

Alteration of somatotrophic function by proinflammatory cytokines¹

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ABSTRACT: Infections direct amino acids away from growth and skeletal muscle accretion toward the hepatic synthesis of acute-phase proteins. The loss of skeletal muscle protein stores results in both a decrease in muscle function and an increase in mortality. In general, muscle protein synthesis is decreased in rodent models of sepsis, as well as after the injection of components of the bacterial cell wall, such as lipopolysaccharide. Although the overexpression of proinflammatory cytokines is known to hasten the loss of skeletal muscle protein, it is not known whether this represents a direct effect of cytokines or results from secondary changes in the IGF system. Plasma concentrations of IGF-I are dramatically lowered by infection in rats, mice, pigs, and steers. The drop in IGF-I often occurs despite an increase in the plasma concentration of somatotropin. Animals are therefore considered to be GH resistant. The IGF bioactivity is determined not only by the plasma concentration of the ligand, but also by IGFBP; IGFBP-3 is the most abundant of these binding proteins and undergoes proteolysis during some catabolic states.

In contrast to IGFBP-3, the plasma concentration of inhibitory IGFBP, such as IGFBP-1, is increased during infection. Insulin-like growth factor-binding protein-1 accumulates in skeletal muscle, where it can potentially inhibit IGF-dependent protein synthesis. Insulin-like growth factor-I and IGFBP-1 are regulated at the level of gene transcription by proinflammatory cytokines. Recent studies demonstrate that bacterial components that activate immune cells also activate the innate immune response in skeletal muscle. Lipopolysaccharide increases proinflammatory cytokine messenger RNA expression in muscle from control mice, but not from mice with a mutation in the lipopolysaccharide receptor. Lipopolysaccharide also increases cytokine expression in human and mouse myoblasts. Local expression of cytokines in skeletal muscle may negatively regulate the autocrine synthesis of IGF-I. Current work is focused on deciphering the mechanism by which muscle becomes GH resistant and the development of therapies to maintain muscle protein stores during infection.

Key Words: Cytokine, Growth Hormone, Insulin-Like Growth Factor, Skeletal Muscle, Tumor Necrosis Factor

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Introduction

Growth hormone and IGF-I are potent regulators of muscle mass in humans and domesticated animals. Transgenic mice that overexpress these proteins exhibit dramatically enlarged skeletal muscles (Knapp et al., 1994; Musaro et al., 2001). In contrast, malnutrition, critical illness, and sepsis are all associated with a dramatic reduction in muscle mass and a decrease in the circulating concentration of IGF-I (Lang and Frost, 2002). Consequently, GH has been used clinically to

increase lean body mass in patients with muscle wasting (Schambelan et al., 1996). The development of GH resistance and increased mortality in trauma patients treated with GH has limited use of this anabolic hormone. A better understanding of the regulation of IGF-I expression in skeletal muscle and muscle cells is therefore of importance. This review summarizes current knowledge of the alterations in the IGF system and somatotrophic function that take place during infection. A series of studies performed by our laboratory and others in humans, rodents, and cell culture are used to illustrate changes that occur from the level of the organism to the level of the cell. This includes lipopolysaccharide (LPS)-induced alterations in GH and IGF-I secretion and changes in IGFBP, such as IGFBP-3 and IGFBP-1. Hepatic production of IGF-I has been found to be dispensable in rodents; therefore, we will also provide evidence that the local synthesis of IGF-I in rat and mouse skeletal muscle is concomitantly depressed during infection. Recent studies that demon-

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strate that LPS can stimulate cytokine expression in skeletal muscle and cultured muscle cells are presented in the context of a Toll-like receptor (TLR)-4 acting as an LPS sensor for peripheral tissues. A special emphasis will be placed on the interaction of IGF-I and proinflammatory cytokines in skeletal muscle and cultured myocytes.

Methods

Experimental Protocol for C3H/HeSnJ and HeJ Mice

All C3H/HeSnJ and HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Control wild-type mice (C3H/HeSnJ) and mice with a mutation in the receptor that binds LPS (HeJ) were used. Mice were housed in a controlled environment and provided water and rodent chow ad libitum for 3 wk before their use. At the time of the study, mice were 8 to 9 wk old and weighed 21.4 ± 0.3 g. In the experiment depicted in Figure 3, C3H/HeSnJ mice were injected intraperitoneally with LPS derived from *Escherichia coli* 026:B6 (DIFCO Laboratories, Detroit, MI; 25 μ g per mouse) or an equal volume of saline (250 μ L/mouse). This dose was based on a preliminary dose-response study and is similar to that used by other investigators (Baumgarten et al., 2001). After 18 h, mice were anesthetized with a mixture of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Bayer, Shawnee Mission, KS) at 90 and 9 mg/kg, respectively. Blood was collected from the inferior vena cava in heparinized syringes. Hindlimb skeletal muscle (gastrocnemius and plantaris) from both legs was dissected from each animal, wrapped in aluminum foil, and flash frozen in liquid nitrogen. Mice were sacrificed by cardiac excision and subsequent exsanguination. Tissues were later powdered under liquid nitrogen using a mortar and pestle and stored at -70°C . All experiments were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine and adhere to the National Institutes of Health guidelines for the use of experimental animals.

Cell Culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, MD). Human skeletal muscle cells were also obtained from Clonetics Corp. (San Diego, CA). Muscle cells were grown in 100-mm petri dishes (Becton Dickinson, Franklin Lakes, NJ) and cultured in minimum Eagle's essential medium containing 10% bovine calf serum, penicillin (100 U/mL), streptomycin (100 μ g/mL), and amphotericin B (25 μ g/mL) (all from Sigma, St. Louis, MO). Cells were grown to confluence and switched to fresh serum-containing media 2 h before addition of LPS, cytokines, or other agents. In general, C2C12 cells were used at the myoblast stage but in some instances the cells were differentiated by incubation for

2 week in medium containing 2% bovine calf serum. Experiments were performed with lipopolysaccharide B derived from *Escherichia coli* 026:B6 (DIFCO Laboratories).

Ribonucleic Acid Isolation and Ribonuclease Protection Assay

Total RNA, DNA, and protein were extracted from C2C12 cells or tissues in a mixture of phenol and guanidine thiocyanate (TRI Reagent, Molecular Research Center, Cincinnati, OH) using the manufacturer's protocol. The RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in formamide, RNA samples were quantified by spectrophotometry. Ten micrograms of RNA was used for each assay. Riboprobes were synthesized from custom multiprobe mouse and human template sets containing probes for IGF-I, interleukin (IL)-6, TNF α , IL-1 β , and SOCS-3 messenger RNA (mRNA) detection (Pharminigen, San Diego, CA). The labeled riboprobe was hybridized with RNA overnight using a ribonuclease protection assay kit and the manufacturer's protocol (Pharminigen). Protected RNA were separated using a 5% acrylamide gel (19:1 acrylamide/bisacrylamide). Gels were transferred to blotting paper and dried under vacuum on a gel dryer. Dried gels were exposed to a phosphor imager screen (Molecular Dynamics, Sunnyvale, CA) and the resulting data were quantified using ImageQuant software and normalized to the mouse ribosomal protein L32 mRNA signal in each lane.

Results and Discussion

The IGF System and the Somatomedin Hypothesis

Insulin-like growth factor-I is a 7.5-kDa peptide that shares approximately 50% AA sequence homology with insulin and 76% homology with IGF-II (Rinderknecht and Humbel, 1978; Jones and Clemmons, 1995; Kim and Accili, 2002; Monzavi and Cohen, 2002; Yakar, 2002). Although the peptides share considerable homology, they tend to preferentially bind to their cognate receptors when present at physiological concentrations (Siddle et al., 2001). The insulin and IGF-I receptors are hetero-tetramers composed of two alpha and beta subunits. Insulin-like growth factor-I and insulin bind to the α -subunit, whereas the β -subunit contains an intrinsic tyrosine kinase activity. Upon ligand binding, the receptor autophosphorylates its opposing β -subunit and transphosphorylates intracellular substrates, such as insulin receptor substrate-1 to -4. Insulin-like growth factor-I and insulin receptors share sufficient homology that their β -subunits can form hybrid IGF-I/insulin receptors. These receptors appear functionally equivalent to IGF-I receptors because they have a reduced affinity for insulin (Pandini et al., 2002). Clinical conditions, such as diabetes, may favor the formation

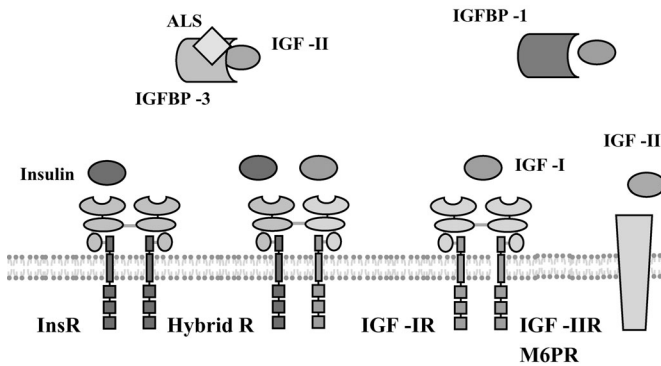


Figure 1. Insulin-like growth hormone-I is a small peptide hormone that exhibits considerable homology to insulin and insulin receptors. It binds to a heterodimeric cell surface receptor that functions as a tyrosine kinase to transduce the binding signal to intracellular proteins and also forms hybrid receptors in some cell types. The IGF-I receptor binds IGF ligands in the order of IGF-I (0.2 nM) > IGF-II (0.6 nM) >> (30 nM) insulin; this can be altered by hybrid receptor formation (Pandini et al., 2002). Insulin-like growth hormone-II can also bind to the IGF-II or mannose-6-phosphate receptor, but this receptor is thought to only clear IGF-II from the circulation and extracellular fluids. Insulin-like growth factor binding is regulated in the extracellular fluid and plasma by IGFBP. The IGFBP bind IGF-I and -II with high affinity, but have very low affinity for insulin. The most abundant IGFBP is IGFBP-3, which forms a heterotrimeric complex with IGF-I or -II and a third acid-labile subunit. The ternary complex extends the half-life of IGF-I and restricts IGF-I to the circulation. Insulin-like growth factor binding protein-1 is the most dynamically regulated IGFBP regulated by insulin (Guo et al., 1999), steroid hormones (Suvanichkul et al., 1994), catecholamines (Hooper et al., 1994), and cytokines (Frost et al., 2000). Free IGF-I is bound by IGFBP and can inhibit muscle protein synthesis both in vitro and in vivo (Frost and Lang, 1999).

of hybrid receptors and impose insulin resistance in some tissues (Federici et al., 1996).

The molar concentration of IGF-I in plasma is approximately 1,000 times that of insulin. However, the majority of IGF-I in the circulation is biologically inactive because it is bound to a family of IGFBP (Figure 1). The most abundant IGFBP in plasma is IGFBP-3. This protein forms a ternary complex with an acid-labile subunit and IGF-I (Firth and Baxter, 2002). Insulin-like growth factor-binding protein-3 extends the half-life of IGF-I and -II and keeps the amount of free or biologically active IGF-I very low. In addition, since the molecular size of the ternary complex is greater than that of molecules that freely diffuse across endothelial barriers, IGF bound to IGFBP-3 are largely restricted to the vascular space. Mechanisms exist to release IGF-I from IGFBP in the circulation, and these may include the proteolysis of IGFBP, which occurs with IGFBP-3

(Binoux et al., 1993) as well as the transfer of IGF from the ternary complex to smaller IGFBP. Insulin-like growth factor-binding proteins may also direct IGF to specific tissues and or cell types (Sandra et al., 1998).

The original somatomedin hypothesis of Daughaday hypothesized that GH stimulated the synthesis of a factor from the liver that mediated the effects of GH on peripheral tissues (Daughaday et al., 1976). Daughaday's GH-inducible factor was isolated from liver perfusates and found to stimulate sulfate uptake in cartilage from hypophysectomized rats and was later revealed to be one and the same with IGF-I. In recent years, the somatomedin hypothesis has been challenged because GH may also have direct effects on peripheral tissues (Daughaday, 2000). In addition, Yakar and colleagues found that mice in which the IGF-I gene was specifically deleted in the liver (e.g., liver IGF-I deficient [LID] mice) exhibited completely normal intrauterine and postnatal growth (Yakar et al., 1999). Normal growth occurred in these mice despite a 75% reduction in the plasma concentration of IGF-I. To many, these data suggested that IGF-I derived from the circulation does not contribute to the growth of peripheral tissues. Yet, the liver may not be the only source of circulating IGF-I. For example, when liver-specific IGF-I knockout mice are fed a low-protein diet, their circulating IGF-I levels fall even further (Naranjo et al., 2002). In addition, crossing LID mice with mice that are deficient in the acid-labile subunit of the ternary complex results in double gene-disrupted mice that have reduced growth, even though each of the individual knockout mice have a normal growth phenotype (Boisclair et al., 2001). Not surprisingly, the double gene-disrupted mice have IGF-I levels that are even lower than either single knockout alone (Yakar et al., 2002). Thus, circulating IGF-I levels may have to fall below a threshold level before a noticeable phenotype can be detected. This threshold may differ between young rapidly growing animals and their more mature counterparts. In addition, it is likely that the threshold level of IGF-I differs between species and that the lowest circulating levels of IGF-I will occur in animals and humans that exhibit a reduction in all 3 components of the ternary complex.

Changes in the GH-IGF-I Axis Due to Infection

Infection, trauma, and critical illness alter the GH-IGF-I axis dramatically (Baxter, 2001). Our laboratory has studied the changes that occur in the GH-IGF axis in response to infection and trauma (Frost and Lang, 1998). Early work by Dahn et al. (1988) showed that septic patients had low circulating levels of IGF-I, and although GH treatment could increase IGF-I in healthy control subjects, septic patients were clearly GH resistant. Growth hormone increases nitrogen balance in healthy control subjects, but septic patients treated with the hormone remain in a negative nitrogen balance. Subsequent studies have shown variable results, and this is thought to depend on the clinical state of

the patient (Gottardis et al., 1991; Ziegler and Leader, 1994; Jenkins and Ross, 1996; Wolf et al., 1996; Unneberg et al., 1997). A testament to our insufficient knowledge in the use of GH in this area came from a multicenter clinical study that was ended prematurely due to an increased incidence of mortality in critically ill patients treated with GH (Takala et al., 1999).

We have used LPS or endotoxin from the bacterial coat of *E. coli* to model some of the early acute changes that occur in response to infection in humans and rodents. Changes in the GH-IGF axis include a transient increase in GH secretion (Lang et al., 1997), a decrease in the circulating concentration of IGF-I (Fan et al., 1994), the development of GH resistance at the liver level (Yumet et al., 2002), proteolysis of IGFBP-3 (Donaghy and Baxter, 1996), and an elevation of IGFBP-1 (Fan et al., 1994; Lang et al., 1997).

Lipopolysaccharide administration to human volunteers results in a rapid increase in the secretion of GH into the blood that is followed temporally by a fall in the plasma concentration of IGF-I (Lang et al., 1997). Although GH levels are not elevated in the rat, LPS injection also decreases the plasma levels of IGF-I; this response appears to reach its maximum 4 h after LPS injection in this species. The depression in IGF-I is maintained for up to 18 h in the rat and mouse (Fan et al., 1994; Lang et al., 2003), and similar changes in GH and IGF-I were observed when sheep were challenged with a high dose of LPS (Briard et al., 2000). The presence of elevated GH levels in the face of falling plasma IGF-I suggests that humans injected with LPS become GH resistant; this is consistent with the original findings of Dahn et al. (1988). Recent work in rats made septic by cecal ligation and puncture suggests that infection generates GH resistance at the liver level, such that the hepatic content of IGF-I mRNA and protein is decreased (Rodriguez-Arno et al., 1996; Hong-Brown et al., 2003). In addition, GH fails to stimulate the phosphorylation of a transcription factor (signal transducer and activator of transcription 5) that is critical for hepatic expression of IGF-I (Hong-Brown et al., 2003). Although endotoxin and the early stages of sepsis are thought to produce a GH-resistant state, the duration of the GH resistance is unclear. Some studies suggest that critically ill patients can respond to low-dose GH, and that in prolonged critical illness, the low activity of the somatotrophic axis can be overcome by coinfusion of thyroid and GH-releasing hormones (Van den Berghe et al., 1998).

Although GH resistance may manifest as a failure of the liver to respond to GH either at the level of the receptor or at the level of molecules that transduce the GH signal, it is also possible that IGF-I is made but cannot be measured due to its rapid loss from the circulation. As noted above, 80 to 90% of circulating IGF-I is bound to IGFBP-3 and the acid-labile subunit (ALS). Insulin-like growth factor-binding protein-3 and ALS expression are also GH-dependent, and disruption of the gene for either protein reduces plasma IGF-I levels

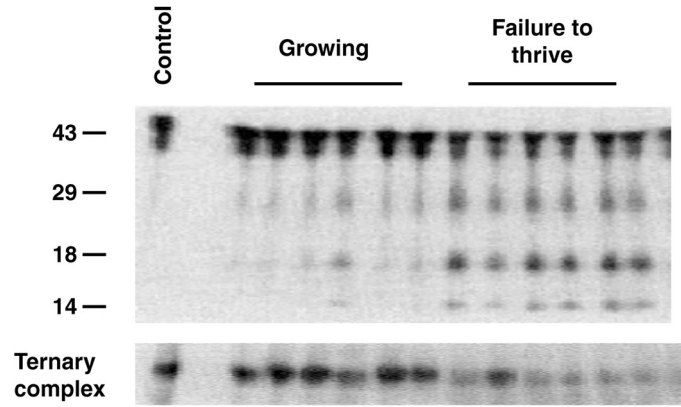


Figure 2. Insulin-like growth hormone-I circulates bound to IGFBP-3 and an acid-labile subunit in vivo. The IGFBP-3 can be proteolyzed during catabolic states by a serum protease. We initially found that the plasma concentration of IGF-I was depressed in HIV-infected children who failed to thrive (Frost et al., 1996) and thus examined whether IGFBP-3 is proteolyzed and whether IGF-I could form a ternary complex with serum from HIV-infected children. Addition of IGFBP-3 to serum from growing children resulted in the generation of 27- and 15-kDa fragments when analyzed by SDS-PAGE, and this was dramatically increased in children who failed to thrive. In contrast, addition of IGF-I to the same plasma samples followed by nondenaturing PAGE resulted in a decreased ability of IGF-I to form a ternary complex compared to age-matched growing children. This figure was modified from data presented by Frost et al. (1996).

(Boisclair et al., 2001). Lipopolysaccharide and inflammatory cytokines, such as IL-1 β , reduce plasma concentrations of both IGF-I (Fan et al., 1996) and ALS in rats (Barreca et al., 1998; Delhanty, 1998), and their gene expression in primary cultures of rat hepatocytes (Thissen and Verniers, 1997; Boisclair et al., 2000). It is therefore likely that under the influence of proinflammatory cytokines, there is a decrease in the endogenous production of each of the ternary complex components.

HIV infection in children is also associated with changes in the GH-IGF axis, and this change manifests itself as a failure to thrive. Children who fail to thrive have dramatically reduced plasma IGF-I levels (Frost et al., 1996b). Additionally, a proteolytic activity is present in the serum of HIV-infected children that degrades IGFBP-3 into smaller fragments that are less able to form a ternary complex with IGF-I and ALS (Figure 2). Although initially this proteolysis may provide a greater proportion of biologically active IGF-I for transport to peripheral tissues, it is also likely that IGF-I is quickly depleted from the circulation. Under such circumstances, we speculate that IGF-I falls below a threshold level of IGF-I that is necessary to support growth. A similar phenomena occurs in HIV-infected adults who exhibit muscle wasting (Frost et al., 1996a).

In HIV-infected adults, IGF-I more freely associates with a highly phosphorylated form of IGFBP-1. Insulin-like growth factor-binding protein-1 is also elevated in humans and rats injected with LPS (Fan et al., 1994; Lang et al., 1997). Phosphorylated IGFBP-1 has a higher affinity for IGF-I than either its nonphosphorylated counterpart or the IGF-I receptor (Jones et al., 1991). Phosphorylated IGFBP-1 complexes with IGF-I and prevents IGF-I receptor binding. Therefore, increased IGFBP-1 inhibits IGF-I bioactivity in vitro, including IGF-I-stimulated glucose uptake and protein synthesis in skeletal muscle cells (Frost and Lang, 1999). Insulin-like growth factor-binding protein-1 can also inhibit muscle protein synthesis when infused in vivo (Lang et al., 2003). Hepatic IGFBP-1 expression is elevated in septic rats, but this can be completely prevented by pretreatment with an IL-1 receptor antagonist (Lang et al., 1996). Interleukin-1 also directly stimulates IGFBP-1 protein and mRNA synthesis in the HepG2 hepatoma cell line (Samstein et al., 1996; Lang et al., 1999; Frost et al., 2000). Collectively, these findings strengthen the causal association between alterations in proinflammatory cytokines and a broad spectrum of IGF system components.

Lipopolysaccharides Induce Local Changes in IGF-I and Proinflammatory Cytokines in Skeletal Muscle

Although sepsis, critical illness, and LPS have a profound effect on the GH-IGF axis, systemically we have focused our attention on the local synthesis of IGF-I and inflammatory cytokines in skeletal muscle per se. This focus has been dictated by two observations: 1) GH stimulates IGF-I expression not only in the liver but also in peripheral tissues, such as skeletal muscle (Turner et al., 1988), and 2) the local production of IGF-I is likely to contribute substantially to the final biologically active pool of IGF-I that surrounds muscle fibers and supporting muscle cells. For example, exercise increases the autocrine synthesis of IGF-I in skeletal muscle without changing systemic IGF-I levels, and this may stimulate hypertrophy and protein synthesis specifically in muscle (Yan et al., 1993; Farrell et al., 1999; Adams, 2002).

Lipopolysaccharides, tumor necrosis factor- α (TNF α), and IL-1 β all decrease the local content of IGF-I peptide in skeletal muscle (Fan et al., 1995, 1996). The decrease in IGF-I is observed in both rats and mice and is paralleled by a concomitant decrease in IGF-I mRNA in skeletal muscle (Fan et al., 1996; Li et al., 1997; Frost et al., 2003b). The LPS-induced decrease in skeletal muscle IGF-I mRNA only occurs in mice with a functional LPS receptor (Figure 3). Intraperitoneal injection of LPS into C3H/HeJ mice that harbor a mutation in the LPS receptor (also known as TLR-4) maintain muscle IGF-I mRNA levels similar to those seen in wild-type mice injected with saline.

In the course of analyzing skeletal muscle from the above mice, we observed that LPS induced the expres-

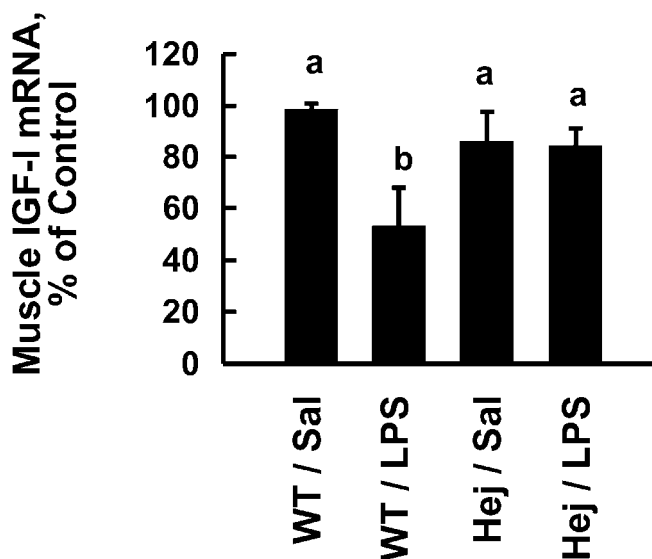


Figure 3. Lipopolysaccharide (LPS) decreases muscle IGF-I messenger RNA (mRNA) in mice with a functional TLR4 receptor. Control wild-type (WT) mice (C3H/HeSnJ) and mice with a mutation in the receptor that binds LPS (Hej) were injected with a nonlethal dose of LPS (25 μ g/mouse). Mice were killed after 18 h and the skeletal muscle analyzed for IGF-I mRNA by ribonuclease protection assay. All data are normalized to the expression of L32 mRNA in each lane and the expression of IGF-I in control animals injected with saline (WT/Sal) set at 100%.

sion of multiple proinflammatory cytokines (IL-6, TNF α , and IL-1 α) not only in immune tissues, such as the liver and spleen, but also in skeletal muscle itself. Once again, cytokines were only induced in mice with a functional TLR4 receptor (C3H/HeSnJ), and not in mutant mice (C3H/HeJ) (Frost et al., 2002). This raised the question of whether skeletal muscle components, such as the muscle fibers themselves and satellite cells, respond to LPS. Alternatively, tissue macrophage trapped within muscle might react to the LPS signal. We are in the process of examining tissue sections from LPS-injected rats for IL-6, TNF α , and IL-1 β mRNA by in situ hybridization and the cytokine proteins by immunohistochemistry.

Skeletal Muscle Cells Respond to LPS

To examine whether muscle cells respond to LPS on their own, we also added LPS directly to C2C12 myoblasts. These cells made copious amounts of IL-6 in response to LPS, and the increase in IL-6 protein was paralleled by a concomitant increase in IL-6 mRNA, as well as increased mRNA content for other proinflammatory cytokines (Frost et al., 2002). Lipopolysaccharides also decreased the expression of IGF-I mRNA in C2C12 cells, suggesting that muscle cells can recapitulate the response of skeletal muscle to LPS observed in

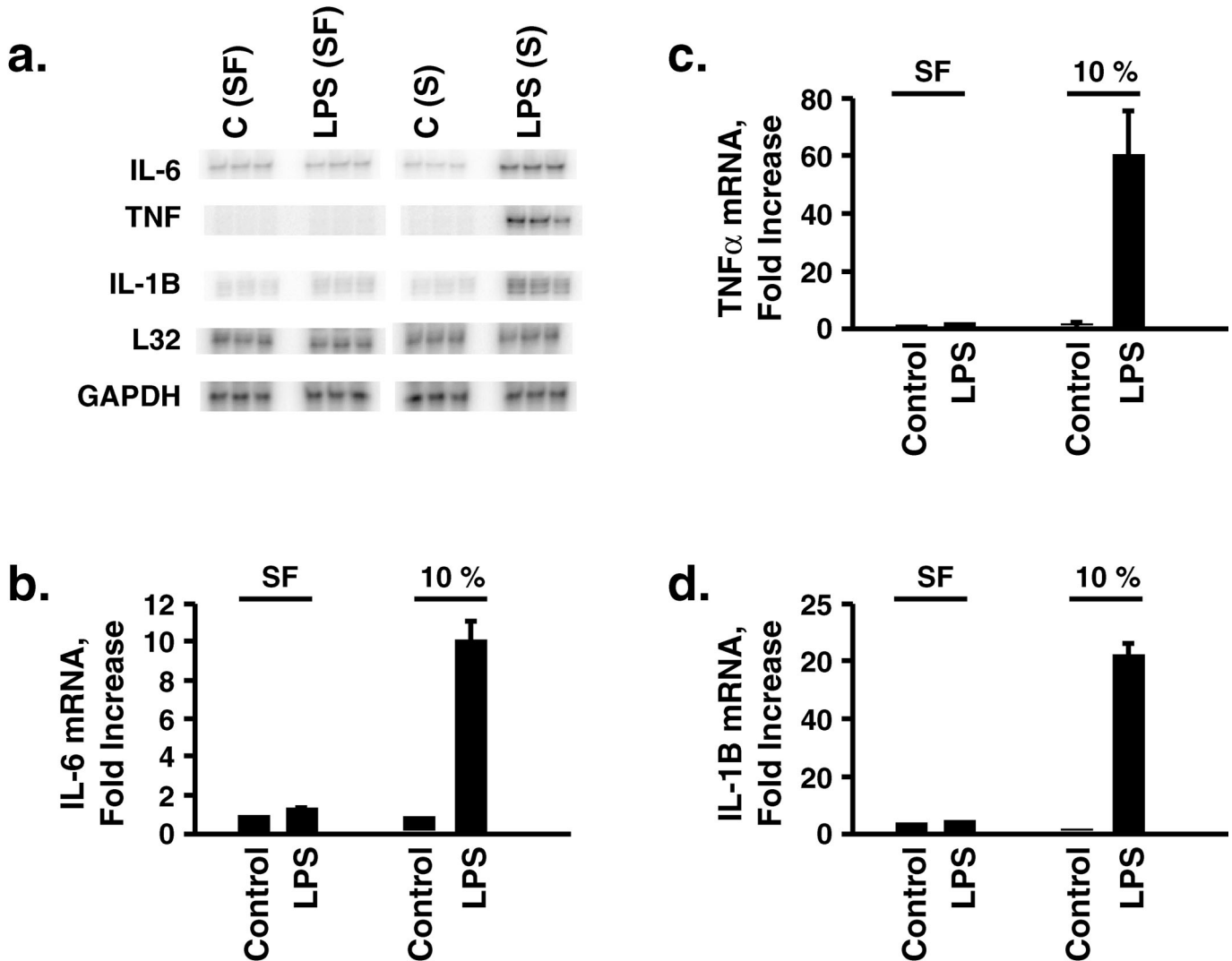


Figure 4. Lipopolysaccharide (LPS) increases interleukin-6 (IL-6), tumor necrosis factor- α (TNF α), and interleukin (IL)-1 β messenger RNA (mRNA) in human skeletal muscle cells. Lipopolysaccharide (4 μ g/mL) induces cytokines in these murine cells in both the absence and presence of serum. Primary cultures of human skeletal muscle cells were also grown in the absence or presence of serum (SF = serum free; 10% = 10% serum); these cells also expressed proinflammatory cytokines. A) Phosphorimage, B) IL-6 mRNA, C) TNF α mRNA, and D) IL-1 β mRNA. Human skeletal muscle cells, compared with C2C12 cells, have a strict dependence on the presence of serum to respond to LPS.

vivo (Frost et al., 2003b). The C2C12 cell line responded to LPS in the absence or presence of serum components. We subsequently tested whether human skeletal muscle cells also respond to LPS. In these cells, LPS stimulated TNF α , IL-1 β , and IL-6 mRNA expression, but the LPS-induced increase in cytokines had a strict requirement for the presence of serum (Figure 4). Human myoblasts may require additional serum components, such as an LPS binding protein or soluble CD14 to respond to LPS, or the human and mouse receptors may have different intrinsic abilities to recognize LPS (da Silva Correia et al., 2001; Hajjar et al., 2002). The overall temporal expression pattern of proinflammatory cytokines in human and mouse skeletal muscle cells were similar to each other, as was their expression in vivo (Figure 5) (Frost et al., 2002).

Lipopolysaccharides and TNF α also decreased IGF-I mRNA expression in C2C12 cells, and this occurred in both myoblasts and differentiated myotubes (Frost et al., 2003b). Currently, it is not clear whether TNF α acts directly or circuitously by disrupting an IGF-I autocrine loop required for the maintenance of skeletal muscle protein stores. Because TNF α decreases global protein synthesis in skeletal muscle, and myocytes and decreases myosin heavy chain in C2C12 cells, we have multiple endpoints with which to test this hypothesis both in vivo and in vitro in the future (Frost et al., 1997; Li and Reid, 2000; Lang et al., 2002).

Not only does TNF α decrease IGF-I mRNA in the basal state, it also prevents GH-inducible expression of IGF-I mRNA in C2C12 cells (Frost et al., 2003b). Unlike the response in liver, which was described ear-

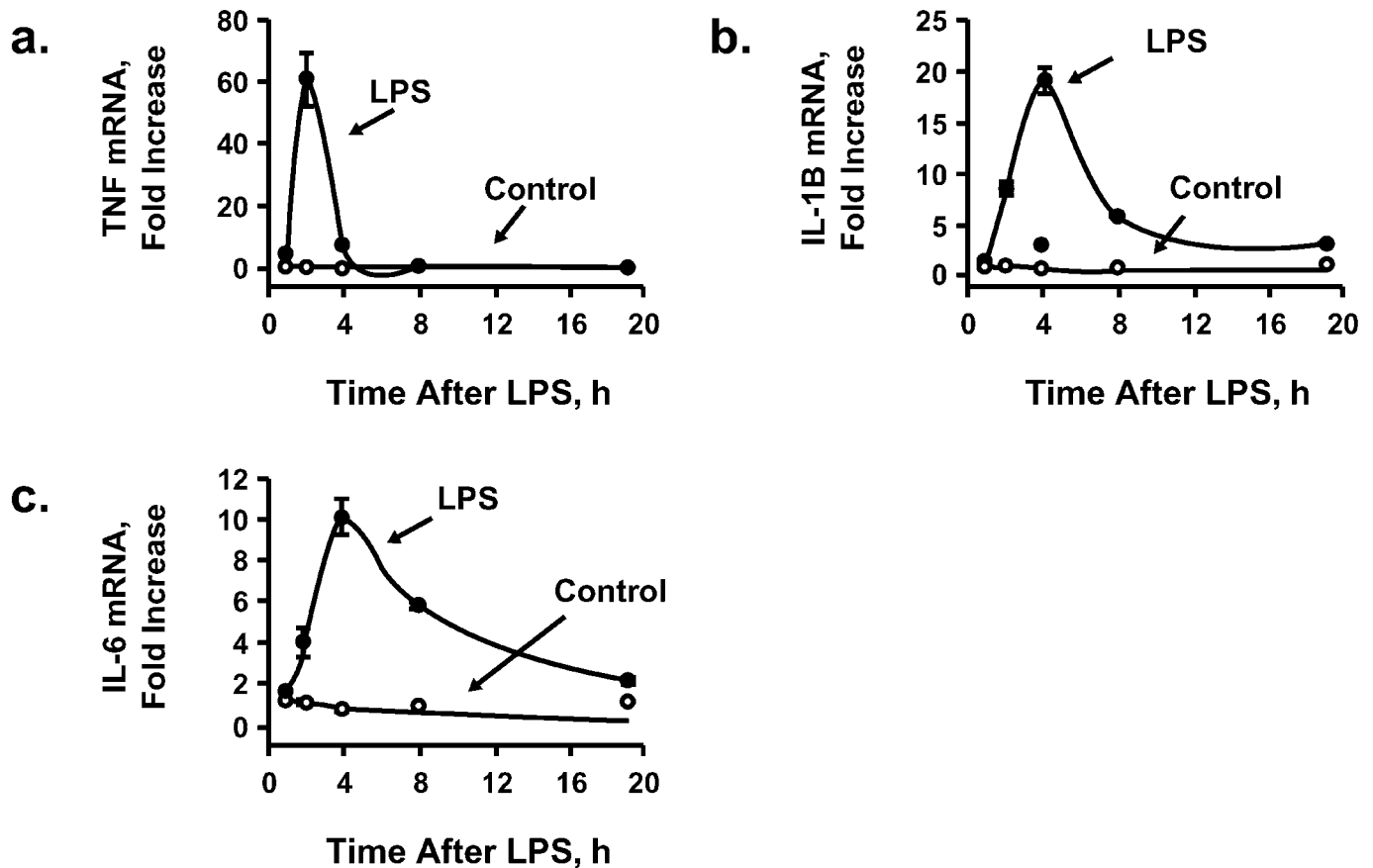


Figure 5. Lipopolysaccharide (LPS) increases tumor necrosis factor- α (TNF α), interleukin (IL)-1 β , and IL-6 messenger RNA (mRNA) in human skeletal muscle cells in a time-dependent manner. Previously, we have shown that LPS stimulates TNF α and IL-1 α mRNA expression in C2C12 cells within 1 h, and that IL-6 expression is maximal by 4 h (Frost et al., 2002). Lipopolysaccharide (LPS) (4 μ g/mL) also stimulated proinflammatory cytokines in a time-dependent manner in human skeletal muscle cells. A) TNF α , B) IL-1 β , and C) IL-6 mRNA.

lier, sepsis does not inhibit GH-inducible Stat5 phosphorylation in skeletal muscle (Hong-Brown et al., 2003), and this was recapitulated in C2C12 cells treated with TNF α (Frost et al., 2003b). Therefore, other mechanisms must explain the GH resistance that is observed in vivo at the level of GH-inducible IGF-I mRNA expression (Hong-Brown et al., 2003). In skeletal muscle from septic rats, there is a small increase in the expression of proteins that can suppress cytokine signaling, including suppressor of cytokines signaling-3 (SOCS-3). Forced expression of SOCS-3 in H4-II-E cells inhibits GH-inducible ALS expression (Boisclair et al., 2000). However, the increase in SOCS-3 protein in vivo is very small and it is not known if this change raises SOCS-3 protein levels to a sufficiently high level to physiologically effect GH signaling. We find that TNF α dramatically down regulates IGF-I mRNA expression in myotubes and myoblasts and that these changes occur independent of changes in the expression of SOCS-3 mRNA (Figure 6).

Growth hormone receptor number decreases in some tissues in response to sepsis, and van Kerkhof et al. (2003) have proposed that if one could prevent the loss

of the GH receptor that one could overcome GH resistance. Growth hormone receptors can be internalized and degraded by a proteasomal pathway, and they are also shed from Chinese hamster lung cells by a matrix metalloprotease. Van Kerkhof (2003) demonstrated that a combination of matrix metalloprotease and proteasome inhibitors increases GH receptor number on the cell surface as well as GH-induced Stat-5 phosphorylation. The combination of inhibitors is a novel method for increasing receptor number at the cell surface, and may be useful in tissues such as the liver, where GH receptor number may be decreased during infection (Defalque et al., 1999; Yumet et al., 2002). The effect of sepsis on GH signaling may be tissue specific because GH-induced Stat-5 phosphorylation is normal in skeletal muscle of septic rats (Hong-Brown et al., 2003).

Although the expression of proinflammatory cytokines and IGF-I is disparately regulated, it is likely that the initial signaling events that decrease IGF-I and increase cytokines in response to LPS are similar. In an effort to characterize these signals, we have found that LPS can activate mitogen-activated protein kinases in C2C12 cells, and that a specific Jun-N-terminal

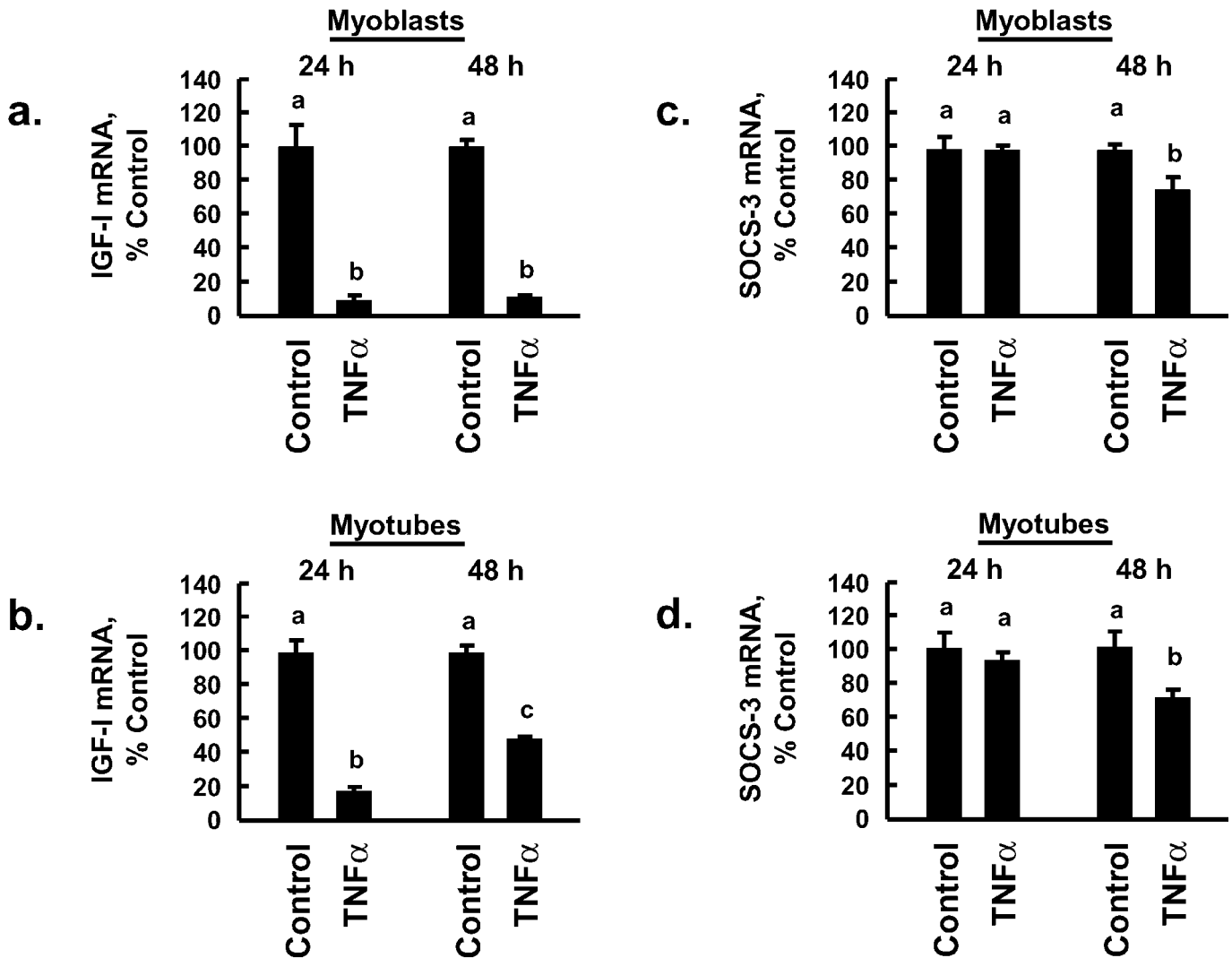


Figure 6. Tumor necrosis factor- α (TNF- α) decreases IGF-I messenger RNA (mRNA) in C2C12 myoblasts and myotubes independent of changes in suppressor of cytokine signaling-3 (SOCS-3) mRNA. The C2C12 cells were grown in either 10% serum to maintain cells as myoblasts or were switched to low serum containing medium to allow the cells to fuse into myotubes. Myotubes and myoblasts were treated with TNF α (40 ng/mL) for either 24 or 48 h, and RNA was isolated. Insulin-like growth factor-I (A and B) and SOCS-3 (C and D) were determined by ribonuclease protection assay. All data were normalized to L32 mRNA in each lane. This figure contains data modified from Frost et al. (2003).

kinase inhibitor (SP600125) can block the TNF-induced fall in IGF-I mRNA (Frost et al., 2003b). In addition, SP600125 is a potent inhibitor of LPS-induced IL-6 expression in C2C12 cells (Frost et al., 2003a). This suggests that the Jun-N-terminal kinase pathway may be a nexus for signaling events that impact on the expression of both catabolic (IL-6, TNF α , and IL-1 β) and anabolic (IGF-I) hormones in skeletal muscle.

Implications

Growth hormone has been used safely for over a decade to increase longitudinal growth in children. In addition, growth hormone has been used to increase lean body mass in human immunodeficiency virus-in-

fectured adults. However, growth hormone is less effective in critically ill patients and in clinical conditions characterized by growth hormone resistance. It is likely that infection also diminishes the efficacy of growth hormone in dairy cattle, pigs, and other domestic livestock. During infection, growth hormone target tissues, such as skeletal muscle, become growth hormone resistant. Infection, therefore, affects not only the immune system, but also peripheral tissues. The combined effect of the loss of anabolic stimuli and an enhanced expression of catabolic cytokines likely tips the metabolic balance of skeletal muscle toward a loss of muscle mass and function. A better comprehension of the regulation of these processes will improve our understanding of muscle physiology and allow us to specifically target these

processes with therapies to prevent the loss of lean body mass.

Literature Cited

- Adams, G. R. 2002. Invited review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. *J. Appl. Physiol.* 93:1159–1167.
- Barreca, A., J. M. Ketelslegers, M. Arvigo, F. Minuto, and J. P. Thissen. 1998. Decreased acid-labile subunit (ALS) levels by endotoxin in vivo and by interleukin-1 β in vitro. *Growth Horm. IGF Res.* 8:217–223.
- Baumgarten, G., P. Knuefermann, N. Nozaki, N. Sivasubramanian, D. L. Mann, and J. G. Vallejo. 2001. In vivo expression of proinflammatory mediators in the adult heart after endotoxin administration: The role of toll-like receptor-4. *J. Infect. Dis.* 183:1617–1624.
- Baxter, R. C. 2001. Changes in the IGF-IGFBP axis in critical illness. *Best Pract. Res. Clin. Endocrinol. Metab.* 15:421–434.
- Binoux, M., C. Lalou, C. Lassarre, C. Blat, and P. Hossenlopp. 1993. Limited proteolysis of insulin-like growth factor binding protein-3 (IGFBP-3): A physiological mechanism in the regulation of IGF bioavailability. *Adv. Exp. Med. Biol.* 343:293–300.
- Boisclair, Y. R., R. P. Rhoads, I. Ueki, J. Wang, and G. T. Ooi. 2001. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: An important but forgotten component of the circulating IGF system. *J. Endocrinol.* 170:63–70.
- Boisclair, Y. R., J. Wang, J. Shi, K. R. Hurst, and G. T. Ooi. 2000. Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1 β on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. *J. Biol. Chem.* 275:3841–3847.
- Briard, N., F. Dadoun, G. Pommier, N. Sauze, Y. Lebouc, C. Oliver, and A. Dutour. 2000. IGF-I/IGFBP system response to endotoxin challenge in sheep. *J. Endocrinol.* 164:361–369.
- da Silva Correia, J., K. Soldau, U. Christen, P. S. Tobias, and R. J. Ulevitch. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. *J. Biol. Chem.* 276:21129–21135.
- Dahn, M. S., M. P. Lange, and L. A. Jacobs. 1988. Insulinlike growth factor 1 production is inhibited in human sepsis. *Arch. Surg.* 123:1409–1414.
- Daughaday, W. H. 2000. Growth hormone axis overview—Somatomedin hypothesis. *Pediatr. Nephrol.* 14:537–540.
- Daughaday, W. H., L. S. Phillips, and M. C. Mueller. 1976. The effects of insulin and growth hormone on the release of somatomedin by the isolated rat liver. *Endocrinology* 98:1214–1219.
- Defalque, D., N. Brandt, J. M. Ketelslegers, and J. P. Thissen. 1999. GH insensitivity induced by endotoxin injection is associated with decreased liver GH receptors. *Am. J. Physiol.* 276:565–572.
- Delhanty, P. J. 1998. Interleukin-1 β suppresses growth hormone-induced acid-labile subunit mRNA levels and secretion in primary hepatocytes. *Biochem. Biophys. Res. Commun.* 243:269–272.
- Donaghy, A. J., and R. C. Baxter. 1996. Insulin-like growth factor bioactivity and its modification in growth hormone resistant states. *Baillieres's Clin. Endocrinol. Metab.* 10:421–446.
- Fan, J., D. Char, G. J. Bagby, M. C. Gelato, and C. H. Lang. 1995. Regulation of insulin-like growth factor-I (IGF-I) and IGF-binding proteins by tumor necrosis factor. *Am. J. Physiol.* 269:1204–1212.
- Fan, J., P. E. Molina, M. C. Gelato, and C. H. Lang. 1994. Differential tissue regulation of insulin-like growth factor-I content and binding proteins after endotoxin. *Endocrinology* 134:1685–1692.
- Fan, J., M. M. Wojnar, M. Theodorakis, and C. H. Lang. 1996. Regulation of insulin-like growth factor (IGF)-I mRNA and peptide and IGF-binding proteins by interleukin-1. *Am. J. Physiol.* 270:621–629.
- Farrell, P. A., M. J. Fedele, T. C. Vary, S. R. Kimball, C. H. Lang, and L. S. Jefferson. 1999. Regulation of protein synthesis after acute resistance exercise in diabetic rats. *Am. J. Physiol.* 276:721–727.
- Federici, M., L. Zucaro, O. Porzio, R. Massoud, P. Borboni, D. Lauro, and G. Sesti. 1996. Increased expression of insulin/insulin-like growth factor-I hybrid receptors in skeletal muscle of noninsulin-dependent diabetes mellitus subjects. *J. Clin. Invest.* 98:2887–2893.
- Firth, S. M., and R. C. Baxter. 2002. Cellular actions of the insulin-like growth factor binding proteins. *Endocrinol. Rev.* 23:824–854.
- Frost, R. A., J. Fuhrer, R. Steigbigel, P. Mariuz, C. H. Lang, and M. C. Gelato. 1996a. Wasting in the acquired immune deficiency syndrome is associated with multiple defects in the serum insulin-like growth factor system. *Clin. Endocrinol. (Oxford)* 44:501–514.
- Frost, R. A., and C. H. Lang. 1998. Growth factors in critical illness: Regulation and therapeutic aspects. *Curr. Opin. Clin. Nutr. Metab. Care* 1:195–204.
- Frost, R. A., and C. H. Lang. 1999. Differential effects of insulin-like growth factor-I (IGF-I) and IGF-binding protein-1 on protein metabolism in human skeletal muscle cells. *Endocrinology* 140:3962–3970.
- Frost, R. A., C. H. Lang, and M. C. Gelato. 1997. Transient exposure of human myoblasts to tumor necrosis factor- α inhibits serum and insulin-like growth factor-I stimulated protein synthesis. *Endocrinology* 138:4153–4159.
- Frost, R. A., S. A. Nachman, C. H. Lang, and M. C. Gelato. 1996b. Proteolysis of insulin-like growth factor-binding protein-3 in human immunodeficiency virus-positive children who fail to thrive. *J. Clin. Endocrinol. Metab.* 81:2957–2962.
- Frost, R. A., G. J. Nystrom, and C. H. Lang. 2000. Stimulation of insulin-like growth factor binding protein-1 synthesis by interleukin-1 β : Requirement of the mitogen-activated protein kinase pathway. *Endocrinology* 141:3156–3164.
- Frost, R. A., G. J. Nystrom, and C. H. Lang. 2002. Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283:698–709.
- Frost, R. A., G. J. Nystrom, and C. H. Lang. 2003a. Lipopolysaccharide and proinflammatory cytokines stimulate interleukin-6 expression in C2C12 myoblasts: Role of the Jun-N-terminal kinase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*
- Frost, R. A., G. J. Nystrom, and C. H. Lang. 2003b. Tumor necrosis factor- α decreases insulin-like growth factor-I messenger ribonucleic acid expression in C2C12 myoblasts via a Jun N-terminal kinase pathway. *Endocrinology* 144:1770–1779.
- Gottardis, M., A. Benzer, W. Koller, T. J. Luger, F. Puhlinger, and J. Hackl. 1991. Improvement of septic syndrome after administration of recombinant human growth hormone (rhGH). *J. Trauma* 31:81–86.
- Guo, S., G. Rena, S. Cichy, X. He, P. Cohen, and T. Unterman. 1999. Phosphorylation of serine 256 by protein kinase b disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. *J. Biol. Chem.* 274:17184–17192.
- Hajjar, A. M., R. K. Ernst, J. H. Tsai, C. B. Wilson, and S. I. Miller. 2002. Human toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol.* 3:354–359.
- Hong-Brown, L. Q., C. R. Brown, R. N. Cooney, R. A. Frost, and C. H. Lang. 2003. Sepsis-induced muscle growth hormone resistance occurs independently of stat5 phosphorylation. *Am. J. Physiol. Endocrinol. Metab.* 285:63–72.
- Hooper, S. B., A. D. Bocking, S. E. White, L. J. Fraher, T. J. McDonald, and V. K. Han. 1994. Catecholamines stimulate the synthesis and release of insulin-like growth factor binding protein-1 (IGFBP-1) by fetal sheep liver in vivo. *Endocrinology* 134:1104–1112.
- Jenkins, R. C., and R. J. Ross. 1996. Acquired growth hormone resistance in catabolic states. *Baillieres Clin. Endocrinol. Metab.* 10:411–419.

- Jones, J. I., and D. R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: Biological actions. *Endocrinol. Rev.* 16:3–34.
- Jones, J. I., A. J. D'Ercole, C. Camacho-Hubner, and D. R. Clemmons. 1991. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. *Proc. Natl. Acad. Sci. USA* 88:7481–7485.
- Kim, J. J., and D. Accili. 2002. Signalling through IGF-I and insulin receptors: Where is the specificity? *Growth Horm. IGF Res.* 12:84–90.
- Knapp, J. R., W. Y. Chen, N. D. Turner, F. M. Byers, and J. J. Kopchick. 1994. Growth patterns and body composition of transgenic mice expressing mutated bovine somatotropin genes. *J. Anim. Sci.* 72:2812–2819.
- Lang, C. H., J. Fan, R. Cooney, and T. C. Vary. 1996. IL-1 receptor antagonist attenuates sepsis-induced alterations in the igf system and protein synthesis. *Am. J. Physiol.* 270:430–437.
- Lang, C. H., and R. A. Frost. 2002. Role of growth hormone, insulin-like growth factor-I, and insulin-like growth factor binding proteins in the catabolic response to injury and infection. *Curr. Opin. Clin. Nutr. Metab. Care* 5:271–279.
- Lang, C. H., R. A. Frost, A. C. Nairn, D. A. MacLean, and T. C. Vary. 2002. TNF-alpha impairs heart and skeletal muscle protein synthesis by altering translation initiation. *Am. J. Physiol. Endocrinol. Metab.* 282:336–347.
- Lang, C. H., G. J. Nystrom, and R. A. Frost. 1999. Regulation of IGF binding protein-1 in HepG2 cells by cytokines and reactive oxygen species. *Am. J. Physiol.* 276:719–727.
- Lang, C. H., V. Pollard, J. Fan, L. D. Traber, D. L. Traber, R. A. Frost, M. C. Gelato, and D. S. Prough. 1997. Acute alterations in growth hormone-insulin-like growth factor axis in humans injected with endotoxin. *Am. J. Physiol.* 273:371–378.
- Lang, C. H., T. Vary, and R. A. Frost. 2003. Insulin-like growth factor binding protein-1 infusion decreases protein synthesis in vivo in rats. *Endocrinology* 144:3922–3933.
- Li, Y. H., J. Fan, and C. H. Lang. 1997. Differential role of glucocorticoids in mediating endotoxin-induced changes in IGF-I and IGFBP-1. *Am. J. Physiol.* 272:1990–1997.
- Li, Y. P., and M. B. Reid. 2000. NF-kappa B mediates the protein loss induced by TNF-alpha in differentiated skeletal muscle myotubes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279:R1165–1170.
- Monzavi, R., and P. Cohen. 2002. IGFs and IGFbps: Role in health and disease. *Best Pract. Res. Clin. Endocrinol. Metab.* 16:433–447.
- Musaro, A., K. McCullagh, A. Paul, L. Houghton, G. Dobrowolny, M. Molinaro, E. R. Barton, H. L. Sweeney, and N. Rosenthal. 2001. Localized IGF-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. *Nat. Genet.* 27:195–200.
- Naranjo, W. M., S. Yakar, M. Sanchez-Gomez, A. U. Perez, J. Setser, and L. E. D. 2002. Protein calorie restriction affects nonhepatic IGF-I production and the lymphoid system: Studies using the liver-specific IGF-I gene-deleted mouse model. *Endocrinology* 143:2233–2241.
- Pandini, G., F. Frasca, R. Mineo, L. Sciacca, R. Vigneri, and A. Belfiore. 2002. Insulin/insulin-like growth factor-I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J. Biol. Chem.* 277:39684–39695.
- Rinderknecht, E., and R. E. Humbel. 1978. The amino acid sequence of human insulin-like growth factor-I and its structural homology with proinsulin. *J. Biol. Chem.* 253:2769–2776.
- Rodriguez-Arnao, J., G. Yarwood, C. Ferguson, J. Miell, C. J. Hinds, and R. J. Ross. 1996. Reduction in circulating IGF-I and hepatic IGF-I mRNA levels after caecal ligation and puncture are associated with differential regulation of hepatic IGF-binding protein-1, -2 and -3 mRNA levels. *J. Endocrinol.* 151:287–292.
- Samstein, B., M. L. Hoimes, J. Fan, R. A. Frost, M. C. Gelato, and C. H. Lang. 1996. IL-6 stimulation of insulin-like growth factor binding protein (IGFBP)-1 production. *Biochem. Biophys. Res. Commun.* 228:611–615.
- Sandra, A., M. Boes, B. L. Dake, J. B. Stokes, and R. S. Bar. 1998. Infused IGF-I/IGFBP-3 complex causes glomerular localization of IGF-I in the rat kidney. *Am. J. Physiol.* 275:32–37.
- Schambelan, M., K. Mulligan, C. Grunfeld, E. S. Daar, A. LaMarca, D. P. Kotler, J. Wang, S. A. Bozzette, and J. B. Breitmeyer. 1996. Recombinant human growth hormone in patients with hiv-associated wasting. A randomized, placebo-controlled trial. Serostim study group. *Ann. Intern. Med.* 125:873–882.
- Siddle, K., B. Urso, C. A. Niesler, D. L. Cope, L. Molina, K. H. Surinya, and M. A. Soos. 2001. Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem. Soc. Trans.* 29:513–525.
- Suwanichkul, A., S. V. Allander, S. L. Morris, and D. R. Powell. 1994. Glucocorticoids and insulin regulate expression of the human gene for insulin-like growth factor-binding protein-1 through proximal promoter elements. *J. Biol. Chem.* 269:30835–30841.
- Takala, J., E. Ruokonen, N. R. Webster, M. S. Nielsen, D. F. Zandstra, G. Vundelinckx, and C. J. Hinds. 1999. Increased mortality associated with growth hormone treatment in critically ill adults. *N. Engl. J. Med.* 341:785–792.
- Thissen, J. P., and J. Verniers. 1997. Inhibition by interleukin-1 beta and tumor necrosis factor-alpha of the insulin-like growth factor I messenger ribonucleic acid response to growth hormone in rat hepatocyte primary culture. *Endocrinology* 138:1078–1084.
- Turner, J. D., P. Rotwein, J. Novakofski, and P. J. Bechtel. 1988. Induction of mRNA for IGF-I and -II during growth hormone-stimulated muscle hypertrophy. *Am. J. Physiol.* 255:513–517.
- Unneberg, K., L. Balteskard, M. Mjaaland, G. Sager, and A. Revhaug. 1997. Growth hormone increases and IGF-I reduces the response to *Escherichia coli* infusion in injured pigs. *Eur. J. Surg.* 163:779–788.
- Van den Berghe, G., F. de Zegher, R. C. Baxter, J. D. Valdhuis, P. Wouters, M. Schetz, C. Verwaest, E. Van der Vorst, P. Lauwers, R. Bouillon, and C. Y. Bowers. 1998. Neuroendocrinology of prolonged critical illness: Effects of exogenous thyrotropin-releasing hormone and its combination with growth hormone secretagogues. *J. Clin. Endocrinol. Metab.* 83:309–319.
- van Kerkhof, P., E. Vallon, and G. J. Strous. 2003. A method to increase the number of growth hormone receptors at the surface of cells. *Mol. Cell. Endocrinol.* 201:57–62.
- Wolf, S. E., R. E. Barrow, and D. N. Herndon. 1996. Growth hormone and IGF-I therapy in the hypercatabolic patient. *Bailliere's Clin. Endocrinol. Metab.* 10:447–463.
- Yakar. 2002. The role of circulating IGF-I: Lessons from human and animal models. *Endocrine* 19:239–248.
- Yakar, S., J. L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer, and D. LeRoith. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor i. *Proc. Natl. Acad. Sci. USA* 96:7324–7329.
- Yakar, S., C. J. Rosen, W. G. Beamer, C. L. Ackert-Bicknell, Y. Wu, J. L. Liu, G. T. Ooi, J. Setser, J. Frystyk, Y. R. Boisclair, and D. LeRoith. 2002. Circulating levels of IGF-I directly regulate bone growth and density. *J. Clin. Invest.* 110:771–781.
- Yan, Z., R. B. Biggs, and F. W. Booth. 1993. Insulin-like growth factor immunoreactivity increases in muscle after acute eccentric contractions. *J. Appl. Physiol.* 74:410–414.
- Yumet, G., M. L. Shumate, P. Bryant, C. M. Lin, C. H. Lang, and R. N. Cooney. 2002. Tumor necrosis factor mediates hepatic growth hormone resistance during sepsis. *Am. J. Physiol. Endocrinol. Metab.* 283:472–481.
- Ziegler, T. R., and I. Leader. 1994. Adjunctive human growth hormone therapy in nutrition support: Potential to limit septic complications in intensive care unit patients. *Semin. Respir. Infect.* 9:240–247.