reversed, even with aggressive nutritional support, and the causative factors are largely unknown (14). However, GH resistance is often observed following trauma, burns, and sepsis, and may play a role in the development of accelerated protein catabolism during critical illness (15-17). Thus, insights into the mechanisms of GH resistance may therefore lead to safer and more effective therapies to aid in recovery from severe injury.

GH is a pituitary hormone with important roles in protein accretion and maintenance of lean body mass (18). GH binding to its receptor (GHR) results in tyrosine phosphorylation and subsequent activation of receptor-associated janus kinase 2 (JAK2) (19;20). Activated JAK2 phosphorylates tyrosine residues within the

cytoplasmic domain of GHR, which serve as docking sites for the SH2 (src-homology 2) domain of signal transducer and activator of transcription 5 (STAT5) (21;22). Once docked, STAT5 is tyrosine-phosphorylated by JAK2, enabling STAT5 dimerization, nuclear translocation (23-25) and regulation of GH-responsive genes. In particular, GH-activated STAT5 stimulates transcription of insulin-like growth factor-I (IGF-I), which mediates many of the anabolic and anti-catabolic effects of GH (26;27). GH resistance is defined as a reduced response of tissues to GH, including reduced transcription of IGF-1. At the cellular level, this can be due to decreased GH-induced phosphorylation of signaling intermediates (28;29), impaired STAT5 DNA binding (30), or inhibition of any other GH-dependent signaling pathway.

Clinical GH resistance is characterized by elevated circulating GH and decreased circulating IGF-I, and is observed during acute systemic inflammation following major injury, surgery, or during infection (15-17). IGF-I is a potent anabolic hormone necessary for maintenance of lean muscle, and reduction of IGF-I likely contributes to protein wasting during critical illness. Several rodent models of sepsis have suggested GH resistance, contributing to decreased hepatic IGF-I gene expression (29-31). However, there have been no studies of the effects of surgery or trauma on GH sensitivity. In the current study, we set out to determine whether surgical trauma alone, or combined with hemorrhage, results in GH resistance, and to characterize the mechanisms of its development. Here we demonstrate that injury results in impaired GH signaling. In addition, trauma combined with hemorrhage results in acute, severe GH resistance that occurs in association with an apparent decrease in size of the GHR.

Materials and Methods

All animal procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama Birmingham.

GH Time Course Study

Mice were anesthetized with isoflurane (Mallinckrodt Veterinary, Mundelein, IL) and immediately injected with saline (75 µL) or 1.5 µg/g bovine GH dissolved in 75 µL saline (obtained from Dr. Albert Parlow, National Hormone and Pituitary Program). Livers were harvested 1, 5, 10, 15, 20, or 25 min after GH injection and immediately stored in liquid nitrogen until further processing.

Animal Model of Trauma and Hemorrhage

Mice were treated as described previously (32) with the exception that in the present study mice were not fasted prior to surgery. Briefly, male C57BL/6 mice (Harlan Laboratories, Inc.), approximately 12 weeks old and 23-25 g, were allowed food and water *ad libitum* prior to experiments. Mice were anesthetized by continuous inhalation of 1.5% isoflurane and 98.5% O_2 throughout all procedures. After mice were clipped and shaved, the surgical trauma procedure began with a 2-cm ventral midline laparotomy, representing soft tissue injury. The abdomen was then closed in layers using 6-0 Ethilon sutures (Ethicon, Somerville, NJ), and the wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) to reduce postoperative pain. Polyethylene-10 catheters

(Clay-Adams, Parsippany, NJ) were placed in the right and left femoral arteries for monitoring mean arterial pressure (MAP; Micro-Med, Louisville, KY) and for experimental hemorrhage, respectively. This completed the surgical trauma portion of the procedure. Mice designated for combined trauma and hemorrhage were then immediately hemorrhaged to a MAP of 35-40 mmHg over a 10 min period. The hemorrhage period began immediately after MAP dropped below 40 mmHg. At the conclusion of the study period, the abdominal incision was re-opened, and either saline or 1.5 µg/g GH (total volume = 75 µL) were injected directly into the inferior vena cava. Livers were harvested 10 min later and stored in liquid nitrogen until further processing (32-35).

Study Design

The primary injury control group consisted of mice subjected to surgical trauma followed immediately by saline or GH injection (referred to in the text as T0' mice) (32;36;37). To study the effects of trauma on GH signaling, mice were subjected to surgical trauma and injected 30 min (T30'), 60 min (T60'), or 90 min (T90') later. Trauma and hemorrhage groups were subjected to surgical trauma, hemorrhaged, and maintained at 35-40 mmHg MAP for 30 min (TH30'), 60 min (TH60'), or 90 min (TH90') before injection with GH or saline. As an additional control group, referred to in the text as "normal", mice were anesthetized and injected immediately with saline or GH followed by tissue removal after 10 min.

Enzymatic Deglycosylation

GHR was deglycosylated as described previously with minor modifications (38). Liver lysates were added to denaturation buffer (New England Biolabs, Ipswich, MA)

and the mixture was boiled at 95ºC for 10 min, and either (1) endoglycosidase H (endoH; New England Biolabs; 1500 U) or (2) PNGase F (New England Biolabs; 1000 U) plus neuraminidase (New England Biolabs; 100 U; combination referred to as F/N) were added and incubated at 37ºC for 16 hours. Samples were then subjected to SDS-PAGE analysis for qualitative assessment of deglycosylation. Nondeglycosylated controls were treated identically, except that incubation was performed in reaction buffer without enzymes.

Western Analysis and Immunoprecipitation

Liver tissue from each animal (0.2 g) was homogenized in extraction buffer, as described previously, and stored at –80°C until use (36;36;37;37). The protein concentration of tissue lysates was determined by the BCA method (Pierce, Rockford, IL) and 22.5 µg of protein were boiled in Laemmli buffer, resolved via SDS-PAGE (3-8% gradient gels; Bio-Rad, Hercules, CA), and transferred to nitrocellulose paper. Membranes were immunoblotted with the following primary antibodies: anti-P-JAK2 (PY1007/1008), anti-total-JAK2, anti-P-STAT5 (PY694/699), anti-total-STAT5, anti-total ERK and anti-Pan-Actin (Cell Signaling, Danvers, MA), anti-GHR (extracellular domain; R & D Systems, Minneapolis, MN) and anti-GHR (cytoplasmic domain; AL-47) was described previously (39).

Immunoprecipitation was performed using 1 mg of total lysate protein incubated with 2 µg of precipitating antibody, overnight at 4ºC with gentle rocking. Sepharose beads conjugated to protein A were added (GE Healthcare), and incubated overnight at 4ºC. Beads were precipitated by centrifugation, washed, resuspended in 2x Laemmli

buffer, boiled at 95ºC for 5 min and the proteins resolved via SDS-PAGE as described above.

Densitometric and Statistical Analysis

Enhanced chemiluminescence images of immunoblots were obtained on CL-X Posure Film (Thermo Scientific, Rockford, IL). Films were scanned using a Hewlett-Packard C5280 Scanner, and relative band intensities were determined using the Quantity One software package (Bio-Rad, Hercules, CA). Data are presented as mean ± SEM. Data was analyzed using the InStat statistical program (GraphPad Software, Inc., San Diego, CA). Differences between groups were determined using one-way ANOVA (Tukey post-test) or Student's *t* test (unpaired, Welch-corrected).

Results

Time Course of Hepatic GH Signaling

In order to determine the time course of hepatic GH signaling, livers were harvested from normal mice 1, 5, 10, 15, 20, and 25 min following injection with GH. Hepatic STAT5 tyrosine phosphorylation increased 28-fold following GH injection, compared with saline-treated mice, within 5 min of GH injection, and remained elevated until at least 25 min post-injection (Fig. 1A and 1B).

Total STAT5 levels did not change significantly at any of the time points following GH injection. However, a progressive change in the distribution of STAT5 between the upper and lower bands of the STAT5 doublet was observed for both P-STAT5 and total STAT5, although more pronounced for P-STAT5 (Fig. 1A). Maximal

phosphorylation of JAK2 was detected even more rapidly, within 1 min following GH injection (11-fold increase over basal, Fig. 1A and 1C), and remained elevated until 15 min post-injection before returning toward control levels.

Acute Traumatic Injury Impairs GH-Induced STAT5 Phosphorylation

Ideally, GH-injected control mice would be completely injury free. However, due to the severe hypotension experienced during the trauma and hemorrhage procedure, it is essential that GH be injected centrally in order to ensure delivery to tissues. This method involves injecting into the inferior vena cava and therefore necessitates an abdominal incision, so there is always some injury to the animal. In our previous studies of insulin signaling following injury, only minor differences in insulin signaling were found between mice that were anesthetized and immediately injected with insulin compared with those that were subjected to the surgical trauma procedure prior to insulin injection (36). Thus, "trauma-only" mice (T0') have been our injury control group. However, the injury incurred during arterial catheterization, as well as the time required for the full procedure, might affect GH sensitivity. Therefore, mice were subjected either to laparotomy alone (termed "normal" mice) or laparotomy and catheterization (T0') prior to injection. GH-induced STAT5 phosphorylation in normal mice was greater than T0' mice (Fig. 2A and 2B). Thus, T0' mice exhibit hepatic GH resistance compared with normal mice, even without additional trauma time or hemorrhage. The level of tyrosine-phosphorylated STAT5 was extremely low and therefore difficult to measure in saline-injected mice (Fig. 2A and 2B). This was true for all saline-injected mice. Thus, saline-treated controls have been omitted from all subsequent figures.

Hemorrhage for 90 Minutes Results in Severe GH Resistance

We next asked whether surgical trauma followed by a 90 min hemorrhage period would result in more severe GH resistance compared with T0' mice. In order to determine the effects of trauma and isolate those of hemorrhage, an additional group was generated, consisting of mice subjected to surgical trauma and then maintained on anesthesia without hemorrhage (T90' mice). T90' mice exhibited a substantial reduction in GH-induced STAT5 phosphorylation compared with T0' mice (59%, Fig. 3A and 3B). Hemorrhage for 90 min (TH90' mice) resulted in even more severe GH resistance evidenced by an 87% reduction in P-STAT5 compared to T0' controls, which is a 93% reduction in P-STAT5 compared with normal mice injected with GH, and a significant reduction in GH-induced P-STAT5 compared with T90' mice (Fig. 3A and 3B).

Fig. 3A also demonstrates a change in the upper-to-lower band ratio of P-STAT5. Whereas in Fig. 1A the ratio increases with increasing time post-GH injection, a decrease in the band ratio is observed following injury. In normal mice following GH injection (Fig. 2A), the upper-to-lower P-STAT5 band ratio was greater than 1, indicating the majority of P-STAT5 detected was at the higher molecular weight (band ratio = 1.81). Similarly, the P-STAT5 band ratio in T0' mice was also greater than 1 (1.35) indicating that in these mice, the majority of P-STAT5 was also at the higher molecular weight following GH (Fig. 3C). However, T90' mice and TH90' mice displayed P-STAT5 band ratios that were reduced to 0.57 and 0.40, respectively. Collectively, these results indicate that, in addition to impairing GH-induced STAT5 phosphorylation, increasing trauma time, with or without hemorrhage, impairs GH-induced P-STAT5 band migration.

GH-induced JAK2 phosphorylation was also impaired following T90' or TH90' (compared with T0'; Fig. 3D and 3E). T90' treatment resulted in GH-induced JAK2 phosphorylation that was 49% of that in T0' mice, and was further reduced to 39% following TH90'. P-JAK2 levels following GH injection of T90' and TH90' mice were not significantly different even though P-STAT5 and the P-STAT5 band ratio were further reduced in TH90' mice.

Hemorrhage-Induced GH Resistance Occurs Within 30 Minutes of Onset

To determine how quickly GH resistance develops following trauma alone or combined trauma and hemorrhage, mice were subjected to trauma alone, or in combination with 30 or 60 min of hemorrhage (T30' or TH30'; T60' or TH60'). Impaired STAT5 phosphorylation was evident as early as 30 min and at 60 min following the onset of hemorrhage (Fig. 4). However, in contrast to T90' mice, T30' and T60' mice did not exhibit any significant change in GH-induced STAT5 phosphorylation compared with T0' mice. Together, these results suggest two distinct mechanisms of GH resistance following injury; a mechanism associated with soft-tissue injury, and a second, more rapidly developing and more severe form of GH resistance associated with hemorrhage (see Fig.4E).

Hepatic Growth Hormone Receptor is Altered Following Hemorrhage

There have been conflicting reports about the role, if any, of the growth hormone receptor (GHR) in experimental models of GH resistance (34;40-42). These include models of sepsis and renal failure, but to date, there have been no reports of GH

resistance following injury. Even though the effects on GH signaling are extremely rapid, we asked whether changes in total GHR protein might account for the GH resistance observed in our model of injury. Western analysis of liver lysates from normal animals yielded a doublet of approximately 95 and 90 kDa that specifically reacted to the AL-47 antiserum directed to the cytoplasmic domain of GHR (Bands 1 and 2, respectively, Fig. 5A). The identities of these bands were confirmed by comparison with liver lysates from $GHR^{-/-}$ mice and their wild type littermates (C57BL/6J mice courtesy of Dr. John Kopchick, Ohio University). Following trauma and hemorrhage for 30 or 90 min, a third GHR band appeared at approximately 85 kDa (Band 3, Fig. 5B). In those same TH30' or TH90' mice, the upper band (Band 1) was much weaker compared to T30' and T90' mice, respectively (Fig. 5B and 5C). The increase in Band 3 intensity coupled with the decrease in Band 1 intensity in TH30' and TH90' mice indicate that hemorrhage resulted in an apparent reduction of GHR size via Western analysis following hemorrhage.

An additional gel was run to compare the level of Band 3 GHR at each TH time point. While Band 3 GHR was significantly increased relative to T0' controls, there were no significant differences found with respect to Band 3 intensity between the three time points studied (Fig. 5C and 5D). In addition, total GHR abundance (all three bands) was unchanged following TH30', TH60', or TH90' compared with T0' controls (Fig. 5E). These results suggest that following hemorrhage, cellular GHR was rapidly altered in a manner that changed the electrophoretic mobility of a fraction of immunoreactive receptors without changing the total number of receptors. Coupled with changes in GH-

induced STAT5 phosphorylation, this suggests that hemorrhage-induced changes in GHR diminish GH-induced signal transduction.

Increased GHR Electrophoretic Mobility is not Due to Changes in N-Linked Glycosylation

GHR protein has a fairly short half-life in the liver (30-40 min) (43). Band 3 GHR, which occurs following 30 or more minutes of hemorrhage, might represent newly synthesized GHR protein with an immature pattern of N-glycosylation. Thus, to determine whether the appearance of Band 3 indicates that hemorrhage interferes with cellular processes, such as receptor maturation, liver lysates were treated with endoH (EH), a combination of PNGase F and neuraminidase (F/N), or a buffer control (B). EndoH is an endoglycosidase that cleaves only immature (high-mannose) N-linked oligosaccharides. F/N is a combination of an exoglycosidase (neuraminidase) and an endoglycosidase (PNGase F); this combination cleaves all N-linked oligosaccharides from glycoproteins, regardless of maturity (mannose content).

Following hemorrhage, (TH30' or TH90'), Band 3 was not affected by treatment with endoH, but was eliminated by treatment with F/N, suggesting that the reduction in GHR size following hemorrhage is not due to an increased proportion of immature receptors (Fig. 6A and 6B). However, Band 2 in TH30' and TH90' mice was almost completely absent following treatment with either endoH or F/N, suggesting that Band 2 GHR is an immature (high-mannose) glycoprotein. Band 2 was also eliminated in lysates from trauma alone mice that were treated with endoH (T0', T30', and T90' mice, Fig. 6A and 6B). Since Band 2 GHR is a prominent feature in livers from normal mice (Fig. 5A), these data suggest that Band 2 represents a precursor form of hepatic GHR as it traverses

the secretory pathway. Since Band 3 is not reduced by endoH treatment, it suggests that the appearance of Band 3 GHR in hemorrhaged liver results from some other post-translational alteration, possibly GHR cleavage. Band 1 GHR was also unaffected by treatment with endoH, suggesting that Band 1 is the mature form of hepatic GHR detected in all groups. In addition, there is often a low level of Band 3 GHR present in livers from trauma alone mice (for instance, see Fig. 6B). This suggests that the Band 3 observed in livers from hemorrhaged mice actually represents a form of GHR that is naturally present at low levels, but is rapidly generated in response to hemorrhage.

Deglycosylated liver lysates were further subjected to Western analysis, with care taken to resolve smaller GHR isoforms (in the 50-75 kDa range) that may have been generated by enzymatic treatment. In order to prevent the high background signal usually observed in this region when probing with AL-47, an anti-GHR antibody raised against the extracellular domain of GHR was substituted for AL-47 (Fig. 6C). In lysates from hemorrhaged mice treated with F/N, a dark band was observed at approximately 65 kDa (asterisk, Fig. 6C, F/N treated TH90' lane). This band was absent in lysates from non-hemorrhaged (T90') mice, indicating that the 65 kDa band in Fig. 6C represents fully deglycosylated Band 3 GHR.

Discussion

The clinical consequences of GH resistance following injury have profound implications in critical care. GH resistance contributes to muscle wasting and treatment with GH reduces the loss of lean mass (44-46). We therefore set out to determine whether soon after trauma alone or combined trauma and hemorrhage, there was an

impairment of GH signaling. To pursue this research, a model of surgical trauma, or the combination of trauma and hemorrhage was used, a model which we have already extensively characterized in the study of the rapid development of insulin resistance (32;47-50). Injection of GH resulted in rapid induction of hepatic JAK2 and STAT5 phosphorylation. JAK2 tyrosine phosphorylation was demonstrated within 1 min, persisting until 15 min after GH injection. GH-induced STAT5 phosphorylation occurred more slowly compared with JAK2 phosphorylation, and was not significantly increased compared to basal until 5 min post-injection. Maximal STAT5 tyrosine phosphorylation was detected 15 min post-injection, which is consistent with previous reports (24;25;33).

Also demonstrated in the present study was a biphasic effect of trauma alone on GH-induced STAT5 phosphorylation. Completion of the trauma procedure requires only 30 min, so the hepatic GH resistance observed immediately after trauma (T0') developed rapidly in response to anesthesia and surgery with minimal blood loss. No further changes in GH sensitivity were observed 30 or 60 min after completion of the trauma procedure, but a more severe impairment of GH-induced STAT5 phosphorylation was demonstrated 90 min after trauma. These studies demonstrate a rapid onset of GH resistance compared to previous reports where GH resistance was observed several hours after the onset of sepsis or intravenous lipopolysaccharide (LPS) injection (29;30). The combination of trauma and hemorrhage resulted in an even more severe impairment of STAT5 phosphorylation that was evident at the earliest time point studied (TH30'), suggesting a separate mechanism of GH resistance following hemorrhage that is distinct from GH resistance due to trauma alone. Unlike STAT5 phosphorylation, no significant difference was detected between trauma alone or combined trauma and hemorrhage with

respect to impaired JAK2 phosphorylation. Thus, following the combination of trauma and hemorrhage, an additional mechanism of GH resistance may occur that affects only signaling elements downstream of JAK2.

In normal, untreated mice, STAT5 was detected as a doublet on Western analysis, with the majority of T-STAT5 localized to the lower STAT5 band. Following injection with GH, the majority of T-STAT5 was detected in the upper band. A similar effect was observed with P-STAT5, suggesting a GH-induced shift in STAT5 banding pattern that was distinct from tyrosine 694/699 phosphorylation recognized by the antibody. Western analysis of total STAT5 and P-STAT5 banding patterns indicated that trauma alone or combined trauma and hemorrhage reduced the GH-induced shifting of total STAT5 and P-STAT5 bands, regardless of the duration of trauma or hemorrhage. We observed a similar shift in STAT5 banding pattern following GH stimulation of rat hepatoma cells (51) as have others in response to GH *in vivo* (25;52). The shift in hepatic STAT5 electrophoretic mobility in response to GH may be due to phosphorylations at other serine or tyrosine sites, which may alter STAT5b DNA binding (53). It is important to realize that these changes in GH signaling due to trauma and hemorrhage are very rapid, and prior to any of the pathophysiological changes that occur, like decreased IGF-1 levels and loss of muscle mass. Thus, these changes in GH signaling are the earliest signs yet found of the development of GH resistance following injury or critical illness.

Additional studies were performed focusing on the GHR to determine a possible role of the GHR in hemorrhage-induced GH resistance. Rodent hepatic GHR is often detected at molecular weights between 100 and 120 kDa (28;29;42;54), as in our own studies of GHR in cultured cells (51;55). In the current study, mouse liver GHR was

detected as a doublet at approximately 95 and 90 kDa (what we refer to as Bands 1 and 2). In order to confirm the identity of the doublet as GHR, we obtained livers from GHR knockout mice ($GHR^{-/-}$) and wild type littermates (Dr. John Kopchick of Ohio University). These 95 and 90 kDa doublet bands were absent in liver lysates from $GHR^{-/-}$ mice but present in livers from their wild type littermates, confirming the identities of Bands 1 and 2 as GHR.

In order to determine whether acute injury resulted in a loss of GHR, liver lysates were probed for GHR following hemorrhage. Unexpectedly, a third GHR band (Band 3) was resolved at 85 kDa via PAGE in lysates from hemorrhaged mice. Following extended exposures, this 85 kDa band was also faintly visible in lysates from normal and trauma alone mice, but was much more abundant in livers from hemorrhaged mice. Thus, hemorrhage may accelerate the generation of an 85 kDa GHR isoform. Densitometric analysis revealed that the amount of Band 3 detected via Western analysis did not change with duration of hemorrhage, and that total immunoreactive GHR was unchanged following hemorrhage, indicating that Band 3 generation occurs at a rate similar to the disappearance of Band 1.

GHR is an extensively glycosylated protein. Mature, fully glycosylated GHR can be as much as 20 kDa larger than its precursor, non-glycosylated forms. Thus, to determine whether Band 3 GHR represented precursor GHR, N-linked oligosaccharides were enzymatically removed. One treatment was with endoglycosidase H (endoH); before glycoproteins leave the Golgi apparatus, mannose residues are removed, resulting in a mature, complex oligosaccharide which cannot be cleaved enzymatically by endoH; however, immature (high-mannose) glycoproteins are endoH sensitive. The second

treatment contained a combination of neuraminidase and PNGase F (F/N), which cleaves all N-linked oligosaccharides from both precursor and mature proteins.

In livers from hemorrhaged mice, neither Band 1 nor Band 3 GHR was affected by treatment with endoH, suggesting they are both mature forms of GHR. The only GHR band affected by treatment with endoH was Band 2, indicating that this 90 kDa band represents the precursor (high-mannose) form of GHR. Treatment with F/N resulted in the absence of all previously observed bands (1, 2, and 3), and the appearance of a fourth band at 75 kDa. Since F/N treatment removes all N-linked oligosaccharides, we conclude that the 75 kDa band represented fully deglycosylated GHR.

In order to resolve individual GHR bands, gels were normally run for prolonged periods of time, resulting in smaller molecular weight proteins being eluted from the gel. This was not an initial concern as the primary focus of our studies was the 75-100 kDa region of the gel. However, since GHR may be cleaved following hemorrhage, resulting in Band 3, we hypothesized that Band 3 GHR would run at an even lower molecular weight following F/N treatment. The difference in apparent molecular weight between Band 1 and Band 3 GHR is approximately 10 kDa (95 and 85 kDa, respectively). The difference in apparent molecular weight between Band 1 GHR and fully deglycosylated GHR is approximately 20 kDa (95 kDa vs. 75 kDa). Thus, we calculated the molecular weight of fully deglycosylated Band 3 GHR to be approximately 65 kDa. Therefore, F/N-treated lysates were re-run under conditions necessary to resolve lower molecular weight proteins. A GHR band was evident at approximately 65 kDa in lysates from hemorrhaged mice. This band was absent in F/N treated lysates from non-hemorrhaged mice, indicating that the 65 kDa band represents fully deglycosylated Band 3 GHR. The

20 kDa reduction of Band 3 GHR size following F/N treatment confirms that untreated Band 3 GHR is fully glycosylated, since enzymatic deglycosylation of mature, glycosylated Band 1 GHR also resulted in a 20 kDa reduction. Together, these observations suggest that Band 3 is a cleavage product of Band 1, likely resulting from alterations to the primary amino acid sequence and not changes in glycosylation. However, another post-translational modification is possible as is an alteration of mRNA splicing, resulting in decreased GHR size following hemorrhage. The GHR half-life in liver is 30-40 min of GHR (43), and a change in mRNA splicing could account for a ~10 kDa reduction in all GHR synthesized during the hemorrhage period. Future studies will need to pursue these possibilities. The appearance of Band 3 occurred with timing similar to the development of the more extensive STAT5 signaling impairment observed following the combination of trauma and hemorrhage, suggesting that Band 3 GHR is not capable of normal GH signaling. Such a hemorrhage-induced inactivation of GHR could explain why hemorrhaged mice displayed a more severe form of GH resistance.

The membrane-proximal Box 1 sequence of GHR is necessary for JAK2 association with GHR (20;56). JAK2 kinase activity, including autophosphorylation, is retained even when large portions of the GHR cytoplasmic domain are removed, as long as Box 1 remains intact (21;23;57). However, STAT5 must associate with the C-terminal end of GHR in order to be tyrosine phosphorylated by JAK2. Therefore, it is possible that C-terminal cleavage or modification of GHR could impair GH-induced STAT5 tyrosine phosphorylation without affecting JAK2 phosphorylation, which would explain our present findings. We are not aware of any reports describing a similar cleavage of the GHR. Proteases known to cleave GHR, such as TNF-α converting enzyme (TACE) or γ-

secretase, result in fragments that are significantly larger than the ~10 kDa fragment described in the present report. Future studies will attempt to confirm whether a portion of the GHR is cleaved, and determine how this might occur following hemorrhage, or whether another modification of the GHR is required to generate Band 3. Unfortunately, this will not be simple, since there is relatively little GHR in the liver of C57BL/6 mice and there is no previous evidence of a similar GHR alteration in cultured cell lines that express higher levels of GHR.

In conclusion, the present study demonstrated severe impairments in hepatic GH signaling following either trauma alone or combined trauma and hemorrhage. Trauma alone resulted in biphasic impairment of GH-induced STAT5 phosphorylation. The initial impairment occurred immediately following completion of the trauma procedure and was sustained for at least 60 min, with more severe resistance 90 min after trauma. These effects on GHR signaling occur rapidly, prior to the pathophysiological changes that are measurable following injury or infection, such as reduced hepatic IGF-1 expression. Thus, the present studies explore the mechanisms leading to the GH resistant state. The mechanism(s) of GH resistance following trauma alone are still unclear, but the absence of a lower molecular weight GHR (Band 3) suggests a separate mechanism from the more severe GH resistance occurring rapidly following the combination of trauma and hemorrhage. These data strongly suggest that structural changes to the GHR are related to the rapid signaling impairment evident in livers from hemorrhaged mice.

Acknowledgments

We thank Dr. John Kopchick and Dr. Darlene Berryman of Ohio University for providing livers from $GHR^{-/-}$ mice and wild type littermates. We also thank John L. Franklin and Ling Zhou for their technical assistance.

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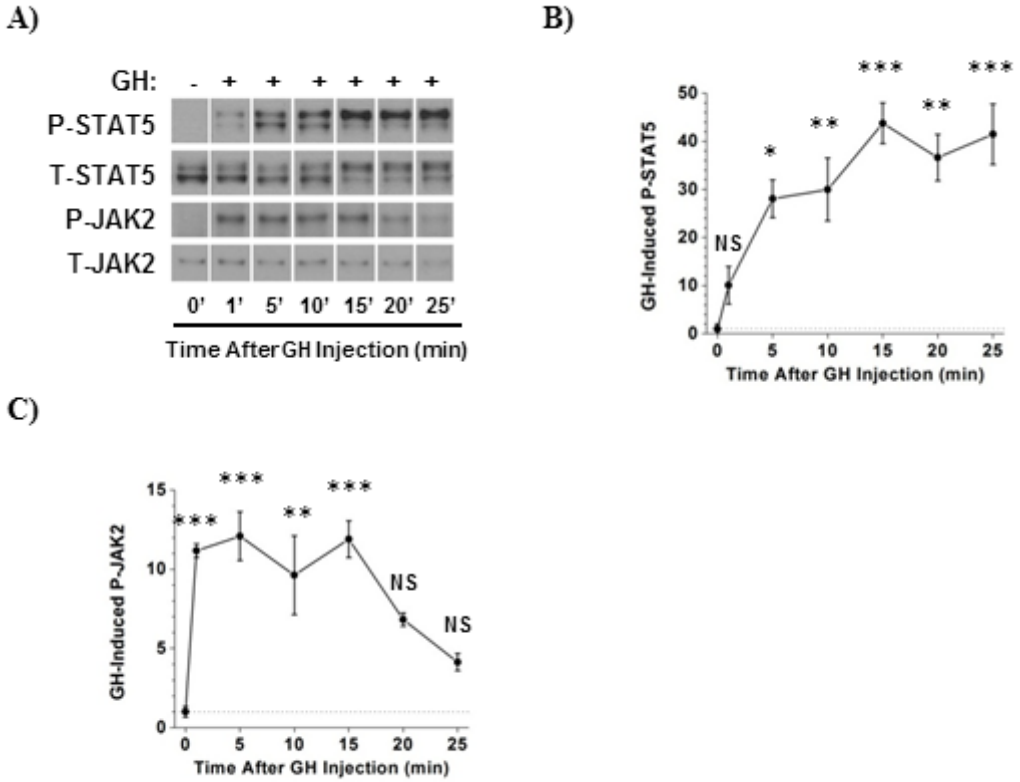


Figure 1. Time course of hepatic GH signaling in response to GH injection. A. Mice were injected with bovine GH (or saline, 1' time point) and livers were removed at the indicated times. Liver tissue lysates were prepared and subjected to Western blot analysis, with specific anti-PY694/699-STAT5 and anti-STAT5 antibodies. In addition, JAK2 was immunoprecipitated from protein extracts with an anti-JAK2 antibody. JAK2 immunoprecipitates were subjected to Western analysis and probed with specific anti-PY1007/1008-JAK2 and anti-JAK2 antibodies. Representative Western blots are presented; scanned images of blots were rearranged for clarity. B-C. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 (B) and P-JAK2 (C) In this figure, the average of 3 measurements at t=0 was arbitrarily set to 1. Data are presented as mean ± SEM fold change in phosphorylation normalized to total protein. In this figure: *, $P < 0.05$ vs. 0'; **, $P < 0.01$ vs. 0'; and ***, $P < 0.001$ vs. 0', all via one-way ANOVA; n = 3 mice per time point.

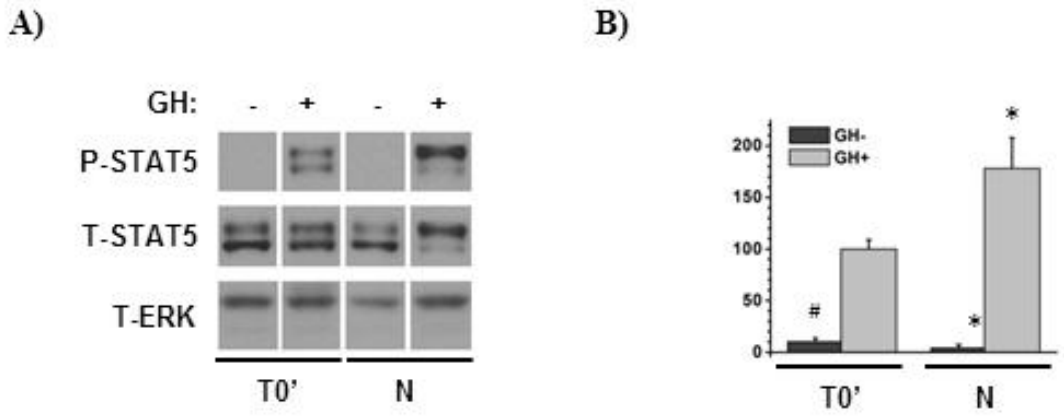
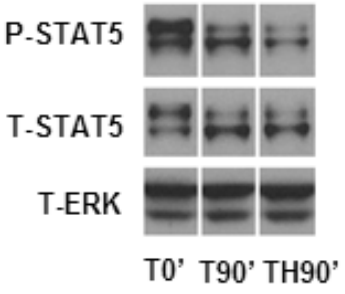
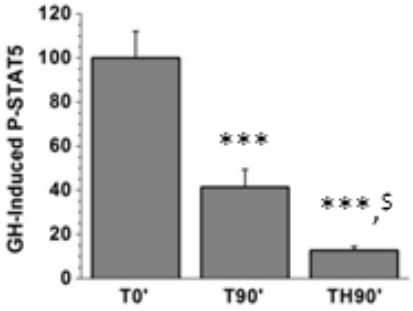


Figure 2. Effect of soft-tissue trauma on GH-induced STAT5 tyrosine phosphorylation. A. Mice were subjected to the full trauma procedure or midline laparotomy alone and GH or saline was injected into the inferior vena cava. Livers were removed 10 min later. Western analysis of liver lysates was performed and blots were probed with specific anti-PY694/699-STAT5, anti-STAT5, and anti-ERK antibodies. Representative Western blots are presented; scanned images of blots were rearranged for clarity. ERK was used to control for loading on separate P-STAT5 and T-STAT5 gels. B. Autoradiographs were quantified by scanning densitometry. Data are presented as mean ± SEM percent change in phosphorylation, normalized to total STAT5. P-STAT5 following T0' was arbitrarily set to 100%, In this figure: *, $P < 0.05$ vs. GH-injected T0' via one-way ANOVA; #, $P < 0.05$ vs. GH-injected T0' via unpaired, two-tailed, Welch-corrected t-test; n = 6 mice per group of GH-injected mice; n = 3 mice per group of saline-injected mice..

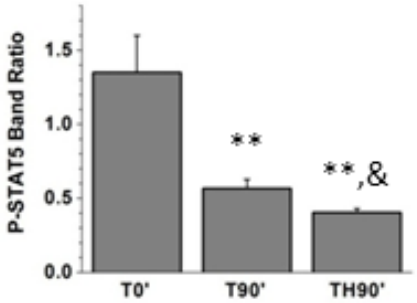
A)


P-STAT5
T-STAT5
T-ERK
T0' T90' TH90'

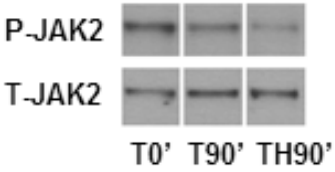
B)


GH-Induced P-STAT5
120
100
80
60
40
20
0
***
***,$
T0'
T90'
TH90'

C)


P-STAT5 Band Ratio
1.5
1.0
0.5
0.0
**
**,&
T0'
T90'
TH90'

D)


P-JAK2
T-JAK2
T0' T90' TH90'

E)

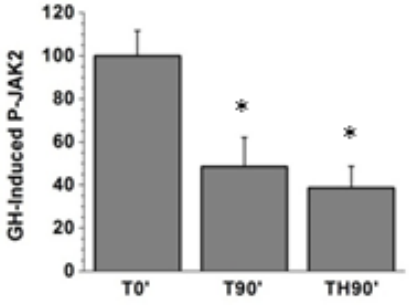
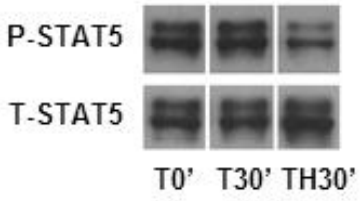
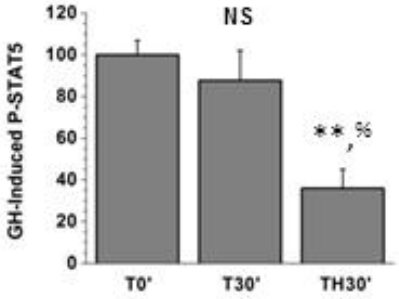

GH-Induced P-JAK2
120
100
80
60
40
20
0
*
*
T0'
T90'
TH90'

Figure 3. Effect of trauma alone and combined trauma and hemorrhage for 90 min on hepatic GH-induced JAK/STAT signaling. A. Mice were subjected to trauma alone and injected with GH or saline immediately (T0'), 90 min later (T90'), or after 90 min hemorrhage (TH90'), and livers were harvested 10 min later. Western analysis of liver lysates was performed and blots were probed with specific anti-PY694/699-STAT5, anti-STAT5, and anti-ERK antibodies. Representative Western blots are presented; scanned images of blots were rearranged for clarity. ERK was used to control for loading. B. Autoradiographs were quantified by scanning densitometry. C. Individual P-STAT5 bands were quantified by scanning densitometry, and the ratio of the upper vs. lower bands were calculated. D. The same liver lysates as in (A) were immunoprecipitated with anti-JAK2 antibody. JAK2 immunoprecipitates were subjected to Western analysis and probed with specific anti-PY1007/1008-JAK2 and anti-JAK2 antibodies. E. Autoradiographs were quantified by scanning densitometry. Except for C., data are presented as mean ± SEM percent change in phosphorylation, normalized to total protein. P-STAT5 following T0' was arbitrarily set to 100%, In this figure: *, $P < 0.05$ vs. T0'; **, $P < 0.01$ vs. T0'; ***, $P < 0.001$ vs. T0'; all via one-way ANOVA; $, $P < 0.05$ vs. T90' via two-tailed, Welch-corrected t-test; &, $P < 0.05$ vs. T90' via one-tailed, Welch-corrected t-test; n = 5 mice per group.

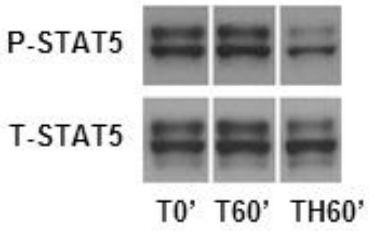
A)


P-STAT5
T-STAT5
T0' T30' TH30'

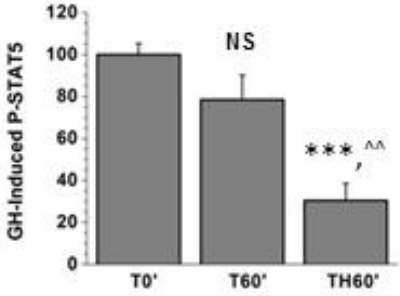
B)


GH-Induced P-STAT5
120
100
80
60
40
20
0
NS
**, %
T0'
T30'
TH30'

C)


P-STAT5
T-STAT5
T0' T60' TH60'

D)


GH-Induced P-STAT5
120
100
80
60
40
20
0
NS
***, ^^
T0'
T60'
TH60'

E)

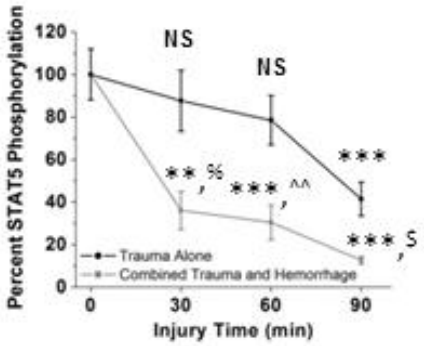
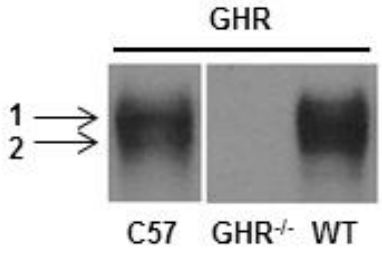
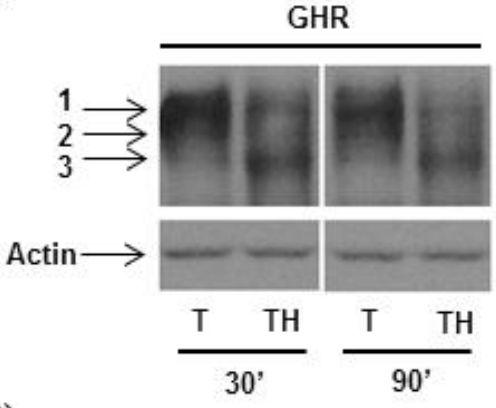

Percent STAT5 Phosphorylation
120
100
80
60
40
20
0
NS
NS
**, %
***, ^^
***
***, $
Trauma Alone
Combined Trauma and Hemorrhage
0
30
60
90
Injury Time (min)

Figure 4. Effect of 30 or 60 min of trauma alone or combined trauma and hemorrhage on GH-induced STAT5 phosphorylation. A,C. Mice were subjected to trauma alone and injected with GH or saline immediately (T0'), 30 or 60 min later (T30' and T60'), or following hemorrhage for 30 or 60 min (TH30' and TH60'). Western analysis of liver lysates was performed and blots were probed with specific anti-PY694/699-STAT5, and anti-STAT5 antibodies. Representative Western blots are presented; scanned images of blots were rearranged for clarity. B,D. Autoradiographs were quantified by scanning densitometry. Data are presented as mean ± SEM percent change in STAT5 phosphorylation, normalized to total STAT5. E. Comparative percentage GH-induced STAT5 phosphorylation following acute injury (from figures 3, 4). Data are expressed as mean ± SEM percent STAT5 phosphorylation. P-STAT5 following T0' was arbitrarily set to 100%, and P-STAT5 following trauma alone and combined trauma and hemorrhage for 30, 60, and 90 min are expressed relative to T0'. In this figure: **, $P < 0.01$ vs. T0'; ***, $P < 0.001$ vs. T0'; %, $P < 0.05$ vs. T30'; ^^, $P < 0.01$ vs. T60', all via one-way ANOVA; $, $P < 0.05$ vs. T90' via two-tailed, Welch-corrected t-test; NS = no statistically significant difference vs. T0'; n = 6 or 7 mice per group.

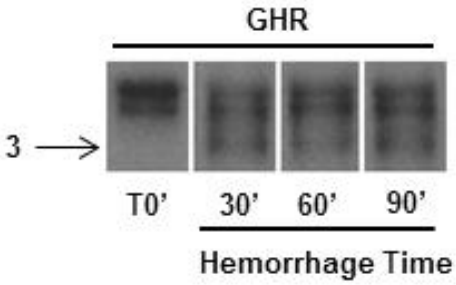
A)


GHR
1
2
C57
GHR-/-
WT

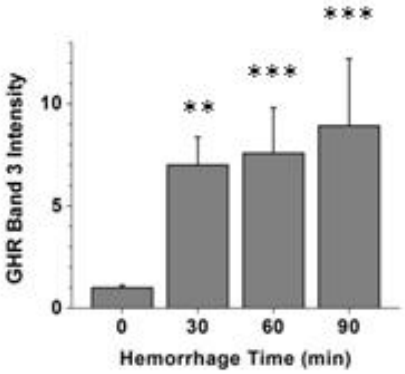
B)


GHR
1
2
3
Actin
T
TH
T
TH
30'
90'

C)


GHR
3
T0'
30'
60'
90'
Hemorrhage Time

D)


GHR Band 3 Intensity
0
5
10
**
***
***
0
30
60
90
Hemorrhage Time (min)

E)

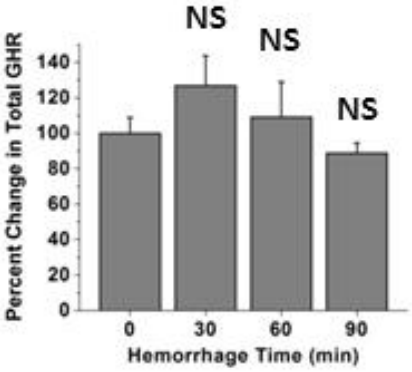
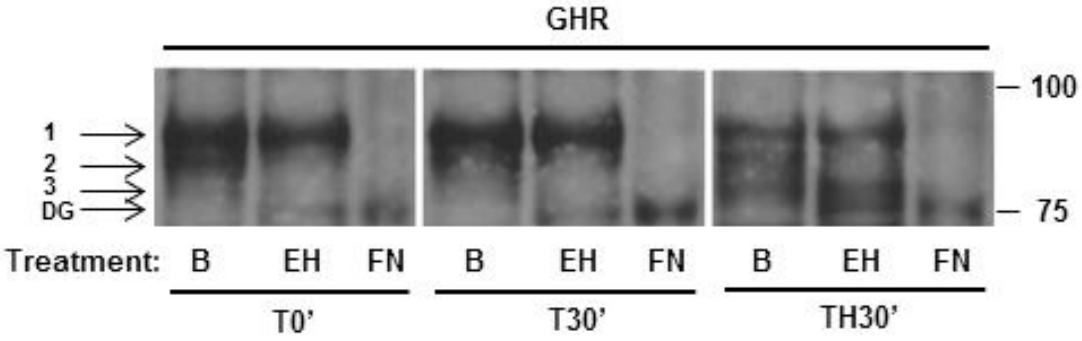
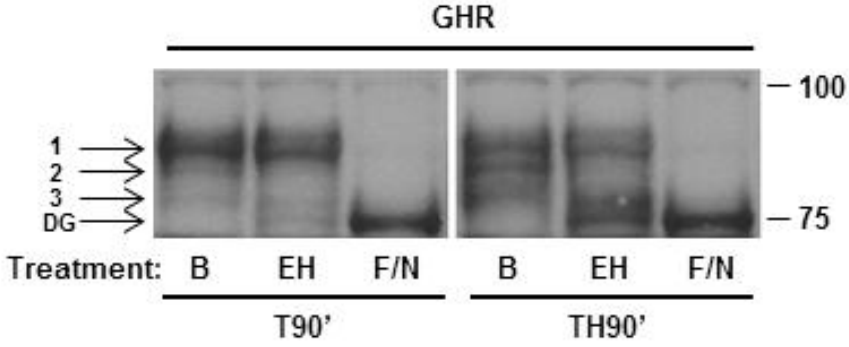

Percent Change in Total GHR
0
20
40
60
80
100
120
140
NS
NS
NS
0
30
60
90
Hemorrhage Time (min)

Figure 5. Effect of combined trauma and hemorrhage on hepatic GHR. Liver lysates were subjected to Western analysis and probed with GHR antiserum raised against the cytoplasmic domain (AL-47; A and B) or a rabbit polyclonal antibody raised against the extracellular domain of GHR (C). Representative Western blots are presented; scanned images of blots were rearranged for clarity. In A-C, the numbers 1, 2, and 3 correspond to GHR Band 1, Band 2, and Band 3, respectively, as described in the text. Autoradiographs were quantified by scanning densitometry. Data are presented as mean ± SEM. D. Band 3 densitometric values were normalized to ERK, and this value was arbitrarily set to 1 for the 0' hemorrhage group. E. Total GHR was calculated by adding the densitometric values for GHR Bands 1, 2, and 3 in each lane, and normalizing those to the total ERK signal for the same lane. The T0' group was arbitrarily set to 100%, and all other groups are expressed relative to T0'. For this figure: **, $P < 0.01$ vs. T0'; ***, $P < 0.001$ vs. T0', both via one-way ANOVA. NS = no statistically significant difference vs. T0'; n = 4 or 7 mice per group.

A)


GHR
100
75
1
2
3
DG
Treatment: B EH FN B EH FN B EH FN
T0'
T30'
TH30'

B)


GHR
100
75
1
2
3
DG
Treatment: B EH F/N B EH F/N
T90'
TH90'

C)

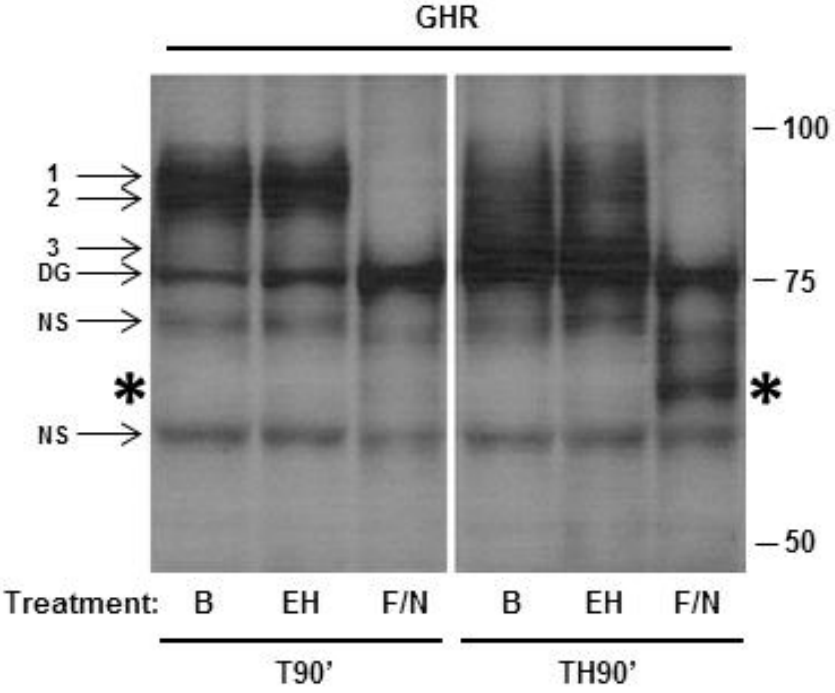

GHR
100
75
50
1
2
3
DG
NS
NS
*
*
Treatment: B EH F/N B EH F/N
T90'
TH90'

Figure 6. Decreased apparent molecular weight of GHR is not due to changes in N-linked glycosylation. Liver lysates were prepared from livers of mice subjected to trauma alone or combined trauma and hemorrhage. Lysates were incubated for 16 h at 37ºC in reaction buffer alone (B), reaction buffer plus Endo H (EH), or reaction buffer plus the F/N combination (F/N). Representative Western blots are presented; scanned images of blots were rearranged for clarity. The numbers 1, 2, and 3 in this figure indicate GHR Bands 1, 2, and 3. DG = fully deglycosylated GHR. NS = nonspecific band. A. Comparison of the effects of enzymatic treatment on livers lysates from T0', T30', and TH30' mice probed with AL-47 antiserum. B. Comparison of the effects of enzymatic treatment on livers from T90' and TH90' mice probed with AL-47. C. Comparison of the effects of enzymatic treatment on livers from T90' and TH90' mice probed with anti-GHR antibody raised against the extracellular domain of GHR. The image in this panel was stretched vertically to improve clarity and to enhance band identification; this change was applied consistently to the entire image.

FASTING PREVENTS ACUTE CARDIAC GROWTH HORMONE RESISTANCE FOLLOWING INJURY

by

RYAN M. CORRICK AND JOSEPH L. MESSINA

In preparation for *Critical Care Medicine*

Format adapted for dissertation

Abstract

Severe injury and infection are frequently followed by an acute state of heart failure, but the mechanisms are not well understood. However, growth hormone (GH) resistance develops rapidly in some tissues following injury and during infection. Since GH regulates calcium homeostasis in myocardium, it is possible that cardiac GH resistance contributes to myocardial dysfunction following injury. We hypothesized that surgical trauma combined with hemorrhage would result in cardiac GH resistance in mice. Impaired GH signaling developed rapidly in response to surgical trauma alone, and was unaltered by the addition of hemorrhage. Unexpectedly, an overnight fast prior to injury prevented the development of cardiac GH signaling deficits. Additional studies revealed that fasting enhances GH sensitivity in the heart, providing an explanation for the effect of fasting on cardiac GH resistance following injury. We conclude that soft tissue trauma results in rapid alterations in GH signaling in the heart.

Introduction

Acute heart failure is a common complication of shock due to injury or sepsis, and is characterized by myocardial contraction and relaxation deficits, as well as impaired cardiac output (1-3). Severe injury and sepsis lead to mitochondrial dysfunction and depletion of ATP stores in the heart, resulting in an energy deficit that contributes to abnormal cardiac function (4-9). In addition, increased myocardial production of pro-inflammatory cytokines (particularly IL-6 and TNFα) and activation of apoptotic signaling in cardiomyocytes are also associated with impaired myocardial contractility and cardiac output following hemorrhage (10-17). Cardiac dysfunction frequently persists after fluid resuscitation and restoration of normal plasma volume, requiring the

use of inotropic therapies. Failure to reverse myocardial dysfunction can contribute to the development of multiple organ failure, which remains the most common cause of death following trauma or major surgery (18;19).

Growth hormone (GH) is a pituitary hormone with important roles in cardiac structure and function. GH binding to its receptor (GHR) results in tyrosine phosphorylation and subsequent activation of receptor-associated Janus kinase 2 (JAK2) (20;21). Activation of JAK2 leads to tyrosine phosphorylation of multiple Signal Transducer and Activator of Transcription (STAT) transcription factors, including STAT5 (22-26). GH-induced STAT5 phosphorylation is necessary to stimulate transcription of insulin-like growth hormone-1 (27), an important mediator of GH action in the heart (28;29). In addition, GH stimulates several other pathways via activation of JAK2, including the PI3K/AKT and MEK/ERK pathways (30-33). However, little is known about the effects of GH stimulation of PI3K/AKT and MEK/ERK in the heart.

GH resistance develops rapidly following trauma, hemorrhage, burns, and sepsis (34;35), and is a complication of several chronic illnesses, including congestive heart failure and chronic renal failure (36-38). GH resistance also develops in response to fasting (39;40). Several rodent models have been used to study the mechanisms of GH resistance, including models of sepsis, kidney failure, and fasting. Employing these models, multiple studies have confirmed the development of GH resistance in several tissues, including the liver and skeletal muscle (38;40-44). In addition, cardiac GH resistance has been reported during experimentally-induced kidney failure (45). However, little is known about the development of GH resistance in the heart, or whether cardiac GH resistance contributes to myocardial dysfunction following injury.

Myocardium expresses receptors for GH and IGF-I (46-49), enabling both hormones to confer a number of important, beneficial effects on the heart. Both GH and IGF-1 directly affect myocardial contractility by influencing intracellular calcium concentrations and modulating the calcium sensitivity of myofilaments (29;50;51). IGF-1 is a potent inhibitor of apoptosis in many cell types, including cardiomyocytes (52-54), and likely assists in modulating the cardiac response to stress and inflammation. In addition, treatment with GH improves cardiac function following burn injury in children (55), and overexpression of IGF-1 in cardiomyocytes reverses LPS-induced cardiac dysfunction and stress signaling (56). A recent report also indicates that low-dose GH administration exerts cardioprotective effects during uremia (57). Due to the important roles of GH and IGF-1 in normal heart function, GH resistance in the heart may have a permissive effect on the development of cardiac dysfunction following injury. However, it is not currently known whether severe injury or hemorrhage result in cardiac GH resistance.

In the present study, we subjected fed and fasted mice to trauma alone or combined trauma and hemorrhage and assessed cardiac GH signaling. We hypothesized that acute traumatic injury would result in cardiac GH resistance. In addition, since fasting alone results in hepatic GH resistance, we also hypothesized that a prior overnight fast would increase cardiac GH resistance following injury. In the present report, we demonstrate the effects of GH on multiple signaling pathways in the heart. We also demonstrate GH resistance following trauma alone and combined trauma and hemorrhage. Finally, we present the novel finding that fasting reverses GH resistance in the heart following acute injury, apparently by increasing basal GH sensitivity.

Materials and Methods

All animal procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama Birmingham.

GH Signaling Time Course and Fasting Studies

Mice were anesthetized with isoflurane (Mallinckrodt Veterinary, Mundelein, IL) and immediately injected with saline (75 µL) or 1.5 µg/g bovine GH dissolved in 75 µL saline (obtained from Dr. Albert Parlow, National Hormone and Pituitary Program). Tissues were harvested 1, 5, 10, 15, 20, or 25 min after GH injection and immediately stored in liquid nitrogen until further processing. To determine the effects of fasting on GH signaling, mice were fed or fasted for 6, 12, or 18 hours before GH or saline injection. Tissues were harvested 10 min later.

Animal Model of Trauma and Hemorrhage

Mice were treated as described previously (58) with minor alterations. Briefly, male C57BL/6 mice (Harlan Laboratories, Inc.), approximately 12 weeks old and 23-25 g, were anesthetized with isoflurane (Mallinckrodt Veterinary, Mundelein, IL), clipped, and shaved. Mice were anesthetized by continuous inhalation of 1.5% isoflurane and 98.5% O_2 throughout all procedures. Mice were then clipped and shaved, and restrained in a supine position. Surgical trauma began with a 2-cm ventral midline laparotomy representing soft tissue trauma. The abdomen was then closed in layers using 6-0 Ethilon

sutures (Ethicon, Somerville, NJ), and the wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) for analgesia. Polyethylene-10 catheters (Clay-Adams, Parsippany, NJ) were placed in the right and left femoral arteries for monitoring of mean arterial pressure (MAP; Micro-Med, Louisville, KY) and for experimental hemorrhage, respectively. This completed the surgical trauma portion of the procedure. Mice designated for combined trauma and hemorrhage were then immediately hemorrhaged to a MAP of 35-40 mmHg over a 10 min period. The hemorrhage time was started immediately after MAP dropped below 40 mm Hg. At the conclusion of the study period, the abdominal incision was re-opened, and either saline or 1.5 µg/g GH (total volume = 75 µL) were injected directly into the vena cava. Tissues were harvested 10 min later and stored in liquid nitrogen until further processing (40;43;58;59).

Study Design

Injury-control mice were anesthetized and injected with GH or saline without additional trauma or hemorrhage, and are hereafter referred to as "normal" mice. Tissues were removed from normal mice 10 min after injection. Trauma-alone mice (T0') were subjected to the surgical trauma procedure, and then immediately injected with GH or saline. Additional trauma-alone groups were injected with GH or saline 30 min (T30'), 60 min (T60'), 90 min (T90'), or 210 min after catheterization. Trauma and hemorrhage groups were subjected to surgical trauma, hemorrhaged and maintained at 35-40 mmHg for 30 min (TH30'), 60 min (TH60'), or 90 min (TH90') before injection with GH or saline. In addition, an "extended hemorrhage" included mice that were subjected to surgical trauma, hemorrhaged and maintained at 35-40 mm Hg for 210 min before

injection with GH or saline. To determine the combined effects of injury and fasting, food (but not water) was withdrawn from mice for 18 hours prior to T0', T90', or TH90' treatment. Tissues were removed from all mice subjected to trauma alone or combined trauma and hemorrhage 10 min after injection.

Western Analysis

Tissues were homogenized in extraction buffer, as described previously, and stored at –80°C until use (60-62). The protein concentration of tissue lysates was determined by the BCA method (Pierce, Rockford, IL) and 7.5 µg of protein were resolved via SDS-PAGE (Criterion System; Bio-Rad, Hercules, CA) and transferred to nitrocellulose paper. Membranes were immunoblotted with the following primary antibodies: anti-P-STAT5 (PY694/699), anti-total-STAT5, anti-P-AKT (PS473), anti-total-AKT, anti-P-ERK (Thr202/Tyr204), anti-total ERK, and anti-Pan-Actin (all from Cell Signaling, Danvers, MA), anti-GHR (extracellular domain; R & D Systems, Minneapolis, MN).

Densitometric and Statistical Analysis

Enhanced chemiluminescence images of immunoblots were obtained on CL-X Posure Film (Thermo Scientific, Rockford, IL). Films were scanned using a Hewlett-Packard C5280 Scanner, and relative band intensities were determined using the Quantity One software package (Bio-Rad, Hercules, CA). Data are presented as mean ± SEM fold change or percent change. Phosphorylated protein amounts were normalized to the respective total protein amounts (i.e. P-STAT5 was normalized to the T-STAT5 signal

for the same animal). Total GHR levels were normalized to total ERK levels. Data was analyzed using the InStat statistical program (GraphPad Software, Inc., San Diego, CA). Differences between groups were determined using one-way ANOVA (Tukey post-test) or Student's *t* test (unpaired, Welch-corrected).

Results

Time Course of Cardiac GH Signaling

In order to characterize the effects of GH on several signaling pathways, mice were anesthetized and injected with saline or GH and hearts were removed 0, 1, 5, 10, 15, 20, and 25 min later. Extracted proteins were separated by SDS-PAGE and immunoblotted for phosphorylated and total forms of STAT5, AKT, and ERK. A 10-fold increase in STAT5 tyrosine phosphorylation (Y694/699) was detected in hearts 1 min after injection with GH compared to hearts injected with saline (Fig. 1A and B). GH-induced P-STAT5 levels peaked 5 min after GH injection with an 18-fold increase compared with saline injection. Cardiac P-STAT5 levels began declining 10 min after GH injection, but remained significantly elevated compared with saline injected-mice until 25 min post-injection (the last time point studied). GH did not induce significant AKT serine phosphorylation (S473) in hearts compared with hearts from mice injected with saline. However, there was a significant decrease in cardiac P-AKT to 0.17 fold compared with saline controls at 25 min following GH injection (Fig. 1A and C). Similarly, ERK phosphorylation in hearts was reduced to 0.61 fold compared to saline 1 min after GH injection, and was further reduced to 0.36 fold compared to saline at 25 min following GH injection (Fig. 1A and D). These data suggest that *in vivo* cardiac GH

signaling is similar to other tissues with respect to STAT5 phosphorylation, but not ERK or AKT.

Rapid GH Signaling Impairment in Heart Following Acute Injury

To determine whether injury resulted in GH resistance in the heart, mice were subjected to surgical trauma, alone or in combination with hemorrhage, and injected with GH or saline. Hearts were removed 10 min later. Protein extracts were immunoblotted and probed for phosphorylated and total STAT5, and total ERK. Compared to normal mice, GH-induced STAT5 phosphorylation in heart was reduced 71% immediately after completion of surgical trauma (T0' mice; Fig. 2A and 2B). Furthermore, GH-induced STAT5 phosphorylation was reduced by 58% and 70% at 30 and 60 min after completion of surgical trauma, respectively (T30' and T60'; Fig. 2C, D, E, and F). No further change in GH-induced P-STAT5 was observed when surgical trauma was followed by 30 or 60 min of hemorrhage compared with surgical trauma alone (TH30'/TH60' vs. T30'/T60'). To further characterize the timing of GH resistance, GH-induced P-STAT5 was studied at extended time points following injury. Similar to studies at 30 and 60 min, cardiac GH-induced P-STAT5 was reduced 80% and 70% at 90 and 210 min after surgical trauma (T90' and T210') compared with normal mice (Fig. 3A, B, C, and D). When surgical trauma was followed by 90 or 210 min of hemorrhage, (TH90' or THH210'), there was no further change in cardiac GH-induced P-STAT5 compared with T90' or T210', respectively. These data suggest that cardiac GH resistance following injury is primarily due to the effects of surgical trauma alone, and is not significantly influenced by hemorrhage.

Prior Overnight Fasting Enhances GH Signaling

To ascertain whether fasting combined with injury would result in a cumulative effect on cardiac GH signaling, mice were fasted for 18 hours prior to surgical trauma. GH-induced P-STAT5 in heart was increased 2.7-fold in fasted T0' mice compared with T0' mice fed *ad libitum* until immediately prior to surgical trauma (Fig. 4A and B). To confirm that fasting was responsible for the enhanced GH signaling in fasted T0'mice, normal mice were subjected to fasting periods of 6, 12, or 18 hours before injection with GH. Normal mice fasted for 6 hours demonstrated a 9.7-fold increase in cardiac GH-induced STAT5 phosphorylation compared to fed controls (Fig. 5A and B). Mice fasted for 12 or 18 hours demonstrated 4.8 and 2.6-fold increases in P-STAT5 compared to fed controls, respectively. P-STAT5 in hearts from mice fasted for 12 or 18 hours was significantly decreased compared with mice fasted for 6 hours, suggesting that GH sensitivity in hearts reaches its peak after approximately 6 hours of fasting. To determine whether the effects of fasting on GH-induced P-STAT5 were specific to heart, adipose tissue lysates from fed and fasted normal mice were subjected to Western analysis. GH-induced STAT5 phosphorylation did not change in adipose tissue following fasting for 6, 12, or 18 hours compared with fed controls (Fig. 5C and D), suggesting that nutrition may not be an important regulator of GH signaling in adipose tissue.

Overnight Fasting Prevents Impaired GH Signaling Following Surgical Trauma

To further confirm the effects of fasting on GH signaling following injury, hearts from fasted T90' and TH90' mice were compared with hearts from fed normal and T90'

mice. As demonstrated previously (Fig. 3A and B), T90' decreased cardiac GH-induced P-STAT5 compared with hearts from normal mice (Fig. 6A and B). However, fasting for 18 hours prior to surgical trauma resulted in cardiac P-STAT5 that was increased in T90' and TH90' mice to 5.6 and 4.8 fold compared with hearts from fed T90' mice. In addition, GH-induced P-STAT5 in fasted T90' and TH90' hearts was increased to 2.0 and 1.7 fold compared with fed normal. Finally, compared with hearts from normal mice, GHR abundance was unchanged in hearts from fed T90', fasted T90', or fasted TH90' mice (Fig. 6C and D). These results suggest that fasting can prevent GH resistance due to injury. In addition, changes in total GHR abundance do not appear to account for decreased GH signaling following injury, or the observed increase in GH signaling due to fasting.

Discussion

Considering the important role of GH action in normal cardiac structure and function, there is surprisingly little known about GH signaling in the heart. In other tissues and in cell culture models, GH stimulates activation of multiple pathways, including STAT proteins, MEK/ERK, and PI3K/AKT. In addition, activation of STAT5 and ERK has been reported in cardiomyocytes following stimulation with GH (30;63), but detailed studies of cardiac GH signaling *in vivo* have not previously been reported. In the present report we provide evidence that, in contrast to *in vivo* studies in other tissues or in cardiomyocytes, injection of C57BL/6 mice with GH results in a time-dependent decrease in cardiac AKT and ERK phosphorylation. However, GH injection also resulted

in a large increase in STAT5 tyrosine phosphorylation in the heart, with kinetics similar to previously published reports of studies in liver and cell culture models (26;64;65).

To date, there has been only one published report of GH resistance in the heart (45), which employed a model of chronic renal failure. We hypothesized that injury also results in cardiac GH resistance. Our studies incorporated two different forms of injury: one that involved only surgical trauma, and the other involving surgical trauma combined with hemorrhage. Our data demonstrates a rapid effect of surgical trauma on GH signaling that was apparent immediately following the procedure. GH-induced STAT5 phosphorylation was impaired at all time points studied following surgical trauma alone, from 0 to 210 min. Although we cannot rule out a neurological mechanism, previous reports of cardiac abnormalities following injury suggest that increased production of one or more soluble mediators (such as the pro-inflammatory cytokines IL-6 and TNFα) are responsible for the profound effects of severe injury on cardiac function (2;11;17;66;67).

Our data indicates that the observed effects of injury on GH signaling in the heart are trauma-dependent, since the addition of hemorrhage did not significantly affect STAT5 phosphorylation at any time point. This was somewhat surprising in that combined trauma and hemorrhage generally results in more severe effects compared to surgical trauma alone. For example, in our studies of acute insulin resistance due to injury, major signaling defects in liver and skeletal muscle were observed only when surgical trauma was combined with hemorrhage, and not following surgical trauma alone (60;61;68-70). However, in those studies it was discovered that TNFα plays a dominant role in the maintenance of the insulin resistant state, following injury, hemorrhage and resuscitation, and that TNFα levels do not rise quickly enough to cause the early

development of insulin resistance. Another group has demonstrated that TNFα increases following combined trauma and hemorrhage, but not trauma alone (71). Thus, it is unlikely that TNFα plays a significant role in the acute development of cardiac GH resistance following surgical trauma alone, or trauma combined with hemorrhage. However, IL-6 is increased rapidly in the blood following surgical trauma alone (71), and reports from other laboratories indicate that IL-6 is a primary contributor to cardiac dysfunction following injury (17;66;67). In addition, IL-6 is implicated in the development of GH resistance in the liver (72), and may therefore also be an important contributor to cardiac GH resistance following injury.

Based on previous reports suggesting that fasting results in hepatic GH resistance (40;73), we hypothesized that fasting would increase the severity of GH resistance in the heart following injury. Surprisingly, when mice were fasted overnight prior to injury, GH-induced P-STAT5 was increased in hearts compared to fed mice from all groups (injury as well as normal controls). Furthermore, short-term fasting (between 6 and 18 hours) significantly enhanced cardiac GH signaling. In a recent report of GH action in rat neonatal cardiomyocytes, it was demonstrated that GH stimulation resulted in an increase in free fatty acid (FFA) uptake while simultaneously decreasing glucose uptake (30). This suggested that a major function of GH in cardiomyocytes is to facilitate an adaptive transition from glucose to fatty acids as the preferred metabolic substrate. Similarly, transgenic mice overexpressing bovine GH exhibited increased cardiac fatty acid transport and oxidation (74). Considering these previous reports, it is possible that increased sensitivity of the heart to GH may be part of a metabolic program, activated by fasting, that alters the ratio of carbohydrates vs. FFA used for metabolic fuel. The

finding that fasting had no effect on adipose tissue P-STAT5 underscores the fact that regulation of GH signaling is not uniform across all tissues.

In conclusion, GH results in increased P-STAT5 and decreased P-AKT and P-ERK, a response that may be unique in mouse heart. Furthermore, surgical trauma resulted in the rapid development of cardiac GH resistance that was sustained for at least 3.5 hours. However, fasting prior to surgical procedures prevented cardiac GH resistance following injury. Due to the importance of GH action in the heart, combined with the frequent development of cardiac dysfunction following injury, future mechanistic studies of cardiac GH resistance are warranted.

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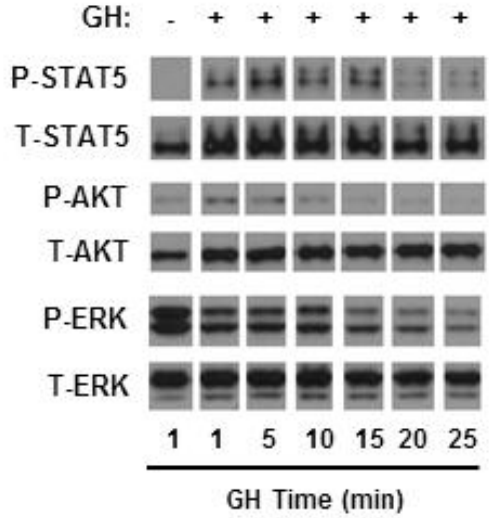
A)


GH: - + + + + + +
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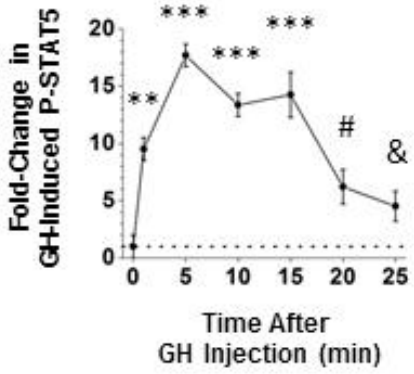
T-STAT5
P-AKT
T-AKT
P-ERK
T-ERK
1 1 5 10 15 20 25
GH Time (min)

B)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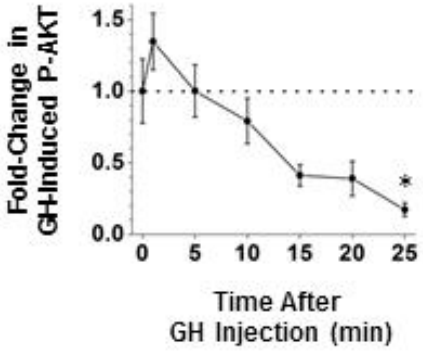
Time After GH Injection (min)

C)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D)

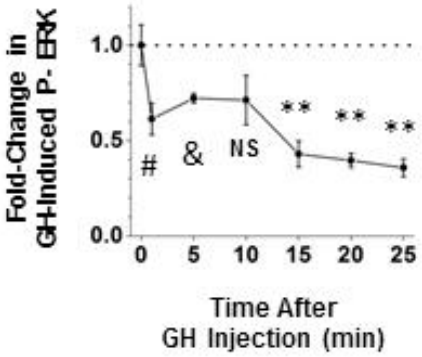
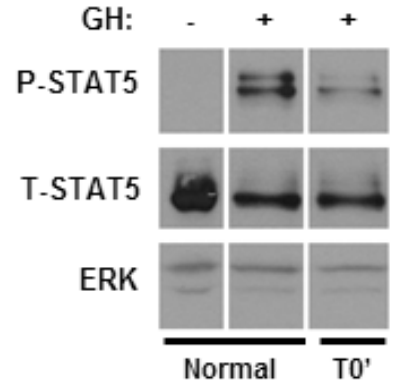
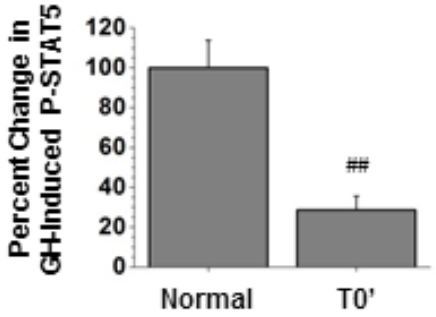

Fold-Change in GH-Induced P-ERK
Time After GH Injection (min)

Figure 1. Time course of GH signaling in the mouse heart following injection with GH. Fed mice were injected with bovine GH (or saline; - GH; see the 1' time point) and hearts were removed at the indicated times. **A**. Protein extracts from hearts were prepared and Western analysis was performed with specific antibodies raised against PY-694/699-STAT5 and total STAT5, PS-473-AKT and total AKT, and PT-202/PY-204-ERK and total ERK. In this and all subsequent figures, blots probed with antibodies against phosphorylated proteins were stripped and reprobed with antibodies against respective total proteins; representative blots are presented; scanned images of individual blots were rearranged for clarity. **B-D**. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 (**B**), P-AKT (**C**), and P-ERK (**D**). The average of the 3 mice at t=0 was arbitrarily set to 1. Data are presented as mean ± SEM fold change in phosphorylated protein normalized to total protein. In this figure: *, $P < 0.05$ vs. 0'; **, $P < 0.01$ vs. 0'; and ***, $P < 0.001$ vs. 0', all via one-way ANOVA; $^{\#}$, $P < 0.05$ vs. 0' via two-tailed t-test; $^{\&}$, $P < 0.05$ vs. 0' via one-tailed t-test; NS = not significantly different vs. 0'; n = 3 mice per time point.

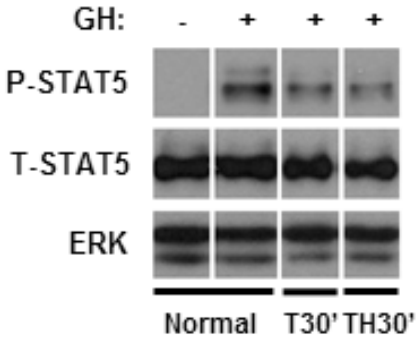
A)


GH: - + +
P-STAT5
T-STAT5
ERK
Normal
T0'

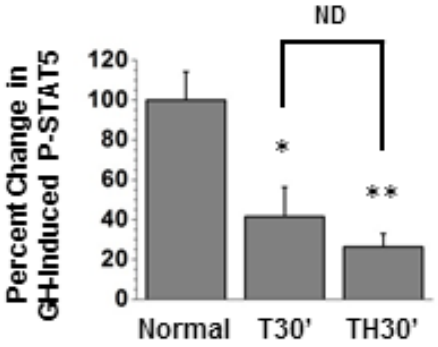
B)


Percent Change in GH-Induced P-STAT5
120
100
80
60
40
20
0
##
Normal
T0'

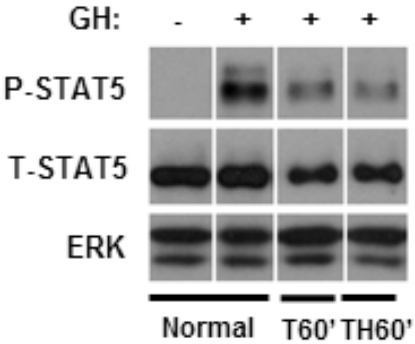
C)


GH: - + + +
P-STAT5
T-STAT5
ERK
Normal
T30'
TH30'

D)


Percent Change in GH-Induced P-STAT5
120
100
80
60
40
20
0
ND
*
**
Normal
T30'
TH30'

E)


GH: - + + +
P-STAT5
T-STAT5
ERK
Normal
T60'
TH60'

F)

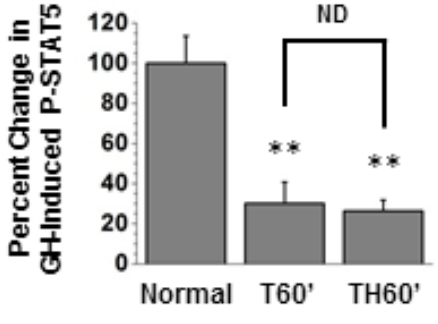

Percent Change in GH-Induced P-STAT5
120
100
80
60
40
20
0
ND
**
**
Normal
T60'
TH60'

Figure 2. Effects of surgical trauma alone or trauma combined with hemorrhage, on GH-induced STAT5 tyrosine phosphorylation. Fed mice were subjected to surgical trauma, alone or combined with hemorrhage, and injected with saline or GH. Hearts were removed 10 min later. Protein extracts from hearts were immunoblotted with antibodies raised against PY694/699-STAT5 and total-STAT5. Total ERK was also probed and is included as a demonstration of even loading. A, C, E: Representative blots are presented with scanned images of individual blots rearranged for clarity. **A**. Western analysis of GH signaling in hearts from mice immediately following surgical trauma (T0') and normal mice. **C**. Western analysis of GH signaling in hearts from mice 30 min after surgical trauma alone (T30') or surgical trauma followed by 30 min hemorrhage (TH30'), compared with normal mice. **E**. Western analysis of GH signaling in hearts from mice 60 min after surgical trauma alone (T60') or surgical trauma followed by 60 min hemorrhage (TH60'), compared with normal mice. **B, D, F**. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5. Data are presented as mean ± SEM percent change in phosphorylated STAT5 normalized to total STAT5. In this figure: $^{\#\#}$, $P < 0.01$ via Welch-corrected t-test; *, $P < 0.05$ vs. normal; **, $P < 0.01$ vs. normal, all via one-way ANOVA; ND = not significantly different; n = 5 for all groups.

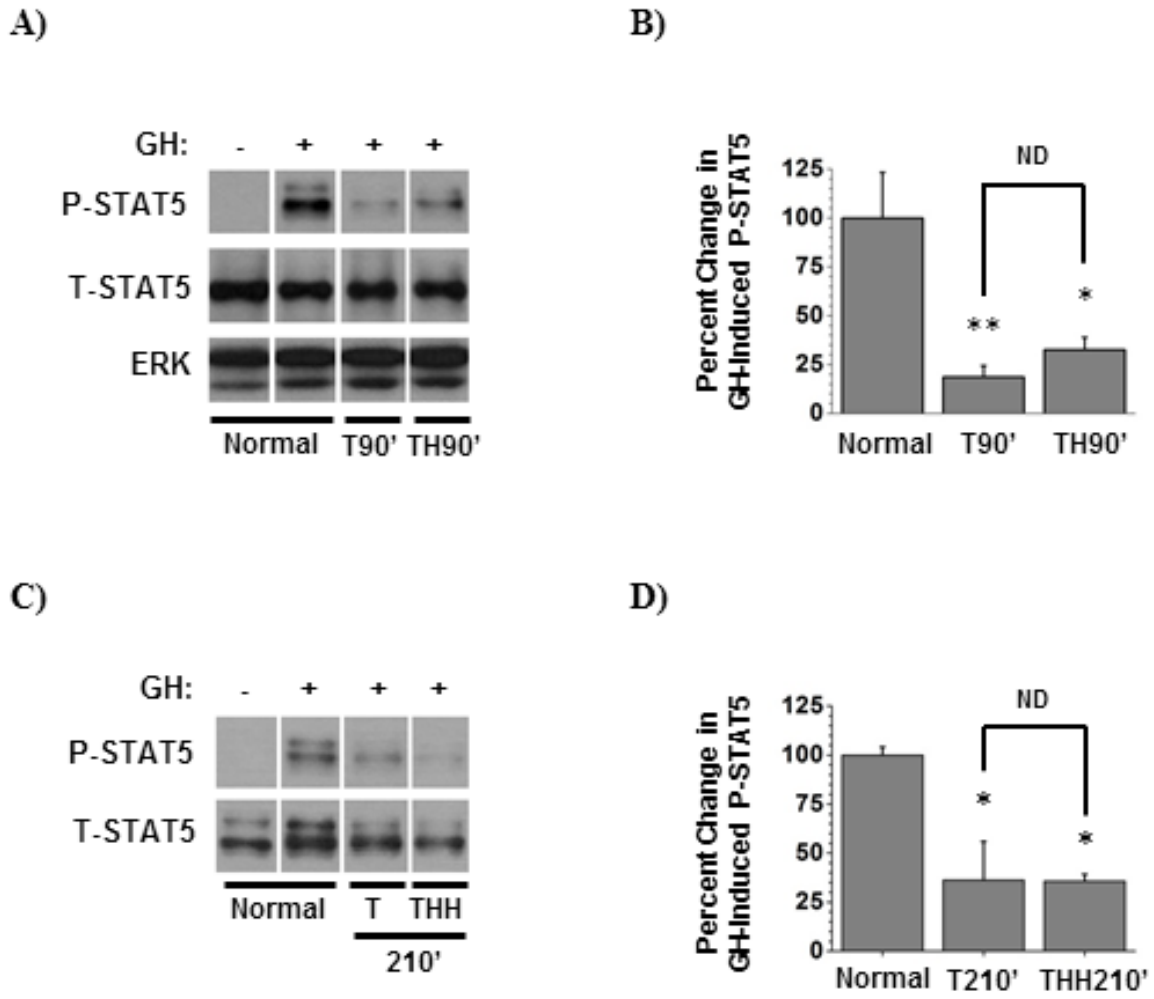


Figure 3. Effects of surgical trauma alone or trauma combined with hemorrhage, on GH-induced STAT5 tyrosine phosphorylation. Fed mice were subjected to surgical trauma, alone or combined with hemorrhage, and injected with saline or GH. Hearts were removed 10 min later. Protein extracts from hearts were immunoblotted with antibodies raised against PY694/699-STAT5 and total-STAT5. A, C: Representative blots are presented with scanned images of individual blots rearranged for clarity. **A**. Western analysis of GH signaling in hearts from mice 90 min after surgical trauma alone (T90') or surgical trauma followed by 90 min hemorrhage (TH90') compared with normal mice. **C**. Western analysis of GH signaling in hearts from mice 210 min after surgical trauma alone (T210') or surgical trauma followed by 210 min hemorrhage (THH210'), compared with normal mice. **B, D**. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5. Data are presented as mean ± SEM percent change in phosphorylated STAT5 normalized to total STAT5. In this figure: *, $P < 0.05$ vs. normal; **, $P < 0.01$ vs. normal, all via one-way ANOVA; $n = 3$ or more for all groups.

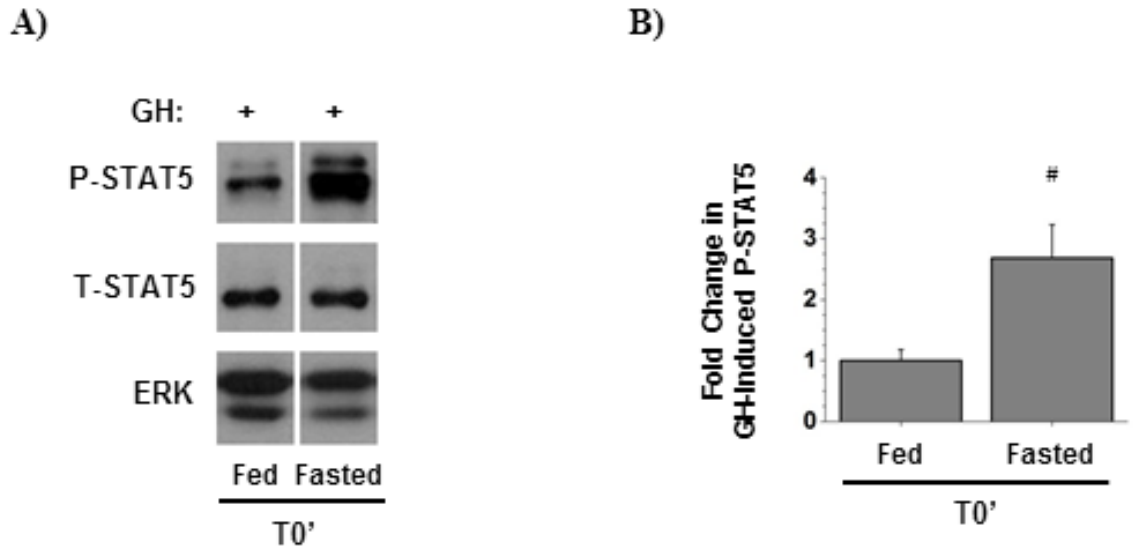


Figure 4. Effects of fasting on GH signaling in mouse heart immediately following surgical trauma. Mice were either fed ad libitum or fasted for 18 hours and immediately following surgical trauma (T0') mice were injected with GH and hearts removed 10 min later. Protein extracts from hearts were immunoblotted with antibodies raised against PY694/699-STAT5 and total-STAT5. Total ERK was also probed and is included as a demonstration of even loading. **A**. Western analysis of GH signaling in fed vs. fasted mice immediately following surgical trauma. Representative blots are presented with scanned images of individual blots rearranged for clarity. **B.** Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5. Data are presented as mean ± SEM fold change in phosphorylated STAT5 normalized to total STAT5. In this figure: $^{\#}$, $P < 0.05$ vs. fed T0' via Welch-corrected t-test; n = 5 for both groups.

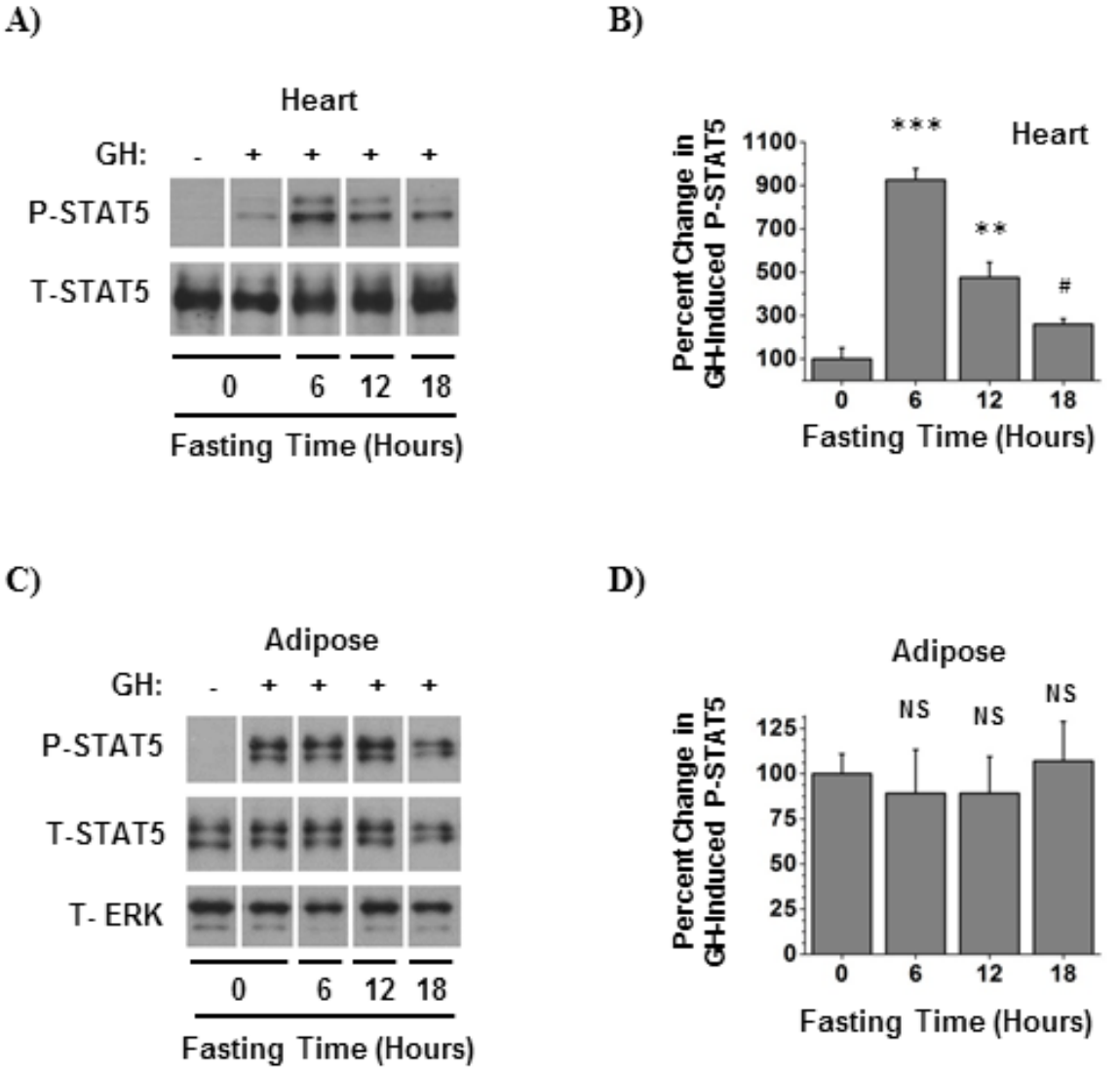


Figure 5. Time course of the effects of fasting on GH signaling in mouse heart. Mice were fed ad libitum (0 fasting time) or fasted for 6, 12, or 18 hours before injection with GH or saline. Hearts and epididymal adipose tissue were removed 10 min later. Protein extracts were immunoblotted with antibodies raised against PY694/699-STAT5 and total-STAT5. Total ERK was also probed and is included as a demonstration of even loading. **A, C**. Western analysis of GH signaling in hearts and adipose tissue, respectively, from fed vs. fasted mice. Representative blots are presented with scanned images of individual blots rearranged for clarity. **B, D**. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5. Data are presented as mean ± SEM fold change in phosphorylated STAT5 normalized to total STAT5. In this figure: **, $P < 0.01$ vs. fed; ***, $P < 0.001$ vs. fed, both via one-way ANOVA; $^{\#}$, $P < 0.05$ vs. fed via t-test; NS = not significantly different from fed mice; n = 3 for all groups.

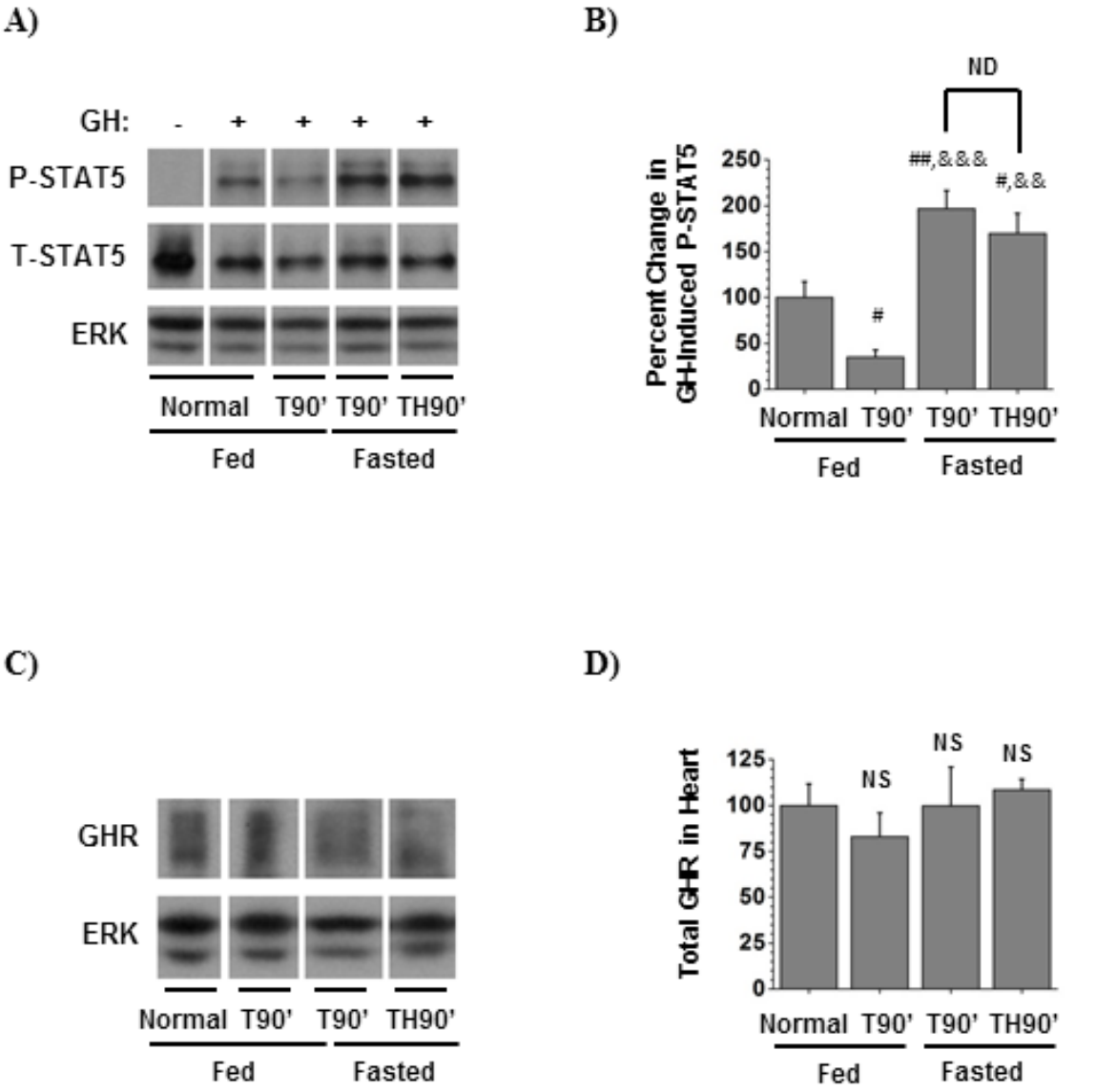


Figure 6. Effects of fasting on GH receptor signaling following surgical trauma alone or trauma combined with hemorrhage. Normal, T90', and TH90' mice were either fed ad libitum or fasted for 18 hours prior to surgical procedures. Hearts were removed 10 min after injection with GH or saline. Protein extracts were immunoblotted with antibodies raised against PY694/699-STAT5 and total-STAT5, GHR, and total ERK. **A, C**. Western analysis of GH-induced STAT5 phosphorylation and total GH in hearts. Representative blots are presented with scanned images of individual blots rearranged for clarity. **B, D**. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 and GHR. Data are presented as mean ± SEM fold change in phosphorylated STAT5 normalized to total STAT5 (**B**) and total GHR normalized to ERK (**C**). In this figure: $^{\#}$, $P < 0.05$ vs. fed normal; $^{\#\#}$, $P < 0.01$ vs. fed normal; $^{\&\&}$, $P < 0.01$ vs. fed T90'; $^{\&\&\&}$, $P < 0.001$ vs. fed T90'; NS = not significantly different from fed normal; ND = not significantly different from each other; n = 5 for all groups.

SELECTED UNPUBLISHED DATA

Introduction

Data presented in the first and second articles in this dissertation demonstrates that acute injury results in GH resistance in both the liver and heart. GH resistance due to fasting has been observed in both humans and rodents (1-3). We therefore hypothesized that GH resistance following injury would be more severe when combined with fasting. Surprisingly, our studies in hearts demonstrated that GH-induced STAT5 phosphorylation was increased relative to normal controls when mice were fasted overnight prior to surgical trauma alone, or combined trauma and hemorrhage. Additional studies demonstrated that fasting enhances GH signaling in the heart, providing a partial explanation for the reversal of injury-induced cardiac GH resistance by fasting. Although the effects of fasting on cardiac GH sensitivity have not been previously studied, the development of GH resistance in the liver during fasting has been well established (2). We therefore investigated the effect of an overnight fast on hepatic GH resistance following injury.

Our primary focus has been on GH sensitivity in the liver. However, the direct effects of GH on other tissues, particularly skeletal muscle and adipose tissue, are important for the beneficial effects of GH on metabolism and maintenance of lean body mass. For example, GH stimulates lipolysis in adipose tissue, and protein retention in skeletal muscle, during fasting (4;5), providing an alternative metabolic fuel while preserving strength and mobility. Following severe injury, skeletal muscle is rapidly

degraded. Adipose stores often remain unaffected during the catabolic state that follows severe injury, despite elevated circulating concentrations of GH, which normally stimulates lipolysis. Taken together, this suggests the development of GH resistance in skeletal muscle and adipose tissue following severe injury. We therefore investigated the effects of acute injury on GH signaling in skeletal muscle and adipose tissue.

The results from the additional studies presented in this section remain to be developed more fully. However, here we demonstrate effects of GH on hepatic STAT3 and AKT phosphorylation, two pathways known to be stimulated by GH that have not been well-studied. We also demonstrate the effects of fasting on hepatic GH receptor signaling, and of fasting combined with injury. Finally, we present separately the effects of fasting and the effects of injury on skeletal muscle and adipose tissue.

Methods

The methods used to study GH signaling in adipose tissue and skeletal muscle were nearly identical to those described for the heart, with only minor exceptions (see Materials and Methods in the second article of this dissertation). Briefly, epidiymal adipose tissue was removed 10 min after injection with GH or saline and stored in liquid nitrogen until further processing. Adipose depots were solubilized in a buffer containing 50 mM Tris at pH 8.0, 150 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 2 nM sodium orthovanadate, 0.1 µg/mL okadaic acid, 1% Igepal, 0.5% sodium deoxycholate, and protease inhibitors (P2714 from Sigma Aldrich, according to manufacturer's instructions). For skeletal muscle, triceps were removed 10 min after injection with GH or saline and were later solubilized in the same muscle extraction buffer described in the

second article of this dissertation for heart. IGF-I protein concentration was measured in triceps using an IGF-I ELISA kit from R&D Systems according to the manufacturer's instructions. Antibodies directed against PY-705 STAT3 and total STAT3 were obtained from Cell Signaling (Danvers, MA). The antibody against EGFR was obtained from Santa Cruz Biotechnology (Boston, MA).

Results

GH-Induced Activation of Hepatic STAT3 and AKT

GH-induced tyrosine-phosphorylation of hepatic STAT3 was statistically significant 10 min after injection with GH (Fig. 1A and 1B). Serine phosphorylation of liver AKT was significantly increased 1 min after GH injection, but returned to basal levels within 10 min after GH injection.

Effects of Fasting on Hepatic GHR, EGFR, and STAT5

Fasting resulted in a time-dependent reduction of hepatic GHR (Fig. 2A), culminating in 75% reduction of GHR after 18 hours (Fig. 2B). In addition, GH injection resulted in greater than 50% reductions in hepatic GHR after 10 min in both unfasted mice and mice fasted for 6 hours. However, there was no significant difference in hepatic GHR in saline- vs. GH-injected mice fasted for 12 or 18 hours. Hepatic EGFR was also significantly decreased following fasting for 6, 12, or 18 hours (Fig. 2C and D). However, even though GHR was significantly reduced, hepatic GH-induced STAT5 phosphorylation was not significantly affected by fasting at any of the time points studied (Fig. 2E and F).

GH-stimulated P-STAT5 band migration was reduced after 12 or 18 hours of fasting (Fig. 2E and G). As discussed in the first article of this dissertation, GH-induced P-STAT5 band migration may indicate P-STAT5 serine phosphorylation, which impacts STAT5 DNA binding (6;7). Therefore, impaired P-STAT5 band migration due to fasting may indicate decreased activity of nuclear STAT5 following GH stimulation.

Effects of Combined Fasting and Injury on Hepatic GH Receptor Signaling

GH-induced P-STAT5 was significantly reduced in livers from fed mice subjected to surgical trauma combined with 90 min hemorrhage (fed TH90'), compared with fed mice subjected to surgical trauma alone (fed T90', Fig. 3A and B). In addition, fasted T90' mice exhibited impaired hepatic GH-induced P-STAT5 compared with fed T90' mice. Hepatic GH-induced P-STAT5 in fasted TH90' mice was significantly reduced compared with fed T90', fed TH90', and fasted T90' mice. Similar to mice subjected to fasting alone (Fig. 2), mice subjected to fasting prior to injury had reduced levels of GHR compared with mice subjected to injury alone (Fig. 3C). In addition, both fed and fasted TH90' mice demonstrated the presence of a third GHR band at a slightly lower molecular weight compared to non-hemorrhaged mice. This third GHR band was previously described in the first article included in this dissertation, where it was referred to as Band 3 GHR. We concluded that this form of GHR likely resulted from a hemorrhage-dependent cleavage of GHR. Therefore, fasted, TH90' mice experienced two distinct reductions in GHR coinciding with the most severe GH signaling impairment observed thus far: the first due to fasting and the second due to hemorrhage.

Effects of Injury on GH Signaling in Triceps

Compared with normal mice, GH-induced P-STAT5 was not changed in mice injected with GH immediately following surgical trauma (T0', Fig. 4A and B). However, GH-induced P-JAK2 was decreased in triceps from TH90' mice (Fig. 4C and D), and GH-induced P-STAT5 was decreased in both T90' and TH90' mice (Fig. 4E and F).

Effects of Fasting on GH Signaling and IGF-I Abundance in Triceps

Fasting increased GH-induced P-STAT5 in triceps at 6, 12, and 18 hours (Fig. 5A and B). However, in contrast to liver, fasting did not result in significant changes in GHR abundance in triceps (Fig. 5C and D). IGF-I protein was reduced in triceps following fasting for 12 or 18 hours (Fig. 5E), despite a clear increase in GH-induced P-STAT5.

Effect of Injury on GH Receptor Signaling in Adipose Tissue

Similar to triceps, surgical trauma alone had no immediate effect on GH-induced P-STAT5 in adipose tissue compared to normal mice (Fig. 6A and B). GH-induced P-JAK2 and P-STAT5 were reduced in adipose tissue by TH90', but not by T90' (Fig. 6C, D, E, and F). However, GHR abundance in adipose tissue was reduced in both T90' and TH90' mice compared with T0' (Fig. 6G and H).

Effect of Fasting on GH Receptor Signaling in Adipose Tissue

Adipose tissue GHR abundance was reduced significantly following a 12 or 18 hour fast compared with fed mice (Fig. 7A and 7B). The data presented in Fig. 7C and

7D is identical to data that in the second article of this dissertation, but is presented again here for ease of comparison. Despite significantly decreased adipose GHR, GH-induced P-STAT5 is unchanged following fasting for 6, 12, or 18 hours (Fig. 7C and D).

Discussion

GH induced significant increases in hepatic P-STAT3 and P-AKT, but with dramatically different kinetics. The activation of hepatic STAT3 by GH occurred with kinetics similar to STAT5 (data presented in the first article of this dissertation). Activation of hepatic AKT by GH occurred with kinetics similar to both AKT and ERK in heart (second article of this dissertation). This suggests that the mechanisms of GH-induced STAT phosphorylation may be similar for different STATs, while the mechanisms of GH-induced AKT and ERK phosphorylation may share similarities that are distinct from STAT activation.

It is not surprising that fasting resulted in a time-dependent decrease in GHR abundance. Fasting-induced hepatic GH resistance has previously been associated with a decrease in hepatic GHR abundance (8;9). In addition, the GH-dependent decrease in hepatic GHR abundance observed in the present studies approximates the decrease in GHR observed in hepatoma cells following stimulation with high concentrations of GH (10). However, it was surprising that no significant change was observed in GH-induced P-STAT5 following fasting, despite the approximately 90% decrease in GHR at 18 hours. It is possible that the supraphysiological GH dose administered to these mice is responsible for the apparently normal signaling in livers from fasted mice. The implications of the decrease in GH-induced P-STAT5 band migration were discussed

previously (second article) and will not be addressed at length again here. The observed decrease in hepatic EGFR approximated the decrease in hepatic GHR following fasting. The liver is exquisitely sensitive to feeding and fasting, and it is possible that decreased GHR and EGRF represent part of a broad reduction in growth factor signaling capabilities is a general response to fasting. However, additional studies will be necessary to verify this.

In the first article of this dissertation we demonstrated that GH-induced hepatic STAT5 phosphorylation was significantly impaired in T90' mice compared with normal and T0' mice. Further, TH90' mice exhibited a more severe impairment in hepatic STAT5 phosphorylation compared with T90' mice. Here we demonstrate that fasting for 18 hours resulted in an even greater decline in hepatic GH signaling in T90' and TH90' mice compared with fed T90' and TH90' mice, respectively. The more severe signaling impairment in fasted T90' and TH90' livers was associated with a significant reduction in hepatic GHR compared with fed mice. This was not surprising since fasting alone had a profound effect on hepatic GHR abundance. It is interesting to note that in both fed and fasted groups, there was a decrease in P-STAT5 in TH90' mice compared with T90' mice. This coincided with the appearance of Band 3 GHR in fed and fasted, hemorrhaged mice, although total GHR was unchanged. These results suggest that the greater signaling impairment in fed and fasted TH90' mice compared with fed and fasted T90' mice, respectively, is related to the observed decrease in GHR size in hemorrhaged mice.

In contrast with heart and liver, GH signaling was not significantly affected by surgical trauma (T0') alone in triceps or adipose tissue. In addition, JAK2

phosphorylation was not affected by T90' in adipose tissue or triceps, but was significantly reduced compared to T0' when mice were hemorrhaged for 90 min (TH90'). GH-induced P-STAT5 was reduced in triceps from both T90' and TH90' mice, but in adipose tissue, P-STAT5 was reduced only in TH90' mice. Triceps was similar to heart in that the effects of injury on P-STAT5 were independent of hemorrhage, and no change was detected in GHR in either tissue regardless of condition. Surprisingly, GHR abundance was reduced in adipose tissue from both T90' and TH90' mice, despite the relatively intact signaling observed in T90' adipose tissue.

Similar to the heart, fasting resulted in an increase in GH-induced STAT5 phosphorylation in triceps. GHR abundance was not altered significantly in triceps. Despite the apparent increase in triceps GH sensitivity following fasting, IGF-I was significantly decreased in triceps after fasting for 12 or 18 hours. In adipose tissue, GHR was significantly reduced by fasting for 12 or 18 hours, but GH-induced STAT5 phosphorylation was not significantly affected. Therefore, the effects of fasting on GHR and P-STAT5 in adipose tissue were strikingly similar to those observed in the liver, with the exception that there was no observable effect of GH stimulation on adipose GHR in fed mice or mice fasted for 6 hours (data not shown).

In conclusion, fasting increased or had no effect on GH-induced P-STAT5 in liver and skeletal muscle. However, acute injury reduced GH signaling in both adipose tissue and skeletal muscle, although surgical trauma alone for 90 min had no significant effect in adipose tissue. In agreement with our original hypothesis, the combination of fasting and acute injury produced a far more severe signaling impairment in liver compared to injury alone. This effect appears to be related to a fasting-induced decrease in hepatic

GHR, and not the appearance of Band 3 GHR. However, Band 3 GHR appears to contribute to the differences in GH-induced P-STAT5 in T90' vs. TH90' liver, in both fed and fasted groups.

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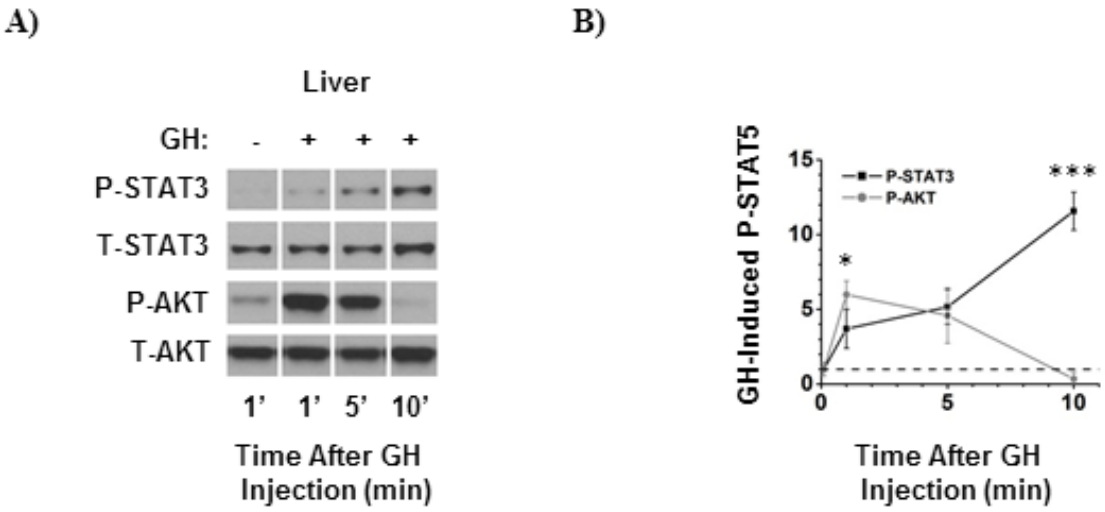
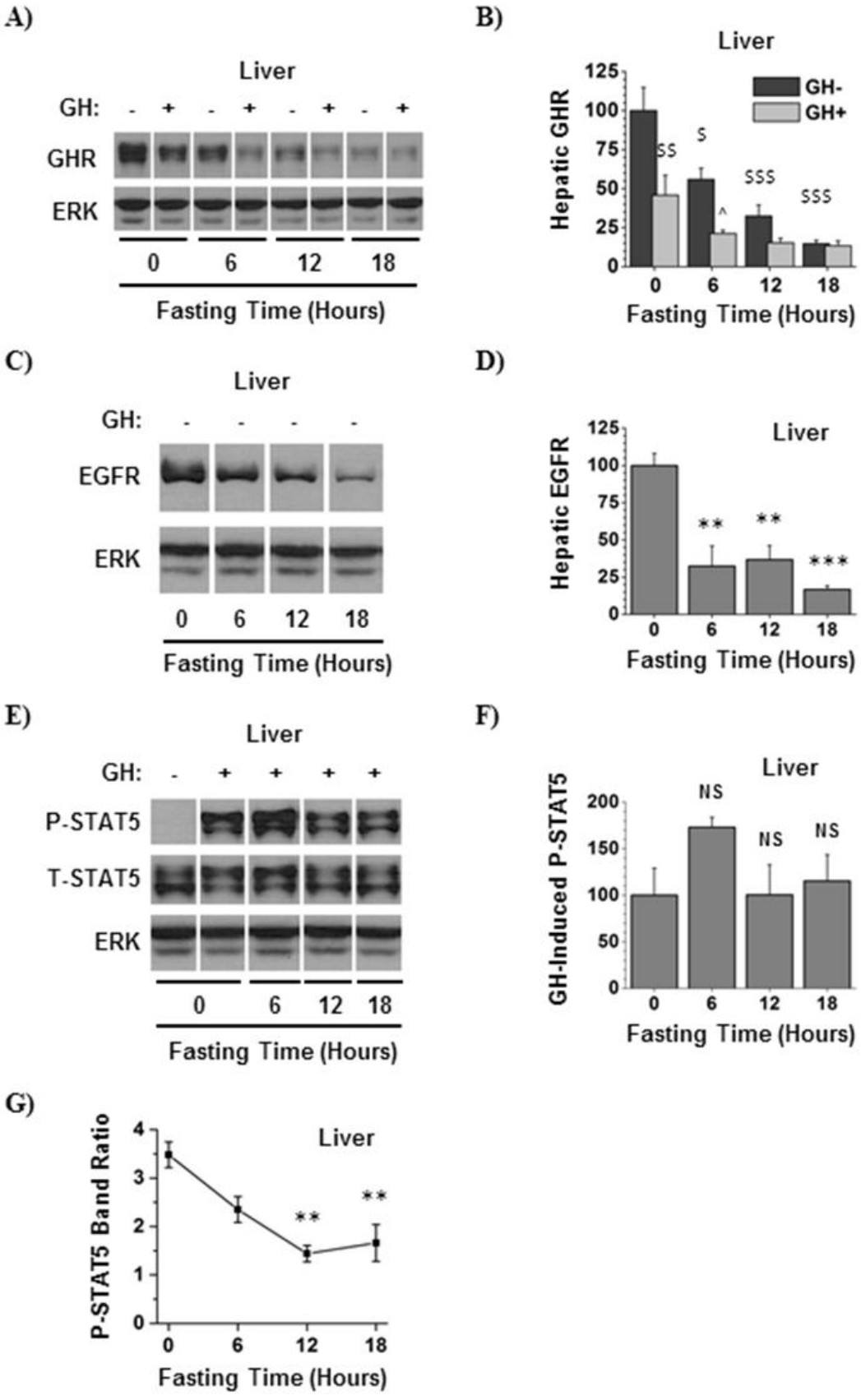


Figure 1. Time course of hepatic GH signaling. Mice were injected with bovine GH (or saline, 1' time point) and livers were removed at the indicated times. A. Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT3 and P-AKT. In this figure, the average of 3 measurements at t=0 was arbitrarily set to 1. Data are presented as mean ± SEM fold change in phosphorylation normalized to total protein. In this figure: *, $P < 0.05$ vs. 0'; and ***, $P < 0.001$ vs. 0' via one-way ANOVA; n = 3 mice per time point.


A)
Liver
GH: - + - + - + - +
GHR
ERK
0 6 12 18
Fasting Time (Hours)
B)
Liver
GH-
GH+
Hepatic GHR
125
100
75
50
25
0
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0 6 12 18
Fasting Time (Hours)
C)
Liver
GH: - - - -
EGFR
ERK
0 6 12 18
Fasting Time (Hours)
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T-STAT5
ERK
0 6 12 18
Fasting Time (Hours)
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Liver
GH-Induced P-STAT5
200
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0
NS
NS
NS
0 6 12 18
Fasting Time (Hours)
G)
Liver
P-STAT5 Band Ratio
4
3
2
1
0
**
**
0 6 12 18
Fasting Time (Hours)

Figure 2. Effects of fasting on hepatic growth factor receptors and GH signaling. Mice were fed *ad libitum*, or fasted for 6, 12, or 18 hours and injected with GH or saline and livers were removed 10 min later. A, C, E. Liver lysates were immunoblotted with antibodies specific to total GHR (R & D Systems), total EGFR, total ERK, PY694/699-STAT5, and total STAT5. Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D, F. Autoradiographs were quantified by scanning densitometry to determine relative changes in GHR, EGFR, and GH-induced P-STAT5. Protein amounts detected in fed mice were arbitrarily set to 100%. Data are presented as mean ± SEM percent change. GHR and EGFR amounts were normalized to ERK, and P-STAT5 was normalized to T-STAT5. G. Upper and lower P-STAT5 bands were quantified by scanning densitometry to determine ratios. Data are presented as mean ± SEM of the upper-to-lower P-STAT5 band ratio. In this figure: NS = not significantly different from fed mice; $^{\$}$, $P < 0.05$ vs. 0 hours -GH; $^{\$\$}$, $P < 0.01$ vs. 0 hours -GH; and $^{\$\$\$}$, $P < 0.001$ vs. 0 hours -GH all via one-way ANOVA. $^{\wedge}$, $P < 0.05$ vs. 0 hours -GH via Welch-corrected t-test. **, $P < 0.01$ vs. 0 hours; and ***, $P < 0.001$ vs. 0 hours via one-way ANOVA. n = 3 mice per group.

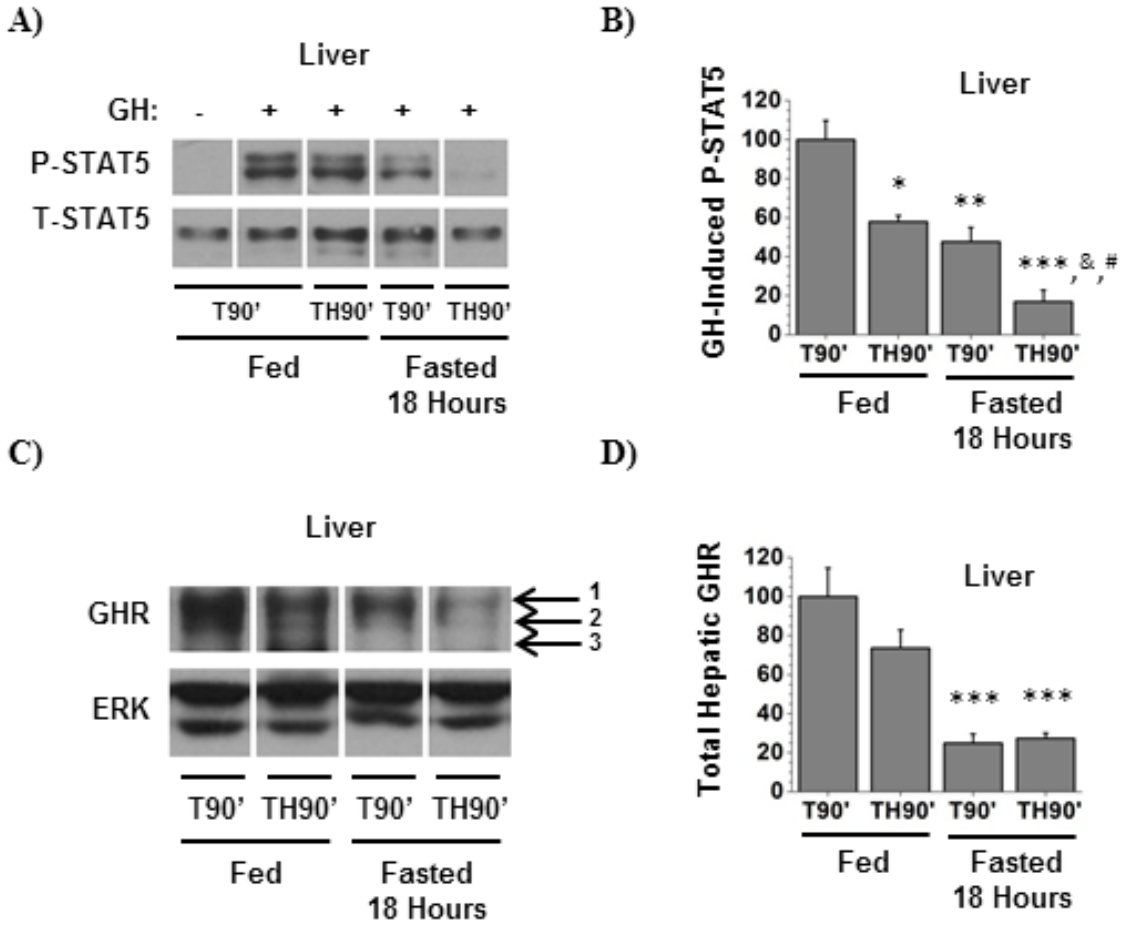


Figure 3. Effects of fasting combined with injury on hepatic GH receptor signaling. Mice were fasted 18 hours or were fed *ad libitum* until immediately before surgical trauma, alone or combined with hemorrhage. At the end of the study period mice were injected with saline or GH and livers were removed 10 min later. A,C. Liver lysates were immunoblotted with antibodies specific to total GHR (R & D Systems), ERK, PY694/699-STAT5, and total STAT5. Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 and GHR. The signal from fed T90' +GH mice was arbitrarily set to 100%, and data are presented as mean ± SEM percent change. P-STAT5 was normalized to T-STAT5 and GHR was normalized to ERK. In this figure: *, $P < 0.05$ vs. fed T90' +GH; **, $P < 0.01$ vs. fed T90' +GH; ***, $P < 0.001$ vs. fed T90' +GH; and $^{\&}$, $P < 0.05$ vs. fed TH90' +GH, all via one-way ANOVA. $^{\#}$, $P < 0.05$ vs. fasted T90' +GH via Welch-corrected t-test; n = 3 or more mice per group.

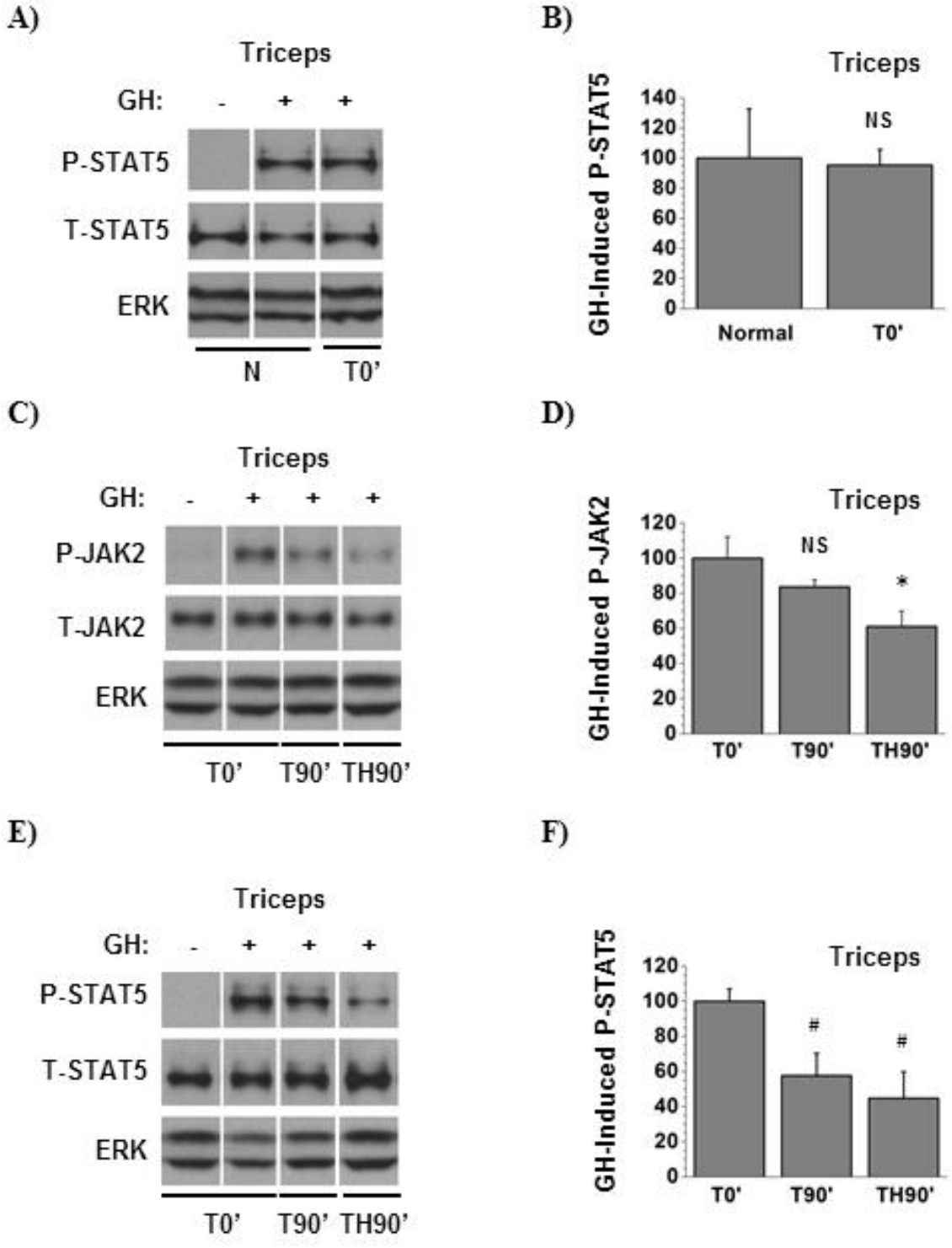
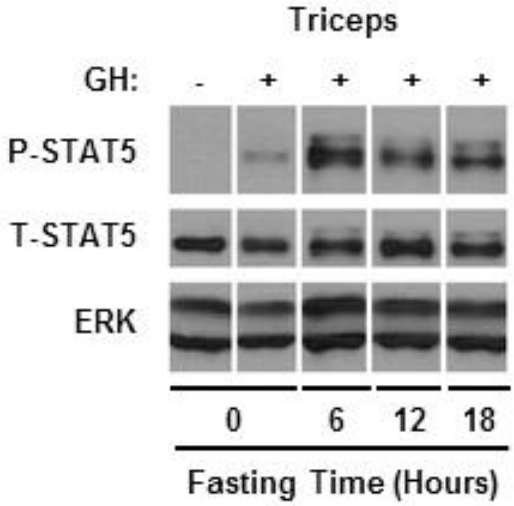
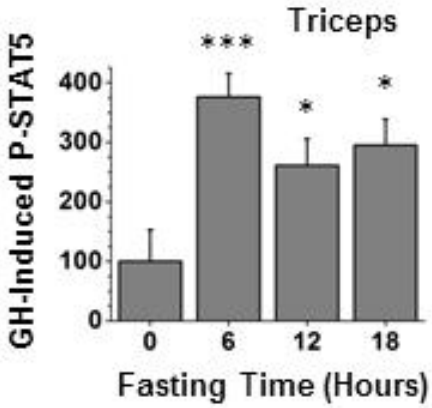

A)
Triceps
GH: - + +
P-STAT5
T-STAT5
ERK
N
T0'
B)
Triceps
GH-Induced P-STAT5
140
120
100
80
60
40
20
0
NS
Normal
T0'
C)
Triceps
GH: - + + +
P-JAK2
T-JAK2
ERK
T0' T90' TH90'
D)
Triceps
GH-Induced P-JAK2
120
100
80
60
40
20
0
NS
*
T0'
T90'
TH90'
E)
Triceps
GH: - + + +
P-STAT5
T-STAT5
ERK
T0' T90' TH90'
F)
Triceps
GH-Induced P-STAT5
120
100
80
60
40
20
0
#
#
T0'
T90'
TH90'

Figure 4. Effects of injury on GH signaling in triceps. Normal mice and mice subjected to surgical trauma, alone or combined with hemorrhage, were injected with GH or saline and triceps were removed 10 min later. A, C, E. Lysates from triceps were immunoblotted with antibodies directed against PY694/699-STAT5 and total STAT5, and PY1007/1008-JAK2 and total JAK2. Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D, F. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 and P-JAK2. The average of 3 measurements in triceps from control mice (normal mice in B, T0' mice in D and F) was arbitrarily set to 100%. Data is presented as mean ± SEM percent change. P-STAT5 and P-JAK2 were normalized to total STAT5 and total JAK2, respectively. In this figure: *, $P < 0.05$ vs. T0' via one-way ANOVA; $^{\#}$, $P < 0.05$ vs. T0' via Welch-corrected t-test; NS = not significantly different from control (Normal in B, T0' in D). n = 3 or more mice per group in B, n = 5 mice per group in D and F.

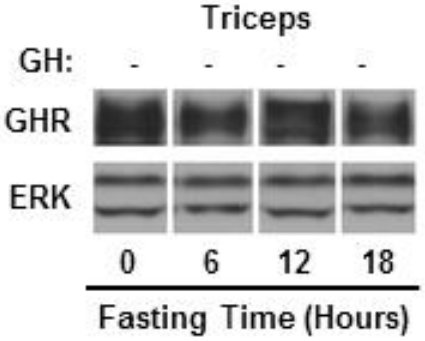
A)


Triceps
GH: - + + + +
P-STAT5
T-STAT5
ERK
0 6 12 18
Fasting Time (Hours)

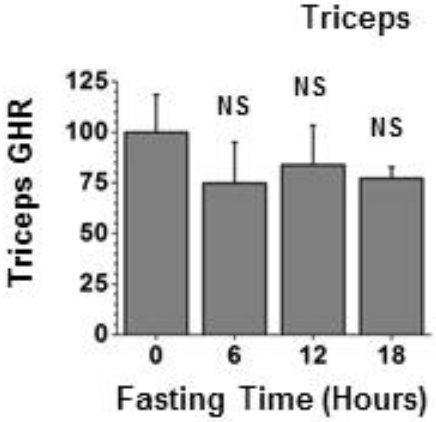
B)


Triceps
GH-Induced P-STAT5
***
*
*
400
300
200
100
0
0 6 12 18
Fasting Time (Hours)

C)


Triceps
GH: - - - -
GHR
ERK
0 6 12 18
Fasting Time (Hours)

D)


Triceps
Triceps GHR
NS
NS
NS
125
100
75
50
25
0
0 6 12 18
Fasting Time (Hours)

E)

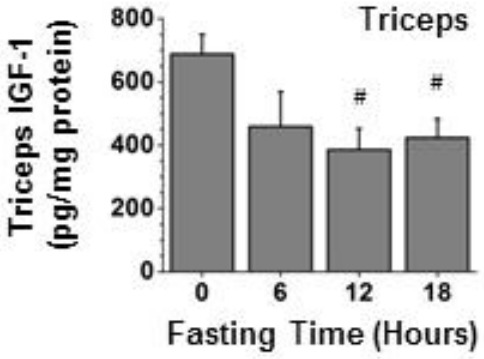
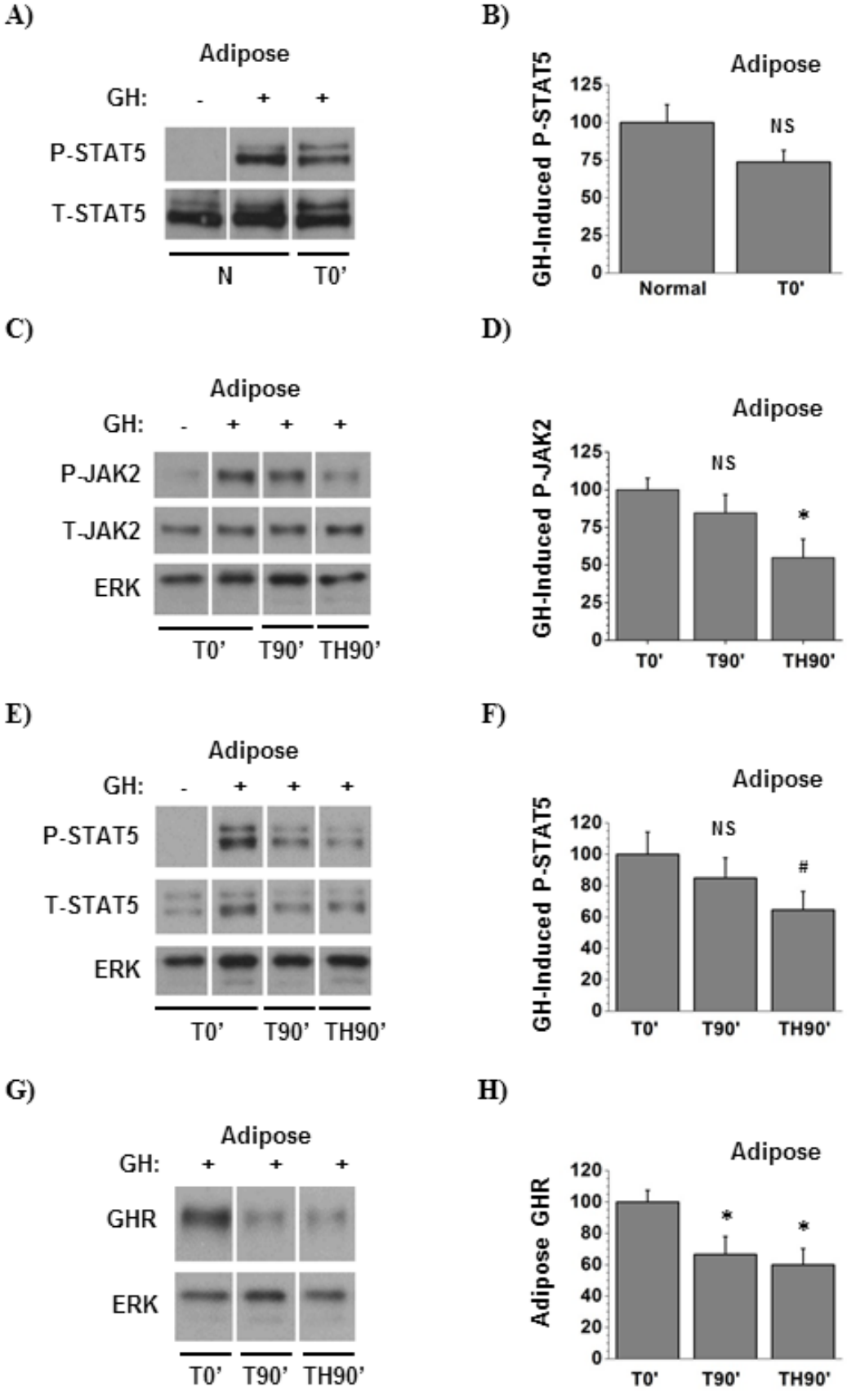

Triceps
Triceps IGF-1 (pg/mg protein)
#
#
800
600
400
200
0
0 6 12 18
Fasting Time (Hours)

Figure 5. Effects of fasting on GH receptor signaling and IGF-I protein in triceps. Mice were fed *ad libitum*, or fasted for 6, 12, or 18 hours and injected with GH or saline and triceps were removed 10 min later. A, C. Lysates from triceps were immunoblotted with antibodies directed against PY694/699-STAT5 and total STAT5, ERK, and GHR (AL-47). Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 and GHR. The average of 3 measurements from fed mice (fasted 0 hours) was arbitrarily set to 100%. Data is presented as mean ± SEM percent change. P-STAT5 was normalized to T-STAT5 and GHR was normalized to ERK. E. Total IGF-I protein in lysates from triceps was measured using an ELISA kit from R&D systems according to the manufacturers instruction. In this figure: *, $P < 0.05$ vs. fed; and ***, $P < 0.001$ vs. fed via one-way ANOVA; $^{\#}$, $P < 0.05$ vs. fed via Welch-corrected t-test; NS = not significantly different from fed mice; n = 3 mice per group.


A)
Adipose
GH: - + +
P-STAT5
T-STAT5
N
T0'
B)
Adipose
GH-Induced P-STAT5
125
100
75
50
25
0
NS
Normal
T0'
C)
Adipose
GH: - + + +
P-JAK2
T-JAK2
ERK
T0' T90' TH90'
D)
Adipose
GH-Induced P-JAK2
125
100
75
50
25
0
NS
*
T0'
T90'
TH90'
E)
Adipose
GH: - + + +
P-STAT5
T-STAT5
ERK
T0' T90' TH90'
F)
Adipose
GH-Induced P-STAT5
120
100
80
60
40
20
0
NS
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T0'
T90'
TH90'
G)
Adipose
GH: + + +
GHR
ERK
T0' T90' TH90'
H)
Adipose
Adipose GHR
120
100
80
60
40
20
0
*
*
T0'
T90'
TH90'

Figure 6. Effects of injury on GH receptor signaling in adipose tissue. Normal mice and mice subjected to surgical trauma, alone or combined with hemorrhage, were injected with GH or saline and epididymal adipose tissue was removed 10 min later. A, C, E, G. Lysates from adipose tissue were immunoblotted with antibodies directed against PY694/699-STAT5 and total STAT5, PY1007/1008-JAK2 and total JAK2, ERK and GHR (R&D Systems).). Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D, F, G. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5, P-JAK2, and GHR. The average of 3 measurements in adipose tissue from control mice (normal mice in B, T0' mice in D, F, and G) was arbitrarily set to 100%. Data is presented as mean ± SEM percent change. P-STAT5 and P-JAK2 were normalized to total STAT5 and total JAK2, respectively, and GHR was normalized to ERK. In this figure: *, $P < 0.05$ vs. T0' via one-way ANOVA; $^{\#}$, $P < 0.05$ vs. T0' via Welch-corrected t-test; NS = not significantly different from control (Normal in B, T0' in D and F). n = 4 or more mice per group in B, n = 5 mice per group in D, F, and G.

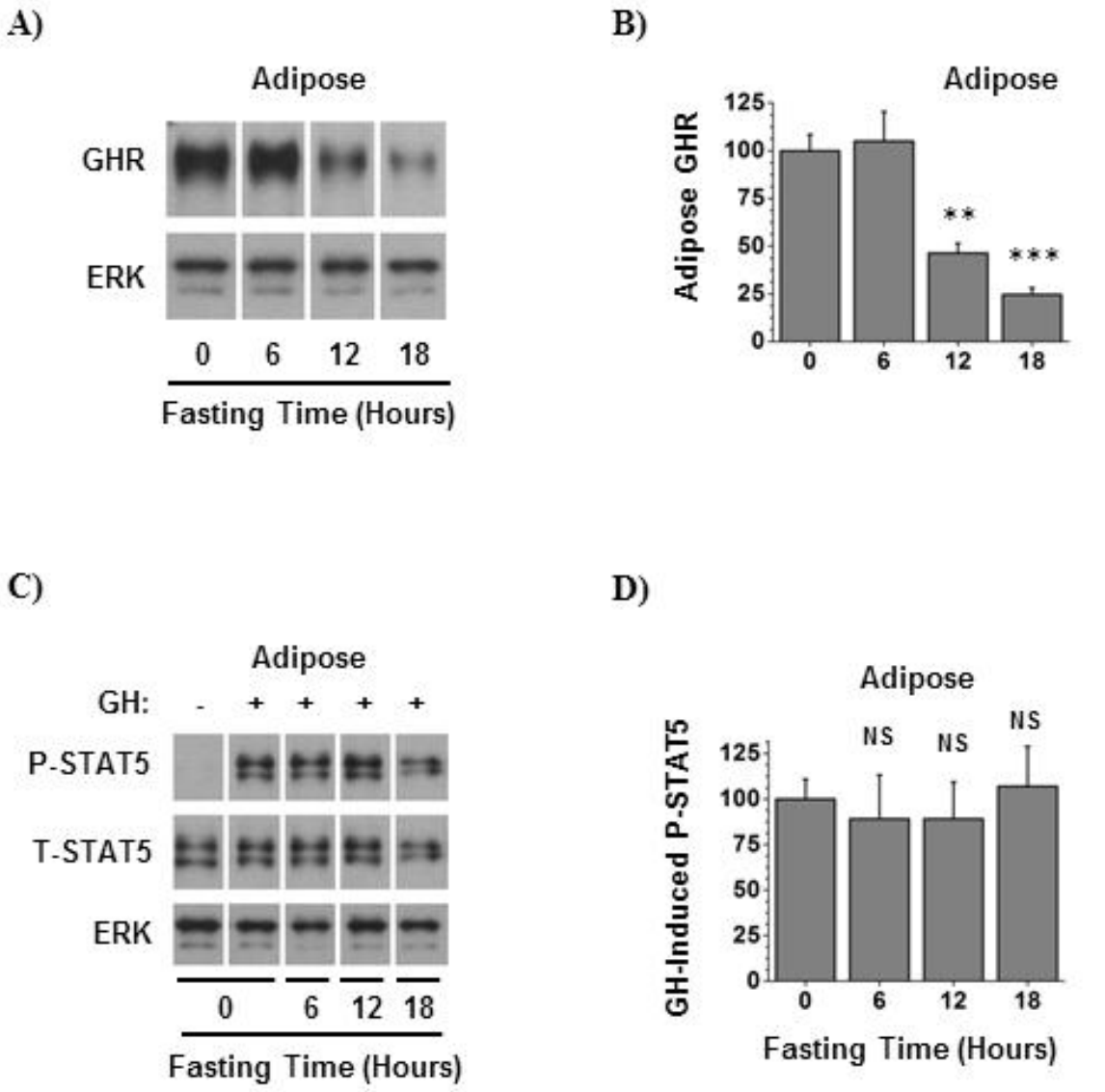

Figure 7. Effects of fasting on GH receptor signaling in adipose tissue. Mice were fed *ad libitum*, or fasted for 6, 12, or 18 hours and injected with GH or saline and epididymal adipose tissue was removed 10 min later. A, C. Lysates from adipose tissue were immunoblotted with antibodies directed at PY694/699-STAT5 and total STAT5, ERK, and GHR (R&D Systems). Representative Western blots are presented; the scanned image of a single blot was rearranged for clarity. B, D. Autoradiographs were quantified by scanning densitometry to determine relative changes in P-STAT5 and GHR. The average of 3 measurements in adipose tissue from fed mice was arbitrarily set to 100%. Data is presented as mean ± SEM percent change. P-STAT5 was normalized to T-STAT5, and GHR was normalized to ERK. In this figure: **, $P < 0.01$ vs. fed mice; and ***, $P < 0.001$ vs. fed mice via one-way ANOVA. NS = not significantly different from fed mice; n = 3 mice per group.

CONCLUSIONS AND FUTURE DIRECTIONS

Effects of Surgical Trauma on GH Receptor Signaling

Presented in this dissertation project is evidence that GH resistance develops rapidly, and in multiple GH-dependent tissues following injury. One of the most striking findings of the studies presented in previous sections was the rapid nature of the effects of injury on GH signaling, particularly in liver and heart. Deficits in GH-induced P-STAT5 were evident in both tissues immediately following completion of surgical trauma. Also surprising was that GH resistance was induced by surgical trauma alone, which is a relatively minor form of injury compared to surgical trauma combined with hemorrhage. In work by our laboratory as well as others, animals subjected to surgical trauma but not hemorrhage have historically been included to control for the effects of soft-tissue injury and anesthesia, and isolate those of hemorrhage. This was appropriate because the major effects under study, whether effects of injury on insulin signaling, cardiac function, or some other variable, were readily apparent following combined trauma and hemorrhage, but minor following trauma alone (93;147). However, the clear differences in GH signaling observed between normal mice and mice subjected to trauma without hemorrhage demonstrated that the effects of surgical trauma merited additional study.

The effects of trauma alone on GH signaling in liver and heart that are presented in this dissertation represent unique examples of relatively minor injury resulting in the

rapid development of major metabolic defects with potentially severe consequences (Table 1). GH signaling deficits in the heart may have an immediate impact on cardiac contraction and relaxation, and similar deficits in the liver may affect glucose homeostasis as well as protein metabolism (74;75;148;149). In contrast, GH signaling was not significantly affected in triceps immediately after surgical trauma; however, measurements 90 min later determined that GH signaling in triceps was reduced by approximately one half. Further studies will be necessary to more precisely determine how rapidly GH resistance developed in triceps following surgical trauma alone.

Table 1

Comparison of Tissue Responses to Surgical Trauma Alone

Surgical Trauma Alone		
Tissue	**STAT5 Signaling Impairment**	**Effect on GHR**
Liver	Immediate, Worse by 90 min	None
Heart	Immediate	None
Triceps	Present at 90 min	None
Adipose	None	Reduction

It is possible that the more rapid development of GH resistance observed in liver and heart reflects higher blood flow to these tissues relative to skeletal muscle. Studies of GH resistance in other models have demonstrated important roles for soluble factors, particularly pro-inflammatory cytokines, in the development of GH resistance (97;137). Such soluble factors may be stimulated in response to surgical trauma alone, but may not achieve sufficient concentrations in blood delivered to skeletal muscle to effect changes

in GH signaling due to the generally decreased blood flow to these tissues relative to central organs like the liver and heart.

It is not clear at this time what mechanisms might explain impaired GH signaling due to surgical trauma alone in liver, heart or triceps, especially since there were no corresponding changes in GHR. Mechanistic answers will not likely be found in studies of gene expression, as GH resistance developed too rapidly to be the result of changes in gene expression. Therefore, other avenues should be explored. Since GH-induced JAK2 phosphorylation is a critical step facilitating GH-induced STAT5 phosphorylation, a more thorough characterization of GH-induced JAK2 phosphorylation should be attempted in these groups. Unfortunately, with the exception of adipose tissue, studies of JAK2 phosphorylation have not been possible without immunoprecipitating JAK2 prior to Western analysis of P-JAK2. Due to low tissue yields in mice, there has generally not been enough tissue available from each animal to complete the studies necessary for measuring GH-induced P-JAK2. For this reason, it may be wise to perform future studies in rats, where adequate tissue will be available to perform the necessary studies.

Obvious changes in the GHR following surgical trauma alone were not apparent in liver or heart and are therefore unlikely to play a role in the impaired STAT5 signaling observed following trauma alone in either tissue. Surprisingly, adipose tissue GHR was significantly decreased in T90' mice, despite apparently normal GH signaling in the same group. In addition, studies of GHR in triceps should be undertaken to determine whether receptor abundance might account for altered GH signaling in T90' mice.

As discussed previously, pro-inflammatory cytokines are reported to play an important role in GH resistance during severe inflammation, particularly IL-6 and TNF-α

(96;97). TNFα levels are increased following trauma and hemorrhage, but not trauma alone; however, IL-6 plasma concentrations rise rapidly following surgical trauma alone (92;94). This might explain our finding of impaired STAT5 tyrosine phosphorylation in both liver and hearts from all groups subjected to surgical trauma alone. An important goal of future studies should include determining the role, if any, of IL-6 in the development of GH resistance following surgical trauma.

We have not completely ruled out the possibility that the effects on GH signaling observed following surgical trauma alone might be due to anesthesia. Although normal mice were also anesthetized prior to injection with GH or saline using the same anesthetic gas (isoflurane) as the injury groups, they were exposed to anesthesia for shorter periods of time. Therefore, normal mice may not represent an optimal control for the effects of anesthesia on mice subjected to surgical trauma alone. An important future control should include groups subjected to anesthesia alone for extended periods of time before injection with GH, to match the amount of time under anesthesia experienced by groups subjected to surgical trauma, alone or combined with hemorrhage. This will be helpful in confirming that the effects of surgical trauma on GH signaling are due to injury and not the anesthesia used to maintain unconsciousness. The primary effects of isoflurane, as well as other halogenated anesthetic gases, are achieved within several seconds even at very low concentrations (150), but the possibility that secondary effects might accumulate over time, potentially leading to effects on signal transduction, cannot be excluded.

The clinical implications of GH resistance following surgical trauma alone are intriguing. Recent studies by other laboratories using models of kidney failure and sepsis

demonstrated the presence of GH resistance days and hours after the primary insult, respectively (137;138). In contrast, the work presented in this dissertation demonstrates profound GH signaling changes occurring immediately after relatively minor injury. The present studies may have particular relevance in clinical scenarios involving injury or major surgery without severe blood loss that are associated with significant loss of lean body mass during the recovery period. It is possible that the metabolic changes facilitating muscle wasting in this group of patients may be established before they have left the operating table. If true, this will likely impact future therapeutic strategies to combat muscle wasting following injury and surgery.

Effects of Surgical Trauma and Hemorrhage on GH Receptor Signaling

The addition of hemorrhage to surgical trauma resulted in a significant effect on GH signaling in both the liver and adipose tissue (see first article and unpublished data section). In adipose tissue, no change was observed in GH-induced STAT5 phosphorylation following trauma alone. However, there was a decrease in adipose GH-induced P-STAT5 when surgical trauma was followed by 90 min of hemorrhage. In liver, the combination of surgical trauma and hemorrhage resulted in a more severe effect on GH signaling compared with trauma alone. Although we cannot yet explain the hemorrhage-dependent effects on GH-signaling in adipose tissue, it is likely that changes to the GHR are responsible for the further decrease in signaling observed in liver following the addition of hemorrhage.

In livers from normal mice and mice subjected to surgical trauma alone, GHR was detected on Western blots primarily as two bands at 95 and 90 kDa which we referred to

as Bands 1 and 2. Hemorrhage resulted in the appearance of an 85 kDa form of GHR we referred to as Band 3. Further, the appearance of Band 3 GHR was associated with a proportional decrease in Band 1 GHR so that there was no change in total GHR. This suggested that hemorrhage resulted in structural alteration of GHR so that it ran at a lower molecular weight on SDS-PAGE (i.e. Band 1 GHR at 95 kDa became Band 3 GHR at 85 kDa). Studies employing enzymatic deglycosylation suggested that Band 1 represented full-length GHR with a mature pattern of N-glycosylation, and that Band 2 represented full-length GHR with incomplete glycosylation. However, Band 3 GHR retained the mature pattern of N-glycosylation, indicating that the structural change in Band 1 GHR resulting in Band 3 GHR was not due to loss of N-linked olicosaccharides.

Based on amino acid sequence, the predicted molecular weight of murine GHR is approximately 73 kDa (151). Our studies determined that complete removal of N-linked oligosaccharides resulted in GHR that ran at 75 kDa. Therefore, deglycosylated GHR retained very little, if any, post-translationally added structures. This suggests that the ~10 kDa reduction in GHR size following hemorrhage is due to loss of primary amino acid sequence. In other words, hemorrhage results in cleavage of a 10 kDa amino acid fragment of the receptor.

JAK2 will associate with truncated forms of GHR even when large portions of the cytoplasmic domain are missing, provided the membrane-proximal Box 1 domain of the receptor remains intact (39;152). In addition, JAK2 can retain kinase activity when associated with truncated forms of GHR, as evidenced by GH-induced phosphorylation of STAT1 and STAT3 (41-43). STAT5 is unique in that, unlike STAT1 and STAT3, it must associate with GHR in order to be tyrosine phosphorylated by JAK2. This

association occurs via interaction of the STAT5 SH2 domain with phosphorylated tyrosines at the C-terminal end of GHR. Therefore, it is possible that C-terminal cleavage of GHR might impair GH-induced STAT5 tyrosine phosphorylation without affecting JAK2 phosphorylation. This would explain our finding in the first article of this dissertation demonstrating reduced STAT5 tyrosine phosphorylation but apparently normal JAK2 tyrosine phosphorylation following hemorrhage (TH90' vs. T90'), and

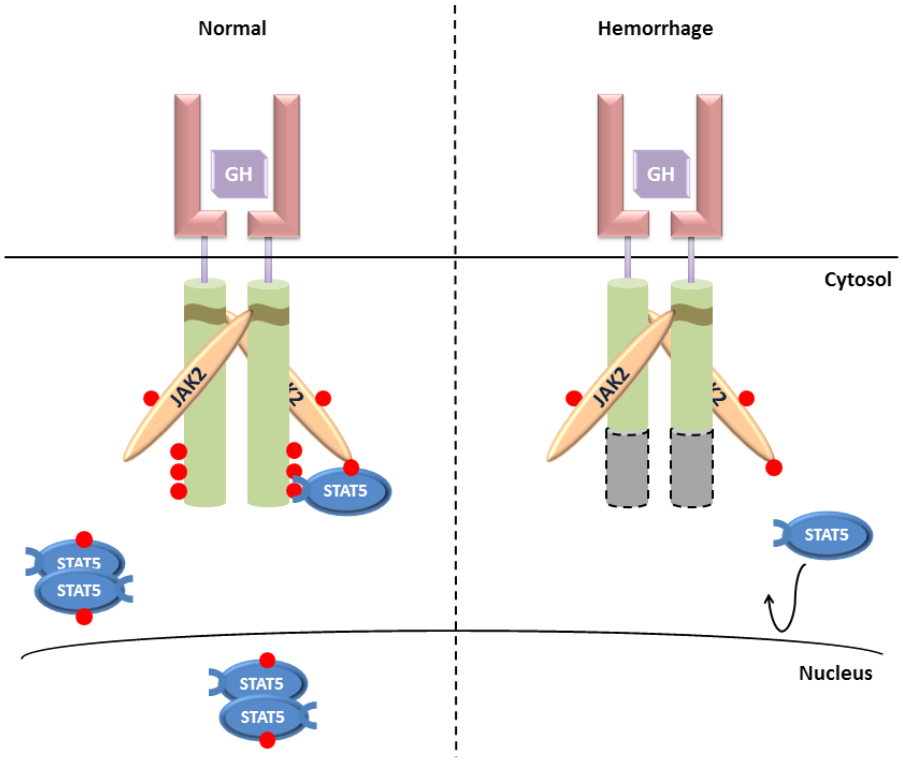

Figure 1. Potential mechanism of severe hepatic GH resistance following hemorrhage.

suggests that the missing ~10 kDa GHR fragment was originally part of the C-terminal end of GHR (Fig 1).

It has been previously demonstrated that a C-terminal fragment of GHR containing 80 amino acids including three phosphorylated tyrosines (548, 577, and 609)

co-precipitates with STAT5; however, the same fragment did not associate with STAT5 when these tyrosines were not phosphorylated (41). In another study, mutational analysis of GHR suggested that the C-terminal region of GHR containing amino acids 455-638 (which includes tyrosines 548, 577, and 609) was necessary for maximal GH-induced STAT5 tyrosine phosphorylation (42). The C-terminal end of mouse GHR containing all three tyrosines has a calculated molecular weight of ~12 kDa, and the region containing only tyrosines 577 and 609 is ~8 kDa. Taken together, cleavage of an approximately 10 kDa, C-terminal GHR fragment likely removes at least two tyrosines that have important roles in GH-induced STAT5 phosphorylation (Fig. 1, next page). In light of this, the hemorrhage-dependent signaling deficit described in the first article of this dissertation may be due to loss of two or more GHR tyrosine residues necessary for maximal GH-induced binding and activation of STAT5.

Since GH-induced STAT1 and STAT3 phosphorylation are not dependent on physical association of either STAT with GHR, an important future experiment would assess whether hemorrhage affects GH-induced STAT1 or STAT3 signaling. The finding that GH-induced STAT1 and STAT3 signaling is not affected by hemorrhage would support the hypothesis that Band 3 GHR lacks the C-terminal portion of the intracellular domain. However, in unpublished experiments we observed that surgical trauma influences STAT3 phosphorylation independent of GH, making it difficult to determine the effects of injury on GH-induced P-STAT3. Therefore, future experiments to determine whether Band 3 GHR retains functional activity should focus on STAT1 phosphorylation.

There are no reports describing a similar cleavage of GHR. Proteases known to cleave GHR, such as TNF-α converting enzyme (TACE) or γ-secretase, result in cleavage of fragments that are significantly larger than the ~10 kDa fragment described in the first article of this dissertation. It is interesting to note that on darker exposures, GHR immunoblots revealed a very low level of Band 3 GHR in normal and trauma-alone livers (see Figure 6, first article). The presence of Band 3 GHR in these mice suggests that Band 3 represents a physiological form of GHR that is generated normally at low levels, but rapidly accumulates following hemorrhage. Additional studies incorporating more sophisticated techniques will be necessary to determine the exact nature of Band 3 GHR.

Results from our studies in liver highlight the importance of GHR in studies of GH resistance. Obviously, GHR plays a central role in GH-induced signal transduction, as several of the initial steps in GH signaling take place on or near the GHR. For this reason, future studies of hepatic GH resistance would incorporate experiments to more precisely characterize the role of GHR. Sequence analysis, particularly of Band 3, would be of enormous value in furthering the present studies. We have already made several attempts to enrich hepatic GHR in protein extracts to levels adequate for analysis by mass spectrometry. Unfortunately, these attempts have been unsuccessful, likely due to the low levels of endogenous GHR in livers from C57BL/6 mice. Another possibility involves experiments designed to determine whether Band 3 GHR can be generated *in vitro* by exposing cell cultures to stresses that are likely increased during hemorrhage, such as reactive oxygen species and reduced oxygen tension. Attempts to produce Band 3 GHR *in vitro* may lead to rapid generation of sufficient quantities for MS analysis

while also providing insights into the mechanisms of Band 3 production during hemorrhage.

Other clues to the role of GHR in GH resistance might be found in studies of GHR subcellular localization. The determination that Band 2 GHR possesses high-mannose oligosaccharides strongly suggests that it is located in the secretory pathway. In addition, Band 1 GHR is likely primarily localized to the plasma membrane where it can function in GH signaling. However, we cannot be certain of the subcellular location of Band 3 GHR. Experiments performed but not included in this dissertation suggested that Band 3 GHR is highly enriched in crude membrane extracts. However, we still do not know whether Band 3 remains in the plasma membrane, or has been internalized in membrane-bound vesicles. For this reason, it may be worthwhile to isolate plasma membranes to determine what fraction of Band 3 GHR, if any, is localized to the cell surface. Alternatively, immunohistochemical studies may also prove valuable in determining Band 3 GHR subcellular location. Studies of GHR tyrosine phosphorylation may also prove valuable, because it is likely that internalized GHR would not become tyrosine phosphorylated in response to GH, while GHR at the plasma membrane should be phosphorylated in response to GH.

Any or all of these possibilities may also prove important in the mechanisms of GH resistance due to injury in heart, triceps, or adipose tissue. Unlike in liver, we have found no changes in GHR abundance or banding pattern that might explain the signaling impairments in these tissues (Table 2, next page). Additionally, insights into protein-protein interactions may be valuable. Co-immunoprecipitation studies to determine co-localization of GHR, JAK2, and STAT5 following GH stimulation, and how these

associations change following injury may provide useful information about the mechanisms of GH resistance. In addition, suppressors of cytokine signaling (SOCS) proteins have demonstrated roles in the down-regulation of GH signaling by physically associating with GHR and/or JAK2 (153). SOCS proteins function by ubiquitinating proteins and targeting them for lysosomal degradation, and SOCS protein expression is increased in several published models of GH resistance (96;154). As mentioned previously it is unlikely that increased gene expression can account for GH resistance at early time points studied in our model. However, the possibility that existing SOCS proteins might somehow be activated by stress should not be ruled out. Therefore, studies to determine whether injury results in increased association of SOCS with GHR and/or JAK2 should be pursued. It should be noted that all co-immunoprecipitation experiments described here require significant quantities of tissue for analysis. Therefore, as mentioned above it may be necessary to perform these studies in larger animals to ensure adequate tissue is available.

Table 2

Comparison of Tissue Responses to Surgical Trauma Combined with Hemorrhage

Surgical Trauma and Hemorrhage		
Tissue	**STAT5 Signaling Impairment**	**Effect on GHR**
Liver	More Severe than Trauma Alone	Structural
Heart	Not Different from Trauma Alone	None
Triceps	Not Different from Trauma Alone	None
Adipose	Present at 90 min	Not Different from Trauma Alone

It should be noted that adipose tissue was the most resistant to the effects of injury on GH signaling, while GH signaling in liver and triceps was far more sensitive to the effects of injury (adipose tissue was the only tissue to maintain normal GH signaling following surgical trauma alone). This would tend to support the idea that GH resistance during severe stress may be an adaptive response. The primary responses in liver and triceps to GH stimulation are tissue anabolism. However, the primary response of adipose tissue to GH is catabolic: fat tissue is broken down into free fatty acids and released into the circulation for use as metabolic fuel. As mentioned in the introduction to this dissertation, it makes teleological sense to suspend anabolic processes while enhancing catabolic processes during periods of high energy demand due to severe stress. This allows survival and repair activities to proceed without interruption until the threat has passed, at which time, tissue anabolism will resume.

The more severe hepatic GH resistance in C57BL/6 mice following the combination of trauma and hemorrhage is strongly associated with a defect in hepatic GHR. Since we do not know the precise mechanism of Band 3 generation, it has not been possible for us to demonstrate causality. Once Band 3 GHR has been more completely characterized, it may be possible in the future to introduce GHR that are altered similarly as in Band 3 GHR into livers of mice that have not been subjected to injury to determine the effects on GH-induced STAT5 phosphorylation. One possible method might employ GHRKO mice, such as those used for GHR identification in the first article. Wild-type or mutant GHR-Band 3 (here defined as a murine GHR with the Band 3 defect) could be introduced into livers (potentially via adenoviral vector) of GHRKO mice, and GH-induced STAT5 phosphorylation could be measured. Impaired

GH-induced P-STAT5 in mice possessing only mutant GHR-Band 3 compared with mice possessing wild-type GHR would provide evidence that defective GH signaling in hemorrhaged mice is due to the appearance of Band 3 GHR at the expense of Band 1 GHR. In addition, if Band 3 characterization reveals a cleavage site for which a protease can be identified, it may also be possible to introduce a mutated version of GHR lacking the cleavage site into the livers of GHRKO mice. Such mice could then be subjected to surgical trauma combined with hemorrhage to determine whether the hemorrhage-dependent GH signaling defect was reversed by deletion of the cleavage site. A similar strategy was successfully employed by Wang et al. to demonstrate that endotoxin-induced decreases in hepatic GHR were due to proteolytic cleavage (146).

We do not currently know how GH signaling defects ultimately affect the murine response to surgical trauma, alone or in combination with hemorrhage. However, the results of our studies suggest that hepatic GH resistance following surgical trauma combined with hemorrhage may be far more severe compared to surgical trauma alone, especially under fasting conditions. Therefore, it should be recognized that significant hemorrhage may predispose the trauma victim to more severe organ dysfunction and insulin resistance. It will be important in future studies to verify that our findings translate to real physiological deficits, such as decreased IGF-I production (in liver or other tissues) or impaired GH-induced lipolysis. Studies of these effects of GH resistance may be difficult, since many intervening steps may occur between the insult and the physiological response. It will therefore be important to determine the effects of resuscitation on GH resistance following trauma and hemorrhage, in order to more closely mirror clinical scenarios where the effects of GH resistance are observed in spite

of aggressive fluid support. It may be that following resuscitation, additional or different mechanisms may occur. For instance, we have discovered that the mechanisms resulting in the development of acute insulin resistance in liver differ from those resulting in the maintenance of the insulin resistant state following resuscitation (93;155-157). These questions must be answered before therapeutic strategies can be designed to address clinical GH resistance following injury.

Table 3

Comparison of Tissue Responses to Fasting Combined with Trauma and Hemorrhage

Fasting Prior to Surgical Trauma and Hemorrhage		
Tissue	**STAT5 Signaling Impairment**	**Effect on GHR**
Liver	Severe	Structural + Reduction
Heart	Enhanced	None

Effects of Fasting on GH Receptor Signaling

Our studies of the effects of fasting on GH resistance following injury provided additional insights into the differences between tissues with respect to GH signaling (Table 3). An overnight fast prior to injury resulted in a complete reversal of cardiac GH resistance due to injury, with no observable change in cardiac GHR (second article). However, when fasting was combined with surgical trauma, alone or combined with hemorrhage, hepatic GH resistance became much more severe (unpublished data). As in the case of hemorrhage, the hepatic response to fasting with respect to GH signaling

appeared to result from additional alterations to GHR. In contrast to the hemorrhage-dependent effect on GH signaling where total hepatic GHR abundance was unchanged, fasting resulted in a pronounced decrease in total GHR. And the appearance of Band 3 in response to hemorrhage was preserved in fasted mice, despite the lower initial level of hepatic GHR. These additional findings illustrated the central importance of GHR in the development of hepatic GHR resistance. However, it is not clear from the studies in this dissertation how such pronounced changes in cardiac GH signaling occur either due to fasting or to injury. Still, results in heart and liver of the combined effects of fasting and injury on GH signaling compelled us to study the effects of fasting alone.

As demonstrated in the second article of this dissertation, fasting for 6 hours prior to GH-injection resulted in a large increase in cardiac P-STAT5. The effect of fasting was less pronounced at later time points and appeared to taper off, although P-STAT5 remained significantly elevated in mice fasted 18 hours compared to fed mice. In previous studies of the effects of GH on cardiomyocytes, it was discovered that many of the genes upregulated in response to GH are related to fatty acid metabolism. Therefore, increased sensitivity of the heart to GH following fasting may be an adaptive strategy to assist in the transition from carbohydrates to fatty acids for metabolic fuel.

Similar to heart, fasting also resulted in increased GH-induced P-STAT5 in triceps. This and other similarities observed between triceps and heart may reflect the similar physiology of these two tissues as both are primarily composed of muscle. Despite the effect of fasting on GH-induced P-STAT5 in triceps and heart, no changes in GHR abundance were observed. However, previous studies have demonstrated effects on GH signaling that are not dependent on changes in GHR structure or abundance and

likely involve a post-receptor mechanism (97;137). It is therefore possible that increased GH signaling in triceps and heart following fasting is also occurs via a post-receptor mechanism. In contrast, GH-induced P-STAT5 was not altered by fasting in either liver or adipose tissue, but fasting was associated with severely decreased GHR in both tissues.

Table 4

Comparison of Tissue Responses to Fasting Alone

Fasting		
Tissue	**STAT5 Signaling Impairment**	**Effect on GHR**
Liver	No Change	Reduction
Heart	Enhanced	None
Triceps	Enhanced	None
Adipose	No Change	Reduction

The apparent lack of correlation between changes in GHR and changes in GH-induced STAT5 phosphorylation in liver and adipose tissue following fasting is also intriguing (Table 4). It is possible that these tissues possess such an abundance of GHR that loss of a significant fraction of total receptors does not affect GH-induced STAT5 phosphorylation (spare receptors). It is also possible that the GH dose used in our studies is so high, that the same signaling response is achieved despite large reductions in receptor availability (the dose used in our studies likely results in plasma concentrations greater than 100-fold the maximum physiological GH concentrations measured in mice). A third possibility is that the combination of high-dose GH and high receptor abundance is responsible for the apparent lack of signal degradation following fasting in spite of reduced receptor number.

Each of these possibilities carries important implications for studies of GH resistance following injury. Therefore, an important suggestion for future studies will include experiments designed to determine the optimal GH dose for assessing GH sensitivity. In other words, it should be determined at what point along the dose response curve the change in response becomes proportional to the change in dose.

Effects of GH on Canonical GH Signaling Pathways in Mice

In preliminary studies to determine which signaling pathways could be reliably detected in C57BL/6 mice, it was discovered that P-STAT3 and P-STAT5 were significantly increased within 10 min of GH injection. Of these, STAT5 phosphorylation was the most easily detectable, with GH often resulting in 40-fold or greater increases in P-STAT5. However, no GH-induced increases were observed in P-AKT or P-ERK in similar studies. In order to determine the timing of peak STAT phosphorylation following GH injection, a signaling time-course study was undertaken. In the two tissues studied thus far, the results were intriguing (data presented for liver and heart in the first and second articles, as well as the unpublished data section) It was discovered that the time course of GH-induced STAT activation was similar for STATs 3 and 5, with peak activation occurring within roughly 10-15 min. However, analysis of AKT phosphorylation in liver demonstrated a peak 1 min after GH injection followed by a rapid return to baseline. In heart, GH resulted in decreased, rather than increased, GH induced AKT and ERK phosphorylation. These findings are important for several reasons. First, they demonstrate that GH signaling under normal conditions (i.e. without injury) is not uniform across all tissues. Second, they demonstrate that C57BL/6 mice

represent a useful model for studying GH signaling in multiple pathways since in general only GH-induced JAK/STAT signaling has been reported in live animals. Finally, the signaling studies presented in this dissertation demonstrate that GH-induced activation of AKT/PI3K and MEK/ERK are not optimal signaling pathways to study in models of GH resistance, since the fold effects on these pathways are much smaller than for STAT5, and the activation kinetics appear to be too different to allow measurement at 10 min following injection with GH as with STAT5 in our studies of injury.

As mentioned previously, STAT3 is not a suitable marker of GH sensitivity in studies of GH due to injury, since injury resulted in STAT3 phosphorylation independent of GH. In addition, STAT3 is phosphorylated in response to IL-6 which is upregulated during inflammation, making STAT3 an unsuitable measure of GH sensitivity in other models of GH resistance, such as sepsis, kidney failure, or heart failure. However, our results suggest that C57BL/6 mice may prove useful in studies of the effects of GH activation of STAT3 phosphorylation, since very little is known about the role of STAT3 signaling in GH action, and there have been few studies of GH-induced STAT3 phosphorylation *in vivo*.

In summary, the studies described in this dissertation have provided a foundation for determining the mechanisms of GH resistance following injury, as well as the study of GH signaling *in vivo*. The model systems described herein should continue to be used to provide novel insights that will impact both basic science and clinical theaters.

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APPENDIX A

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: May 8, 2012

TO: JOSEPH L MESSINA, Ph.D.
VH -G019 0019
FAX: (205) 934-1775

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Hepatic Insulin Resistance Following Hemorrhage
Sponsor: NIH
Animal Project Number: 120609145

As of June 3, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	225
Mice	C	75
Rats	B	125
Rats	C	75

Animal use must be renewed by June 2, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120609145 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: May 8, 2012

TO: JOSEPH L MESSINA, Ph.D.
VH -G019 0019
FAX: (205) 934-1775

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on May 8, 2012.

Title of Application: Hepatic Insulin Resistance Following Hemorrhage
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019